Fate of pathogenic organisms in a whey beverage treated with high pressure processing

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Fate of pathogenic organisms in a whey beverage treated with high pressure processing

by

Shawna Simpson

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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To my family—Mom, Dad, Travis and Micah
# Table of Contents

Abstract .......................................................................................................................... v

Chapter 1. General Introduction ..................................................................................... 1

Chapter 2. Literature Review .......................................................................................... 3

  High Pressure Processing ............................................................................................... 3
  High Pressure Concepts and Background .................................................................... 4
  High Pressure Processing of Non-Sporeforming Bacteria ........................................... 6
  High Pressure Processing of Spores ............................................................................ 10

Pathogens ....................................................................................................................... 11

  *Listeria monocytogenes* .............................................................................................. 11
  *Salmonella enterica* .................................................................................................. 16
  *Escherichia coli* O157:H7 ......................................................................................... 20

Whey Beverages .............................................................................................................. 24

  Whey Protein Concentrate and Whey Protein Isolate .................................................. 24
  High Pressure Modification of WPC and WPI for Improved Functional Properties ...... 26
  Health Benefits of WPC and WPI ............................................................................. 27
  Whey Protein Beverage Sensory Attributes ................................................................ 28

References ....................................................................................................................... 29

Chapter 3. High Pressure Processing of a Whey Beverage to Reduce Pathogens ........... 45

  Abstract ....................................................................................................................... 45
  Introduction .................................................................................................................. 46
  Materials and Methods ............................................................................................... 49
  Results and Discussion ............................................................................................... 52
  Conclusion ................................................................................................................... 57

Acknowledgements ......................................................................................................... 58

References ....................................................................................................................... 58

Tables and Figures .......................................................................................................... 62
Chapter 4. General Conclusions

Acknowledgments
ABSTRACT

The resistance of *Listeria monocytogenes*, *Salmonella* spp. and *Escherichia coli* 0157:H7 to high pressure processing (HPP) in an acidic whey protein beverage (pH 3.8) was evaluated. Five strains of each pathogen were grown to stationary phase at 35°C following two 24-hour transfers. Cells were harvested by centrifugation (2450 rpm, 25°C, 15 min), washed and re-suspended in 0.85% (w/v) sodium chloride. The whey beverage was inoculated with approximately 6-7 log₁₀ CFU/mL with one of the cocktails. Samples were subjected to pressurization treatments for 2, 4, 6, or 8 min at pressures ranging from 200 to 500 MPa depending on organism sensitivity to HPP. Cell survivors were enumerated by serial dilution of the beverage in buffered peptone water (to reduce acidic beverage effect) and plated on tryptic soy agar with 0.6 % yeast extract (TSAYE) and selective media: Modified Oxford Medium (MOX) for *L. monocytogenes*, Sorbitol MacConkey agar (SMAC) for *E. coli*, and Xylose Lactose Tergitol 4 agar (XLT-4) for *Salmonella* spp. D-values were calculated from log transformed survival curves. The *E. coli* 0157:H7 cocktail exhibited the greatest resistance to high pressure over *L. monocytogenes* and *Salmonella* spp. A shelf life study of *E. coli* 0157:H7 in a whey beverage containing 50 ppm nisin, 50 ppm nisin and 0.04% (w/v) potassium sorbate, 0.04% (w/v) potassium sorbate and a control (without nisin and/or potassium sorbate) was performed. The beverage was stored at 25°C following HPP at 400 MPa for 4 minutes. Samples were assayed by spread plate on TSAYE and SMAC on days 0, 1, 3, and 5 following pressure treatment. Five mL of the remaining sample was mixed with sterile double strength tryptic soy broth with 0.6 % yeast extract (TSBYE) for enrichment
and incubated at 35°C for 48 hours. Enrichment was streaked on TSAYE and SMAC to confirm \textit{E. coli} O157:H7 growth. Antimicrobial containing samples treated with HPP were more effective at inactivation of \textit{E. coli} O157:H7. At day 1 of storage all antimicrobial samples contained \textit{E. coli} O157:H7 counts below the detection limit of 1 CFU/mL by plating and were undetectable by enrichment. Unpressurized controls contained \textit{E. coli} O157:H7 counts above 6 log CFU/mL at day 5 on TSAYE, reconfirming this organism’s ability to persist in an acidic environment. Overall, HPP was effective in reducing \textit{E. coli} O157:H7 6.79 log\textsubscript{10} CFU/mL in a low pH whey beverage. The acidity of the whey beverage alone was not enough to reduce \textit{E. coli} O157:H7 counts 1 log after 5 days of storage.
Chapter 1. GENERAL INTRODUCTION

Whey protein is a nutritious, cost effective source of amino acids while also being low in fat and sugar. It is very digestible and has a high biological value. These properties make whey protein a popular food supplement among athletes and those wanting to build muscle. Whey protein may also lower cholesterol. These positive health benefits have led to the use of whey protein in the fortification of juices and in functional beverages.

Consumer demand for safe, fresh-like foods with high nutrient retention gave rise to the development of high pressure processing (HPP). This food technology is an alternative to thermal processed foods to reduce pathogens and spoilage organisms. HPP has been used effectively in low pH foods such as ranch dressing (pH 4) to produce shelf stable products at room temperature for up to two months (Waite et al. 2009). The effects of HPP on pathogens in numerous food systems have been studied, however each food “environment” is unique. Survival of these organisms during pressurization is dependent on the formulation of the food being assessed as pH, water activity, and antimicrobials may enhance HPP effectiveness in the reduction of pathogens.

The first goal of this study was to evaluate the effects of HPP on *Listeria monocytogenes*, *Salmonella* spp., and *Escherichia coli* O157:H7 survival in a low pH (3.8) whey beverage. These three pathogens have a large economic impact as well as cost to human life. They are also three of the most common bacterial pathogens worldwide. Each of these pathogens has the ability to survive at low (<4.6) pH. D-values were calculated at pressures between 200 and 500 MPa as the sensitivity of each organisms to HPP and low pH varied. D-values were calculated from log transformed survival curves. Injury was evaluated by comparison of CFUs/mL on selective and non-selective media.
The second goal of this study was to determine if antimicrobials (nisin and potassium sorbate) could be used in conjunction with high pressure to reduce *E. coli* O157:H7, the most pressure resistant cocktail used in this research. Nisin, a bacteriocin, is known to target and inactivate gram positive organisms. Potassium sorbate is a common preservative used to reduce mold and yeast growth. These antimicrobials used alone are not effective against *E. coli* O157:H7, however we hypothesized high pressure could damage cells to allow entry of antimicrobials into the cell cytoplasm. Also, low pH enhances the activity of nisin and potassium sorbate. Therefore, a whey beverage containing no potassium sorbate, potassium sorbate and 50 ppm nisin, and 50 ppm nisin was inoculated with *E. coli* O157:H7. These beverages were HPP treated at 400 MPa for 4 minutes. Samples were stored at 25°C and counts were monitored over 0, 1, 3, and 5 days. Enrichment was performed to confirm complete inactivation of *E. coli* O157:H7.
Chapter 2. LITERATURE REVIEW

HIGH PRESSURE PROCESSING

HPP technology, also known as high hydrostatic pressure (HHP), ultra high pressure processing (UHP), or Pascalization after Blaise Pascal, was not initially developed for the processing of foods, but rather resulted from military weapons research (Patterson et al. 2007). Other forms of high pressure technology are integral to the shaping of powdered ceramics and growth of quartz crystals (Mertens 1995). HPP was first utilized to treat a food product by Hite (1899) in an attempt to preserve milk. HPP was used as a preservation technique in order to circumvent the cooked taste and smell that comes with thermal treatment of milk while still reducing spoilage microorganisms. Though pressures as low as 1 to 4 tons per square inch (14-55 MPa) were tested, the most effective treatments were between 30 and 90 tons per square inch (414-1241 MPa) for an hour to several days at 75 to 80°F (24-27°C). These treatments delayed the souring of the milk up to 4 days. Hite (1914) went on to publish another paper regarding the effect of high pressure on various fruits and vegetables as well as the reduction in growth of various spoilage microorganisms including spores and yeasts. In the same year, Bridgman (1914) went on to use varying pressures at room temperature to coagulate egg whites to the consistency of a hardboiled egg.

Though several other papers were published in regards to the effects of HPP on foods, major interest did not resume until 1990, with the production of high pressure processed acidic fruit jam from the Japanese company Meidi-Ya (Mertens 1992). High pressure processed foods did not catch on immediately on an international scale. It was
not until 1997 that Avomex Inc. introduced a high pressure processed guacamole made from fresh avocado in the United States. This product had the advantages of an increased shelf life, fresh-like taste, and decreased browning. Today, the list of products treated with high pressure has expanded considerably. The percentages of industrial HPP machines in different food industries are as follows: 35% vegetable products, 25% meat products, 15% juice and beverages, 15% seafood and fish, and 6% other (Campus 2010).

**High Pressure Concepts and Background**

High pressure processing involves pressurization of foods between “100 and 900 MPa, with pressures used in commercial systems between 400 and 700 MPa” (San Martin et al. 2002). There are numerous advantages associated with HPP, in comparison to traditional thermal processing, such as retention of fresh-like flavors, color, and texture; retention of heat labile nutrients/vitamins; altered enzyme activity (this may or may not be a disadvantage) and reduction of spoilage and pathogenic organisms. Unlike thermal processing, HPP does not affect the covalent bonds in foods, therefore flavor and color compounds remain unchanged. Also, these effects are uniform through the product regardless of the initial shape of the product. This can be explained by the main principle behind this process, Pascal’s law, that states “pressure exerted anywhere in a confined incompressible fluid is transmitted equally in all directions throughout the fluid such that the pressure ratio (initial difference) remains the same” (Louis 2006).

A disadvantage of HPP is that spores are very resistant to high pressure, and in some cases high pressure can induce germination of spores. Foods with air cells are not good candidates for HPP treatment. Air cells are more compressible than liquid or solid food components, therefore when pressure is released the rapid expansion of air causes
cell structure damage (Otero and Préstamo 2008). For example, HPP treated strawberries can undergo cell damage causing redistribution of water in strawberry tissue (Otero and Préstamo 2008).

A HPP system includes a pressure vessel, closure, yoke to hold down the closure during processing, high pressure intensifier, monitoring system for pressure and temperatures, and a product handling system (Balasubramaniam et al. 2008). There are two types of common processing systems: batch and semi-continuous. Liquid foods can be pressurized in both manners (Balasubramaniam et al. 2008). In batch systems food is packaged in a flexible pouch or bottle then loaded into the pressurization chamber which can contain 30 to 600 or more liters. Semi-continuous processing can be made by assembling several pressure vessels in a series. During semi-continuous processing, the food is pumped into one chamber of the pressurization vessel and pressurization fluid is pumped into the other chamber, compressing the food. Then the HPP treated food is pumped into a sterile chamber from which containers can be filled aseptically.

During HPP, gas, liquid and solid components of foods are compressed to varying levels which generates heat. Gasses are more compressible than liquids and solids. The increase in temperature due to compressive work on intermolecular forces is known as adiabatic heating or compression heating. Upon decompression, the temperature decreases. The increase in adiabatic heat varies with initial temperature, food composition, pressure level, and pressure transmitting fluid. Water generates the least amount of compression heat (2.6-2.9°C at 149-592 MPa) in comparison to linolenic acid (9-5.9°C at 170-602 MPa) and soy oil (8.3-6.3°C at 172-617 MPa) and olive oil (8.7-6.9°C at 173-501 MPa) (Rasanayagam et al. 2003). The differences in oil and water
compressibility are caused by differences in molecular structure and interactions. Water, a small polar molecule, can be compressed more easily than the large unsaturated and non-polar fats contained in oils leading to less adiabatic heat generated. The increase in adiabatic heat may be beneficial. For example, the increase in temperature during pressure assisted thermal sterilization (PATS) can result in reduction of processing time, higher product quality and lower energy consumption (Campus 2010).

Pressure assisted thermal processing (PATP), or alternately known as PATS, combines high pressures (500 to 700 MPa) and high temperatures (90 to 120°C) to produce low acid shelf stable foods (Ratphitagsanti et al. 2009). Advantages of PATP are greater retention of color, flavor and nutrients in comparison to thermal processing. Also, PATP lasts from 1 to 5 minutes which is shorter than thermal treatments. Gupta et al. (2010) found PATP and HPP of hot break tomato juice increased extraction of trans-lycopene as well as resulted in better product color, in comparison to thermal treatments. Trans-lycopene is the most common isomer of lycopene found in red tomatoes and is more thermodynamically stable. The advantages of using PATP are due to the uniform compression heating and expansion cooling which reduces the severity of thermal effects (Nguyen and Balasubramaniam 2011). Spores tend to be much more resistant to HPP than vegetative cells, so current research is investigating PATP efficacy on inactivation of spores (Ratphitagsanti et al. 2009).

**High Pressure Processing of Non-Sporeforming Bacteria**

Inactivation of bacteria by HPP is dependent on numerous factors including the composition of the food (water activity, pH, use of hurdle technology), bacterial factors (growth stage, strain pressure sensitivity, growth temperature), and pressurization level.
Food composition is an important factor when testing the sensitivity of microorganisms to HPP. Complex matrices, such as milk and oysters, in comparison to buffer or broth have been shown to have a protective effect on microorganisms (Black et al. 2007; Smiddy et al. 2005). Foods with low water activity have also exhibited protective effects on microorganisms; effective HPP treatment requires water activity near 1 to reduce microorganisms. Goodridge et al. (2006) observed a $\log_{10}$ reduction of \textit{Salmonella Enteriditis} of 0.83 in HPP treated raw almonds compared to a $\log_{10}$ reduction of 3.37 in HPP treated raw almonds suspended in water. The protective effect of low water activity during HPP has also been observed with yeasts and molds (Oxen and Knorr 1993; Palou et al. 1998). Low pH has been used in combination with HPP to cause enhanced reduction of microorganisms. Pressure pre-treatment, between 300 and 500 MPa, at neutral pH followed by exposure to TSBYE (pH 3.5) caused enhanced reduction of \textit{E. coli O157} compared to non-pressurized cells which showed no loss of viability (Pagan et al. 2001). Low pH enhances inactivation during HPP treatment and inhibits growth of sublethally injured cells (Smelt 1998). The use of a combination of “preservation techniques in order to establish a series of preservative factors (hurdles) that any microorganism present should not be able to overcome” is known as hurdle technology (Leistner and Gorris 1995). Examples of hurdles include temperature, water activity, pH, radiation, and the use of preservatives, bacteriocins and antibiotics. Nisin, a bacteriocin produced by \textit{Lactococcus lactis}, targets gram positive organisms. However, HPP can facilitate inactivation of gram positive organisms by nisin (Lee and Kaletunç 2010). The disruption of the cell membrane may allow for entry of nisin into cells at a greater rate than without HPP (Whitney et al. 2008).
It is important to take into account the effect of growth stage, growth temperature and organism strains when measuring pressure sensitivity. Exponential phase cells are significantly less pressure resistant than stationary phase cells (Smelt 1998; McClements et al. 2001). The growth temperature of microorganisms has been shown to affect pressure resistance. Several authors have observed that as growth temperature increased there was a corresponding increase in pressure resistance of stationary phase cells and a corresponding decrease in pressure resistance of exponential phase cells (McClements et al. 2001; Casadei and Mackey 1997). Juck et al. (2012) observed that the temperature during growth, treatment, and recovery significantly affected *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella Typhimurium* inactivation and recovery in turkey meat. Pressure resistance can vary greatly between strains (Simpson and Gilmour 1997; Alpas et al. 1999; Chung and Yousef 2010). Alpas et al. (1999) observed a viability loss of 0.7 to 7.8 log10 between *Staphylococcus aureus* strains pressurized at 345 MPa at 25°C for 5 min.

The HPP treatment parameters affect the viability decrease of microorganisms. As a general rule, as the pressurization level increases the cell inactivation increases (Campus 2010, Waite et al. 2009). Cell inactivation may also be increased by increasing pressurization temperature (Juck et al. 2012). Temperature increase during HPP caused by adiabatic heating also must be taken into account. Varying fat concentration and pressurization fluid composition can alter the amount of adiabatic heat generated during HPP. The temperature increase generated from adiabatic heating may cause enhanced inactivation.
HPP causes alteration of bacterial cell structures such as ribosomes, enzymes, the nucleoid, and the cell membrane (Casadei et al. 2002). The major site of injury is the cell membrane which in turn alters cell permeability, transport systems, osmotic responsiveness, organelle disruption and loss control of intracellular pH (Campus 2010). Examples of pressure induced membrane damage have been demonstrated by leakage of intracellular components such as ATP and uptake of the fluorescent dyes ethidium bromide and propidium iodide (Smelt et al. 1994; Benito et al. 1999).

Sublethal injury by HPP has been observed in various food pathogens. However, the extent of this injury varies and in some cases may be reparable during storage. Antimicrobials in selective media can inhibit the growth of pressure injured cells which would normally be able to grow. Lower decimal reduction values (D-values) of *E. coli* were calculated from counts spread plated on violet-red bile agar (selective) to brain heart infusion agar (non-selective) (Ramaswamy et al. 2003). D-values are the treatment time at a given pressure that results in a 1 log<sub>10</sub> reduction in CFUs. A decrease in D-value indicates an enhanced rate of destruction of organisms. Injury can be categorized as primary (I1) where cells can form visible colonies on non-selective agar, but not selective agar and secondary (I2) where cells cannot form visible colonies on either type of agar (Bozoglu et al. 2004). These patterns in recovery suggest two types of injury: damage to the cytoplasmic membrane or physiological damage (Bull et al. 2005). In some cases, these pressure injured cells may recover following storage. The extent of this recovery is dependent on the type of food, processing conditions, and storage conditions (Bull et al. 2005; Bozoglu et al. 2004).
High Pressure Processing of Spores

Destruction of spores by high pressure has been and continues to be a challenge in the food industry. In 1918, Larson et al. utilized a direct pressure method in an attempt to destroy bacteria and sporeformers. They found pressures that were able to destroy non-sporeformers were ineffective against *B. subtilis*, which they tested up to 12,000 atm (approximately 1200 MPa).

Current research on spore inactivation with high pressure is focused on HPP utilized in conjunction with another method to synergistically inactivate spores. There have been many papers which utilize a two-step strategy for decreasing spore viability; the initial step is germination followed by an inactivation step which may include a combination of heat treatments, antimicrobials, chemicals, low pH and high pressure. Germination of spores prior to HPP has been found to increase inactivation as dormant spores are very resistant to many antimicrobial agents such as heat, radiation and high pressure. This resistance is hypothesized to be related to the lack of water in the core of the spore. Method of germination and the pH at which spores are germinated can have an increased effect on the sensitization of spores. Pressure and alanine-germinated *Bacillus subtilis* spores, which were germinated at neutral pH, were inactivated by exposure to low pH (3-3.5) at room temperature (Wuytack and Michiels 2001). However pressure-induced germination was inhibited at low pH. Pressures of approximately 500 atm can be used to germinate *B. pumilus* spores leading to sensitization to irradiation (Clouston and Wills 1969). Pretreatment with heat to induce germination of spores was also found to increase inactivation of spores at 1000-3000 atm in conjunction with heat treatments ranging from 35-75°C (Sale et al. 1970).
Chemicals and antimicrobials can have a synergistic effect to reduce bacteria when utilized with high pressure. Nisin, an antimicrobial peptide, in conjunction with high pressure treatments and heat has been found to cause increased inactivation, compared to high pressure or heat alone, against *Bacillus cereus* spores in cheese (Lopez-Pedemonte et al. 2003) and *Clostridium perfringens* in milk (Gao et al. 2011). Sucrose laurate, an emulsifier, in conjunction with HPP and heat has also been found to reduce several *Bacillus* and *Alicyclobacillus* species by 3 to 5.5 log$_{10}$ CFU/mL (Shearer et al. 2000).

**PATHOGENS**

Food safety is an ever present concern in the food industry. *Listeria monocytogenes*, *Salmonella* spp., and *Escherichia coli* O157:H7 are three bacterial foodborne pathogens that have a large impact on consumer health. Outbreaks and recalls cost the food industry billions of dollars each year. Non-typhoidal *Salmonella* (30.6%) and *L. monocytogenes* (27.6%) cause the two highest percentages of foodborne deaths in the United States (Mead et al. 1999). *E. coli* O157:H7 causes the 4th highest amount of foodborne deaths at 2.9%. Each of these organisms has been shown to persist at low pH (<4.6) and have been isolated in unpasteurized juices.

**LISTERIA MONOCYTOGENES**

*Listeria monocytogenes* is a gram positive, facultative anaerobe and non-spore forming rod. It exhibits tumbling motility at incubation temperatures near 20°C. This bacterium is ubiquitous in nature due to its ability to thrive in a wide variety of environmental stresses. *L. monocytogenes* is capable of growing from a pH of 4.3 to 9.4,
but may be able to grow at a lower pH at refrigeration temperatures. Sado et al. (1998) detected *L. monocytogenes* in unpasteurized apple and apple raspberry juice (pH 3.78 and 3.74 respectively). *L. monocytogenes* was also detected in cranberry concentrate at pH 2-2.2 after two weeks of storage at -23°C (Nogueira et al. 2003). This demonstrates that *L. monocytogenes* may survive at this pH, even if it is unable to grow. Generally, this organism can grow at temperatures as low as -0.1 up to 50°C (Walker et al. 1990).

This psychrotrophic organism is responsible for the disease listeriosis. The primary route of infection is by consumption of contaminated food. In healthy individuals, the disease results in flu-like symptoms, but in immunocompromised people, such as children, the elderly and pregnant women, the disease can lead to septicemia, meningitis, and death. Its ability to grow in harsh conditions has led to outbreaks in a variety of foods.

As of 2011, the Center for Disease Control and Prevention reports about 1,600 cases of listeriosis annually in the United States. Though few individuals are infected each year, it was estimated that 28% of all deaths resulting from food illnesses are caused by *L. monocytogenes* (Buchanan and Lindqvist 2000). This organism is a serious problem in the food industry because of its ability to grow at refrigeration temperatures in ready to eat (RTE) foods and its tenacious survival in processing equipment and niches in processing facilities.

*L. monocytogenes* outbreaks have been associated with a variety of foods ranging from dairy products, deli meats, raw vegetables, raw meats, cooked and raw poultry. It is considered an adulterant in RTE foods. This organism’s presence in RTE foods is particularly problematic because in these types of food no further cooking or processing
to kill pathogens is required by the consumer. In September 2011, a multi-state outbreak in pre-cut cantaloupe occurred with a total of 139 people infected. This outbreak resulted in 29 deaths and one miscarriage (CDC 2011). At this time, no specific source of contamination in this incident has been identified. In 1998, contaminated frankfurters and deli meats from Bil Mar Foods, a subsidiary of Sara Lee, caused 108 reported cases of listeriosis, 14 deaths and 4 miscarriages (Mead et al. 2006). These products were traced to a single processing plant, resulting in a recall and destruction of 35 million pounds of product. In total, the recall cost Sara Lee Corp. 76 million dollars (Licking and Carey 1999). Mexican soft cheese contaminated with unpasteurized milk caused listeriosis in 142 people in 1985 (Linnan et al. 1988). Most of these cases (65.5%) occurred in pregnant women and led to 40 deaths in total, including 20 fetuses and 10 infants. In order to decrease the costly effect of this pathogen on product recall and human life, processors must focus their energy on preventative strategies through sanitation and microbial inactivation through examples such as heat treatment, antimicrobials, freezing, irradiation, acid, and high pressure processing.

Improper or insufficient sanitation to remove *L. monocytogenes* has been cited as the contamination source in several outbreaks. In regards to the Bil Mar outbreak, Mead et al. (2006) postulated the source of contamination was the ceiling refrigeration unit in the frankfurter hopper room. Autio et al. (1999) found contamination of cold smoked trout occurred in the brining and post brining area and equipment of one processing facility. Five months later, following a facility wide eradication program, the number of positive samples decreased to 0 of 93 in all areas and critical control points (CCPs) sampled (equipment, brine solution, final product). Proper sanitation, identification of
CCPs and screening are imperative to insure product safety. The selection of proper sanitizers and monitoring concentration used is important to ensure pathogen inactivation.

*L. monocytogenes* has the ability to stay in a processing environment for many years due to its ability to form biofilms. Common locations of biofilm formation are in niche areas such as dead ends, joints, valves and gaskets as well as pits and cracks in surfaces (Wong 2006). Attachment to stainless steel, glass, polypropylene, rubber, Teflon®, and polyester floor sealant has been demonstrated (Mafu et al. 1990; Blackman and Frank 1996). *L. monocytogenes* biofilms have been shown to be more resistant to sanitizers than planktonic cells (Frank and Koffi 1990; Blackman and Frank 1996; Wong 1998). Factors such as temperature, water activity, and pH have been found to have an effect on the resistance of the biofilm to sanitizers (Briandet et al. 1999; Wong 1998). Resistance also depends on type of sanitizer used (Pan et al. 2006).

The exact infectious dose to cause listeriosis in humans is unknown, however, it is thought that the infectious dose is <1000 CFU in at risk individuals. The infectious dose in mice is extremely variable $10^3$-$10^8$ CFU/mL due to differences in mouse and human E-cadherin (Farber and Peterkin 1991; Lecuit et al. 1999). E-cadherin is a human protein that binds internalin, a surface protein of *L. monocytogenes* which is responsible for internalization of this bacterium into host cells. Therefore, alternative animal models such as gerbils and zebra fish with E-cadherin homologous to humans have been investigated (O’Byrne and Utratna 2010). This pathogen can colonize the gut of a healthy individual asymptotically, between 2 and 6% of the population are reported to be fecal carriers (Olier et al. 2003, Rocourt et al. 2000).
Once *L. monocytogenes* is consumed, it enters epithelial cells via production of internalin A and B which interact with the receptor E-cadherin in the host (Lecuit 1999). This organism is unusual in that it induces phagocytosis in normally non-phagocytic cells (Cossart and Lecuit 1998). Once the pathogen enters the cell and lyses the vacuole it reproduces in the cytosol. Lysis of the vacuole is enabled by cytolysis listeriolysin O (Radtke et al. 2011). There the bacteria uses the host’s actin, similar in appearance to “comet tails,” that move the bacteria to the surface of the plasma membrane (Tilney and Portnoy 1989). The bacteria produce “surface protrusions” causing neighboring cells to engulf the infected cell, restarting the infectious cycle.

Listeriosis may manifest as gastroenteritis, meningitis and septicemia. Common symptoms associated with gastroenteritis are fever, watery diarrhea, nausea, headache, and pain in joints and muscle. The most prevalent symptom associated with infection is diarrhea. In a study observing the bacterial meningitis rates in the United States from 1998-2007 there were 105 confirmed cases and 19 deaths attributed to *L. monocytogenes* (Thigpen et al. 2011). Of the total cases of bacterial meningitis in the U.S., *Listeria* accounted for only 3.4%. A high fever, headache and stiff neck are associated with meningitis. Septicemia is characterized by chills, high fever, rapid breathing and heart rate. *Listeria* infections are more likely to occur in pregnant women. *L. monocytogenes* is unusual in that it is able to cross the placenta and may cause miscarriages or infection of the fetus. Demonstration of this ability was previously mentioned in the Mexican soft cheese outbreak.

HPP to eliminate the growth of *L. monocytogenes* and *Listeria innocua* has been studied in numerous food systems such as milk, raw pork, salami, and smoked salmon.
(Koseki et al. 2008; Porto-Fett 2010; Ritz 2008). Pressure treatments on *L. monocytogenes* at 4°C in citrate buffer at pH 4.5 resulted in 5.5 log$_{10}$ CFU/mL inactivation at 200 MPa (Ritz 2008). Acid resistant strains (growth at pH 2) did not exhibit significant cross-protective resistance against pressure (Chen et al. 2009). Dogan and Erkman (2004) observed lower *L. monocytogenes* D-values in orange juice (pH 3.55) pressurized at 300, 400 and 600 MPa than those in peach juice (pH 5.21) and raw whole milk (pH 6.64) treated under the same HPP parameters. Alpas and Bozoglu (2003) observed *L. monocytogenes* CA viability loss of 3.90-4.34 log$_{10}$ in apple (pH 3.5), apricot (pH 3.8), orange (3.76), and cherry juice (pH 3.30) at 250 MPa, 30°C, for 5 minutes. They observed increased viability loss (6.80-7.44) at higher pressures (350 MPa, 30°C, for 5 minutes).

**SALMONELLA ENTERICA**

*Salmonella enterica* is a gram negative facultative anaerobe. This flagellated rod is capable of surviving in a wide variety of harsh environments. *Salmonella* spp. is capable of growing at pH of 4-9.5, but its optimal growth range is 6.5 to 7.5. Its ability to persist in low pH juices has been previously demonstrated by outbreaks in unpasteurized apple cider and orange juice (Parish 1997). *Salmonella* spp. has a broad temperature growth range from 10 to 49°C with an optimum growth temperature of 37°C (Balamurugan 2010). *Salmonella enterica* is divided into six subspecies: *S. enteric* subsp. *enterica*, *S. enteric* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *indica*, and *S. enterica* subsp. *houtenae* (Porwollick et al. 2004). Of these subspecies, only *S. enterica* subsp. *enterica* can cause disease in warm blooded animals. This subspecies is further divided into over 2,500 serovars, although
few of these cause disease in animals and humans. A 2008 Morbidity and Mortality Weekly Report (MMWR) analysis of the FoodNet surveillance data identified 10 serovars as the cause of 91% of the Salmonella infections in U.S. patients. The percentage of infection caused by each serovar is as follows: Enteritidis, 20.1%; Typhimurium, 16.0%; Newport, 10.1%; Javiana, 6.3%; Saintpaul, 6.0%; I 4,[5],12:i:-, 4.0%; Muenchen 3.2%; Heidelberg, 2.9%; Montevideo, 2.9%; and Braenderup, 1.6%.

Although *Salmonella* is often associated with eggs, meat, milk and poultry, this organism continues to be an obstacle in the food industry in a wide variety of foods. In 2011 the CDC reported food-related outbreaks in ground beef, broiled chicken livers, Turkish pine nuts, ground turkey, whole fresh papayas, alfalfa, sprouts, turkey burger, and cantaloupe. In 2010, the CDC reported food outbreaks of *Salmonella* in alfalfa sprouts, shell eggs, chicken frozen entrees, frozen fruit pulp, red/black pepper and Italian-style meats.

*Salmonella* spp. outbreaks continue to be costly to the food industry as well as in regards to human life. A 1999 FoodNet analysis of the number of foodborne deaths in the United States reported 72% of the deaths were associated with bacterial pathogens and 31% of these were cause by *Salmonella* spp. (Mead). In 2010, the FoodNet MMWR showed that *Salmonella* spp. was the pathogen “furthest from its national target” at 17.6 cases per 100,000 population compared to the Healthy People 2010 target of 6.8 or fewer cases per 100,000 population. In 2011, there were almost 1.4 million cases of salmonella reported to the CDC in the U.S. While FoodNet collects data from laboratory diagnosis of patients in 10 U.S. states to extrapolate these nationwide statistics, these do not
account for asymptomatic cases or cases of foodborne illness which go undiagnosed and or unreported. In total, the USDA’s Economic Research Service estimates the economic cost of *Salmonella* spp. in the U.S. to be near 2.7 billion dollars.

One example of a nationwide outbreak began in November of 2008. Peanut butter, from the Peanut Corporation of America, contaminated with *Salmonella* spp. was distributed in bulk to institutions, food service industries and private label food companies even though safety testing results had not been received. This outbreak resulted in 9 deaths and 529 laboratory confirmed cases reported to the CDC, as well as led to the bankruptcy of the Peanut Corporation of America. This was not the first incidence of salmonella in peanut butter. In 2007, Peter Pan peanut butter was linked to 425 cases of salmonella.

Salmonella infection is divided into two common manifestations. The first is enteric (typhoid) fever caused by *Salmonella* Typhi. This type of infection is transmitted person to person as humans are the only known hosts. The second manifestation is gastroenteritis caused by nontyphoidal salmonellosis (NTS). NTS is spread via contaminated food and water. While transmission of Salmonella is primarily foodborne, it may also be passed through contact with infected pets, birds, livestock and fish (Hoelzer et al. 2011). This organism’s normal reservoir is in the intestinal tracts of animals which is why it is considered a zoonotic disease. Person to person spread via the fecal oral route is also possible, as this organism is shed in the feces of some infected individuals, however, few cases have been documented (Musher and Musher 2004).

Children, the elderly and those with compromised immune systems, are most at risk for contracting salmonellosis. An example of a compromised immune system
increasing susceptibility to salmonellosis can be demonstrated by the association of severe malaria and NTS bacteremia (Bronzan et al. 2007). The reported infectious dose varies greatly with serovar from $10^3$-$10^9$ organisms (Kothary and Babu 2000). Once ingested, Salmonella enters the intestinal epithelium via two type III secretion systems (TTSS), which is encoded on the *Salmonella* pathogenicity island 1 (Schlumberger et al. 2005). *Salmonella* enters host cells through bacterial mediated endocytosis causing a phenomenon known as membrane ruffling. The host’s proinflammatory cytokine response to Salmonella within the host cells which attracts neutrophils is the “hallmark of gastroenteritis caused by nontyphoidal *Salmonella* serovars” (Andrews-Polymenis et al. 2010). After *Salmonella* has passed through the epithelium, it reproduces intracellularly in macrophages, dendritic cells, neutrophils and the underlying tissue. In order to cause systemic infection, *Salmonella* is transported to other organs within macrophages via lymphatics and blood (Ohl and Miller 2001). The incubation period for gastroenteritis ranges from 12 to 72 hours and infection lasts 2-7 days. Gastroenteritis caused by NTS is considered self limiting. Treatment is supportive, with fluids or IVs being administered in cases of extreme dehydration unless the infection spreads to other sites in the body or bacteremia. In this instance, antibiotics are administered.

HPP has been shown to significantly reduce *Salmonella* in a number of acidic food products. Diced and whole tomatoes (pH 3.99-4.37) subjected to HPP for 2 minutes reduced *Salmonella* Branderup significantly at 350 ($0.46 \log_{10}$ CFU/g reduction), 450 ($1.44 \log_{10}$ CFU/g reduction) and 550 MPa ($3.67 \log_{10}$ CFU/g reduction) (Maitland et al. 2011). HPP of grapefruit juice (pH 3.0) at 615 MPa and 15°C for 2 minutes decreased five *Salmonella* serovars to undetectable levels (Teo et al. 2001). HPP at 345 MPa, 50°C
for 5 minutes reduced *S. enteriditis* FDA and *S. typhi* E21274 greater than 8 log$_{10}$ CFU/mL in orange juice (pH 3.76). Unlike the rest of the examples in the literature, following HPP treatment at 300 MPa for 2 minutes at 6°C in orange juice (pH 3.9), Whitney et al. (2007) observed a population reduction of 0.26-0.62 log$_{10}$ CFU/mL after 0 hours which was significantly less than the reductions seen in Tryptic Soy Broth (0.53-3.04 log$_{10}$ CFU/mL reduction) or distilled water (1.72-2.52 log$_{10}$ CFU/mL reduction) demonstrating a protective effect by the juice. This was suggested to be due to the activation of the acid tolerance response which is common in both *Salmonella* and *E. coli*.

Antimicrobials in combination with high pressure have resulted in an enhanced synergistic reduction of *Salmonella* serovars. Addition of nisin prior to HPP treatment reduces viable counts compared to HPP or nisin alone in deionized water and TSBYE (Gou et al. 2010; Lee and Kaletunç 2010). Storage at 4°C for 7 days following HPP in this nisin solution caused an additional decrease in recoverable viable cells (Gou et al. 2010).

**ESCHERICHIA COLI O157:H7**

*Salmonella* and *E. coli* share many common traits as they are both members of the family Enterobacteriaceae. Both are rod-shaped gram negative facultative anerobes. *E. coli* can be found in the intestinal tract of animals (mammals). It is a coliform, which is an indicator of fecal contamination in foods and water. While most strains of *E. coli* are nonpathogenic, *E. coli* O157:H7 is the most common enterohemorrhagic serovar, known to cause hemolytic uremic syndrome and hemorrhagic colitis. A second enterohemorrhagic strain, *E. coli* O104:H4, was discovered to be the cause of an outbreak
in Germany in 2011 from consumption of contaminated raw sprouts (CDC 2011). The temperature range for growth is 19.3°C to 42.5°C with an optimum growth temperature at 37°C (Raghubeer and Matches 1990). This organism is capable of growing from a pH of 4.5-9.0 (Glass et al. 1992). Optimum growth of *E. coli* O157:H7 occurs at a neutral pH 7.0. However, *E. coli* O157:H7 has been shown to persist in apple cider at 8°C (pH 3.6-4.0) for 31 days, in ketchup at 23°C (pH 3.6) for 2 days or at 5°C for over 7 days, and in Ranch salad dressings stored at 25°C (pH 3.21-3.69) for over 3 days, but less than 6 days (Zhao et al 1993; Tsai and Ingham 1997; Beuchat et al. 2006).

Several *E. coli* O157:H7 outbreaks have taken place in recent years. In 2012, there were 12 persons infected from consumption of raw clover sprouts traced back to Jimmy John’s restaurants (CDC 2012). In 2011, there were three outbreaks in the U. S. associated with romaine lettuce, bologna and hazelnuts. Cattle and other food animals are major reservoirs of *E. coli* O157:H7, therefore *E. coli* O157:H7 outbreaks are often associated with meat, however, outbreaks in fresh foods that are sold without a kill step are becoming more common. These foods include bean sprouts, cantaloupe, apples and leaf lettuce (Solomon et al. 2002). These foods can be contaminated when fertilized with contaminated manure or when washed with water contaminated with cattle feces. Solomon et al. (2002) demonstrated that this pathogen can be internalized within lettuce by uptake through the roots of a plant following irrigation with contaminated water. Fecal contamination of milk can lead to contamination of dairy products, which is why pasteurization is so important. According to the CDC, in 2010 a multistate outbreak occurred of both *Listeria monocytogenes* and *E. coli* O157:H7 in Gouda cheese, which resulted in 38 persons infected and 15 reported hospitalizations. *E. coli* O157:H7 caused
several cases of gastroenteritis in unpasteurized apple cider in 1991 and 1997. Though apple cider is acidic (pH 3-4), *E. coli* O157:H7 was detected in cider after storage at 8°C for 31 days or 25°C for 2 to 3 days (Zhao 1993).

The USDA’s Economic Research Service estimates that the number of annual cases of *E. coli* O157:H7 (73,480 cases) costs the U. S. $488 million a year. The estimated percent of underreported cases is 20-fold (Mead 1999). 85% of these cases are estimated to be acquired from foodborne sources. *E. coli* O157:H7 also causes 3% of the foodborne related deaths.

*E. coli* pathovars can be classified as diarrhoeagenic or extraintestinal (ExPEC) (Croxen and Finlay 2010). *E. coli* strains that can cause diarrheal disease can be divided into six categories (Kothary and Babu 2000). These categories are enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EaggEC), diffuse adherence *E. coli* (DAEC) and enterohemorrhagic *E. coli* (EHEC). *E. coli* O157:H7 falls into the EHEC category. *E. coli* may also be categorized by the toxin they produce, for example, *E. coli* may produce shiga-like toxins (STEC) or necrotoxin (NTEC).

*E. coli* O157:H7 is most often spread by foodborne contamination, but can be spread by swimming in contaminated water or via person to person contact via the fecal-oral route. The infectious dose is estimated to be very low, less than 100 CFU. In an outbreak in beef liver, the ingestion dose by persons exposed was less than 9 CFU/g (Hara-Kudo and Takatori 2011).

*E. coli* O157:H7 resistance to acid in comparison to many other *E. coli* strains may be due to the fact that it must survive the low pH of the stomach to pass into the gut.
Once in the gut, it adheres to intestinal epithelial cells by forming attaching and effacing lesions. Underneath the attached bacteria, “pedestals” are formed from an accumulation of host cytoskeletal components (Paton and Paton 1998). The symptoms associated with \textit{E. coli} O157:H7 infections are due to Shiga like toxin (SLT). STL are named for their similarity to toxins produced by \textit{Shigella dysenteriae}. The structure of the SLT-I and SLT-II form consists of an enzymatic subunit A with five copies of the B binding subunit (Ling et al. 1998). SLT binds the globotriaosylceramides on the surface of Paneth cells and kidney epithelial cells (Croxen and Finlay 2010). There is also evidence that SLT can bind globotriaosylceramides on platelets. After SLT is internalized by a cell, the A subunit is activated by cleavage from the B subunit, causing cell death. Following cell lysis, SLT is released to be internalized by other cells.

The most common manifestation of \textit{E. coli} O157:H7 is hemorrhagic colitis which is a type of gastroenteritis. Common symptoms of hemorrhagic colitis include watery then bloody diarrhea, cramps and fever. In some cases, infection worsens and becomes hemolytic-uremic syndrome (HUS). HUS is characterized by fatigue, hemolytic anemia, and sudden kidney failure. HUS is a severe syndrome which may result in death in children and the elderly. Treatment is generally supportive and without antibiotics.

The use of HPP to reduce \textit{E. coli} O157:H7 has been shown effectively in apple juices (Whitney et al. 2007, 2008). Whitney et al. (2007) demonstrated an enhanced reduction of \textit{E. coli} O157:H7 when HPP was used with apple juice in comparison to HPP and distilled water or tryptic soy broth at 300 and 550 MPa. This is possibly because of the low pH of the apple juice. Whitney et al. (2008) demonstrated significantly higher reduction in \textit{E. coli} O157:H7 when using dimethyl dicarbonate, hydrogen peroxide,
potassium sorbate and sodium benzoate in combination with HPP. Interestingly, 500 ppm of potassium sorbate or sodium benzoate resulted in greater log reductions than 1000 ppm of either antimicrobial. As stated before, pressure resistance varies within strains of a species, and *E. coli* is no exception. There is some evidence that pressure resistance can be roughly correlated with heat resistance (Benito et al. 1999). Heat shock prior to pressurization can increase pressure resistance of *E. coli* (Aertson et al. 2004).

**WHEY BEVERAGES**

**Whey Protein Concentrate and Whey Protein Isolate**

Precipitation of caseins from milk during cheese production results in a greenish tinged liquid known as whey (Panesar et al. 2010). At 9 liters of whey for every kilogram of cheese produced, large scale cheese manufacturers may produce greater than one million liters of whey per day (Jelen 2003). Historically, whey was dumped in rivers in the US until environmental regulations halted this water pollution (Tunick 2008). Today, this nutritious by-product is utilized for its advantageous functional properties such as improved foaming, viscosity, gelling and browning in baked goods, ice creams, whipping creams, and cheeses. Whey protein concentrate and whey protein isolate are also used to enhance juice-based sports beverages due to stability at low pH (King 1996).

Whey contains 55% of the nutrients from the milk (Marwaha and Kennedy 1988). Following cheese-making where many of the salts, lactose, fats and caseins are removed, whey is concentrated by ultrafiltration and ion exchange chromatography. Two products formed from whey are whey protein isolate (WPI) and whey protein concentrate (WPC). The concentration of WPC can range from 20 to 89%, but WPI must contain 90% or
greater protein (Tunick 2008). The differences in composition of WPI and WPC are shown in Table 1.

Table 1—Typical compositions of whey protein concentrates and isolate

<table>
<thead>
<tr>
<th>Component</th>
<th>WPC 34</th>
<th>WPC 80</th>
<th>WPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, %</td>
<td>34</td>
<td>80</td>
<td>92</td>
</tr>
<tr>
<td>Lactose, %</td>
<td>53</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Ash, %</td>
<td>7</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Fat, %</td>
<td>3</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Moisture, %</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Adapted from Huffman and Harper 1999.

The major proteins found in dairy whey are β-lactoglobulin and α-lactalbumin which make up 80% of the proteins, as well as a small amount of immunoglobulins, bovine serum albumin and α-caseins, see Table 2.

Table 2—Physical characteristics of major whey proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration (g/L)</th>
<th>Isoelectric pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Lactoglobulin (monomer)</td>
<td>2.7</td>
<td>5.2</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>1.2</td>
<td>4.5-4.8</td>
</tr>
<tr>
<td>Immunoglobulin</td>
<td>0.65</td>
<td>5.5-8.3</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.4</td>
<td>4.7-4.9</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>0.1</td>
<td>9.0</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>0.02</td>
<td>9.5</td>
</tr>
</tbody>
</table>

Adapted from Zydney 1998.
The advantageous functional properties of WPI such as stabilizing foams, result from β-lactoglobulin’s binding and gelling abilities. Approximately half of the proteins found in whey are β-lactoglobulin. Though the exact function of β-lactoglobulin is not known, it has been suggested that its purpose is ligand binding as it is known to bind many compounds such as retinol, cholesterol, mercury ions, and Vitamin D₂ (Kontopidis et al. 2002, 2004). β-lactoglobulin structure and binding properties are dependent on pH; at a pH above 5.2 it exists as a dimer (Liu et al. 2009). Heat and high pressure can cause protein aggregation of β-lactoglobulin to produce gels in foods. Reversible denaturation of β-lactoglobulin and α-lactalbumin will occur between pressures of 100 and 300 MPa (Sazuki and Taniguchi 1972). Increase of pH during thermal aggregation of β-lactoglobulin causes increased aggregate diameter (Zúñiga et al. 2010).

The second most common protein in milk and whey, α-lactalbumin, was previously identified as the B protein subunit of lactose synthetase complex (Brodbeck et al. 1967). This protein is essential in catalyzing the following reaction in mammary tissue (Watkins and Hassid 1962):

\[
\text{UDP-}D\text{-galactose} + \alpha\text{-D-glucose} \xrightarrow{\text{glactosyl transferase}} \text{lactose} + \text{UDP}
\]

The lactose synthase complex is formed from β1-4-galactosyltransferase interaction with α-lactalbumin (Ramakrishnan 2002). Unlike β-lactoglobulin, α-lactalbumin does not contain any free thiol groups to initiate aggregation under high pressure so it does not produce gels unless a reducing agent is added (Jegouic et al. 1996). High pressure treatment and decreased pH of α-lactalbumin results in increased thermostability, suggesting a more stable structure is formed under these parameters (Rodiles-López et al. 2010).
High Pressure Modification of WPC and WPI for Improved Functional Properties

HPP has been used to modify whey proteins for improved functional properties in foods. Improved overrun, viscosity and foam stability was observed in low-fat whipping cream when HPP-treated WPC was included in formulation compared to two control low-fat whipped creams without WPC and with untreated WPC (Padiernos et al. 2009). Similar results were found in low-fat ice cream where increased overrun, foam stability and ice cream hardness were observed (Lim et al. 2008). Gels have also been formed from HPP treatment of milk proteins and whey concentrate (Famelart et al. 1998; Van Camp and Huyghebaert 1995). While the gelling properties of milk proteins have been utilized beneficially in foods, they also form “proteinaceous milk-fouling deposits” on pasteurization and sterilization equipment that trap food particles (Mercadé-Prieto et al. 2006).

Health Benefits of WPI and WPC

The natural health benefits of whey have been recognized for centuries. The use of cheese whey to treat “an assortment of human ailments” can be traced back to Hippocrates in 460 B.C. (Holsinger et al. 1974). Whey supplementation is popular among those trying to build muscle as it is high in protein, but low in fat and sugar. Ingestion of whey protein following a workout has the ability to increase net leucine and phenylalanine concentrations in the human body, leading to a net increase in muscle synthesis (Tipton et al. 2004). Leucine is sensed by the insulin signaling pathway, triggering muscle synthesis (Etzel 2004). WP decreases insulin resistance as well as has heart health benefits. Consumption of WPI daily by overweight/obese individuals for 12 weeks significantly decreased total cholesterol levels, LDL cholesterol levels, serum
triacylglycerols, and plasma insulin levels (Pal et al. 2010). Postoperative feeding of patients with WP also decreased insulin resistance (Perrone et al. 2011)

**Whey Protein Beverage Sensory Attributes**

Astringency is the mouth-drying sensation caused by some foods. There are many astringent beverages such as coffee and red wine, however low pH whey protein beverages are strongly associated with an astringent mouth sensation. Childs and Drake (2010) found that 95% of their test subjects (n=49) liked their acidified whey beverage the least (pH 3.4) when compared to their sweet (pH 2.6) and sour model controls (pH 2.4) and 81% found the whey beverage “very astringent.” Functional beverages containing whey proteins may be made at neutral or low pH depending on desired appearance. Neutral pH beverages containing whey protein are opaque due to denaturation of proteins, therefore beverages are often made at low pH in order to optimize clarity and heat stability (Beecher et al. 2008). The astringency associated with whey protein is more pronounced at low pH. Various sensory analyses have been performed to determine the cause of astringency in these beverages. Beecher et al. (2008) proposed that “negatively charged salivary proteins interacting with positively charged whey proteins to form aggregates, similar to polyphenolic compounds and saliva proteins interacting to cause astringency.” These results were consistent with the work of Kelly et al. (2010), who also found perceived astringency was dependent on whey protein concentration, pH and buffering capacity of protein solution. Both research groups found that buffering the whey protein solution reduced astringency significantly, which should
be considered when formulating a new beverage. Viscosity of a beverage does not affect perceived astringency (Beecher et al 2008).

Thermal treatment at 88°C for 2 minutes is a precaution to eliminate growth of yeasts and molds in hot-filled and shelf stable beverages, even beverages below pH 4.6 (Etzel 2004). Consumer acceptance of beverages containing whey protein requires low turbidity (La Clair and Etzel 2010). Etzel noted that thermal treatment of WPI solutions results in turbidity and/or sedimentation at a pH above 4.0, but that turbidity may be eliminated by addition of lauryl sulfate, a food additive, prior to treatment. Careful beverage formulation is essential in promotion of clarity. Use of sugar alcohols, as opposed to sugar, as well as inclusion of glycerol, propylene glycol, ethanol and urea significantly reduced turbidity in thermally treated WPI solutions while addition of salts significantly increased turbidity (LaClair and Etzel 2010).

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CHAPTER 3. High Pressure Processing to Inactivate *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella enterica* in an Acidic Whey Beverage

A paper to be submitted to the Journal of Food Protection

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**Keywords:** High pressure processing, whey beverages, pathogenic microorganisms

ABSTRACT

Whey protein (WP) is used in functional beverages because of its nutrient-rich qualities and health benefits. High pressure processing (HPP) has been shown to effectively reduce pathogens in foods while maintaining nutrients. The objective of this study was to determine the effect of HPP at ambient temperature (25°C) on viability of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* spp. in a low pH (3.8) WP isolate beverage. The WP beverage was inoculated with one of the 5-strain culture cocktails for a final concentration of ~6-7 log10 CFU/mL. HPP treatments were 200, 250, and 350 MPa for *L. monocytogenes*, 300, 400, and 500 MPa for *E. coli* and 350 MPa for *Salmonella* spp. Pathogens were enumerated by plating on selective and non-selective media to determine injury. D-Values on TSAYE were 4.86, 1.20, and 0.64 at
200, 250, and 300 MPa for *L. monocytogenes*, 1.49 at 350 MPa for *Salmonella* spp., and 5.24, 2.64, and 1.80 at 300, 400, and 500 MPa for *E. coli*. Sublethal injury of *L. monocytogenes* was observed. *E. coli* O157:H7 was most resistant of the 3 bacterial cocktails to HPP treatments. A storage study combining HPP treatment with 50 ppm nisin and/or 0.04% w/v potassium sorbate to inactivate was performed to test the storage safety of WP beverages. Inactivation of >6 log$_{10}$ CFU/mL *E. coli* O157:H7 was observed by day 1 of storage in all HPP treated samples. Unpressurized controls were able to persist at levels above 6 log$_{10}$ CFU/mL by day 5 of storage.

**INTRODUCTION**

No longer considered a waste byproduct of cheese-making, whey is a profitable food ingredient with valuable nutritional and sensory characteristics. Whey contains 55% of the nutrients from the milk (Marwaha and Kennedy 1988). These nutrients may be further concentrated and dried to form whey protein isolate (WPI) and whey protein concentrate (WPC). Both WPC and WPI contain high protein and are low in sugar and fat making them ideal supplements in functional and sports beverages.

Whey protein beverages that contain more water than all the other ingredients combined are defined as water-based liquids. To optimize clarity, whey beverages are formulated at low pH (Beecher 2008). At pH 4.6 or below they are considered acidified foods under the 21 CFR 114.3. The FDA requires these foods to be “thermally processed to an extent that is sufficient to destroy the vegetative cells of microorganisms of public health significance and those of non-health significance capable of reproducing in the food under the conditions in which the food is stored, distributed, retailed and held by the
user” (21 CFR 114.8). However, hot-fill treatment to reduce microorganisms at a pH near the isoelectric point of whey (pH 4.6) produces heavy precipitate and sediment formation (Etzel 2004). At pH 4.0, hot-fill treatment results in a turbid beverage. Consumer acceptance of beverages containing whey protein requires low turbidity (La Clair and Etzel 2010).

High pressure processing (HPP) is an alternative to traditional thermal treatments for inactivation of pathogens and spoilage organisms. This process is independent of product geometry, and pressure is isostatic and transmitted instantaneously through the food. Advantages of HPP include fewer changes in texture, color and flavor than other conventional technologies (Torres and Velasquez 2005). Inactivation of microorganisms by HPP is dependent on numerous variables such as treatment temperature, pressure level, duration of treatment, initial microbial load, pH, growth conditions, suspending media, species and strain (Cheftel 1995; Patterson 1999).

*Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp. are amongst the most common and deadly food pathogens worldwide. *L. monocytogenes* is a gram positive pathogen capable of growing at refrigeration temperatures. Approximately 2,518 human cases are reported annually in the U.S. each year (Mead et al. 1999). It is estimated that *L. monocytogenes* causes 28% of the deaths foodborne illness (Buchanan and Lindqvist 2000). *E. coli* O157:H7 causes approximately 73,480 human cases annually in the U.S. with a hospitalization rate of 29.5% and a 0.83% fatality rate (Mead et al. 1999). Nontyphoidal *Salmonella* causes the greatest number of estimated food illnesses at approximately 1.4 million per year with a hospitalization rate of 22% and a fatality rate of 0.78%. Both *Salmonella* and *E. coli* O157:H7 foodborne outbreaks have
occurred in unpasteurized apple juice and apple cider (pH ~ 3.5) (Parish 1997). *L. monocytogenes* was detected in unpasteurized apple and apple raspberry juice (pH 3.78 and 3.75) (Sado 1998).

Hurdle technology, such as antimicrobials, may be used in conjunction with HPP to enhance microbial destruction (Lee and Kaletunç 2010; Whitney et al. 2008). Potassium sorbate is a generally recognized as safe (GRAS) chemical preservative used primarily to inhibit yeasts and molds. It forms sorbic acid which can be found naturally in rowan berries. Nisin, a bacteriocin produced by *Lactococcus lactis*, can bind and inactivate gram positive bacteria. Inactivation of gram negative bacteria by nisin in conjunction with a membrane chelating agent such as EDTA has also been observed (Stevens et al. 1991). HPP alteration of the outer membrane in combination with nisin, the only bacteriocin approved by the FDA (GRAS), has been shown to synergistically increase inactivation of *E. coli*, a gram negative bacterium (Masschalck et al 2000; Hauben et al. 1996).

This research was conducted to determine the effect HPP parameters pressure and holding time on the viability of three pathogens. The first objective was to determine growth curves and D-values for these pathogens at varying pressure levels. The second objective was to analyze the safety of the beverage in a 5 day storage period at room temperature in conjunction with antimicrobials. At this time, virtually no work has been reported on the effects of HPP on pathogens in low pH whey beverages.
MATERIALS AND METHODS

Whey beverage preparation

A whey beverage was prepared based on a formulation provided by the Midwest Dairy Association. Whey protein isolate (WPI) (Hilmar Ingredients, Hilmar, CA, U.S.A.) was mixed in a flask at half maximum speed on a stir plate (Corning Inc., Corning, NY, U.S.A.) with half the total volume of distilled water until dissolved. This solution was allowed to hydrate for 1 hour at 20°C. The remaining ingredients except phosphoric acid were combined with the whey solution (Table 1). The final beverage was pH adjusted with 85% phosphoric acid to pH 3.8. Percent phosphoric acid in Table 1 is an estimate. The beverage was refrigerated at 4°C 24 hours prior to testing then brought to 25°C in a water bath before packaging.

Growth of pathogens for culture preparation

Five pure strains of *Escherichia coli* O157:H7 (ATCC 43894 [human feces isolate], ATCC 43895 [raw hamburger isolate], ATCC 35150 [human feces isolate], FRIK 125, and 93-062) were grown in 9 mL Tryptic Soy Broth (TSB)(Becton Dickson, Sparks, MD, U.S.A.) with 0.6% yeast extract (YE)(MP Biomedicals, Solon, OH., U.S.A.) (TSBYE) tubes. These strains were streaked for isolation on Sorbitol MacConkey agar (SMAC)(Becton Dickson, U.S.A.) and TSA (Becton Dickson, U.S.A.) with 0.6% YE prior to testing and grown at 35°C to confirm purity. Two culture transfers were performed. Cultures were grown for 24 hours at 35°C, with 0.1 mL transfer to fresh 9 mL TSBYE tubes. Five milliliters of each strain were aseptically removed and placed in a sterile centrifuge vial. This five strain cocktail was subjected to centrifugation for 15 minutes, at 2450 rpm and 25°C (Sorvall, Newton, CT, U.S.A.). The supernatant was
decanted and the cells were resuspended in 25 mL sterile saline (0.85% sodium chloride). Centrifugation and addition of saline was repeated.

Five pure strains of *Listeria monocytogenes* (Scott A, H7969, H7962, H7596, and H7762) were grown in TSBYE and streaked for isolation on Modified Oxford Medium (MOX) and TSAYE prior to testing. Five strains of *Salmonella enterica* (S. Heidleberg, S. Typhimurium, S. Gaminara, S. enteriditis, and S. Oranienburg) were grown in TSBYE and streaked for isolation on TSAYE and Xylose Lactose Tergitol 4 agar (XLT-4). The same growth and centrifugation procedure was used on the *Listeria* and *Salmonella* cocktails as the *E. coli* cocktail.

**Preparation of samples and high pressure processing treatment**

The whey protein beverage was combined with the five-strain cocktail (1:100 dilution of culture) for a final concentration of cells of approximately $10^6$ to $10^7$ CFU/mL. The beverage was divided into 10 mL aliquots and heat sealed into 2.5 millimeter thick Kapak bags (KAPAK Corporation, Minneapolis, MN, USA) with minimum air headspace. In case of leakage, samples were packaged and sealed in 4 layers of Kapak bags; the innermost bag contained the beverage, a second bag, a bag containing 20 mL of a 1% bleach solution and a final bag. Samples were packaged in duplicate. Samples were divided into pressure treatments, 300, 400, 500 MPa for *E. coli*, 350 for *Salmonella spp.* and 250, 300, 350 MPa for *L. monocytogenes*, and dwell time treatments of 2, 4, 6, and 8 min at an initial temperature of 20°C. These times do not include come-up and pressurization release times. Pressurization was performed with a FOOD-LAB 900 High-Pressure Food Processor (Stansted Fluid Power Ltd., Essex,
Pressure transmission fluid was a 1:1 mixture of DI water and 1,2 propanediol 95-100% by volume (Global Water Technology Koilguard, Oakbrook Terrace, IL, U.S.A).

Microbiological analyses

Sample packs were opened with sterile scissors. Aliquots of each sample were serially diluted (10 fold) in buffered peptone water (Becton Dickson, U.S.A.) immediately after HPP treatment. Samples and original culture cocktails were spread plated in duplicate on non-selective media (TSAYE) and selective media (SMAC for *E. coli*, MOX for *L. monocytogenes* and XLT-4 for *Salmonella* spp). A 1 mL aliquot was spread plated on 4 plates when the counts fell below the detection limit (10 CFU/mL). Plates were incubated at 35°C for 48 hours. Percent injury was calculated using the following equation:

\[
\text{Percent injury} = \left( \frac{(\text{CFU/mL on TSAYE} - \text{CFU/mL on selective media})}{(\text{CFU/mL on TSAYE})} \right) \times 100
\]

Storage study

Five strains of *E. coli* O157:H7 cultures were prepared as before. The whey beverage was divided into 5 groups following pH adjustment: 50 ppm nisin, 50 ppm nisin and 0.04% (w/v) potassium sorbate, 0.04% (w/v) potassium sorbate and a control (without nisin and/or potassium sorbate), and a control without pressure treatment. Nisin (0.1 g) was diluted in 1 mL DI water and added to the beverage for a final concentration of 50 ppm. The pH was retested after nisin addition, however a change in pH was not observed. Samples were packaged as before and pressurized at 400 MPa for 4 min at 25°C. Stored samples were placed in a 25°C incubator and sampled on days 0 (immediately following pressurization), 1, 3, and 5. Two separately packaged samples
per treatment were tested each day and two reps were performed. Following serial dilution and plating, 5 mL of the remaining sample was added to 5 mL double strength TSBYE and incubated for 48 hours at 35°C for enrichment. A sterile loop was used to streak enrichment on TSAYE and SMAC. Plates were then observed for presence/absence of *E. coli* O157:H7.

**Statistical analysis**

The experiment was run in two replications. Each treatment combination was tested in duplicate in each replication. Samples were randomly assigned to treatments. Statistical analysis was performed using analysis of covariance (ANCOVA) using R version 2.14.2. For counts equaling 100% inactivation (=0), an arbitrary addition of “1” was made in order to perform log transformations. Statistical differences between treatments were calculated using Student’s t test with Bonferroni correction. Statistical significance was determined at $P < 0.05$.

**Results**

The average beverage pH was 3.80 (SD 0.02). Populations of aerobic mesophilic background microflora were <1 CFU/mL (data not shown).

**HPP in conjunction with a low pH whey beverage: *Salmonella* spp.**

Figure 1 shows the survival of the 5 strain cocktail of *Salmonella* spp. on TSAYE following pressurization at 350 MPa. The initial average inoculation level was 6.73 $\log_{10}$ CFU/mL. A 3.62 (± 0.93 SD) $\log_{10}$ CFU/mL reduction was observed at 350 MPa, 2 min dwell time. This reduction was greater than the 1.97 reduction in *Salmonella* Agona observed in orange juice (pH 3.9) pressure treated at 400 MPa for 2 min at 6°C (Whitney et al. 2008). However, this could be due to multiple factors such as variations in strain
pressure resistance, pressurization parameters and differences in juice and WP beverage properties. By 4 minutes dwell time a 5.94 (± 0.62 SD) 1og10 CFU/ml reduction had been achieved. Counts at dwell times of 4, 6, and 8 min were not significantly different, but complete inactivation on non-selective media was not observed. At 8 min holding time, *Salmonella* was reduced to 0.32 log10 CFU/mL indicative of tailing. Tailing has been observed in various microorganisms during HPP of foods (Guan et al. 2005; De Lamo-Castellvi et al. 2005). This phenomenon is thought to exist due to variation in pressure resistance among subpopulations. Injury can be categorized as primary (I1) where cells can form visible colonies on non-selective agar, but not selective agar and secondary (I2) where cells cannot form visible colonies on either type of agar (Bozoglu et al. 2004). Percent injury of *Salmonella* was 100.00% in pressure treated samples (dwell times of 2 to 8 min) demonstrating this organisms’ inability to recover on XLT-4, indicating primary injury. In some cases, these pressure injured cells may recover following storage. The extent of this recovery is dependent on the type of food, processing conditions, and storage conditions (Bull et al. 2005; Bozoglu et al. 2004). Recovery microorganisms can be inhibited at low pH. Yuste and Fung (2004) observed a 4 log10 reduction of *Salmonella Typhimurium* following storage at 20°C in apple juice, pH 3.71. It is possible that the low pH of the whey beverage could inhibit recovery of pressure injured *Salmonella*.

**HPP in conjunction with a low pH whey beverage: *E. coli* O157:H7.**

As the pressure level increased, the viability of the 5 strain cocktail of *E. coli* O157:H7 decreased on non-selective media (Figure 2). The initial average inoculation level was 7.20 1og10 CFU/mL. At 300 MPa, dwell times 2, 4, 6, and 8 were not significantly different. The log10 reduction at 300 MPa by 8 min was 1.77 (± 0.1 SD).
Pressurization at 400 MPa for 2 min resulted in an initial drop of \( \sim 2 \log_{10} \text{CFU} \). At the same pressure, dwell times of 4, 6 and 8 min were not significantly different with counts ranging from \( 4.49-3.70 \log_{10} \text{CFU/mL} \). At 500 MPa with a 6 min dwell time a \( 5.97 (\pm 1.39 \text{ SD}) \log_{10} \text{CFU/ml} \) reduction had been achieved. Counts at 500 MPa and dwell times of 2, 4, 6, and 8 min were not significantly different, but all were significantly different from the initial count. Pressurization at 500 MPa for 4 min or longer was required for \( >5 \log_{10} \) reduction. Injury (I1) of \textit{E. coli} O157:H7 on SMAC and pressurized at 300 MPa was 99.99% at 2 min dwell and 100% at 4 to 8 minutes. All treatments at 400 and 500 MPa demonstrated 100.00% injury. Of the strains tested in this study, the \textit{E. coli} O157:H7 strains were the most barotolerant in the low pH whey beverage.

**HPP in conjunction with a low pH whey beverage: \textit{L. monocytogenes}**

Survival of the 5 strain cocktail of \textit{L. monocytogenes} on TSAYE following pressurization at 200, 250, and 300 MPa was investigated (Figure 3). At 200 MPa, significant difference in counts in relation to the initial count, \( 7.44 \log_{10} \text{CFU/mL} \), did not occur until the 6 min dwell time treatment with a log reduction of \( 1.27 (\pm 0.42 \text{ SD}) \log_{10} \text{CFU/ml} \). A reduction of \( 3.45-4.92 \log_{10} \text{CFU/mL} \) at 250 MPa for 4 and 6 min dwell time was observed. These results are similar to the findings of Alpas and Bozoglu (2003) with \textit{L. monocytogenes} CA viability loss of \( 3.90-4.34 \log_{10} \) in apple (pH 3.5), apricot (pH 3.8), orange (3.76), and cherry juice (pH 3.30) at 250 MPa, 30°C, for 5 minutes. At 300 MPa, 6 min dwell time, 100% log reduction and injury was observed (Table 2). Percent injury of \textit{L. monocytogenes} ranged from 13.97 to 88.17% at 200 MPa and 42.66 to 93.17% at 250 MPa. Colony growth on selective media could result from two populations: pressure injured cells that were able to recover or uninjured cells. Lower
levels of injury observed could have resulted from lower pressure levels used in comparison to *Salmonella* and *E. coli* O157:H7, differences in sensitivity to selective media, or differences in antimicrobial potency against pressure injured cells.

**D-values of *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* spp.**

The log$_{10}$ transformed survival counts of each organism were plotted against holding time at each pressure tested. A best fit line was applied to the counts and D-values were calculated from the slope. D-values within organism groups were significantly different (not calculated for *Salmonella*). *E. coli* O157:H7 was the most pressure resistant cocktail of the organisms used followed by *Salmonella* and *L. monocytogenes*. D-values of *E. coli* O157:H7 were (min) were 5.24 (300 MPa), 2.64 (400 MPa), and 1.80 (500 MPa). In general gram positive organisms are more resistant to HPP than gram negative organisms (Smelt 1998). Though the *E. coli* O157:H7 cocktail proved to be the most pressure resistant cocktail, surpassing *L. monocytogenes* in survival, Alpas et al. (1999) reported overlapping levels of destruction between strains of *E. coli* O157:H7 and *L. monocytogenes*. *E. coli* was observed to be more pressure resistant than *L. monocytogenes* when pressurized in a buffer at low pH (4.0) (Somolinos et al. 2008).

Overall, all three pathogens showed decreased viability (CFU/mL) with increased pressure. This is illustrated more clearly in the significant differences between D-values at varying pressures. *E. coli* O157:H7 was selected as the target organism for the 5 day study, because it proved to be the most resistant to high pressure.

**5 day storage study**
The WP beverage was inoculated with an average of 6.79 log_{10} CFU/mL. Log transformed viable cell counts were graphed against sampling day following pressurization (Table 4). All pressure treated samples were significantly different from the initial counts. Pressure treated control and pressure treated potassium sorbate samples did not contain significantly different levels of *E. coli* O157:H7 suggesting that potassium sorbate did not significantly enhance microbial destruction in combination with high pressure. Non-pressurized controls were not significantly different and the log reduction of these treatments ranged from 0.16-0.74 log_{10} CFU/mL on day 0 to day 5. The low pH of the beverage did not inhibit pathogen persistence following 5 days of storage at 25°C. Persistence and outbreaks of *E. coli* O157:H7 in apple cider (pH 3.5) is well documented (Parish 1997; Yuste and Fung 2004). Yuste and Fung (2004) observed *E. coli* O157:H7 persistence in apple cider after 14 days of storage at 25°C. Nisin and nisin in combination with potassium sorbate showed significantly greater log reduction over pressurized control and potassium sorbate treatments. Both treatments that contained nisin on day 0 displayed enhanced reductions at 4.28 (nisin) and 4.89 (nisin and potassium sorbate) log_{10} CFU/mL respectively, though these treatments were not significantly different from each other. Nisin and HPP have been used to synergistically inactivate microorganisms (García-Graells et al. 1999; Masschalck et al. 2000). Previous work has shown that HPP treatments and low pH enhances the efficacy of nisin (Linton 1999). Nisin without a chelating agent is considered ineffective against gram negative organisms because it cannot bind to the surface of these organisms. However, HPP treatment causes outer membrane permeability and leakage of intracellular enzymes, leading to sensitization to nisin (Hauben et al. 1996). On day 1 through day 5 of storage,
all pressure treated samples were not significantly different from each other. Nisin, nisin and potassium sorbate, and potassium sorbate treatments reached 100% log reduction by day 1. *E. coli* O157:H7 was not detected in these treatments by enrichment. *E. coli* O157:H7 persisted in pressure treated controls through day 5 and were detected by plating and enrichment, though counts were below 10 CFU/mL by day 3. Pressure treated samples exhibited 100% injury on SMAC indicating sublethal injury on day 0. Following storage, *E. coli* O157:H7 was unable to repair in this beverage.

**Conclusions**

In conclusion, the findings of this study support those of Alpas et al. (1999) that microorganism sensitivity to HPP varies greatly between species. As pressure increased, microbial inactivation increased except in cases where tailing occurred. D-values were dependent on microorganism and pressure. Of the three pathogenic culture cocktails used in this study, *E. coli* O157:H7 was the most resistant to pressure. Nisin treatments were the most effective at reducing *E. coli* O157:H7 on day 0, but all pressurized treatments containing nisin and/or potassium sorbate were undetectable by plate counts or enrichment. *E. coli* O157:H7 persisted in control treatments (pressure and non-pressure treated) after 5 days of storage. This suggests that another hurdle technology in conjunction with HPP at parameters used in this study is required to completely inactivate 6.79 log\(_{10}\) CFU/mL *E. coli* O157:H7. However, the D-values and log reductions observed are unique to the resistances of the five strains used per pathogen. Overall, our research suggests that HPP can be used effectively to reduce *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella* in a low pH whey beverage. Further research to optimize nisin level and pressure level still needs to be performed.
Acknowledgements

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References


http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm


TABLES AND FIGURES

Table 1. Whey beverage ingredients listed as a percent of w/v

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>88.51</td>
</tr>
<tr>
<td>Fructose</td>
<td>5.60</td>
</tr>
<tr>
<td>whey protein isolate</td>
<td>4.59</td>
</tr>
<tr>
<td>apple juice concentrate</td>
<td>0.80</td>
</tr>
<tr>
<td>potassium sorbate</td>
<td>0.04</td>
</tr>
<tr>
<td>25% sucralose solution</td>
<td>0.02</td>
</tr>
<tr>
<td>85% phosphoric acid</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Figure 1. Average survival of *Salmonella* spp. subjected to HPP at 350 MPa and plated on TSAYE (●). Error bars represent standard deviation.
Figure 2. Average survival of *E. coli* O157:H7 subjected to HPP at 300 (◆), 400 (■), and 500 (▲) MPa and plated on TSAYE. Error bars represent standard deviation.
Figure 3. Average survival of *L. monocytogenes* subjected to HPP at 200 (♦), 250 (■), and 300 (▲) MPa and plated on TSAYE. Error bars represent standard deviation.
Table 2. Average percent injury of *L. monocytogenes*.

<table>
<thead>
<tr>
<th>Pressure (Mpa)</th>
<th>Dwell Time (min)</th>
<th>Percent Injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>2</td>
<td>13.97%</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>23.11%</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>62.90%</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>88.17%</td>
</tr>
<tr>
<td>250</td>
<td>2</td>
<td>42.66%</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>97.26%</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>98.17%</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>93.48%</td>
</tr>
<tr>
<td>300</td>
<td>2</td>
<td>93.71%</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>90.00%</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>100.00%</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>100.00%</td>
</tr>
</tbody>
</table>
Table 3. D-Values (min) of *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* after exposure to pressure.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Pressure</th>
<th>D Value (min)</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli O157:H7</em></td>
<td>300</td>
<td>5.24&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>2.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>1.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.13</td>
</tr>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>350</td>
<td>1.49</td>
<td>0.11</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>200</td>
<td>4.86&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>1.20&lt;sup&gt;y&lt;/sup&gt;</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0.64&lt;sup&gt;x&lt;/sup&gt;</td>
<td>0.17</td>
</tr>
</tbody>
</table>

D-Values with different letters were significantly different within organism groups (p<0.05).
Figure 4. Viable counts of *E. coli* O157:H7 in a whey protein beverage following HPP treatment (400 MPa, 4 min, 20°C) and storage at 25°C. Significant differences (*P*<0.05) between counts within days are denoted by letters a, b and c, significant differences within treatments are denoted by letters x-z.
CHAPTER 4. GENERAL CONCLUSIONS

The overall goal of this study was to produce a safe shelf stable whey beverage that could be stored at room temperature. These goals were accomplished by determining sensitivities of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* to HPP in an acidified whey beverage. Though previous research has shown enhanced reduction of pathogens in acidic drinks, such as fruit juices, following HPP treatment each food system being tested is unique in its effect on cell viability. Several researchers have noted protective effects of complex food matrices such as milk and meat, that cause reduced log reduction when compared with laboratory buffers or broth. Therefore, it was necessary to determine the optimum pressure ranges to inactivate each organism, to enhance efficacy while reducing cost. The *E. coli* O157:H7 proved to be the most pressure resistant of the pathogens tested in this study. Complete inactivation was observed in HPP treated samples that contained nisin and/or potassium sorbate. These antimicrobials worked in conjunction with HPP to reduce *E. coli* O157:H7 population levels completely. This research also established HPP treatment (400 MPa, 4 min, 25°C) alone was not enough to reduce *E. coli* O157:H7 beyond detection.

Future research to optimize HPP treatment in conjunction with nisin still needs to be performed. Lower levels of pressure caused 100% damage on SMAC. Therefore, it is possible that lower pressures could effectively sensitize *E. coli* O157:H7 to nisin. Nisin activity increases as pH decreases, so less is required in high acid foods to reduce spoilage and other microorganisms. The effect of HPP on the turbidity of the whey beverage in comparison to thermal treatments needs to be investigated. Other ideas for
research include use of pressure resistant and acid tolerant cultures which may be less
affected by HPP and nisin treatments.
ACKNOWLEDGEMENTS

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