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Tara K. Stiles
tstiles11@gmail.com

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THE EFFECTS OF HIGH PRESSURE PROCESSING ON PEANUT SAUCE

INOCULATED WITH *SALMONELLA*

By

Tara Kristin Stiles

A THESIS

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THE EFFECTS OF HIGH PRESSURE PROCESSING ON PEANUT SAUCE INOCULATED WITH *SALMONELLA*

Tara Kristin Stiles, M.S.

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Advisor: Jayne Stratton

Recent *Salmonella* outbreaks have prompted the need for new processing options for peanut products. Traditional heating kill-steps have shown to be ineffective in lipid-rich matrices such as peanut products. High pressure processing is one such option for peanut sauce because it has a high water activity, which has proved to be a large contributing factor in microbial lethality due to high pressure processing. Four different formulations of peanut sauce were inoculated with a five strain *Salmonella* cocktail and high pressure processed. Results indicate that increasing pressure or increasing hold time increases $\log_{10}$ reductions. The Weibull model was fitted to each kill curve, with b and n values significantly optimized for each curve (p-value < 0.05). Most curves had an n parameter value less than 1, indicating that the population had a dramatic initial reduction, but tailed off as time increased, leaving a small resistant population. ANOVA analysis of the b and n parameters show that there are more significant differences between b parameters than n parameters, meaning that most treatments showed similar tailing effect, but differed on the shape of the curve. Comparisons between peanut sauce formulations at the same pressure treatments indicate that increasing amount of organic peanut butter within the sauce formulation decreases $\log_{10}$ reductions. This could be due to a protective effect from the lipids in the peanut butter, or it may be due to other factors such as nutrient availability or water activity. Sauces pressurized at lower temperatures had decreased $\log_{10}$ reductions, indicating that cooler temperatures offered some protective effect. $\log_{10}$ reductions exceeded 5 logs, indicating that high pressure processing may be a suitable option as a kill-step for *Salmonella* in industrial processing of peanut sauces. Future research should include high pressure processing on other peanut products with high water activities such as sauces and syrups as well as research to determine the effects of water activity and lipid composition with a food matrix such as peanut sauces.

Dedication

I would like to dedicate this thesis to my parents, John and Patti Stiles. They are my inspiration, my mentors, and my best friends.

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Chapter 1: Literature Review

Introduction

Recent peanut butter and peanut paste product outbreaks involving *Salmonella* have caused major recalls and over a thousand illnesses across the United States (CDC, 2007; CDC, 2009). The demographic of the affected were mostly children or elderly persons because of the vast quantities of peanut products served at schools and nursing homes (Morrone, 2008). These outbreaks have led to studies on the survivability of *Salmonella* in peanut butter and other high fat, low water activity foods and the results indicate that the processing parameters currently used for peanut butter production are not sufficient. *Samonella* Tennessee, the serotype responsible for the 2007 peanut butter outbreak, was found to have a D-value of 120 minutes at 90°C, a condition that is not met with current production practices (Ma et al., 2009). Other studies have found that *Salmonella* is more potent in high fat, low water activity foods due to filamentation (Mattick et al., 2000b). All these findings suggest that a new processing step is needed to ensure that future outbreaks do not occur

High pressure processing (HPP) is an alternative processing method to heating in that it is currently being used on products that cannot withstand heating but require a microbial reduction processing step. Such products include guacamole, oysters, and fruit juices (Alpas et al., 2000; Bayindirli, et al, 2006; Caner et al., 2004; Casadei et al., 2002; Considine et al., 2008; Cruz-Romero, Kerry, Kelly, 2008; Jimenez Colmenero, 2002; Matser, et al., 2004; Palou et al., 2000; Rastogi et al., 2007). Products that are processed using HPP also retain more nutrients and flavors than heated products, which is favorable to the consumer (Considine et al., 2008; Metrick et al., 1989).

The use of high-throughput sequencing methods to obtain information on microbial ecologies present in food matrices has been limited and lacking. Pyrosequencing various peanut butter products available to consumers will provide snapshots of the natural microbial ecology. Information on the natural flora can then be used to determine if the natural flora interacts with pathogens such as *Salmonella* in the instance of contamination or if there are inherent differences between types of peanut butter.

Thus, this literature review covers information on high pressure processing, the outbreaks in peanut products associated with *Salmonella*, the research being done on peanut products, and how pyrosequencing is being used in the food industry.

I. High Pressure Processing

A. High Pressure Technology

High pressure processing is a technology that has until recently been used for non-food related purposes. Applications include crystal and diamond production, ceramic production, and stability testing for weapons manufacturing (Barbosa-Canovas et al., 1998; Doona & Feeherry, 2007; Farr, 1990; San Martin, 2002; Rastogi et al., 2007). Research for the application of high pressure processing on foods began with Hite, who applied pressure to milk, concluding that it kept its sweetness longer (Considine et al., 2008; Earnshaw et al., 1995; Metrick et al., 1989; Rastogi et al., 2007). The interest in high pressure processing on foods was spotty until the 1980's when renewed interest was sparked due to the processing of acidic foods and jams in Japan (Considine et al., 2008; Farr, 1990).

A high pressure system consists of a pressure vessel, a liquid medium that is compressed, hydraulic compressors, and a heating/cooling unit (See Appendix A: High Pressure Processing Machinery Set-Up) (Patterson, 2005). There are three ways that pressure can be generated: direct compression, indirect compression, and heating of the liquid medium. Direct compression machines are set up such that the hydraulic piston is applying pressure directly on the vessel and its contents. This allows for fast compression, but the size restricts its use to a laboratory or pilot plant (Barbosa-Canovas et al., 1998; Mertens, & Deplace, 1993). Indirect compression machines are set up such that the hydraulic pistons are held in a side compartment (Mertens, & Deplace, 1993). The pistons pressurize the fluid, typically water, in a reservoir attached to the vessel (Caner et al., 2004; Le-Bail et al., 2006; San Martin et al., 2002; Westerlund, 1994). This allows for industrial sized applications and is the type of machine used for this project. The third method, heating of the liquid medium, relies on the expansion of the medium as it is

heated to produce the pressure and must be carefully controlled to achieve the correct pressure desired and can only be used for heat applications (Barbosa-Canovas et al., 1998; Mertens, & Deplace, 1993).

The temperature at which the medium and sample are pressurized and cycle duration can be controlled and modified according to product specifications. Temperatures can range from -20°C to upwards of 100°C and pressures can reach 900MPa (See Table 1. Pressure Conversions). Pressures can be held for as little as twenty seconds or it can be held for weeks, but wear on the machine must be considered (Donsi, et al., 2007). The ramp up rate and ramp down rate refer to the time it takes to achieve the desired pressure and how fast the pressure is released (Donsi, et al., 2007). The holding time refers to the time the desired pressure is held, and does not include the ramp up and ramp down times (Donsi, et al., 2007). The pressure cycles can be repeated multiple times, or pulsed for short periods of time, which is used for the destruction of pressure resistant structures such as spore or prions (Donsi, et al., 2007). When the chamber is pressurized, a phenomena known as adiabatic heating occurs, in which the change in pressures causes an increase in temperature, typically three to nine degrees Celcius per one-hundred megapascals (Caner et al., 2004; Considine et al., 2008; Doona and Feeherry, 2007; Patterson, 2005; Rastogi et al., 2007; Ting, Balasubramaniam, & Raghubeer, 2002). The exact temperature increase induced by adiabatic heating can be roughly estimated using the thermophysical properties of food and resulting heat capacity (Matser, et al., 2004; Otero and Sanz, 2003). The temperature of the vessel can be cooled to offset the adiabatic heating if needed.

Table 1.1: Pressure Conversions			
Atms	MPas	Bar	PSI
1	0.101	1.01	14.7
9.87	1	10	0.0069
1000	101	1013	14696
3948	400	4000	58015
4935	500	5000	72519
5922	600	6000	87023
6415	650	6500	94275
6908	700	7000	101526
7402	750	7500	108778
7895	800	8000	116030
8882	900	9000	130534

Products can be processed in a batched manner or a continuous process. Continuous processing is used for liquids such as juices and is referred to as a dry bag process (Mertens & Deplace, 1993; Mertens, 1994). In this process, the juice is the compression liquid and there are no packaging barriers between the product and the machine (Caner et al., 2004; Mertens & Deplace, 1993; Mertens, 1994). All surfaces that come in contact with the product must be able to be cleaned and sterilized as necessary, which may or may not pose problems (Mertens, 1994). In contrast, batched systems are used for bulk products and are processed in cycles (Mertens & Deplace, 1993; Mertens, 1994). This method, referred to as a wet bag process, is preferred for food applications because it is considered more sanitary since the products are sealed prior to processing and are not opened during processing (Caner et al., 2004; Mertens & Deplace, 1993; Zimmerman & Bergman, 1993). The products are packaged within flexible materials and sealed; materials such as rigid metal cans or glass containers are not appropriate for high pressure processing because the materials can break or deform under the high pressures (Caner et al., 2004; Le-Bail et al., 2006; Westerlund, 1994). A typical batch cycle begins by loading the product or sample into a basket, lowering the basket into the pressure chamber, and sealing the pressure chamber (Mertens & Deplace, 1993). As the pressure cycle begins, pressure is increased to the desired pressure (ramp up rate), holding the pressure at the desired time (hold time), and releasing the pressure (ramp down rate) (Mertens & Deplace, 1993). Once the cycle is complete and all pressure has been released, the product can be retrieved from the basket and the next sample is loaded (Barbosa-Canovas et al., 1998). The high pressure unit can be horizontally or vertically orientated, depending on what is desired (Mertens & Deplace, 1993; Mertens, 1994). The vertical orientation requires that the basket be lowered into the chamber, using a pulley or a crane system, whereas the horizontal orientation allows the baskets to be slid into place using a platform or rail system (Mertens & Deplace, 1993; Mertens, 1994).

The pressure applied during high pressure processing is isostatic, meaning that the entire product has a uniform and instantaneous amount of pressure applied, whereas in temperature processing, there is a gradient of temperature through the product (Alpas, et al., 2000; Caner et al., 2004; Considine et al., 2008; Doona & Feeherry, 2007; Farr, 1990; Patterson, 2005; Rastogi et al., 2007; San Martin et al., 2002; Ting et al., 2002; Westerlund, 1994). This is a major advantage to other processing methods such as thermal

treatments because heat treatments are not necessary (depending on the food product) which allows producers to make a food product that retains more nutrients and natural colors, tastes fresh, does not require additives and preservatives, has a long shelf life, and is microbiologically safe (Caner et al., 2004; Considine et al., 2008; Donsi, et al., 2007; Doona & Feeherry, 2007; Mackey et al., 1994; Mertens, 1994; Metrick et al., 1989; Rastogi et al., 2007; Swientek, 1992; Ting et al., 2002; Westerlund, 1994).

B. The Effect of High Pressure on Biological Systems

The effect of high pressure on microorganisms has been investigated from a molecular level to a cellular level. The membrane has been found to be the primary site of high pressure damage (Abee & Wouters, 1999; Bowman et al., 2008; Casadei et al., 2002; Earnshaw et al., 1995; Farr, 1990; Hartmann et al., 2006; Hauben et al., 1997; Kalchayanand, Sikes, Dunne, & Ray, 1998; Metrick et al., 1989; Smelt et al., 1994). Membranes are composed of a lipid bilayer and it has been shown that this lipid bilayer thickens as pressure increases (Casadei et al., 2002; Farr, 1990; Kalchayanand et al., 1998). The lipid bilayer goes through lipid to gel and gel to interdigitated gel transitions as pressure increases under steady temperature (Alpas et al., 1999; Alpas et al., 2000; Hartmann et al., 2006; Pilavtepe-Celik, et al., 2008; Winter & Dzwolak, 2005). The liquid to gel transition increases the membrane volume, violating Le Chatelier's principle which states that under increasing pressures, reactions will favor a reduction in volume (Caner et al., 2004; Earnshaw et al., 1995; Farr, 1990; Kitching, 1957; Rastogi et al., 2007; Winter & Dzwolak, 2005; Zhang & Mittal, 2008). The second transition from a gel to an interdigitated gel occurs at pressures above 200 Mega Pascals and results in a five-percent decrease in volume, conforming to Le Chatlier's principle (Pilavtepe-Celik et al., 2008; Winter & Dzwolak, 2005). The literature conflicts over whether an increase in pressure results in an increase or decrease in fluidity of the membrane, but it may be because of the various stages of transformation the membrane undergoes and the pressures at which the studies focus on. Despite this incongruency, it is generally accepted that pressure causes the membrane to leak, allowing intracellular and extracellular components to exchange without regulation (Considine et al., 2008; Earnshaw et al., 1995; Farr, 1990; Matser, Krebbers, van den Berg, & Bartels, 2004; Patterson, 2005; San Martin et al.,

2002; Smelt, 1998). The membrane protein ATPase is denatured, destroying the proton motive force, and making a low pH environment more harmful because the cell cannot regulate the gradient of protons across the membrane (Casadei et al., 2002; Earnshaw et al., 1995; Farr, 1990; Hoover, et al., 1989; Smelt et al., 1994; Smelt, 1998; Tholozan, et al., 2000).

The type of outer membrane also affects the ability to survive under high pressures in that gram positive bacteria are generally more resistant to high pressures than gram negative bacteria (Alpas et al., 1999; Alpas, et al., 2000; Barbosa-Canovas et al., 1998; Bayindirli et al., 2006; Considine et al., 2008; Hartman, et al., 2006; Patterson, 2005; Prestamo et al., 1999; Smelt, 1998; Wuytack, et al., 2002). This is due to the complexity and abundance of protein, phospholipids, and lipopolysaccharide in the gram negative outer wall (Patterson, 2005; Pilavtepe-Celik et al., 2008; Ritz, et al., 2000). Scanning electron microscope images have revealed that after recovery from exposure to high pressure, the outer membrane has scars from where damage occurred (Hartmann et al., 2006; Mackey et al., 1994; Ritz et al., 2002; Ritz et al., 2000).

Protein denaturation under high pressure is influenced by electrostatic interactions, hydrogen bonding, hydrophobic interactions, and the volume occupied in solution. Electrostatic interactions are dissociated by an increase in pressure so that water may access more ions, which decreases overall volume and also has the effect of lowering the pH (Earnshaw et al., 1995; Hoover et al., 1989; Rastogi et al., 2007). Hydrogen bond formation is favored by high pressures because it decreases volume, which stabilizes α-helices and β-sheets in proteins at lower pressures (<1000 atm. or <101.3MPa) and reduces denaturation (Hoover et al., 1989; Patterson, 2005; Winter & Dzwolak, 2005). Hydrophobic reactions increase in volume under pressures of 1000 atmospheres, but reduce in volume at pressures over 1000 atmospheres because the intense pressure stabilizes the unfolded state (Hoover et al., 1989). It has been demonstrated that unfolded proteins expose more nonpolar residues and amino acids to water, which allows water to bind to previously unavailable sites, decreasing the overall volume (Hoover et al., 1989; Kornblatt & Kornblatt, 2002). Proteins can be divided into three classes of denaturation: compact intermediate states (molten globules), partially unfolded states, and fully unfolded states (Hoover et al., 1989). Protein denaturation in

the compact intermediate state has a large volume than the folded or unfolded states, similar to the volume transitions of the lipid membrane (Pilavtepe-Celik et al., 2008). Depending on the severity of unfolding, protein denaturation can be reversible such that function is fully restored (Hoover et al., 1989). Covalent bonds are not broken by high pressure, meaning that only the primary structure of proteins is unaffected by high pressure processing (Considine et al., 2008; Farr, 1990; Rastogi et al., 2007; Winter & Dzwolak, 2005). This is relevant for the preservation of flavors, vitamins, and color compounds in food matrices (Considine et al., 2008; Kalchayanand, et al., 1998; Patterson, 2005; Rastogi et al., 2007; San Martin et al., 2002; Trujillo, et al., 2002).

Nucleic acids are more resistant to high pressures than proteins due to hydrogen bonding. The effects of pressure on hydrogen bonding help stabilize DNA under high pressure, which in turn raises the melting point of DNA and protects the DNA from temperature-related degradation (Barbosa-Canovas et al., 1998; Heden, 1964; Winter & Dzwolak, 2005). The increased pressure also causes the structure of DNA to contract, reducing the overall volume, and thus making it favorable due to Le Chatelier's principle (Hoover et al., 1989; Smelt, 1998; Winter & Dzwolak, 2005). Ribosomes dissociate at high pressures; it is the 30S subunit that determines the pressure at which dissociation occurs (Abee & Wouters, 1999; Casadei et al., 2002; Considine et al., 2008; Earnshaw et al., 1995; Hoover et al., 1989; Mackey et al., 1994; Metrick et al., 1989). Cellular processes critical to growth and proliferation are hindered by high pressures, especially the production of protein from mRNA (Abee & Wouters, 1999; Earnshaw et al., 1995; Hoover et al., 1989). High pressures induce SOS temperature response systems as well as oxidative stress systems (Abee & Wouters, 1999; Considine et al., 2008; Earnshaw et al., 1995). Plasmids are transferred at a higher rate when the bacteria are under stresses including pressure, low pH, high salt, or high temperature, which has implications for antibiotic resistance (McMahon, et al., 2007). The effects are seen in the recipient cell being more fertile rather than the donor cell (McMahon et al., 2007). Metabolic processes such as fermentation in yeasts and bioluminescence in bacteria are known to be depressed under pressures of 100-1000 atmospheres (10.1-101.3 MPa) and virulence genes have been shown to be depressed (Bowman, Bittencourt, & Ross, 2008; Kitching, 1957). There is more research needed to fully understand the genetic response to pressure-related stress.

Other characteristics that affect survivability include the species, the shape of the microorganism, the growth phase, the temperature the culture was grown, compounds in the growth media, the pressure applied, and the length of time the pressure is applied (Abee & Wouter, 1999; Alpas et al., 1999; Alpas et al., 2000; Considine et al., 2008; Doona & Feeherry, 2007; Earnshaw et al., 1995; Goodridge et al., 2006; Heden, 1964; Metrick et al., 1989; Patterson, 2005; San Martin et al., 2002; Westerlund, 1994). Microorganisms fall within a wide range of pressure resistance, from being extremely resistant under high pressures to relatively susceptible to high pressure. Microorganisms that are resistant include prions, bacterial spores, some mold ascospore, and some viruses (Alpas et al., 1999; Alpas et al., 2000; Considine et al., 2008; Doona & Feeherry, 2007; Earnshaw et al., 1995; Metrick et al., 1989; Patterson, 2005; Rastogi et al., 2007; Winter & Dzwolak, 2005). The pressures required for these microorganisms exceed 1000 MegaPascals and the temperatures required can exceed 80°C (Abee & Wouters, 1999; Considine et al., 2008; Rastogi et al., 2007). Yeasts, molds, and vegetative bacteria are considered to be susceptible in that they can be killed or lethally injured by lower pressures (300-800 MPa) and temperatures at or below room temperature (Abee & Wouters, 1999; Alpas et al., 1999; Alpas et al., 2000; Considine et al., 2008; Doona & Feeherry, 2007; Goodridge et al., 2006; Mackey et al., 1994; Matser et al., 2004; Metrick et al., 1989; Rastogi et al., 2007; Smelt, 1998). There are species that are barotolerant, and it has been proposed that there is a link between heat tolerance and barotolerance, but there are exceptions (Abee & Wouters, 1999; Earnshaw et al., 1995; Smelt, 1998; Winter & Dzwolak, 2005). Membrane fluidity used to survive high temperatures is also important to survive high pressures, but there are other factors that come into play (Casadei et al., 2002; Smelt et al., 1994). Cultures grown at ambient temperatures prior to being exposed to high pressures tend to survive more so than cultures grown in colder or sub-optimal temperatures (Abee & Wouters, 1999; Barbosa-Canovas et al., 1998; Casadei et al., 2002; Hoover et al., 1989). The shape of bacteria greatly affects survivability; cocci shaped bacteria survive better than rod or spirochete shaped bacteria (Considine et al., 2008; Hartmann et al., 2006; Patterson, 2005; San Martin et al., 2002). Rod shaped bacteria such are *E. coli* elongate under pressure, extending from 1-2μm to 10-100μm (Barbosa-Canovas et al., 1998). Bacteria in the lag or stationary phase of growth survive better than bacteria in the log phase of growth, perhaps because the cellular membrane is more robust and stress induced genes can be

turned on more readily in the stationary phase cells (Alpas et al., 1999; Bayindirli et al., 2006; Bowman et al., 2008; Considine et al., 2008; Doona & Feeherry, 2007; Patterson, 2005; San Martin et al., 2002; Smelt, 1998).

Compounds in the growth medium, or the food matrix, can protect or injure bacteria during and after pressurization. Bacteria are more sensitive to lower pH after pressurization, in part because high pressure causes the environment and the cytoplasm to become more acidic and also because the cellular defense against acidic conditions, the membrane, is the first site of damage from high pressure (Alpas et al., 1999; Considine et al., 2008; Doona & Feeherry, 2007; Patterson, 2005; Smelt, 1998). Cells are also more sensitive to salt post pressurization due to membrane damage (Hoover et al., 1989). Bacteria can be protected by a wide range of compounds, including carbohydrates, lipids, proteins, and cations (Bowman et al., 2008; Koseki & Yamamoto, 2007; Patterson, 2005; San Martin et al., 2002). Glycerol, which is known to protect bacteria against extreme temperatures, also protects bacteria from the effects of high pressures by stabilizing cellular membrane functionality (Considine et al., 2008; Corry, 1974; Patterson, 2005). Cations such as calcium and magnesium work to protect and stabilize ribosomes, allowing cellular functions to continue or recover quicker (Buzrul et al., 2008; Considine et al., 2008; Doona & Feeherry, 2007; Hauben et al., 1997; Koseki & Yamamoto, 2007; Patterson, 2005; San Martin et al., 2002). Rich mediums with rapidly available sources of nutrients protect bacteria by providing the energy sources needed to recover (Considine et al., 2008; Hoover et al., 1989). Low water activity matrices protect microorganisms during pressurization, but injured cells are more sensitive to low water activities after high pressure exposure (Barbosa-Canovas et al., 1998; Considine et al., 2008; Goodridge et al., 2006; Koseki & Yamamoto, 2007; San Martin et al., 2002; Smelt, 1998).

Operation parameters such as temperature, ramp up rate, hold time, release rate, and cycle number all affect microorganisms and must be considered for high pressure treatments. High pressure treatments above or below room temperatures tend to inactivate more organisms than pressure treatments held at ambient temperatures (Alpas et al., 1999; Considine et al., 2008; Matser, et al., 2004). Refrigeration temperatures can be used on products such as milk that cannot be heated but still requires microbial

inactivation (Considine et al., 2008; Matser, et al., 2004). Parameters where the pressurized environment is heated are useful for spore inactivation because a heated environment promotes sporulation (Considine et al., 2008; Matser, et al., 2004). High pressure treatments are more effective at killing germinated cells than spores; therefore, heating the medium is a logical step to inactivate spores unless sporulation produces toxins (Considine et al., 2008). The rate at which pressure is applied and released is important for cell membrane rupture (Donsi et al., 2007). Applying and releasing pressure quickly is more effective at breaking cellular membranes than slower rates, and thus, this is preferred for high pressure treatments (Donsi et al., 2007). Quick release of pressure is thought to be responsible for bursting the cellular membrane open (Donsi et al., 2007; Matser, et al., 2004; Smelt, 1998). A slow ramp rate may allow time for stress response in the microorganisms, thus making the high pressure process less effective (Donsi et al., 2007; Smelt, 1998).

High pressure can be applied in multiple pulse cycles where the pressure is increased and released multiple times and the holding time is very short (Donsi et al., 2007; Matser, et al., 2004). In general, holding time can vary from a few seconds to a week or more, although for industrial food processing purposes, hold times should be minimized to keep cost of production low (Alpas et al., 2000; Donsi et al., 2007; Matser, et al., 2004; Mertens, 1994; Westerlund, 1994). Longer hold times and higher pressures are correlated with high inactivation rates (Alpas et al., 1999; Donsi et al., 2007; Matser, et al., 2004). Inactivation may follow either a log-linear curve, similar to what is seen for temperature death curves or inactivation may not be linear, in which case the Weibull model is used (Barbosa-Canovas et al., 1998; Matser, et al., 2004; Buzrul et al., 2008). The Weibull model predicts whether a population is increasingly sensitive to pressure (downward concavity or shouldering) or whether a population consists of sensitive species, which die off quickly, leaving a resistant species that are able to adapt to high pressures (upward concavity, or tailing) (Abee & Wouters, 1999; Alpas et al., 2000; Buzrul et al., 2008). When a resistant subpopulation is exposed to high pressures a second time, there is more pressure resistance than in the original population and therefore more survivors than in the original population, suggesting that this is a baroresistant population and survival is not due to other factors such as matrix composition (Abee & Wouters, 1999; Buzrul et al., 2008; Hauben et al., 1997). This tailing effect is also seen in temperature

death curves, but is more pronounced in high pressure death curves and must be considered when processing foods (Bowman et al., 2008; Hauben et al., 1997). To combat the survival of the tailing population, antibiotic compounds, low pH foods, and high salt concentrations are being used in addition to high pressure to provide a synergistic effect (Alpas et al., 1999; Alpas et al., 2000; Kalchayanand, et al., 1998; Ogihara, et al., 2009; Patterson, 2005; Smelt, 1998; Yuste, et al., 2002).

While there has been research on the effects of high pressure on many food products, there is a lack of research on peanut butter, peanut sauces, and coconut milk. Some of the foods that are currently using high pressure processing include salsa, jams, fruit juices, salad dressings, oysters, guacamole, luncheon meat and other meat emulsion products, dog food products, and cubed meat products (Alpas et al., 2000; Bayindirli, et al, 2006; Caner et al., 2004; Casadei et al., 2002; Considine et al., 2008; Cruz-Romero, Kerry, Kelly, 2008; Jimenez Colmenero, 2002; Matser, et al., 2004; Palou et al., 2000; Rastogi et al., 2007). While most applications of high pressure are for microbial inactivation, high pressure is also used to inactivate the enzymes responsible for browning guacamole and to open oysters without the use of knives, thus reducing human labor and injury during processing (Caner et al., 2004; Considine et al., 2008; Crus-Romero et al., 2008; Le-Bail et al., 2006; Palou et al, 2000; Rastogi et al., 2007). High pressure processing of fruit juices has been shown to help retain vitamin C, or ascorbic acid, more so than conventional heat processing (Matser, et al., 2004). Some future products that are currently being researched include sauerkraut, spices, and cereal grains and legumes (Estrada-Giron, Swanson, & Barbosa-Canovas, 2005; Penas, et al., 2010). High pressure processing of spices is promising because the lack of heat needed to sterilize the spices helps retain the flavor components (Matser, et al., 2004). Preliminary studies have been conducted on basil. The pressurized basil exhibited flavor and aroma of fresh basil and had retained more essential oils than dried or frozen basil (Matser, et al., 2004). Cereal grain proteins have been found to be more digestible after high pressure processing and some of the allergenic proteins are solubilized and removed without removing a significant amount of vitamins that are otherwise destroyed by heat treatments (Estrada-Giron et al., 2005; Farr, 1990; Matser, et al., 2004). The number of products processed by high pressure treatments is growing rapidly, and research is needed for complex food systems.

II. Pyrosequencing

High throughput sequencing is used for a wide range of applications relating to bacteria, including identification of viruses, bacteria, and fungi. Identification can be used to determine possible treatments for pathogens, identify genes of interest for metabolite production, or to determine ecological relationships within a niche, among others (Diggle, 2004). A more recent application has been the analysis of food matrices. This approach provides information on the resident flora of a food, which is important for producing high quality, low risk foods (Juste et al., 2008). Many foods also require microorganisms for flavor and development, such as sourdough bread, yogurt, cheese, sausage, and wine, among others (deVos, 2001; Juste et al., 2008; Kuipers, 1999). Ensuring these foods are being developed using optimal cultures is also important for industry to ensure quality and consistency (deVos, 2001; Kuipers, 1999).

High throughput sequencing and other molecular techniques do not require the microorganism of interest to be culturable, so there is little selection on what is being analyzed (Juste et al., 2008). These techniques also eliminate the possibility of not detecting injured microorganisms that may have been damaged by food processing steps (Juste et al., 2008). Analysis at each processing step allows for better contamination risk assessment and control, with the hopes of a reduction in the occurrence of contaminations, recalls, and illnesses associated with food-borne outbreaks.

The general procedure for high throughput sequencing is to first extract DNA from the food matrix (Juste et al., 2008). It is important to remove inhibitors such as proteins and lipids because these can inhibit PCR reactions (Juste et al., 2008). The DNA is then amplified using PCR, in which the primers used target a conserved region, such as the 16s gene (Juste et al., 2008). It is also important for the primers to encompass a variable region of the genome such that the amplified fragment can give information on the species from which it came from. Each sample is "barcoded" by labeling one of the primers with a specific tag that can be read by the sequencer. All sequences with the same barcode come from the same sample, allowing the sequences to be pooled based on which original sample they came from. The amplified DNA

is then sequenced according to pyrosequencing protocols and the data is analyzed using databases that

match the variable regions of the DNA to specie information (Juste et al., 2008).

More specifically, Pyrosequencing is a real-time sequence by synthesis method of DNA

sequencing that uses luciferase and extremely sensitive cameras to detect light from the incorporation of

deoxynucleotides in a DNA sequencing reaction (Diggle, 2004; Rahman et al., 2009; Ramon, 2003,

Ronaghi, 1998, Ronaghi, 2003). The apparatus consists of many tiny wells that contain a probe that

hybridizes with a sample of single stranded DNA (ssDNA) (Diggle, 2004; Ramon, 2003, Ronaghi, 1998,

Ronaghi, 2003). The ssDNA is then used as a template for the addition of complementary nucleotides

(Diggle, 2004; Rahman et al., 2009; Ramon, 2003, Ronaghi, 1998, Ronaghi, 2003). Each cycle consists of

adding a specific deoxynucleotide along with DNA polymerase, ATP sulfurase, luciferase, and apyrase

(Diggle, 2004; Ramon, 2003, Ronaghi, 1998, Ronaghi, 2003; Shendure et al., 2008). During a cycle, if the

specific base is incorporated, the PPi remaining after base incorporation is used to make ATP via ATP

sulfurase which then drives the luciferase to oxyluciferin reaction, giving off light (Diggle, 2004; Rahman

et al., 2009; Ramon, 2003, Ronaghi, 1998, Ronaghi, 2003; Shendure et al., 2008). The magnitude of light

emitted is directly proportional to the number of bases incorporated (Diggle, 2004; Ramon, 2003, Ronaghi,

1998, Ronaghi, 2003). If no bases are incorporated, the apyrase degrades the luciferase and the next base

cycle is performed (Diggle, 2004; Ramon, 2003, Ronaghi, 1998, Ronaghi, 2003).

This method has some drawbacks that should be considered. First is the short base pair (bp) length

that is read. Originally, pyrosequencing had a read length of 200-300 bp, meaning that from primer end to

primer end in the PCR amplification, there is a 200-300bp limit from which to read the barcode, primers,

conserved region, and variable region (Morozova and Marra, 2008). Newer chemical advances allow for

read lengths to be increased to 500bp, which may allow for more information about variable regions

(Morozova and Marra, 2008). This translates to roughly 80-120Mb to be sequenced in one run using the

original chemistry, which is powerful if shotgun sequencing an entire genome (Morozova and Marra,

2008). For amplicon sequencing, or a metagenomic approach, the read length is the crucial information

limit, and thus the amount sequenced per run can be translated into the number of samples sequenced at once (Morozova and Marra, 2008).

Another limit to pyrosequencing is that the genetic information that is sequenced may have originated from viable or non-viable organisms. This may cause problems if monitoring the effectiveness of kill-steps on a food processing line. However, it has been suggested that an enrichment step be added prior to DNA extraction to allow the viable cells to proliferate and to allow the non-viable cells to degrade completely or to use chemicals to differentiate between the non-viable cells and the viable cells (Juste et al., 2008). Typically, degradation occurs rapidly due to endogenous enzymes, but in some food matrices, such as sugar thick juice, which is an intermediate in extracting sugar from sugar beets, degradation of dead microbes is extended out to 40 days (Juste et al., 2008).

Analysis of food products using molecular techniques such as pyrosequencing have included ready to eat (RTE) salads, pork, biofilms in a beer processing plant, and atlantic cod (Rudi et al., 2002). The study conducted on the RTE salads discovered that there was a difference in the flora based on where the salad was grown as well as storage conditions (Rudi et al., 2002). Microorganisms found consisted mostly of plant pathogens from either the genus *Pseudomonas* or the family *Enterobacteriaceae* (Rudi et al., 2002). The study conducted on pork consisted of sampling from fresh pork as well as pork stored at 4°C for 4 days (Olsson et al., 2003). The diversity of genera decreased in the stored sample, perhaps due to exposure to oxygen (Olsson et al., 2003). The genera for both samples consisted mostly of *Acinetobacter, Staphylococcus, Macrococcus,* and *Pseudomonas,* which was consistent with culture-based methods (Olsson et al., 2003). The prevalence of unknown genera increased with the stored samples (Olsson et al., 2003). The study on the biofilm formation at a beer bottling plant investigated the efficiency of the sanitation protocols at removing biofilms (Timke et al., 2005). This study found a large variety of biofilm-forming genera that were previously not found using culture-based methods, such as *Methylobacterium* (Timke et al., 2005). Some of these genera were capable of breaking down formaldehyde, which was an ingredient in the sanitizers being used (Timke et al., 2005). The study did not find spoilage organisms within their biofilm samples and thus the sanitation protocols were accepted as suitable (Timke et al.,

2005). The study on cod dealt with the biofilm formation of bacteria within the epidermal mucous that cod produce to reduce drag as they swim (Wilson et al., 2008). Samples were taken from three different bodies of water at three different seasons to test whether bacterial populations change with location and/or with seasons (Wilson et al., 2008). They found that *γ-proteobacteria* and *Cytophaga-Flavobacter-Bacteroides* species were prominent for all samples, indicating that bacterial populations do not differ significantly according to geographical location (Wilson et al., 2008). The bacterial populations did not change significantly for two of the three locations over time, indicating that the cod maintained bacterial communities through the seasons (Wilson et al., 2008). These studies show the diversity of food samples that have been tested with molecular techniques such as pyrosequencing. They demonstrate the vast diversity that can is not always captured using culture-based techniques, and thus that the use of non-culture based techniques plays a vital role in food microbiology.

III. Peanuts and Peanut Butter

The peanut plant (*Arachis hypocaea L.*) is considered to be a nitrogen-fixing legume that is important to many agricultural practices around the world, ranging from Africa to the Americas (Akcai, Ince, & Guzel, 2006; Woodroof, 1983). The peanut itself is formed from pollinated flowers that are forced underground to mature, forming a peanut shell with the kernel and skin inside (Woodroof, 1983). Peanuts are mostly composed of protein and unsaturated fatty acids, with linoleic acid (C18:2) and oleic acid (C18:1) accounting for roughly 83% of the fatty acids in peanuts (Maguire et al., 2004, Lokko et al., 2007). Peanuts have high levels of squalene, α-tocopherol, stigmasterol, campesterol, and β-sitosterol that are believed to reduce chronic heart disease by reducing LDL cholesterol, inhibiting LDL cholesterol oxidation that leads to atherogenesis, and providing antioxidant protection against singlet oxygen oxidation that is damaging to nucleic acids (Maguire et al., 2004). The folic acid content has been associated with lower homocysteine levels, which are linked to a lower risk of coronary heart disease (Lokko et al., 2007). Peanut butter, a processed food made from peanuts, also displays these health benefits.

Peanut butter is a paste produced from roasted peanuts that, by definition, must contain at least 90% peanuts and no more than 55% oil (APV, 2008; Woodroof, 1983; Grange, 1972). The 10% of ingredients that may be added may not include artificial flavors, preservatives, or vitamins (APV, 2008, Woodroof, 1983). Peanut butter is graded based on color, consistency, absence of defect, flavor, and aroma and may have textures ranging from smooth to crunchy (Grange, 1972). Peanut butter is also classified as stabilized or non-stabilized based on the presence or absence, respectively, of any ingredient that is added to prevent oil separation (Grange, 1972). Unstabilized peanut butters are typically termed natural or organic peanut butters and do not differ significantly in composition than its stabilized counterpart but may have sensory differences (McWatters & Young, 1978). The nutritional content of peanut butter is similar to that of peanuts, with only sodium and chloride being elevated in peanut butter due to the addition of salt (see Table 1.2: Essential Elements Found in Peanut Butter) (Galvao et al., 1976, Miller et al., 1942). Peanut butter provides over a quarter of the recommended daily allowance (RDA) of chloride, magnesium,

sodium, phosphorous, chromium, copper, manganese, and zinc based on a one-hundred gram serving size (Galvao et al., 1976).

Table 1.2: Essential Elements Found In Peanut Butter			
Element	RDA (mg)	PB (mg/100g)	% of RDA
Chloride	2300	581	25.3
Potassium	4700	685	14.6
Magnesium	400	150	37.5
Sodium	1500	388	25.9
Phosphate	700	188	26.9
Chromium	0.03	0.26	866.7
Copper	0.9	0.67	74.4
Iron	10	1.8	18.0
Manganese	2	1.4	70.0
Zinc	10	2.9	29.0
			(Galvao et al., 1976)

The process of making peanut butter involves many steps to take raw peanuts, which are unpalatable to humans, and turn them into a palatable paste (See Figure 1.1: Peanut Butter Production Process). First, the raw peanuts are shelled and roasted to about 200°C (392°F) for roughly thirty minutes (APV, 2008; Ma et al., 2009). Roasting causes a Maillard reaction to occur, creating the desired roasted flavor from the bitter taste of raw peanuts (APV, 2008). The extent of the roasted flavor can be adjusted depending on the duration of roasting. During roasting, volatile compounds and moisture are lost, the enzyme lipoxygenase is inactivated, and the relative oil content is increased (Felland & Koehler, 1997; Woodroof, 1983). The peanuts are then cooled to 100°C and blanched in water to separate the skins, the cotyledons, and the hearts (APV, 2008; Ma et al., 2009). In most peanut butter, only the cotyledons are used to make peanut butter because the skins and the heart give off a bitter flavor that is not desired (APV, 2008). Burnt, rotten, undercooked, and foreign materials are removed as the peanuts travel down a conveyor belt and the remainder is ground into paste (APV, 2008). It is during the grinding process that ingredients such as salts and stabilizers are added (APV, 2008; Ma et al., 2009). The pieces are ground into different sizes, according to their final destinations (APV, 2008). Chunky peanut butter incorporate larger chunks of peanuts into the finely ground paste typical of a smooth peanut butter (APV, 2008). After

grinding, the product remains under nitrogenous conditions to reduce fatty acid oxidation (APV, 2008). The

peanut butter is packaged and then the containers are left for two days at 50°C (122°F) to allow the peanut

butter fats to crystallize and to prevent shrinkage which causes the peanut butter to crack in the container

(Woodroof, 1983). For natural peanut butter, it may be assumed that the same process occurs, but there is

no addition of salt or stabilizers and oil separation may be expected after packaging. The shelf life of

peanut butter may extend to 2 years, provided that lipid autoxidation has not occurred to a noticeable extent

(Woodroof, 1983). Storage in a cool, dark place and the use of higher quality peanut extends shelf life

(Woodroof, 1983). The peanut butter used in this study is made from organically grown peanuts that were

dry roasted but were not blanched and contain the skins. There were no additional ingredients added, so

there is a natural oil separation that occurs.
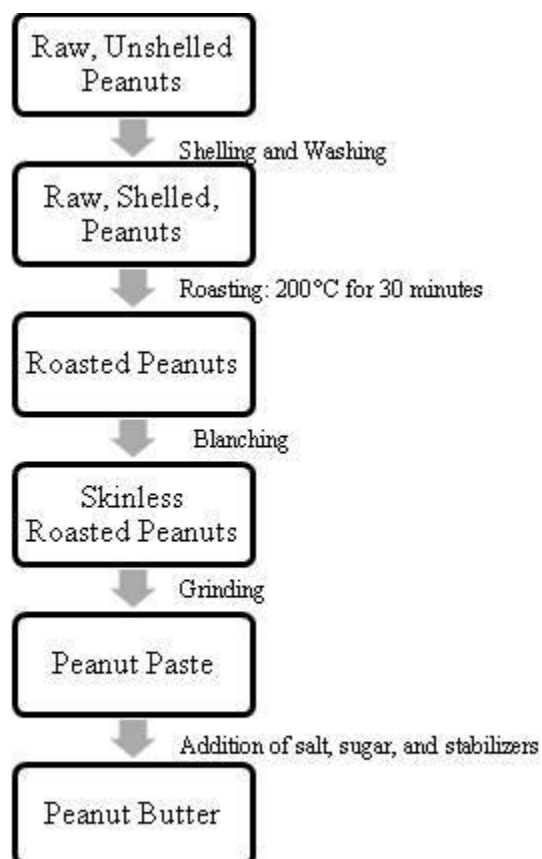
Figure 1.1: Peanut Butter Production Process

IV. *Salmonella*

A. Organism Information

Salmonella are facultative anaerobic gram-negative, motile rods that are responsible for a wide range of foodborne and waterborne illnesses including gastroenteritis, enteric fever, septicaemia, and bacteraemia (Bell &Kyriakides, 2002). *Salmonella* belong to the *Enterobacteriaceae* family and are divided into seven subspecies. The first subspecies is *S. bongori* while the remaining six are grouped together under the subspecies *S. enterica* (Bell &Kyriakides, 2002; Brenner, Villar, Angulo, Tauxe, & Swaminathan, 2000; Iwen; Morrone, 2008). Within these subspecies, there are serogroups that are named based on the somatic, or O, antigen. *Salmonella* is divided even further into serotypes, of which there are roughly 2500 currently (Brenner et al*., 2000; Iwen).

Salmonella infections are characterized by watery diarrhea, fever, abdominal pain, headache, emesis and nausea and may lead to intestinal bleeding, intracranial infection, septicemia, organ failure, and even rheumatoid arthritis (D'Aoust, 2000; Lee, et al., 1994; Morrone, 2008; Park, 2008). While the infection is typically self-limiting, infection affects the young, elderly, and immunocompromised in a more severe manner (Buchwald & Blaser*., 1984; Chalker & Blaser, 1988; Lee, et al., 1994; Whitney, 2007). There are roughly 1 million *Salmonella* infections per year in the United States, with 500 of those resulting in death (Brenner et al., 2000; Mattick et al., 2000a; Mattick et al., 2000b; Park et al., 2008). *Salmonella* has traditionally been implicated in outbreaks involving raw poultry and egg, but has been found to cause illness from products such as raw vegetables, juices, cereals, spices, cheeses, chocolate, and ice cream (Arumugaswamy, et al., 1995; Bell, 2002; D'Aoust, 2000; Metrick et al., 1989; Park et al., 2008). Most of these products have water activities within a viable range (>0.95) for *Salmonella*, but products such as chocolate and peanut butter have low water activities (<0.70) that are not typically associated with pathogen viability (Bell, 2002; Burnett et al., 2000; D'Aoust, 2000; Mattick et al., 2001; Park et al., 2008).

B. Outbreaks

There have been several outbreaks associated with peanuts and peanut products, including peanut butter, with the earliest outbreak occurring in 1996. This outbreak was due to *Salmonella* Mbandaka and *Salmonella* Seftenberg from peanut butter products in Australia (Burnett et al., 2000; Ng et al., 1996). This outbreak caused 54 cases of salmonellosis in South Australia and Victoria, with over half of the infected people being under the age of five (Burnett et al., 2000; Ng et al., 1996). This was the first documented case of a *Salmonella* outbreak associated in peanut butter. Five years later, in 2001, health departments in Australia and Canada recognized an increase in *Salmonella* Stanley infections (Kirk et al, 2004). Pulse Field Gel Electrophoresis (PFGE) analysis revealed four different serotypes (Newport, Stanley, Kottbus, and unknown) responsible for the outbreak (Kirk et al, 2004). Sharing of this information linked outbreaks in England and Scotland that had the same serotypes and PFGE patterns (Kirk et al, 2004). Unshelled peanuts and flavored peanuts imported from a single Asian country were discovered to be the cause of the outbreaks and in total, 97 people were ill due to *S*.Stanley, and 12 were due to *S.* Newport (Kirk et al, 2004). The source of contamination was impossible to determine and recalls were issued in the affected countries (Kirk et al, 2004).

In 2006, the United States experienced its first peanut butter outbreak when PulseNet discovered an increasing trend in *Salmonella* Tennessee isolates (CDC, 2007; Ma et al., 2009). Further investigation led to discovering the source of the contamination was from Peter Pan peanut butter produced from a single ConAgra plant in Sylvester, Georgia (CDC, 2007; Maki, 2009; Morrone, 2008). Opened and unopened jars of peanut butter tested positive for *Salmonella* Tennessee (CDC, 2007). The plant also produced Great Value peanut butter and all peanut butter of either brand was recalled and consumers were informed not to consume any peanut butter with lot number starting with 2111(CDC, 2007). The source of the contamination was unknown, but raw peanuts were ruled out as the source since none of the other plants using the same raw peanuts tested positive for *Salmonella* (CDC, 2007; Ma et al., 2009). This outbreak caused illness in 628 people, with 20% of those being hospitalized (CDC, 2007). There were no deaths associated with salmonellosis (CDC, 2007).

The next peanut butter outbreak was also discovered by PulseNet beginning in November, 2008. *Salmonella* Typhimurium was the source of illness for 529 people from 43 states having illness from September, 2008 to January, 2009 (CDC, 2009; Maki, 2009). Of these patients, 116 were hospitalized and 8 people died (CDC, 2009; Maki, 2009). The Minnesota Department of Health interviewed patients for a history on their diets and concluded that the common source of infection was King Nut creamy peanut butter (CDC, 2009). A container of King Nut peanut butter from a long term care facility tested positive for *Salmonella* Typhimurium, but it was yet to be determined if all the illnesses reported came from the King Nut peanut butter or if was from an ingredient (CDC, 2009). The King Nut peanut butter was produced in a single facility in Blakely, Georgia by Peanut Corporation of America (PCA) and was distributed to institutions, food service industries and private companies, but was not labeled for retail sale (CDC, 2009). Peanut paste was also produced at the same facility and was distributed to numerous food companies for production of peanut paste containing products that were distributed to at least 23 countries and within the U.S.(CDC, 2009). Packages of peanut butter crackers made from the peanut paste tested positive for *Salmonella* and it was concluded that the peanut paste was contaminated as well (CDC, 2009). A recall of more than 400 peanut containing products and pet food products from 54 companies was issued, making it one of the largest recalls in the United States (CDC, 2009; Maki, 2009).

FDA inspections later revealed that the PCA plant failed to withhold contaminated product and failed to properly clean production lines (DeVault & McKilligin, 2009). Reports were found that indicated that twelve individual samples, including peanut pastes and peanut meals, tested positive for *Salmonella* upon initial testing (DeVault & McKilligin, 2009; Maki, 2009). The products were then retested for *Salmonella* and were shipped after negative results (DeVault & McKilligin, 2009). Therefore, the plant was aware of *Salmonella* being found in peanut products, but failed to withhold product (DeVault & McKilligin, 2009). The FDA found upon further inspection that the plant failed to properly clean peanut paste production lines and failed to store food in proper conditions (DeVault & McKilligin, 2009; Maki, 2009). Areas as close as three feet away from production lines tested positive for *Salmonella* Seftenberg and Mbandaka (DeVault & McKilligin, 2009). Mold growth was found within coolers and water drips were observed from overhead cooling fans (DeVault & McKilligin, 2009).

Table 1.3: Salmonella Outbreaks					
Year	Place	Product(s)	Number of Infected	Serotype(s) Responsible	Reference
1996	Australia	Peanut Butter	54	Mbandaka and Seftenberg	Burnett et al., 2000; Ng et al, 1996; Oliver, 1996
2001	Australia, Canada, England	Unshelled Flavored Peanuts	109	Newport, Stanley, Kottbus, and Unknown	Kirk et al.,2004
2006	United States	Peanut Butter	628	Tennessee	CDC, 2007; Maki, 2009; Morrone, 2008
2008-2009	United States	Peanut Butter and Peanut Paste Products	529	Typhimurium	CDC, 2009; Maki, 2009

C. Peanut Butter and *Salmonella* Studies

There have been two studies to date on the survival of *Salmonella* in various peanut butter formulations in response to the outbreaks. The first study, conducted by Burnett et al., studied the survival of a five strain *Salmonella* cocktail in five different peanut butters and two peanut spreads for a duration of twenty-four weeks either at 5°C or 21°C (2000). The second study by Park et al. studied the survival of a three strain *Salmonella* Tennessee cocktail in five different peanut butter brands that were stored at either 4°C or 22°C for up to 14 days (2008). These studies showed that *Salmonella* was able to survive in the high fat, low water activity environment of peanut butter and peanut spreads (Burnett et al., 2000; Park et al., 2008). Survivability was greater at lowered temperatures, but there was not any significant difference in the survivability in most of the storage temperatures from the Park et al. study (Burnett et al., 2000; Park et al., 2008). Both studies concluded that *Salmonella* is capable of surviving in peanut butter for the duration of its shelf life (Burnett et al., 2000; Park et al., 2008). *Salmonella's* ability to survive is due to the water droplet size and prevalence within the colloidal matrix of peanut butter, as well as other high fat, low water activity foods such as margarine, chocolate, cheese, and mayonnaise (Burnett et al., 2000; Delamarre

&Batt, 1999; Park et al., 2008). Smaller droplets of water are associated with a finer emulsion, fewer

nutrients available, and fewer organisms that can survive within these foods (Delamarre &Batt, 1999;).

Studies have been conducted on *Salmonella*'s survivability in chocolate, another high fat, low

water activity product that has been subject to outbreaks. Studies have found that in these chocolate

outbreaks, the level of *Salmonella* contamination was as low as two organisms per gram of chocolate (Bell,

2002; Burnett, 2000; Craven et al*.,* 1975). The chocolate bars involved in the outbreak were consumed at

least seven month after production, and *Salmonella* was isolated from the chocolate bars after twelve

months, demonstrating the incredible survivability of *Salmonella* in high fat, low water activity products

(Bell, 2002; Burnett, 2000; Craven et al., 1975). The high fat content in chocolate is thought to protect

Salmonella as it passes through the acidic environment of the stomach, making the infectious dose less than

one hundred cells, and since peanut butter also has a high fat content, it is reasonable to assume the same

low infectious dose for peanut butter outbreaks (Bell, 2002).

Products such as peanut butter go though high temperature processing, and it has been generally

assumed that this step would aid in killing *Salmonella* that had contaminated the raw product. However,

studies on the heat tolerance of *Salmonella* in a low water activity environment such as peanut butter

suggest that *Salmonella* may be able to survive these high temperature processes (Goepfert and Biggie,

1968; Goepfert et al., 1970; Ma et al., 2009; Mattick et al., 2000b; Mattick et al., 2001; Shachar and Yaron,

2006). *Salmonella* Tennessee inactivation rates in peanut butter were compared with other serotypes

(Enteriditis, Heidelberg, Typhimurium) and it was determined that Tennessee had greater heat tolerance,

with a D-value of 120 minutes at 90°C, indicating that Tennessee would not be killed under typical heat

processing during peanut butter production (Ma et al., 2009).The heat inactivation of *Salmonella* in peanut

butter is not a linear relationship, but instead follows the tailing Weibull model in which initially there is a

rapid inactivation of organisms, but this tapers out into an asymptotic tail (Ma et al., 2009; Mattick et al.,

2001; Shachar and Yaron, 2006). This tailing effect results in about 0.05% to 0.1% of *Salmonella* surviving

and can be explained by the heterogeneous matrix of peanut butter causing an aggregation of *Salmonella* in

the water phase of peanut butter (Ma et al., 2009; Park et al., 2008; Shachar and Yaron, 2006). The fat or

oil phase then acts as a protective bubble surrounding *Salmonella*, protecting it from temperature and, perhaps, high pressures (Ma et al., 2009; Shachar and Yaron, 2006). Other components in the food matrix, such as sugars, have a protective effect on heating and may also have a baroprotectant effect (Corry, 1974; Mattick et al., 2000b).

Other effects of low water activity conditions such as those in peanut butter include filamentation of *Salmonella* (Kieboom et al., 2006; Mattick et al., 2000a). This makes *Salmonella* more potent in low water activity foods because biomass increases, but the number of cells does not increase due to incomplete cell division and septa formation caused by stress induced from low water activity (Kieboom et al., 2006; Mattick et al., 2000a). Once *Salmonella* is exposed to higher water activities, the filamentous structure forms septa, and rapidly divides (Kieboom et al*.,* 2006; Mattick et al., 2000a). Traditional plating methods do not detect the rapid division of cells, and the large filamentous organism results in one single colony (Kieboom et al., 2006; Mattick et al., 2000a). This implies that traditional enumeration methods are not accurate and worst-case scenario data may not be accurate in deciding precautionary processing steps (Kieboom et al., 2006; Mattick et al., 2000a).

The effects of increasing water activity in peanut butter have been studied for the sensory attributes and the microbiological consequences. Such increased water activity products may include peanut sauces or peanut mixtures such as peanut butter and jelly mixed together. Peanuts contain an endogenous lipoxygenase, but it is destroyed during roasting, therefore leaving the fatty acids susceptible to lipid oxidation via a non-enzymatic method (Felland & Koehler, 1997). Peanuts contain metallic pro-oxidants such as iron that oxidize the fatty acids in peanuts and peanut products and result in a rancid flavor (Felland and Koehler, 1997). The effect of water on the rate of lipid oxidation varies according to the amount of water present. Very low amounts and high amounts of water have a pro-oxidant effect while the middling ranges of water have an antioxidant effect (Felland & Koehler, 1997). The addition of water reduces the roasted aroma and taste of peanut butter and makes the peanut butter darker in color (Felland and Koehler, 1997). Increasing the water activity of a peanut product such as peanut butter may allow for microbial growth such as mold, spoilage microorganisms and pathogenic microorganisms (Clavero, 2000).

One study found that increasing peanut butter to a water activity above 0.96 allowed for growth of *Clostridium botulinum*, but the peanut butter was inedible by the time toxin was produced due to the presence of molds and lactic acid bacteria that produced off odors (Clavero, 2000).

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Chapter 2: Preliminary Studies

I. Abstract

Recent *Salmonella* outbreaks in peanut butter and peanut products have prompted the need for new processing methods. High pressure processing offers an alternative to heating and retains more nutrients and flavors than heating. Thus, affect of high pressure processing on inoculated organic, minimally processed peanut butter, and peanut products was investigated. High pressure processing of organic peanut butter inoculated with a 5-strain *Salmonella* cocktail resulted in $\log_{10}$ reductions of under 1.17 CFU/g when processed at 600MegaPascals for 30 minutes. It was determined that the water activity was providing a protective effect. Trials were conducted in which the water activity of the organic peanut butter was increased incrementally. Lecithin was initially used as an emulsifier, but only a maximum water activity of 0.69 was obtained, resulting in a 0.58 $\log_{10}$ reduction. Lite coconut milk was tested and was found to be an appropriate emulsifier as water activities >0.99 were obtainable. Peanut sauces composed of equal parts organic peanut butter, lite coconut milk, and distilled water (33% peanut sauce) were processed at pressures of 400, 475, 500 and 550MPa with hold times ranging from 2 to 7 minutes. Results indicated that there were design flaws in the enumeration of processed sauces or that the sauces became contaminated through laboratory equipment. However, $\log_{10}$ reductions >5.00 were obtained, indicating that high pressure processing may be suitable for peanut sauces.

II. Introduction

There have been several outbreaks associated with peanuts and peanut products, including peanut butter, with the earliest outbreak occurring in 1996. This outbreak was due to *Salmonella* Mbandaka and *Salmonella* Seftenberg from peanut butter products in Australia (Burnett et al., 2000; Ng et al., 1996). In 2006, the United States experienced its first peanut butter outbreak when PulseNet discovered an increasing trend in *Salmonella* Tennessee isolates (CDC, 2007; Ma et al., 2009). Further investigation led to discovering the source of the contamination was from Peter Pan peanut butter produced from a single ConAgra plant in Sylvester, Georgia (CDC, 2007; Maki, 2009; Morrone, 2008). The next peanut butter

outbreak was also discovered by PulseNet beginning in November, 2008. A container of King Nut peanut butter from a long term care facility tested positive for *Salmonella* Typhimurium and packages of peanut butter crackers made from peanut paste tested positive for *Salmonella* (CDC, 2009). It was concluded that the peanut paste that was produced in a single facility in Blakely, Georgia by Peanut Corporation of America (PCA) was contaminated (CDC, 2009). This prompted a recall of more than 400 peanut containing products and pet food products from 54 companies, making it one of the largest recalls in the United States (CDC, 2009; Maki, 2009).

Major components of peanut butter go though high temperature processing (i.e. roasting), and it has been generally assumed that this step would aid in killing *Salmonella* that had contaminated the raw product. However, studies on the heat tolerance of *Salmonella* in low water activity environments such as peanut butter suggest that *Salmonella* may be able to survive these high temperature processes (Ma et al., 2009; Mattick et al., 2000b; Mattick et al., 2001; Shachar and Yaron, 2006). *Salmonella* Tennessee inactivation rates in peanut butter were compared with other serotypes (Enteriditis, Heidelberg, Typhimurium) and it was determined that *S.* Tennessee had greater heat tolerance, with a D-value of 120 minutes at 90°C, indicating that *S.* Tennessee would not be killed under typical heat processing during peanut butter production (Ma et al., 2009).

Low water activity conditions such as those in peanut butter cause filamentation of *Salmonella* (Kieboom et al., 2006; Mattick et al., 2000a). This makes *Salmonella* more potent in low water activity foods because biomass increases, but the number of cells does not increase due to incomplete septa formation caused by stress induced from low water activity (Kieboom et al., 2006; Mattick et al., 2000a). Once *Salmonella* is exposed to a higher water activity environment, the filamentous structure forms septa, and rapidly divides (Kieboom et al., 2006; Mattick et al., 2000a). Traditional plating methods do not detect the rapid division of cells, and the large filamentous organism results in one single colony (Kieboom et al., 2006; Mattick et al., 2000a). This implies that traditional enumeration methods are not accurate and worst-case scenario data may not be accurate in deciding precautionary processing steps (Kieboom et al., 2006; Mattick et al., 2000a).

High fat matrices also provide protection to microorganisms such as *Salmonella* that are often overlooked. For example, the high fat content in chocolate is thought to protect *Salmonella* as it passes through the acidic environment of the stomach, making the infectious dose less than one hundred cells, and since peanut butter also has a high fat content, it is reasonable to assume the same low infectious dose for peanut butter outbreaks (Bell, 2002). Thus it is reasonable to conclude that high fat, low water activity foods such as peanut butter are vulnerable to dangerous outbreaks due this combination of increased heat tolerance, filament formation, and protection from digestive barriers.

High pressure processing has recently been employed as an alternative processing method to heating in foods such as guacamole, oysters, and fruit juices (Alpas et al., 2000; Bayindirli, et al, 2006; Caner et al., 2004; Casadei et al., 2002; Considine et al., 2008; Matser, et al., 2004; Palou et al., 2000; Rastogi et al., 2007). High pressure processing is an isostatic process in which the pressure is instantaneous without a gradient (Alpas, et al., 2000; Considine et al., 2008; Patterson, 2005). When the processing vessel is pressurized, the processing fluid and samples undergo adiabatic heating, in which the change in pressure also causes a change in temperature (Considine et al., 2008; Doona et al., 2007). High pressure processing does not break covalent bonds, which preserves endogenous nutrients, colors, and flavors (Considine et al., 2008; Mackey et al., 1994; Metrick et al., 1989). High pressure processing is effective against microorganisms, but severity depends on the type of microorganism, growth stage, and environmental components such as salts and sugars (Alpas et al., 2000; Barbosa-Canovas et al., 1998; Considine et al., 2008). It is generally accepted that longer hold times and/or greater pressures increase lethality (Alpas et al., 1999; Considine et al., 2008).

The objectives of this research were to (i) determine if high pressure processing is a suitable process to lethally injure *Salmonella* in organic peanut butter and peanut butter sauce, (ii) to determine what factors affect *Salmonella's* viability in these matrices, and (iii) to determine if high pressure processing of these foods is feasible for manufactures.

III. Materials and Methods

Preliminary Work

Initial studies on the effect of high pressure processing on *Salmonella* included the following food matrices: 1x phosphate buffered saline (PBS), handmade peanut butter made in the University of Nebraska-Lincoln Food Processing Center's pilot plant, organic peanut butter (100%), organic peanut butter with lecithin, and organic peanut butter with multiple milk types.

The high pressure unit used throughout the study was the Stansted ISO Lab high pressure unit (Stansted, UK). The unit is capable of reaching pressures of 900MPa and temperatures of 90°C. The sample basket has a 2.5 inch diameter and a length of 15 inches. The processing fluid is a 30:70 mixture of propylene glycol and water. The thermocouple for measuring the processing temperatures is located at the bottom of the pressure vessel and measures the temperature of the processing fluid. The vessel is not insulated, and thus, there is heat transfer between processing fluid and pressure barrel. Pressures and temperatures were recorded in real-time with the sensors responsible for these reading calibrated regularly (See Appendix A: High Pressure Processing Machinery Set-Up).

The cultures used include five *Salmonella* serotypes obtained from the frozen stock cultures of the Food Processing Center at University of Nebraska-Lincoln. The serotypes included *Salmonella* Branderup NVSL 96-12528 from chicken, *Salmonella* Oranienburg NVSL 96-12608 from chicken, *Salmonella* Typhimurium ATCC 14028, *Salmonella* Enteriditis IV NVSL 94-13062 Liquid chicken egg, and *Salmonella* Heidelberg/Sheldon 3347-1. Cultures were kept in 10% glycerol at -80°C until use. Cultures were restored by suspending 20μl of thawed culture into 5 ml of Tryptic Soy Broth (TSB, Difco, MD), vortexed and incubated at 37°C for 20-24 hours before use.

Equations

Equation 2.1: $LogS = Log_{10} {}^{N}/_{N_0}$ where, N is the final population (CFU/mL) and N_0 is the initial population (CFU/mL).

Equation 2.2: Weibull Model $Log\ S = -bt^n$ where, b and n are the scale and shape factors and t is time in minutes.

a. Phosphate Buffered Saline Validation Study

An initial test using phosphate buffered saline (PBS) was performed to validate the data found in the literature on the lethal pressures of *Salmonella*. This tested the validity of the equipment, the plating techniques, and the safety protocols. For this test, 20 mL of overnight *Salmonella* cultures were centrifuged at 6000RPM for 5 minutes and re-suspended in1x PBS. Cultures were combined to make a *Salmonella* cocktail and 5mL of cocktail was pressurized at 200MPa for 5 minutes as well as 275MPa for 5 minutes. A control was also used as a baseline for the initial inoculum. The samples and controls were spread plated on trypic soy agar (TSA, Neogen, MI) and incubated at 37°C overnight and enumerated by hand. The processing fluid and the pressure vessel were also tested for contamination.

b. *Salmonella* Isolation

Work was done using the five serotypes of *Salmonella* that composed the cocktail to determine the appearance of each strain as well as the cocktail on all mediums used in the FDA-BAM method for *Salmonella* detection. This detection method was used to detect *Salmonella* when it was not detected via plating. A cocktail was prepared by combining 4mL of each overnight serotype culture and spinning down the cocktail at 6,000RPM for 5 minutes, decanting the liquid, and re-suspending the pellet in 1x PBS, re-centrifuging the cocktail and re-suspending the pellet in 2mL of 1x PBS to create a 10x cocktail solution. This cocktail solution was diluted using 9mL tubes of butterfield's dilution water to dilutions of 10^{-2}, 10^{-4}, and 10^{-6} which were spiral plated on xylose lysine deoxycholate agar (XLD, Neogen, MI) and TSA. Each serotype was streaked out onto XLD and TSA. TSA plates were overlaid with XLD. All the plates were incubated overnight at 37°C. Pictures of each plate were taken for future reference. Samples from each serotype and the cocktail mixture were transferred to Tetrothionate broth (TT, Difco, MD) and Rappaport-Vassiliadis (RV, BAM Media M132) broths, left to incubate for the appropriate time, and then loopfuls were streaked for isolation onto Hecktoen enteric (HE, Oxoid, Unipath, UK) agar and Bismuth Sulfate (BS,

Difco, MD) agar. Colonies from these plates were then used to inoculate Lysine Iron Agar (LIA, Neogen, MI) and Triple Sugar Iron (TSI, Neogen, MI) agar slants that were incubated overnight. Colony morphologies and general observations were taken for each serotype as well as the cocktail mixture. This served as a reference for future enumerations and identifications of *Salmonella*.

c. High Pressure Studies on Organic Peanut Butter

Hand-milled peanut butter made by the University of Nebraska-Lincoln Food Processing Center Pilot Plant was tested for oil separation or color change due to high pressure processing. The peanuts used were not organic, but there were no additives like the organic peanut butter used and thus the peanut butter was considered a natural peanut butter. The natural peanut butter was mixed and roughly 30 grams was bagged into polyethylene high pressure pouches and vacuum sealed. Two control bags were held without processing and two sample bags were processed at 600MPa for 10 minutes with a ramp rate of 600MPa min^{-1} and depressurization rate of 900MPa min^{-1}. The bags were held at room temperature and changes in oil separation or color were observed continuously over a month.

The next preliminary project was to high pressure process inoculated samples of the organic, minimally processed peanut butter from Once Again Nut Butters (NY) that would be used for the remainder of the project. For this, the cocktail was prepared as described above, with centrifugation set at 6,000RPM for 5 minutes. Water activities and total plate counts were measured from each of the freshly opened jars, with three jars being used, one for each replication (Aqua Lab, Decagon, WA). Each replication was done in triplicate. For high pressure processing, bags were filled by adding 2.5g of peanut butter, then adding 0.100 mL of 10x cocktail and adding another 2.5g of peanut butter, such that the peanut butter created a sandwich around the inoculum. The bags were vacuum sealed from both ends and then the inoculum was mixed by hand into to peanut butter. The sample bags were subjected to a pressure of 420MPa for 5 minutes. Control bags were held without pressurization. For enumeration, 5g of sample was diluted with 45g of buffered peptone water (BPW, Neogen, MI) in a sterile stomacher bag and homogenized in a stomacher (Stomacher Lab Blender 400, Seward Tekmar Co, OH) for 2 minutes and then diluted to the needed dilutions in 9mL dilution tubes made from Butterfield's stock solution. The samples

were then plated using the Eddy Jet Spiral Plater on TSA and XLD agar. The TSA plates were kept at room temperature for two hours and then overlaid with XLD that was no warmer than 50°C. The plates were then incubated overnight at 37°C. The diluted samples in the stomacher bags were incubated at 37°C overnight in case the *Salmonella* enumerations were below detection limits. In the cases where no *Salmonella* was detected by plating, the FDA BAM method was followed for detecting *Salmonella*. Briefly, this included recovery in Tetrothionate broth (TT) and Rappaport-Vassiliadis (RV) broth and isolation of colonies on XLD agar, HE agar, and BS agar. Questionable colonies were then confirmed using TSI agar slants and LIA slants (Andrews & Hammack, 1998).

A range of pressures and hold times were investigated to determine appropriate combinations of pressures and times that could be used with the pure organic peanut butter as the food matrix. Duplicate bags of inoculated peanut butter were subjected to pressures of 480, 540, and 600MPa with a 5 minute hold time at each pressure. Controls and samples were processed and plated as described above. After this, another trial was conducted with duplicate bags of inoculated peanut butter processed at 600MPa for 30 minutes. Samples and controls were plated on XLD and TSA-XLD overlay plates.

d. Water Activity Studies

The effect on changing the water activity of peanut butter on the survivability of *Salmonella* was investigated. The first water activity determination trial was done by stomaching 6g of organic peanut butter and adding amounts of distilled water that ranged from 0.60mL to 2.0mL. Water activities were measured as well as the ability of the mixture to homogenize.

Lecithin was added to peanut butter-distilled water mixtures to determine if this was a suitable emulsifier. First, room temperature lecithin (Soy Lecithin, ADM, IL) was added to peanut butter and mixed in prior to adding water using a mortal and pestle. A total mixture weight of 10g was maintained, with the ratio of organic peanut butter to distilled water being altered and the amount of lecithin being added held constant at 0.01grams, making 0.1% lecithin mixtures. The distilled water was originally added as one aliquot, but was later added in smaller aliquots to help obtain a finer emulsion.

Lecithin was increased to 0.5% of the total mixture weight. Lecithin was heated to 60°C using a water bath and peanut butter was mixed in using a mortal and pestle. The organic peanut butter and lecithin mixture was kept heated in the water bath as distilled water was added in 0.05mL increments and mixed in. 100μl aliquots of 1x PBS were added to the organic peanut butter mixtures after they cooled to room temperature to determine if the water activity would be altered from the addition of inoculum. Water activities were measured after the mixture cooled to room temperature, after the addition of 100μl 1xPBS, and after 72 hours. Increasing amounts of lecithin were used until the FDA limit on lecithin was exceeded. All mixtures were analyzed based on the water activity and the ability to remain homogenous.

Four 0.5% lecithin mixtures with water activities ranging from 0.63 to 0.93 were made according to the heating method proposed above. 10g of each mixture was packaged into polyethylene bags, sealed, and stored overnight. Each bag had 100μl of either *Salmonella* cocktail or 1xPBS added and mixed into the mixture using a spoon. Inoculated samples and negative controls in which 1xPBS was added were high pressure processed at 600MPa for 5 minutes. Water activity was measured on an uninoculated control held at room pressure and on an uninoculated control that was high pressurized. Inoculated samples and controls were spiral plated on XLD and TSA-XLD overlay and incubated at 37°C.

40mg of dimodan (Danisco, DK) was mixed into 13g of organic peanut butter and 7mL of distilled water were added and mixed together using a mortal and pestle and the ability of the mixture to homogenize was studied.

Next, using various milk products as emulsifiers was investigated. Whole milk (Roberts Dairy, NE), 2% milk (Roberts Dairy, NE), and canned coconut milk (Thai Kitchen, CA) were used. The milk and organic peanut butter were mixed together using a wire whisk and once homogenized, distilled water was whisked into the mixture. The amounts of distilled water and milk were changed to determine the possible water activity changes. The samples were divided into two subsamples, one which was heated to 90°C and one which was not heated. Water activities were measured on the samples and the appearance of each mixture was noted. All subsamples were kept at 8°C overnight to determine how well the sample remained homogenous.

Lite coconut milk was combined in various amounts with organic peanut butter and distilled water by whisking together the lite coconut milk and organic peanut butter until thoroughly mixed and whisking in the distilled water. Portions of each mixture were heated to 90°C and left to cool to room temperature. Water activity was measured on the pre-heated sample and post-heated sample of each mixture. Each mixture was kept refrigerated overnight and the color and separation of each mixture was observed.

Lite coconut milk was used in a high pressure trial in which organic peanut butter, distilled water, and lite coconut milk were combined in different amounts (See Table 2.1: Preliminary Data: Peanut Sauce Formulations). The mixtures were then divided into two sub samples, with one sample being unheated and the other sample being heated for 4 minutes until the mixture started to boil. Viscosities were measured using a Brookfield LV viscometer (Brookfield Engineering Laboratories, MA). Each mixture was inoculated with the standard *Salmonella* cocktail mentioned above and pressurized at 600MPa for 5 minutes with a ramp rate of 600MPa min^{-1} and a depressurization rate of 1000MPa min^{-1}. Samples and controls were processed and plated as mentioned above for preliminary samples.

Table 2.1: Peanut Sauce Formulations

Sample ID	Organic Peanut Butter (g)	Lite Coconut Milk (mL)	Distilled Water (mL)	% of Organic Peanut Butter
A	20.0	20.0	20.0	33.4
B	20.1	30.0	10.0	33.4
C	20.0	10.0	30.0	33.4
D	30.0	15.0	15.0	50.0
E	10.0	25.0	25.0	16.7

e. High Pressure Processing on 33% Peanut Sauce

A 1:1:1 mixture by weight of organic peanut butter, lite coconut milk, and distilled water was chosen for preliminary high pressure studies. For this mixture, the organic peanut butter and lite coconut milk were mixed together with a whisk until homogenized and then the distilled water was whisked in until homogenized. This created an emulsion that did not separate when held at 8°C overnight. Water activity was measured on each sauce after preparation. Samples and controls had three replicates done in triplicate, such that there were nine bags per data point. Sauces were inoculated with *Salmonella* cocktail that was

prepared by centrifuging combined overnight cultures at 7500RPM for 10 minutes, washing with 1x PBS, centrifuging again, and re-suspending the cocktail in 2mL of 1x PBS to create a 10x cocktail solution. The parameters at which the sauces were tested is as follows: 550MPa at times of 2, 4, 5, and 7 minutes; 500MPa at times of 2, 4, 5, and 7 minutes; 475MPa at times of 4, 5, and 7 minutes; and 400MPa at times of 5, 6, and 7 minutes. Controls were held without pressurization. Temperature of the pressurization vessel was held at ambient temperature (22°C) and ramp rates and depressurization rates were held constant at 600MPa min^{-1} and 1000MPa min^{-1}, respectively. Five grams of samples and controls were diluted initially with 45g of BPW, stomached for 2 minutes and aliquots removed for enumeration. The stomacher bags were incubated overnight for further *Salmonella* detection if enumeration was below the detection limit. Samples and controls were enumerated on TSA-XLD overlay and XLD using the Eddy Jet Spiral Plating system and Flash and Grow colony counting software.

IV. Results

a. Phosphate Buffered Saline Validation Study

TSA plates were enumerated by hand and averages were calculated to determine the colony forming units per milliliter (CFU/mL). The starting concentration of *Salmonella* was $1.1x10^9$ CFU/mL. After high pressure processing at 200MPa for 5 minutes, *Salmonella* was enumerated at $1.5x\ 10^7$CFU/mL. This is a log_{10} reduction of 1.88 CFU/mL. At high pressure processing at 275MPa for 5 minutes, there was no growth on the lowest dilution plate. The estimated amount of *Salmonella* in this sample was <10 CFU/mL and the log_{10} reduction was >8.05 CFU/mL. Results are summarized in Table 2.2: Phosphate Buffered Saline Validation Study.

Fluid samples and swabs taken from the inside of the pressure vessel were all negative for *Salmonella*.

Table 2.2: Phosphate Buffered Saline Validation Study

Sample ID	Plate 1*	Plate 2*	Average*	Log Reduction
Control	1.04×10^9	1.22×10^9	1.13×10^9	
200 MPa	1.32×10^7	1.63×10^7	1.48×10^7	1.88
275 MPa	<10	<10	<10	>8.05

* = CFU/mL

b. *Salmonella* Isolation

All serotypes used proliferate on all mediums used for *Salmonella* recovery and enumeration, including all mediums used in the FDA-BAM method for *Salmonella* isolation and identification (Andrews & Hammack, 1998). From this work, it was discovered that the *S. Heidelberg/Sheldon* culture does not product hydrogen sulfide on XLD, LIA or TSI medias. All serotypes had very similar morphologies on HE agar, with colonies appearing black or clear with blue/green halos around the colonies. The colonies on BS agar were black with a distinct silver shimmer. Growth in TSI slants mimicked colony morphology on XLD agar because all serotypes produced acid in TSI, giving the butt of the agar a yellow color. All serotypes except *S.* Heidelberg produced hydrogen sulfide (H_2S) and therefore had black coloring in the agar as well. Morphology on LIA slants was uniform across serotypes for coloring, with the agar having a purple color. The cocktail did not produce hydrogen sulfide on LIA slants. Observations can be found in Table 2.3: Salmonella Morphology on XLD Agar.

Table 2.3: *Salmonella* Morphology on XLD Agar

Serotype	H_2S Production	Yellow Halo
Heidelberg	No	Yes
Oranienburg	Yes	Yes
Typhimurium	Yes	Yes
Branderup	Yes	Yes
Enteriditis	Yes	Yes
Cocktail	Yes and No	Yes

c. High Pressure Studies on Organic Peanut Butter

The natural peanut butter produced in the University of Nebraska-Lincoln's Pilot Plant was processed at 600MPa for 10 minutes to determine if there was any significant change in color or oil separation. Due to the safety protocols for the high pressure machine used, the samples could not be opened and tested and thus only visual observations were used. It was determined that the color was not changed significantly by high pressure processing and that there was not any increase in oil separation beyond what naturally occurs with natural peanut butter without emulsifiers and additives. This indicated that high pressure processing may not alter these sensory characteristics of peanut butter and that high pressure processing may be acceptable for other sensory attributes as well.

For organic peanut butter processed at 420MPa for 5 minutes, the water activity of each jar of organic peanut butter used was taken once the jar was open and mixed (See Table 2.4: Water Activity of Organic Peanut Butter). The peanut butter was at an average temperature of 24.9°C and the average water activity was 0.31. There was an average $\log_{10}$ kill of 0.55 and a log S value of -0.55 (See Table 2.5: High Pressure Processsed Organic Peanut Butter). All suspected colonies were confirmed according to the FDA-BAM method (Andrews & Hammack, 1998).

Resident flora in the organic peanut butter was enumerated using Total Plate Count Petrifilm®. All organic peanut butter samples had little to no growth, with an average of 1.7×10^{1} CFU/g of organic peanut butter.

A range of pressures and times were tested using the organic peanut butter as the matrix to determine a possible range for further study. The pressures used were 480, 540, and 600MPa. All these pressures were investigated at hold times of 5 minutes and a worst-case trial was conducted at 600MPa and a hold time of 30 minutes. Samples processed at higher pressures had larger $\log_{10}$ reduction values. High pressure processing inoculated organic peanut butter at 600MPa for 30 minutes resulted in a 1.17 $\log_{10}$ reduction, which was the maximum $\log_{10}$ reduction value for these processing parameters. The results are

found in Table 2.5: High Pressure Processsed Organic Peanut Butter Enumeration Data and Table 2.6: High

Pressure Processed Organic Peanut Butter.

Table 2.4: Water Activity of Organic Peanut Butter

Jar	Water Activity (a_w)	Temperature (°C)
Jar 1	0.33	25.0
	0.33	24.9
Jar 2	0.31	25.0
	0.30	24.8
Jar 3	0.30	24.9
	0.31	24.8

Table 2.5: High Pressure Processed Organic Peanut Butter Enumeration Data

Pressure (MPa)	Time (Minutes)	Sample Population (logCFU/g)		Control Population (Log CFU/g)	
		TSA-XLD	XLD	TSA-XLD	XLD
420	5	7.26 ± 0.42	6.92 ± 0.63	7.81 ± 0.24	7.49 ± 0.16
480	5	7.02 ± 0.00	6.17 ± 0.00	6.65 ± 0.16	5.94 ± 0.42
540	5	6.83 ± 0.00	6.13 ± 0.00	6.65 ± 0.16	5.94 ± 0.42
600	5	6.62 ± 0.00	6.10 ± 0.00	6.65 ± 0.16	5.94 ± 0.42
600	30	5.95 ± 0.01	5.12 ± 0.33	7.12 ± 0.23	6.62 ± 0.68

Table 2.6: High Pressure Processsed Organic Peanut Butter

Pressure	Hold Time (Minutes)	Survivors (log CFU/g)	Initial Population (log CFU/g)	Log Killed
420	5	7.81	7.26	-0.55
480	5	7.02	6.65	-0.37
540	5	6.83	6.65	-0.18
600	5	6.62	6.65	0.03
600	30	5.95	7.12	1.17

d. Water Activity Studies

The first attempt to increase organic peanut butter's water activity involved stomaching organic peanut butter with distilled water and then measuring water activity on the mixture. The original jars of organic peanut butter had an average water activity of 0.39. The addition of 0.60mL of distilled water increased the water activity to 0.82. The addition of 1.0mL of distilled water increased the water activity to 0.90, which was the highest water activity that was obtained. Two milliliters of distilled water was added to organic peanut butter, but the mixture could not be homogenized. All the results are found in Table 2.7: Organic Peanut Butter Mixed with Distilled Water.

The addition of lecithin improved the ability to emulsify organic peanut butter and distilled water together. Adding 0.01g of lecithin to 8g of organic peanut butter and 2.0mL of distilled water had an average water activity of 0.96 and the mixture could be homogenized. The addition of 2.5mL of distilled water to 7.5g of organic peanut butter was not able to homogenize and thus water activity was not measured. Complete results can be found in Table 2.8 Organic Peanut Butter Mixed with Lecithin at Room Temperature.

Mixtures containing 0.5% lecithin (0.25g) were heated while mixing had initial water activities ranging from 0.60 to 0.93 with the addition of 5% water and 20% water, respectively. Water activities increased with the addition of 0.1mL of 1xPBS, with an average increase of 0.03. After 72 hours at room temperature, the water activities of the mixtures decreased below their original measurements by an average of 0.01. Lecithin amounts were increased to 0.7% in order to obtain a 35% organic peanut butter and distilled water mixture, but water activity was not recorded because legal limits for lecithin in food were exceeded. It was also noted that the mixtures had oil separation overnight. See Table 2.9: Organic Peanut Butter Mixed with Lecithin at 60°C.

High pressure processed organic peanut butter and 0.5% lecithin mixtures had initial water activities ranging from 0.63 to 0.93 with the addition of 5% and 20% distilled water, respectively. After high pressure processing, water activity measurements increased by an average of 0.01. Log reductions

decreased with increasing water activity measurements, with the 5% water and organic peanut butter

mixture having a 0.51 $\log_{10}$ reduction and the 20% water and organic peanut butter mixture having a 0.35

$\log_{10}$ reduction. These results are from one replication and are thus not statistically significant, but the trend

was noted for future work. Full results are found in Table 2.10: High Pressure Processed Organic Peanut

Butter Mixed with Lecithin at 60°C.

Since legal limits for lecithin had been exceeded, Dimodan was used as an emulsifier. However,

13g of organic peanut butter, 7.0mL of distilled water and 0.04g of Dimodan could not homogenize when

mixed together. The mixture did not mix very well and thus it was decided not to continue using Dimodan.

Organic peanut butter mixed with various types of milks in various amounts resulted in water

activity levels in the range of 0.99 to 1.00, with both of these mixtures containing coconut milk. Mixtures

with other kinds of milk had water activities that fell within this range. Mixtures containing whole milk and

2% milk had an average water activity of 0.99. Separation occurred in pre heated mixtures containing

whole milk and 2% milk, but not coconut milk, when stored at 8°C overnight. All heated samples were

more viscous and did not separate when stored at 8°C overnight. See Table 2.11: Organic Peanut Butter

Mixed with Various Milk Types.

Data from the trials using lite coconut milk in combination with organic peanut butter and distilled

water can be found in Table 2.12: Organic Peanut Butter Mixed with Lite Coconut Milk. It was observed

that samples using 20g of organic peanut butter were not runny before heating whereas all the other

formulations were runny before heating. The 20g organic peanut butter samples also had oil separation.

Other samples did not have oil separation and remained homogenous after being stored at 8°C overnight.

Data from high pressure processing sauces made from lite coconut milk, organic peanut butter and

distilled water in various amounts can be found in Table 2.13: High Pressure Treatment of Organic Peanut

Butter and Lite Coconut Mixtures. For all the sauces, heating caused an increase in viscosity, or no change

in viscosity. There is also a trend for more *Salmonella* to be lethally injured from high pressure processing

in the native sauces that were not heated. Sauces with the same viscosities did not necessarily have similar

log reductions associated with them. It was also noted that there was separation in the sauce composed of 10g organic peanut butter, 25g distilled water, and 25g lite coconut milk.

Table 2.7: Organic Peanut Butter Mixed with Distilled Water

Organic Peanut Butter (g)	Distilled Water (mL)	Water Activity 1	Water Activity 2	Average Water Activity (a_w)	Notes
6.00	0.00	0.43	0.35	0.39	
6.04	0.60	0.81	0.83	0.82	
6.00	0.70	0.87	0.85	0.86	
6.00	0.80	0.84	0.90	0.87	
6.01	0.90	0.89	0.92	0.91	
6.00	1.00	0.88	0.93	0.90	
6.02	2.00	N/A	N/A	N/A	Could not homogenize

Table 2.8: Organic Peanut Butter Mixed with Lecithin at Room Temperature

Organic Peanut Butter (g)	Distilled Water (mL)	Lecithin (g)	Water Activity 1	Water Activity 2	Average Water Activity (a_w)	Notes
9.51	0.50	0.01	0.69	0.68	0.68	
9.01	1.00	0.01	0.84	0.83	0.84	
8.01	2.00	0.01	0.93	0.95	0.94	
8.00	2.00	0.01	0.96	0.95	0.96	
7.51	2.50	0.01	N/A	N/A		Could Not Homogenize

Table 2.9: Organic Peanut Butter Mixed with Lecithin at 60°C

Organic Peanut Butter (g)	Distilled Water (mL)	Lecithin (g)	Average Initial Water Activity	Average Water Activty +0.1mL 1xPBS	Average Water Activty After 72 Hours
47.51	2.50	0.25	0.60 ± 0.01	0.66 ± 0.01	0.59 ± 0.01
46.05	3.75	0.25	0.74 ± 0.00	0.77 ± 0.01	0.73 ± 0.01
45.03	5.00	0.25	0.81 ± 0.00	0.82 ± 0.01	0.79 ± 0.00
40.06	10.00	0.25	0.93 ± 0.00	0.94 ± 0.00	0.93 ± 0.00

Table 2.10: High Pressure Processed Organic Peanut Butter Mixed with Lecithin at 60°C

Organic Peanut Butter (g)	Distilled Water (mL)	Lecithin (g)	Initial Water Activity	Water Activity: HPP	Log Reduction
47.50	2.50	0.25	0.63 ± 0.01	0.63 ± 0.00	0.51 ± 0.10
46.25	3.25	0.25	0.69 ± 0.02	0.71 ± 0.00	0.58 ± 0.28
45.00	5.00	0.25	0.79 ± 0.01	0.79 ± 0.01	0.48 ± 0.26
40.02	10.00	0.25	0.93 ± 0.00	0.94 ± 0.00	0.35 ± 0.12

Table 2.11: Organic Peanut Butter Mixed with Various Milk Types

Organic Peanut Butter (g)	Distilled Water (mL)	Milk Type	Milk (mL)	Post-Heating Water Activity
10.03	10.00	10.00	Whole	0.99
10.01	15.00	5.00	2%	0.99
10.03	5.00	15.00	2%	0.99
10.03	10.00	10.00	Coconut	0.99
10.08	15.00	5.00	Coconut	1.00
10.02	5.00	15.00	Coconut	0.99

Table 2.12: Organic Peanut Butter Mixed with Lite Coconut Milk

Organic Peanut Butter (g)	Distilled Water (mL)	Lite Coconut Milk (mL)	Initial Water Activity	Post-Heating Water Activity
10.02	10.00	10.00	0.99	1.00
10.02	15.00	5.00	1.00	0.99
10.02	5.00	15.00	1.00	1.00
15.02	10.00	10.00	0.99	0.99
15.03	15.00	5.00	0.99	0.99
15.02	5.00	15.00	0.99	0.99
20.01	10.00	10.00	0.99	0.99
20.04	5.00	15.00	0.99	0.99

Table 2.13: High Pressure Treatment of Organic Peanut Butter and Lite Coconut Mixtures

Organic Peanut Butter (g)	Distilled Water (mL)	Lite Coconut Milk (mL)	Initial Viscosity (cps)	Post Heating Viscosity (cps)	Log Reduction in Native Sauce (CFU/g)	Log Reduction in Heated Sauce (CFU/g)
20.04	20.00	20.00	500	5000	7.39 ± 0.20	7.31 ± 0.20
20.06	30.00	10.00	250	5000	5.24 ± 0.00	5.73 ± 0.00
20.02	10.00	30.00	4000	37000	7.4 ± 0.17	6.08 ± 0.17
30.04	15.00	15.00	22500	>50000	7.39 ± 0.49	7.37 ± 0.49
10.00	25.00	25.00	500	500	5.35 ±0.17	5.6 ± 0.17

e. High Pressure Processing on 33% Peanut Sauce

Peanut sauces composed of equal part organic peanut butter, lite coconut milk, and distilled water were exposed to pressures of 550, 500, 475 and 400MPa with various hold times at each pressure. There was a trend for $\log_{10}$ reductions to increase with hold time and then decrease as hold time approached 7 minutes. Average water activity from all sauces was 0.99. There is a trend of increased injury with a decrease in $\log_{10}$ reductions. See Table 2.14: High Pressure Processing of 33% Peanut Sauce: Enumeration Data and Table 2.15: High Pressure Processing of 33% Peanut Sauce for complete results.

Figures 2.1 to 2.4 are graphs of the log reductions according to processing pressure and hold time and the $\log_{10}$ of injured *Salmonella*. LogS is a function which is defined as the $\log_{10}$ of the initial population, or N_0, subtracted from the $\log_{10}$ of the final population, or N. The $\log_{10}$ of injured *Salmonella* is the $\log_{10}$ of survivors enumerated on XLD plates subtracted from the $\log_{10}$ of survivors enumerated on TSA-XLD overlay plates. The population enumerated on XLD represents the uninjured population while those enumerated on TSA-XLD overlay represent the uninjured as well as the injured which recovered during the 2 hours prior to overlaying with XLD.

Table 2.14: High Pressure Processing of 33% Peanut Sauce: Enumeration Data

Pressure (MPa)	Time (Minutes)	Sample Population (Log$_{10}$CFU/g)		Control Population (Log$_{10}$CFU/g)	
		TSA-XLD	XLD	TSA-XLD	XLD
550	2	5.27 ± 0.26	3.64 ± 0.20	8.05 ± 0.07	7.75 ± 0.07
550	4	2.32 ± 0.24	2.05 ± 0.08	8.05 ± 0.08	7.83 ± 0.10
550	5	2.97 ± 0.56	3.08 ± 0.61	8.23 ± 0.08	7.38 ± 0.04
550	7	4.00 ± 0.20	2.23 ± 0.25	7.32 ± 0.14	7.03 ± 0.18
500	2	6.16 ± 0.14	5.42 ± 0.28	8.05 ± 0.07	7.75 ± 0.07
500	4	5.15 ± 0.19	4.10 ± 0.30	8.12 ± 0.10	7.95 ± 0.12
500	5	2.86 ± 0.58	2.26 ± 0.36	8.05 ± 0.08	7.83 ± 0.10
500	7	4.73 ± 0.19	3.14 ± 0.51	7.32 ± 0.14	7.03 ± 0.18
475	4	3.12 ± 0.76	2.64 ± 0.55	8.05 ± 0.08	7.83 ± 0.10
475	5	5.19 ± 0.41	4.46 ± 0.63	8.23 ± 0.05	8.18 ± 0.07
475	7	4.62 ± 0.28	4.02 ± 0.35	8.00 ± 0.15	7.92 ± 0.12
400	5	5.94 ± 0.28	5.76 ± 0.35	8.23 ± 0.05	8.18 ± 0.07
400	6	6.01 ± 0.18	5.53 ± 0.24	8.12 ± 0.10	7.95 ± 0.12
400	7	5.71 ± 0.13	5.56 ± 0.25	8.00 ± 0.15	7.92 ± 0.12

Table 2.15: High Pressure Processing of 33% Peanut Sauce

Pressure (MPa)	Time (Minutes)	Log$_{10}$ Reduction (CFU/mL)	Log S = (N/N$_0$)	Log$_{10}$ Injured (CFU/mL)	Water Activity
550	2	1.89	-1.89	1.64	0.99
550	4	5.73	-5.73	0.22	0.99
550	5	5.26	-5.26	0.04	0.99
550	7	3.32	-3.32	1.78	0.99
500	2	1.89	-1.89	0.73	0.99
500	4	2.97	-2.97	1.05	0.99
500	5	5.19	-5.19	0.6	0.99
500	7	2.59	-2.59	1.6	0.99
475	4	4.93	-4.93	0.48	0.99
475	5	3.04	-3.04	0.73	0.99
475	7	3.38	-3.38	0.61	0.99
400	5	2.29	-2.29	0.18	0.99
400	6	2.11	-2.11	0.48	0.99
400	7	2.29	-2.29	0.16	0.99

Figure 2.1: 33% Peanut Sauce High Pressure Processed at 550MPa. Closed squares represent the LogS value and the closed triangles represent the $\log_{10}$ injured.
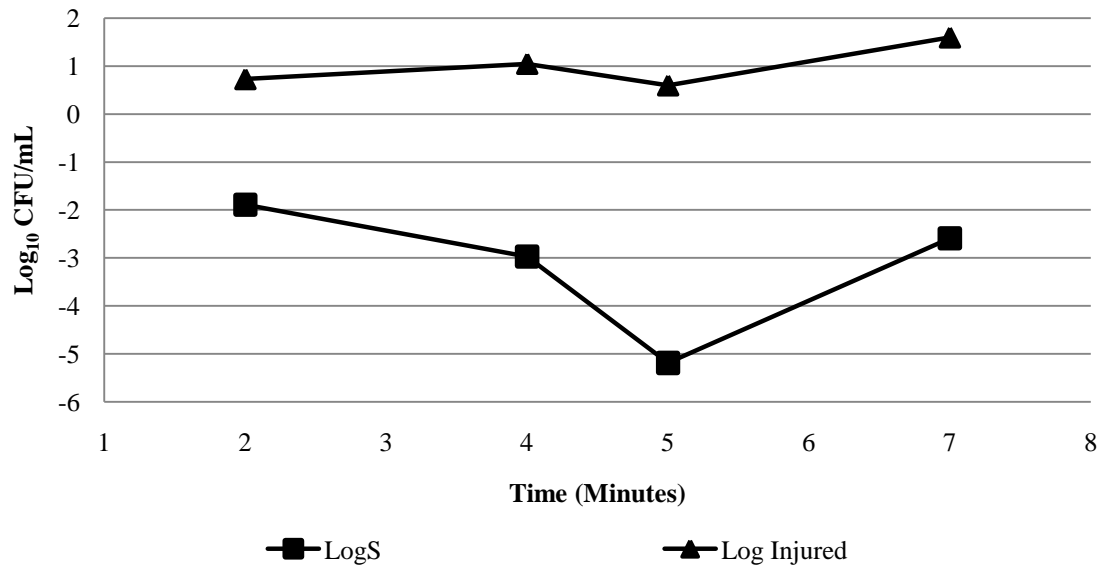
Figure 2.2: 33% Peanut Sauce High Pressure Processed at 500MPa. Closed squares represent the LogS value and the closed triangles represent the $\log_{10}$ injured.
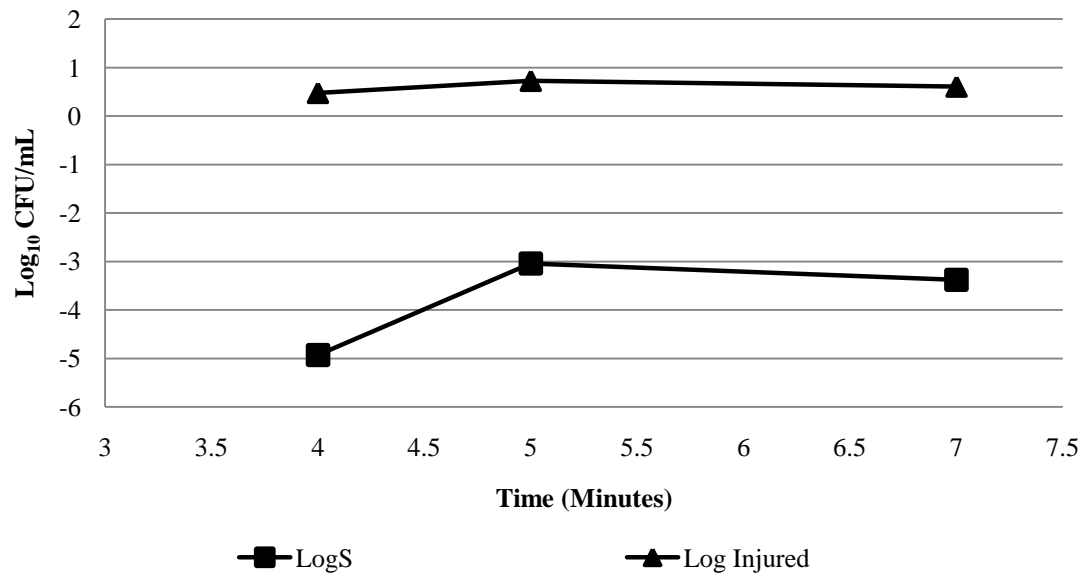
Figure 2.3: 33% Peanut Sauce High Pressure Processed at 475MPa. Closed squares represent the LogS value and the closed triangles represent the $\log_{10}$ injured.
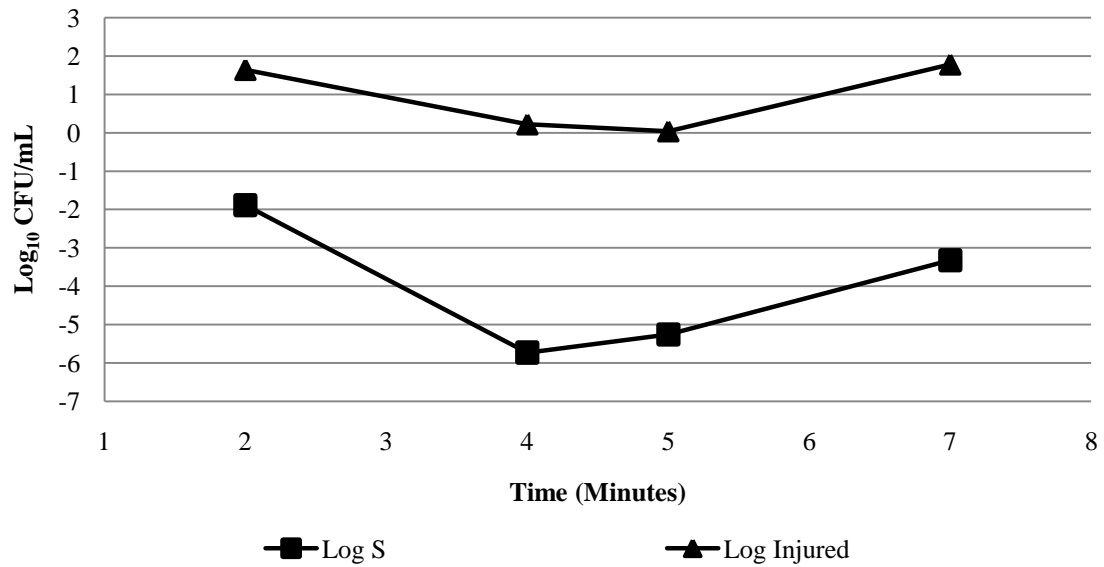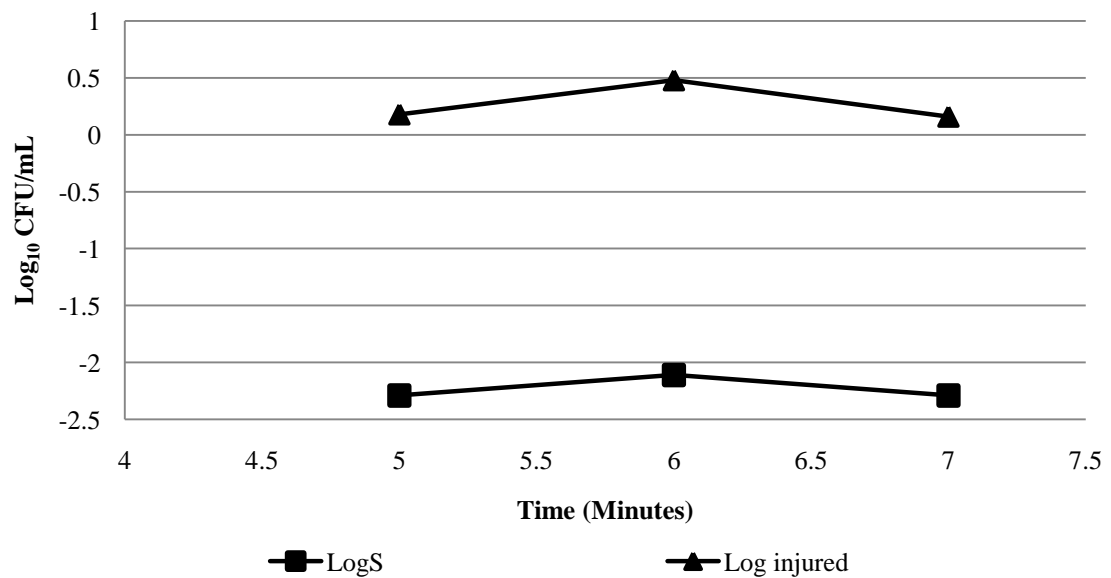

Figure 2.4: 33% Peanut Sauce High Pressure Processed at 400MPa. Closed squares represent the LogS value and the closed triangles represent the $\log_{10}$ injured.

V. Discussion

a. Phosphate Buffered Saline Validation Study

Numerous studies have been performed to test the effects of high pressure processing on *Salmonella* serotypes because there is a great interest in using HPP for an alternative processing step in the manufacturing of minimally processed foods. One study conducted by Alpas et al. used a matrix of oil and water to test *Salmonella* Typhimurium serotypes (2000). This matrix can be considered a minimally nutritive matrix that is similar to phosphate buffered saline (PBS). They reported a 1.54 $\log_{10}$ reduction when processed at 207MPa for 5 minutes and a 5.01 $\log_{10}$ reduction at 245MPafor 5 minutes. These values can be seen in Table 2.16: High Pressure Processing Effect on Salmonella. These values correspond to our findings, but we had a larger $\log_{10}$ reduction at 245MPa for 5 minutes (>8.05). Difference in specific serotypes used, the lack of nutrients in PBS, or a change in pH may have contributed to the inability of cells to recover after exposure to high pressure. This study demonstrated that our mixture of serotypes for our cocktail can be killed or injured by high pressure processing and our safety protocols are sufficient.

Table 2.16: High Pressure Processing Effect on *Salmonella*						
Culture Name	Pressure (MPa)	Time (min)	Temp (°C)	Matrix	Reduction (logs)	Reference
S. Enteritidis FDA	345	5	25	TSB	5.45	Alpas, 1999
S. Typhimurium E21274	345	5	25	TSB	5.71	Alpas, 1999
S. Typhimurium ATCC 14028	345	5	25	TSB	7.3	Alpas, 1999
S. Enteriditis VL	345	5	25	TSB	7.48	Alpas, 1999
S. choleraesius ssp. Choleraesuis Typhi ATCC6539	345	5	25	TSB	7.7	Alpas, 1999
S. choleraesuis ssp. Choleraesuis ATCC 10708	345	5	25	TSB	8.34	Alpas, 1999
S. Typhimurium E21274 VL	207	5	25	oil/water	1.54	Alpas, 2000
S. Typhimurium E21274 VL	276	5	25	oil/water	3.56	Alpas, 2000
S. Typhimurium E21274 VL	245	5	25	oil/water	5.01	Alpas, 2000
S. Enteriditis	350	5	30	Orange juice	>8	Doona, 2007
S. Senftenberg	340	10	23	PBS	4	Doona, 2007
S. Typhimurium	400	15	20	PBS	>6	Doona, 2007
S. Agona	300	2	6	orange juice	0.53	Whitney, 2007
S. Michigan	300	2	6	orange juice	3.95	Whitney, 2007

b. *Salmonella* Isolation

The morphology of each serotype was investigated to create a reference for identifying *Salmonella* against any background growth during enumeration studies in the event that there was a complete lack of growth on either TSA-XLD or XLD agars. Each serotype was carried through the FDA-BAM method for isolation and identification of *Salmonella*.

Typical growth of *Salmonella* on XLD agar is production of a pink colony that may or may not have a black center due to hydrogen sulfide production (Andrews & Hammack, 1998). All serotypes used in this study fall within this definition. The serotypes used also produced acid due to the fermentation of xylose and had a yellow halo due to a change in pH (Andrews & Hammack, 1998). This characteristic was important because it was discovered that potential spore-forming contaminants do no ferment xylose and thus have a dark red to purple color around their colonies, distinguishing them from *Salmonella* colonies.

Growth on HE agar and BS agar were as described by the FDA-BAM method as typical *Salmonella* colony morphology. More specifically, growth on HE agar produces blue/green colonies that may have black centers and growth on BS agar produces brown or black colonies with or without a metallic sheen and a brown halo around the colony that may turn black with prolonged incubation (Andrews & Hammack, 1998).

For a final confirmation, LIA and TSI slants were used, as recommended by the FDA-BAM method. Production of a yellow color in the butt of the TSI slant with or without hydrogen sulfide production is considered a positive for *Salmonella* (Andrews & Hammack, 1998). All cultures used demonstrated this morphology. Acid production in LIA resulting in a purple butt with or without hydrogen sulfide is also considered a positive reaction, but it is advised that only colonies that produce a distinctive yellow color in the butt of an LIA tube be considered negative for *Salmonella* (Andrews & Hammack, 1998). All cultures used produced a purple butt in LIA tubes, with hydrogen sulfide production being present in 4 of the 5 cultures. *S.* Heidelburg/Sheldon was the only culture that did not product hydrogen sulfide.

c. High Pressure Studies on Organic Peanut Butter

The lack of color change and significant oil separation of the hand-milled natural peanut butter led to the conclusion that natural peanut butter would potentially be a suitable food matrix for high pressure processing. There were no further studies on sensory attributes of high pressure processed peanut butter because the high pressure machine is not considered food grade, and thus all our samples are not for human consumption due to possible biohazard contamination.

Initial water activity measurements suggest that *Salmonella* will not proliferate in organic peanut butter, but may survive in a vegetative state. A water activity level below 0.85 is considered to be an environment not suitable for microbial growth, but not lethal. The literature confirms that *Salmonella* can survive in low water activity matrices such as peanut butter (Ma et al., 2009). Initially, it was proposed that a low water activity environment may inhibit recovery after high pressure treatment. However, initial

results showed that *Salmonella* survived well in organic peanut butter after high pressure treatment, indicating that organic peanut butter had a strong protective effect.

The results shown in Table 2.6: High Pressure Processed Organic Peanut Butter imply that *Salmonella* can survive pressures up to 600MPa in organic peanut butter and that the length of exposure to high pressure needs to be increased beyond 30 minutes to obtain a $\log_{10}$ reduction that would be useful for industrial use, that being at least a 5 $\log_{10}$ reduction. The processing hold time for high pressure processing on industrial products should be <5minutes in order to maintain reasonable costs and productivity, and thus, organic peanut butter would not be a suitable food matrix for industrial high pressure processing (Balasubramaniam et al., 2004). These results also indicate that there may be a link between water activity and the survivability of *Salmonella*. The enumeration data from 480 and 540MPa indicate that there was no reduction in *Salmonella* populations, which may have been due to a difference in initial inoculum levels.

Further investigation revealed three possible reasons for *Salmonella* survival in organic peanut butter. First, the low water activity may be inducing a vegetative state that makes *Salmonella* more resistant to pressure. It has been shown that stationary phase cells survive better when stressed than log phase cells (Alpas et al., 1999; Bayindirli et al., 2006; Bowman et al., 2008; Considine et al., 2008; Doona & Feeherry, 2007; Patterson, 2005; San Martin et al., 2002; Smelt, 1998). Another possible reason is that low water activity may induce filamentation, in which the biomass of *Salmonella* increases, but cellular number does not increase due to a lack of septum formation between cells (Kieboom et al., 2006; Mattick et al., 2000a). Filamentation in low water activity conditions may cause incorrect enumeration of *Salmonella* because an entire string of *Salmonella* give rise to one colony rather than one cell giving rise to one colony, which is the basis for modern enumeration techniques (Kieboom et al., 2006; Mattick et al., 2000a). Exposing the *Salmonella* to a high water activity such as the dilution step before plating may cause septum to form and therefore a rapid increase of cells. The time needed to form septa after exposure to stressful conditions is roughly 50 minutes, according to a study done by Ahmed and Rowbury (1971). This does not necessarily span the time for plating, but it may have an impact for confirmation protocols (FDA-BAM) since the peanut butter is kept in BPW overnight. The next possible reason for *Salmonella*'s survival in organic

peanut butter may be that the dense, lipid rich matrix may be forming protective pockets around the water droplets in the emulsion. *Salmonella* most likely resides in the water droplets in the peanut butter emulsion because it increases nutrient access as opposed to residing in the lipid portion where nutrient availability is limitied (Ma et al., 2009). It is not known what happens to an organic peanut butter emulsion under high pressure, but examining emulsions properties before and after high pressure processing may give insight into why organic peanut butter provides protection to *Salmonella* from high pressure.

To investigate the exact affect water activity had on *Salmonella*'s survivability in organic peanut butter, it was decided that the water activity should be raised above 0.95 because it is in this range that *Salmonella* will proliferate. This would also ensure that filamentation does not occur, improving enumeration methods. It would also reduce the density of the matrix and create an emulsion that would be more favorable for growth and less favorable for protective pocket formation.

d. Water Activity Studies

In order to raise the water activity of organic peanut butter, distilled water was mixed into the organic peanut butter using a stomacher. However, the water activity could not exceed 0.93 by only adding water and the mixtures separated. Separation in this context refers to peanut butter particles settling to the bottom of the container with a layer of water or oil on top. Segregation would not be an accurate representation for traditional peanut sauces and could lead to the creation of multiple matrices within a mixture in which *Salmonella* could survive high pressure processing. Thus, it was therefore concluded that an emulsifier was needed to increase the water activity of organic peanut butter while creating a uniform food matrix. It was difficult to maintain similar water activity measurements within the same sample without an emulsifier, perhaps because the water tended to pocket in various regions instead of being homogenous throughout the organic peanut butter.

The addition of 0.1% lecithin helped to improve the ability to mix distilled water and organic peanut butter, but there was a limit to the amount of water that could be added, with the water activity only

reaching 0.96. The initial lecithin mixtures were darker in color than the original organic peanut butter and were very dense and brittle.

The amount of lecithin being added was increased to 0.5% and the lecithin and organic peanut butter were heated in hopes that lecithin would coat the organic peanut butter particles in a more uniform manner to create a more stable emulsion. Water was added in smaller aliquots to ensure that the distilled water was being uniformly distributed. The addition of 0.1mL of 1xPBS caused water activity levels to increase, but allowing the mixture to equilibrate for 72 hours caused the water activity levels to drop below their original values. This indicates that water was being lost from the mixture, perhaps because it evaporated or condensed onto the package, or perhaps because water was not being tightly bound and integrated into the emulsion. This could be remedied by storing the packages in a refrigerator or by mixing the samples prior to opening the packages for inoculation. It was noted that oil separation occurred when the mixtures were stored, again indicating that the emulsion was not stable.

A high pressure study on the organic peanut butter and lecithin mixtures was conducted to continue the investigation on the relationship between *Salmonella* survivability and water activity of the food matrix. There was a marked decrease in $\log_{10}$ reductions at a water activity of 0.94. There were no studies performed on the effect of lecithin on *Salmonella*'s survival under stressful conditions, and thus the increased survival in increased water activity sauces may be due to lecithin rather than the water activity. The lecithin mixtures were still very dense, despite the addition of water. A sauce with lower viscosity and density was desired, with a peanut sauce made with 35% water being the goal. The levels of lecithin were increased to 0.7% in order to obtain an organic peanut butter mixture that contained 35% distilled water, but these levels exceeded the allowable limit of lecithin in food.

A more suitable emulsifier was needed, so a trial was conducted using dimodan as an emulsifier. However, this mixture could not be homogenized and a search for new emulsifiers continued.

Milk products proved to be suitable emulsifiers as they created a uniform food matrix when mixed with organic peanut butter and distilled water. Most of the unheated milk sauces separated overnight, but

coconut milk was the only milk based sauce that did not separate when unheated. Using coconut milk as an emulsifier also allowed the water activity to reach 1.00, which was higher than any of the other milks used, or any of the previous emulsifiers used. Regular coconut milk was used in the first sauces, but lite coconut milk was used in later trials because it was not as thick as regular coconut milk, but still had optimal emulsifying characteristics. The emulsion characteristics presumably come from the protein composition in coconut milk, so using a lite coconut milk where some of the fat is replaced with water should not affect the emulsion properties.

Trials using lite coconut milk in which the water activity was measured before and after heating had more consistent water activity measurements than previous mixtures. Heating the mixture did not have a uniform effect on the water activity because some sauces showed an increase in water activity after heating while other sauces had a decrease in water activity. The effect of heating on the emulsion properties of coconut milk were investigated and was shown that heating coconut milk caused the proteins within to coagulate and denature when heated above 80°C, which results in high viscosities (Tangsuphoom et al., 2005). The increase in viscosity helped to stabilize the emulsion and prevent separation when stored at 8°C overnight.

A high pressure study was conducted to determine if $\log_{10}$ reductions could reach 5logs or more, and to determine if there was a link between viscosity and *Salmonella* survival in organic peanut sauce. Viscosity readings before and after heating show that heating increases viscosity, due to the denaturation and coagulation of coconut proteins (Tangsuphoom et al., 2005). There were higher $\log_{10}$ reductions in native sauces than heat treated sauces, perhaps due to the increased viscosity in heated samples protecting *Salmonella* from the effects of high pressure. Sauces of similar viscosities did not have similar $\log_{10}$ reductions, indicating that viscosity is not the only factor responsible for determining *Salmonella* survival in organic peanut sauce. For example, the first and last sauce both had initial viscosity readings of 500cps, but there was a $2\log_{10}$ difference between the $\log_{10}$ reductions. It is also interesting to note that the sauce with the highest viscosity readings had an average $\log_{10}$ reduction of 7.38 and that there was relatively little

difference between $\log_{10}$ reductions in the native and heated samples for this sauce. Since the data shows a higher $\log_{10}$ reduction in native sauces, sauce preparation for future studies did not include a heating step.

e. High Pressure Processing on 33% Peanut Sauce

The data show that with increasing pressure and/or hold time, the $\log_{10}$ reductions increase, and the more *Salmonella* is lethally injured. Water activity was consistent throughout sauce formulation. Since $\log_{10}$ reductions increased compared to previous high pressure trials, the factor responsible may be the increase in water activity or the decrease in viscosity. Since high pressure processing is an isostatic process in which there is no gradient of pressure within a food matrix, the reason for increased $\log_{10}$ reductions is most likely water activity (Alpas et al., 2000; Considine et al., 2008). The increase in water activity may have also prevented filament formation, resulting in more accurate enumeration. Colony growth on XLD agar was consistently less than colony growth on TSA-XLD agar, indicating that there was an ubiquitous injured population. Differences between TSA-XLD and XLD enumerations for controls also indicate that the process of preparing samples and plating causes injury to some *Salmonella*. There was a slight decrease in injured cell counts in 33%peanut sauce samples than in organic peanut butter samples, indicating that increasing water activity provides a better environment for recovery. It is accepted that low water activity matrices provide an initial protection against high pressure but also inhibit injury recovery (Barbosa-Canovas et al., 1998; Considine et al., 2008; Goodridge et al*.,* 2006; Koseki & Yamamoto, 2007; San Martin et al., 2002; Smelt, 1998). Thus, it is reasonable to assume that high water environments such as the 33% peanut sauce provide less initial protection, but provide a better environment for recovery.

The following data points are not congruent with the data: 550MPa at 7 minutes, 500MPa at 7 minutes, 475MPa at 4 minutes, and 400MPa at 6 minutes. There are many possible reasons for these incongruencies. The first is that there may have been possible contamination during plating, possibly from a contaminated pipette tip or pipette barrel. Since then, pipette barrels are routinely sprayed with 90% ethanol and the end of the pipette is dipped in 90% ethanol to ensure that contamination is eliminated. The second possibility is that when the sauces were packaged in the polyethylene bags, a range of weights were used and the dilution factor this may have introduced was not accounted for. It was assumed that the

original amount of peanut sauce would not impact the final enumerations. The amount of peanut sauce used on future work was standardized to ensure there is no compounding dilution factor that was not taken into account. The method in which the cocktail was prepared may have caused clumping of cells due to the pellet not being completely dissolved after centrifugation. This may have resulted in variations in inoculation concentrations that could not be accounted for. The preparation of the cocktail was revised such that the pellet was completely dissolved before the cocktail was used to inoculate future peanut sauces. The standard deviations of the raw enumeration data suggest that control enumerations were very consistent whereas the sample enumerations were more variable, which may have contributed to data error.

This preliminary data exposed some flaws in the methodology of this high pressure study, which was necessary to ensure that the project would be accurate and reliable.

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Chapter 3: High Pressure Studies

I. Abstract

Recent *Salmonella* outbreaks have prompted the need for new processing options for peanut products. Traditional heating kill-steps have shown to be ineffective in lipid-rich matrices such as peanut products. High pressure processing is one such option for peanut sauce because it has a high water activity, which has proved to be a large contributing factor in microbial lethality due to high pressure processing. Four different formulations of peanut sauce were inoculated with a five strain *Salmonella* cocktail and high pressure processed. Results indicate that increasing pressure or increasing hold time increases $\log_{10}$ reductions. The Weibull model was fitted to each kill curve, with b and n values significantly optimized for each curve (p-value < 0.05). Most curves had an n parameter value less than 1, indicating that the population had a dramatic initial reduction, but tailed off as time increased, leaving a small resistant population. ANOVA analysis of the b and n parameters show that there are more significant differences between b parameters than n parameters, meaning that most treatments showed similar tailing effect, but differed on the shape of the curve. Comparisons between peanut sauce formulations at the same pressure treatments indicate that increasing amount of organic peanut butter within the sauce formulation decreases $\log_{10}$ reductions. This could be due to a protective effect from the lipids in the peanut butter, or it may be due to other factors such as nutrient availability or water activity. Sauces pressurized at lower temperatures had decreased $\log_{10}$ reductions, indicating that cooler temperatures offered some protective effect. Log_{10} reductions exceeded 5 logs, indicating that high pressure processing may be a suitable option as a kill-step for *Salmonella* in industrial processing of peanut sauces. Future research should include high pressure processing on other peanut products with high water activities such as sauces and syrups as well as research to determine the effects of water activity and lipid composition with a food matrix such as peanut sauces.

II. Introduction

There have been several outbreaks associated with peanuts and peanut products, including peanut butter, with the earliest outbreak occurring in 1996. This outbreak was due to *Salmonella* Mbandaka and *Salmonella* Seftenberg from peanut butter products in Australia (Burnett et al., 2000; Ng et al., 1996). In 2006, the United States experienced its first peanut butter outbreak when PulseNet discovered an increasing trend in *Salmonella* Tennessee isolates (CDC, 2007; Ma et al., 2009). Further investigation led to discovering the source of the contamination was from Peter Pan peanut butter produced from a single ConAgra plant in Sylvester, Georgia (CDC, 2007; Maki, 2009; Morrone, 2008). The next peanut butter outbreak was also discovered by PulseNet beginning in November, 2008. A container of King Nut peanut butter from a long term care facility tested positive for *Salmonella* Typhimurium and packages of peanut butter crackers made from peanut paste tested positive for *Salmonella* (CDC, 2009). It was concluded that the peanut paste that was produced in a single facility in Blakely, Georgia by Peanut Corporation of America (PCA) was contaminated (CDC, 2009). This prompted a recall of more than 400 peanut containing products and pet food products from 54 companies, making it one of the largest recalls in the United States (CDC, 2009; Maki, 2009).

Major components of peanut butter go though high temperature processing (i.e. roasting), and it has been generally assumed that this step would aid in killing *Salmonella* that had contaminated the raw product. However, studies on the heat tolerance of *Salmonella* in low water activity environments such as peanut butter suggest that *Salmonella* may be able to survive these high temperature processes (Ma et al., 2009; Mattick et al., 2000b; Mattick et al., 2001; Shachar and Yaron, 2006). *Salmonella* Tennessee inactivation rates in peanut butter were compared with other serotypes (Enteriditis, Heidelberg, Typhimurium) and it was determined that *S.* Tennessee had greater heat tolerance, with a D-value of 120 minutes at 90°C, indicating that *S.* Tennessee would not be killed under typical heat processing during peanut butter production (Ma et al., 2009).

Low water activity conditions such as those in peanut butter cause filamentation of *Salmonella* (Kieboom et al., 2006; Mattick et al., 2000a). This makes *Salmonella* more potent in low water activity foods because biomass increases, but the number of cells does not increase due to incomplete septa formation caused by stress induced from low water activity (Kieboom et al., 2006; Mattick et al*.,* 2000a). Once *Salmonella* is exposed to a higher water activity environment, the filamentous structure forms septa, and rapidly divides (Kieboom et al., 2006; Mattick et al*.,* 2000a). Traditional plating methods do not detect the rapid division of cells, and the large filamentous organism results in one single colony (Kieboom et al., 2006; Mattick et al., 2000a). This implies that traditional enumeration methods are not accurate and worst-case scenario data may not be accurate in deciding precautionary processing steps (Kieboom et al., 2006; Mattick et al., 2000a).

High fat matrices also provide protection to microorganisms such as *Salmonella* that are often overlooked. For example, the high fat content in chocolate is thought to protect *Salmonella* as it passes through the acidic environment of the stomach, making the infectious dose less than one hundred cells, and since peanut butter also has a high fat content, it is reasonable to assume the same low infectious dose for peanut butter outbreaks (Bell, 2002). Thus it is reasonable to conclude that high fat, low water activity foods such as peanut butter are vulnerable to dangerous outbreaks due this combination of increased heat tolerance, filament formation, and protection from digestive barriers.

High pressure processing has recently been employed as an alternative processing method to heating in foods such as guacamole, oysters, and fruit juices (Alpas et al., 2000; Bayindirli, et al, 2006; Caner et al., 2004; Casadei et al., 2002; Considine et al., 2008; Matser, et al., 2004; Palou et al., 2000; Rastogi et al., 2007). High pressure processing is an isostatic process in which the pressure is instantaneous without a gradient (Alpas, et al., 2000; Considine et al., 2008; Patterson, 2005). When the processing vessel is pressurized, the processing fluid and samples undergo adiabatic heating, in which the change in pressure also causes a change in temperature (Considine et al., 2008; Doona et al., 2007). High pressure processing does not break covalent bonds, which preserves endogenous nutrients, colors, and flavors (Considine et al., 2008; Mackey et al., 1994; Metrick et al., 1989). High pressure processing is effective against

microorganisms, but severity depends on the type of microorganism, growth stage, and environmental components such as salts and sugars (Alpas et al., 2000; Barbosa-Canovas et al., 1998; Considine et al., 2008). It is generally accepted that longer hold times and/or greater pressures increase lethality (Alpas et al., 1999; Considine et al., 2008).

The objectives of this research were to (i) determine if high pressure processing is a suitable process to lethally injure *Salmonella* in peanut butter sauce, (ii) to determine what factors affect *Salmonella's* viability in this matrix, and (iii) to determine if high pressure processing of this food is feasible for manufactures.

III. Material and Methods

Cultures

Five serotypes of *Salmonella* were obtained from frozen stock cultures from the Food Processing Center at University of Nebraska-Lincoln. The serotypes included *Salmonella* Branderup NVSL 96-12528 from chicken, *Salmonella* Oranienburg NVSL 96-12608 from chicken, *Salmonella* Typhimurium ATCC 14028, *Salmonella* Enteriditis IV NVSL 94-13062 Liquid chicken egg, and *Salmonella* Heidelberg/Sheldon 3347-1. Cultures were kept in 10% glycerol at -80°C until use. Cultures were restored by suspending 20μl of thawed culture into 5mL of Tryptic Soy Broth (TSB, Difco, MD), vortexed, and incubated at 37°C for 20-24 hours before use. For cocktail preparation, 4mL of each overnight serotype culture were combined into a centrifuge tube (20mL total) and spun down at 7,500 RPM at 4°C for 10 minutes (Beckman Coulter GS-15R, Beckman Coulter, CA). The liquid was decanted off and 20mL of 1x phosphate buffered saline (PBS) was added. The mixture was vortexed until the pellet was resuspended and the mixture was then centrifuged at 7,500 RPM at 4°C for 10 minutes. The liquid was decanted off and 2mL of 1x PBS was added. The cocktail was vortexed until the pellet was resuspended and was then used for inoculations. There were three independently prepared cocktails, one for each repetition. This ensured that there was an equal representation among the serotypes of *Salmonella* among the repetitions. Each cocktail was at a level of 10^{7-8} CFU mL^{-1} throughout the study.

Peanut Sauces

For the peanut sauce, organic peanut butter (Once Again NutButter, NY) and lite coconut milk (Thai Kitchen, CA) were first weighed out into a stainless steel bowl. These were mixed with a wire whisk until thoroughly homogenized and the sauce became a light tan color. Then distilled water was added and whisked into the sauce until homogenized. There were three independently prepared sauces, one used for each repetition. After each sauce was prepared, water activity (a_w) was measured (Aqua Lab, Decagon, WA) in duplicate and 7mL of the sauce was pipetted into each polyethylene high pressure bag to ensure that the inoculum was equally diluted in each peanut sauce bag (Prime Source Packaging, CA). The bags were then vacuum sealed and stored for 12-24 hours at 8℃ until inoculated. For the control and samples, there were 3 replications with each sample done in triplicate, totaling 9 bags per sample and 9 bags per control.

For our purposes, four different sauces were prepared using 0%, 15%, 20%, and 33% organic peanut butter. The amount of lite coconut milk and distilled water were kept equal to each other for all sauce formations. For example, the 20% peanut sauce was composed of 40% lite coconut milk and 40% distilled water. The 0% peanut sauce consisted of 50% lite coconut milk and 50% distilled water and was considered the base matrix

When the sauces were inoculated, the bags were aseptically cut open, 100μl of cocktail was pipetted into the bag, the bags were vacuum sealed and the contents were thoroughly mixed, ensuring the cocktail did not pocket in one region of the bag. The bags were then packaged into two additional bags, totaling three polyethylene layers as is required by safety protocols (Appendix H). The sauces were then brought to the high pressure room where the samples were high pressure processed and the controls were held at room temperature.

High Pressure Studies

The high pressure unit used throughout the study is the Stansted ISO Lab high pressure unit (Stansted, UK). The unit is capable of reaching pressures of 900MPa and temperatures of 90°C. The sample

basket has a 2.5 inch diameter and a length of 15 inches. The processing fluid is a 30:70 mixture of propylene glycol and water. The thermocouple for measuring the processing temperatures is located at the bottom of the pressure vessel and measures the temperature of the processing fluid. The vessel is not insulated, and thus, there is heat transfer between processing fluid and pressure barrel. Pressures and temperatures were recorded in real-time with the sensors responsible for these reading calibrated regularly (See Appendix A: High Pressure Processing Machinery Set-Up).

Pressures of 250, 400, and 600MegaPascals were used with a constant ramp rate of 600MPa min^{-1} and a depressurization rate of 1000MPa min^{-1}. Hold times at each pressure were 1, 2, 5 and 7 minutes. See Table 3.1: High Pressure Processing Parameters. Each high pressure cycle, or run, was analyzed to determine starting temperature, peak temperature, and final temperature of the processing vessel. For each run, there were 9 sample sauce pouches loaded into the basket, ensuring that they were placed in such as way to prevent any package ruptures that could lead to contamination.

Table 3.1: High Pressure Processing Parameters				
		Pressure (Mpa)		
		250	**400**	**600**
Percentage of Peanut Butter	**0%**	X	X	
	15%		X	X
	20%		X	X
	33%		X	X
X indicates processing at 1, 2, 5, and 7 minutes				

Salmonella **Enumeration**

After all the samples were processed, all the peanut sauce pouches were washed and stored at 8°C until plating began. The outer 2 layers were cut open and the original sauce pouches were ordered according to the plating scheme and placed in a Styrofoam cooler with cold packs to keep growth of *Salmonella* to a minimum. Each sauce pouch was mixed thoroughly prior cutting open the pouches by running the pouch back and forth on the edge of the bench-top, moving the sauce from one end of the pouch to the other, to ensure accurate enumeration. The sauce pouches were aseptically cut open and sauce was pippetted out using a sterile pipette tip or a serological pipette. The appropriate dilutions were plated

using the EDDY Jet spiral plater (IUL Instruments EDDY Jet, IUL S.A., ESP) on trypic soy agar (TSA, Neogen, MI). All samples and controls were spiral plated because spiral plating allows for there to be multiple dilutions on one plate, reducing the time between platings and reducing materials. The plates were kept at room temperature for two hours and then overlaid with xylose lysine deoxycholate agar (XLD, Neogen, MI) that was no warmer than 50°C to prevent any heat shock damage. Allowing *Salmonella* to recover on the TSA plates for 2 hours allowed for enumeration of the injured and uninjured *Salmonella* cells to give a worst-case scenario for contamination incidences. The plates were incubated at 37°C for 24 hours. Plates were counted using the Flash and Grow software and counter (IUL Instruments, IUL, S.A, ESP), which takes a digital image of the plate, and calculates the colony forming units per gram (CFU/g) based on the size of the plate being counted, the amount of sample spread on the plate, and the spiral pattern used. The colony forming units per gram were adjusted using the initial dilution factor to obtain more accurate *Salmonella* counts.

Statistical Analysis

Three replications of each experiment were performed with each sample done in triplicate. The data was analyzed in three progressive steps using Statistical Analysis Systems (SAS; SAS Institute, Cary, NC). First the data was checked for outliers by examining the standardized residuals. An observation was termed an outlier if it's Bonferroni corrected p-value was less than 0.05 (p-value < 0.05). Next, outliers were removed from the data set and nonlinear regression was used to fit the log transformed Weibull function to the logS data (Equation 3.1) for each repetition, pressure, and percentage combination. Finally, the estimated b and n parameters were analyzed using an ANOVA. Least significant differences were used for mean comparisons.

Equations

Equation 3.1: $LogS = Log_{10} {}^{N}/_{N_0}$ where, N is the final population (CFU/mL) and N_0 is the initial population (CFU/mL).

Equation 3.2: Weibull Model $Log\ S = -bt^n$ where, b and n are the scale and shape factors and t is time in minutes.

IV. Results

Enumeration data can be found in Tables 3.2-3.5. There were two outlying data points from the original plates which were removed for the final analysis because their Bonferroni corrected p-values were less than 0.05, or 5%. The data from each percentage of peanut sauce indicate that increasing pressure results in higher $\log_{10}$ reductions. Increasing the hold time also increases the $\log_{10}$ reductions. Increasing the percentage of organic peanut butter in the sauce matrix decreases the $\log_{10}$ reduction. Thus, *Salmonella* survived better in the 33% peanut sauce when compared to other sauce formulations. Controls had smaller standard deviations than samples, perhaps because high pressure processing introduced greater differences in enumeration data.

The average water activity for 0% peanut sauce was 1.0, for 15% the average water activity was 0.99, for 20% the average water activity was 0.99, and for 33% the average water activity was 0.99. All water activity measurements were above 0.99, which is above the 0.95 limit needed for *Salmonella* growth in a food matrix. Water activities in comparison to LogS values can be found in Tables 3.6-3.9.

The temperatures at which the high pressure vessel and samples were exposed to during high pressure processing can be found in Table 3.10-3.13. The initial temperature is the temperature of the vessel prior to high pressure. Peak temperature is the maximum temperature the samples were exposed to during high pressure processing, which was typically at the time which the target pressure was reached, or when hold time began. The final temperature was the temperature of the vessel after depressurization. Temperatures were recorded to assess if there was any protective effect associated with a change in temperature, and to determine if there were any changes in high pressure conditions that should be noted.

Each replication was fitted with the Weibull model (Equation 3.2), a non-linear regression model with parameters b and n. The b term dictated the degree of curvature, with a larger b indicative of a more

pronounced curvature and a smaller b indicative of a gradual curvature. The n value provided information

on how the population reacted to the high pressure. An n value greater than 1 indicated that there was a

shouldering effect, an n value equal to 1 indicated a linear relationship and an n value less than 1 indicated

a tailing effect. In most of the data, the b values were large and the n values were less than one, which

means that there was an initial drop in population, but that the population stabilized over time and there was

a residual tailing population that survived high pressure processing. All parameters for each replication had

p-values less than 0.05, indicating that the fits were not significantly different between replications. A table

of the parameters, the standard errors, and the p-values can be found in Table 3.14. Graphic representations

of each peanut sauce formulation and pressure combination can be found in Figures 3.1-3.6. The b and n

variables were also plotted versus pressure to determine how each variable changed when pressure was

altered. From Figures 3.7 and 3.8, it was concluded that as pressure increased, the b variable increased

while the n variable decreased. Final Weibull curves were constructed by averaging b and n values from the

replicates.

Analysis of b and n variables of the Weibull model were analyzed using ANOVAs. The first

ANOVA analyzed the significant differences between b values between pressures and percentages. In this

ANOVA, there was a significant interaction between pressure and peanut sauce percentage (p-value =

0.0252). The differences between treatments by comparing the least square means can be found inTable

3.15: Significant Differences of b-parameters Based on Least Square Means. By comparing the least square

means, it is noted that there is a significant difference between all treatments except treatment at 400MPa

between 15% and 20% peanut sauces (p-value = 0.5671). The standard error was 0.0915 for all least square

mean values.

The second ANOVA analyzed the n variable of the Weibull model. In this ANOVA, the type 3

interactions dictated that there was a significant effect between pressure and peanut sauce percentage (p-

value = 0.019). When each of the parameters is held constant, there is not a significant difference between

peanut sauce percentages when treated at 400MPa (p-value = 0.494), when the peanut sauce percentages

are held at 20% (p-value = 0.792) and when the peanut sauce percentage were held at 33% (p-value =

0.352). The differences between the least square means were analyzed to determine if there was a significant difference between combinations of treatments. The standard error for the least square means was 0.0424. A table of results can be found in Table 3.16: Significant Differences of n-parameters Based on Least Square Means.

Table 3.2: Enumeration Data: 0% Peanut Sauce

Time (Minutes)	250MPa		400MPa	
	$N_0 \pm SD*$	$N \pm SD*$	$N_0 \pm SD*$	$N \pm SD*$
1	8.25± 0.10	7.67 ± 0.07	8.25± 0.10	5.58 ± 0.12
2	7.98 ± 0.11	7.65 ± 0.06	8.01 ± 0.11	5.96 ± 0.12
5	7.98 ± 0.11	6.85 ± 0.10	8.01 ± 0.11	5.20 ± 0.11
7	7.98 ± 0.11	6.09 ± 0.10	8.01 ± 0.11	4.33 ± 0.32

*= (Log CFU/mL)

Table 3.3: Enumeration Data: 15% Peanut Sauce

Time (Minutes)	400MPa		600MPa	
	$N_0 \pm SD*$	$N \pm SD*$	$N_0 \pm SD*$	$N \pm SD*$
1	8.25± 0.10	6.30 ± 0.18	8.25± 0.10	2.90 ± 0.32
2	8.04 ± 0.09	5.74 ± 0.13	8.19 ± 0.07	3.82 ± 0.30
5	8.04 ± 0.09	5.48 ± 0.22	8.19 ± 0.07	3.27 ± 0.19
7	8.04 ± 0.09	4.50 ± 0.14	8.19 ± 0.07	2.81 ± 0.25

*= (Log CFU/mL)

Table 3.4: Enumeration Data: 20% Peanut Sauce

Time (Minutes)	400MPa		600MPa	
	$N_0 \pm SD*$	$N \pm SD*$	$N_0 \pm SD*$	$N \pm SD*$
1	8.25± 0.10	6.22 ± 0.24	8.14 ± 0.06	3.76 ± 0.31
2	8.14 ± 0.07	5.88 ± 0.12	8.14 ± 0.06	2.96 ± 0.28
5	8.14 ± 0.07	5.83 ± 0.11	8.14 ± 0.06	1.93 ± 0.40
7	8.14 ± 0.07	4.90 ± 0.33	8.14 ± 0.06	1.68 ± 0.34

*= (Log CFU/mL)

Table 3.5: Enumeration Data: 33% Peanut Sauce

Time	400MPa		600MPa	
(Minutes)	$N_0 \pm SD*$	$N \pm SD*$	$N_0 \pm SD*$	$N \pm SD*$
1	8.15 ± 0.08	6.95 ± 0.15	8.35 ± 0.08	4.27 ± 0.24
2	8.15 ± 0.08	6.62 ± 0.15	8.35 ± 0.08	4.01 ± 0.24
5	8.15 ± 0.08	6.19 ± 0.17	8.35 ± 0.08	2.23 ± 0.23
7	8.15 ± 0.08	5.94 ± 0.26	8.35 ± 0.08	2.21 ± 0.32

*= (Log CFU/mL)

Table 3.6: Water Activity Data: 0% Peanut Sauce

Time	250MPa	Water Activity	400MPa	Water Activity
(Minutes)	Log S	(a_w)	Log S	(a_w)
1	-0.58	1.00	-2.67	1.00
2	-0.33	1.00	-2.05	1.00
5	-1.13	1.00	-2.81	1.00
7	-1.89	1.00	-3.68	1.00

Table 3.7: Water Activity Data: 15% Peanut Sauce

Time	400MPa	Water Activity	600MPa	Water Activity
(Minutes)	Log S	(a_w)	Log S	(a_w)
1	-1.95	0.99	-5.35	0.99
2	-2.3	0.99	-4.37	0.99
5	-2.56	0.99	-4.92	0.99
7	-3.54	0.99	-5.38	0.99

Table 3.8: Water Activity Data: 20% Peanut Sauce

Time	400MPa	Water Activity	600MPa	Water Activity
(Minutes)	Log S	(a_w)	Log S	(a_w)
1	-2.03	0.99	-4.38	0.99
2	-2.26	1.00	-5.18	0.99
5	-2.31	1.00	-6.21	0.99
7	-3.24	1.00	-6.46	0.99

Table 3.9: Water Activity Data: 33% Peanut Sauce

Time	400MPa		600MPa	
(Minutes)	Log S	Water Activity (a$_w$)	Log S	Water Activity (a$_w$)
1	-1.2	0.99	-4.08	0.99
2	-1.53	0.99	-4.34	0.99
5	-1.96	0.99	-6.12	0.99
7	-2.21	0.99	-6.14	0.99

Table 3:10: High Pressure Vessel Temperatures: 0% Peanut Sauce

Time	250MPa				400MPa			
(Minutes)	Log S	Initial °C	Peak °C	Final °C	Log S	Initial °C	Peak °C	Final °C
1	-0.58	18.5	24.7	14.2	-2.67	20.9	25.8	13.6
2	-0.33	24.0	27.4	15.2	-2.05	19.1	23.7	9.2
5	-1.13	23.3	23.9	14.3	-2.81	18.6	22.6	6.4
7	-1.89	22.6	26.2	14.7	-3.68	18.4	22.6	5.6

Table 3:11: High Pressure Vessel Temperatures: 15% Peanut Sauce

Time	400MPa				600MPa			
(Minutes)	Log S	Initial °C	Peak °C	Final °C	Log S	Initial °C	Peak °C	Final °C
1	-1.95	21.1	24.9	13.1	-5.35	21.2	26.3	11.2
2	-2.3	23.2	28.6	17.0	-4.37	19.0	24.0	6.4
5	-2.56	22.1	28.1	15.3	-4.92	18.0	23.6	4.2
7	-3.54	22.1	27.8	15.1	-5.38	17.7	23.1	2.9

Table 3:12: High Pressure Vessel Temperatures: 20% Peanut Sauce

Time	400MPa				600MPa			
(Minutes)	Log S	Initial °C	Peak °C	Final °C	Log S	Initial °C	Peak °C	Final °C
1	-2.03	21.1	25.5	13.6	-4.38	24.3	30.5	16.0
2	-2.26	23.6	29.1	17.6	-5.18	23.4	29.8	16.8
5	-2.31	23.0	28.9	16.1	-6.21	23.5	30.0	13.6
7	-3.24	22.6	28.3	15.6	-6.46	22.1	30.1	13.3

Table 3:13: High Pressure Vessel Temperatures: 33% Peanut Sauce

Time (Minutes)	400MPa				600MPa			
	Log S	Initial °C	Peak °C	Final °C	Log S	Initial °C	Peak °C	Final °C
1	-1.2	18.2	25.2	13.6	-4.08	23.4	29.3	15.9
2	-1.53	18.9	25.0	11.8	-4.34	22.8	29.3	14.7
5	-1.96	20.5	24.5	10.6	-6.12	22.6	29.0	11.7
7	-2.21	19.5	24.6	10.3	-6.14	22.1	29.2	11.8

Table 3.14: Weibull Model Parameters

Replication	Pressure (MPa)	Percent Organic Peanut Butter (%)	b	SE	n	SE	p-value
1	250	0	0.1721	0.1233	1.1641	0.3952	0.0277
2	250	0	0.2267	0.1543	1.0967	0.3777	0.0287
3	250	0	0.3454	0.2157	0.8533	0.3555	0.0408
1	400	0	2.0835	0.5181	0.3269	0.1602	0.0219
2	400	0	2.2678	0.4597	0.2413	0.1354	0.0176
3	400	0	2.3735	0.3978	0.2131	0.1134	0.0128
1	400	15	1.779	0.1751	0.3192	0.0636	0.0036
2	400	15	1.802	0.2821	0.2878	0.1025	0.0096
3	400	15	2.0085	0.372	0.251	0.1231	0.0144
1	600	15	4.8208	0.4236	0.0312	0.0658	0.005
2	600	15	5.0317	0.4626	0.00804	0.0698	0.0057
3	600	15	4.9942	0.5751	0.0082	0.0884	0.009
1	400	20	1.74	0.1762	0.2489	0.0674	0.0044
2	400	20	1.9913	0.4175	0.2401	0.1401	0.0188
3	400	20	2.085	0.35	0.1484	0.1173	0.0145
1	600	20	4.5113	0.1027	0.193	0.0155	0.0002
2	600	20	4.7726	0.3142	0.1641	0.0456	0.0022
3	600	20	4.1615	0.2073	0.232	0.0334	0.0011
1	400	33	1.1656	0.0301	0.3608	0.0164	0.0002
2	400	33	1.2012	0.0894	0.3364	0.0478	0.002
3	400	33	1.3027	0.0123	0.2054	0.00643	0.0001
1	600	33	4.0407	0.294	0.2214	0.049	0.0024
2	600	33	3.8283	0.3755	0.2486	0.0653	0.0041
3	600	33	3.8933	0.174	0.26	0.0296	0.0008

Table 3.15: Significant Differences of b-parameters Based on Least Square Means[a]

Pressure (MPa)	Peanut Sauce Percentage			
	0%	15%	20%	33%
250	0.2481[a, x]			
400	2.2416[a, y]	1.8632[b, x]	1.9388[b, x]	1.2232[c, x]
400		4.9489[a, y]	4.4818[b, y]	3.9208[c, y]

Standard Error = 0.09146

[a]Differences between LSMeans in the same column are significant when preceding letters (x-z) are not the same (P<0.05). Differences between LSMeans in the same row are significant when preceding letters (a-c) are not the same (P<0.05).

Table 3.16: Significant Differences of n-parameters Based on Least Square Means[a]

Pressure (MPa)	Peanut Sauce Percentage			
	0%	15%	20%	33%
250	1.0380[a,x]			
400	0.2604[a, y]	0.2860[a, x]	0.2125[a, x]	0.3009[a, x]
400		0.01581[a, y]	0.1964[b, x]	0.2433[b, x]

Standard Error = 0.04244

[a]Differences between LSMeans in the same column are significant when preceding letters (x-z) are not the same (P<0.05). Differences between LSMeans in the same row are significant when preceding letters (a-c) are not the same (P<0.05).

Table 3.17: Optimal Parameters

| Sauce %	Pressure (MPa)	Time (Minutes)	LogS
0	400	7	-3.68
15	600	7	-5.38
20	600	7	-6.46
33	600	7	-6.14
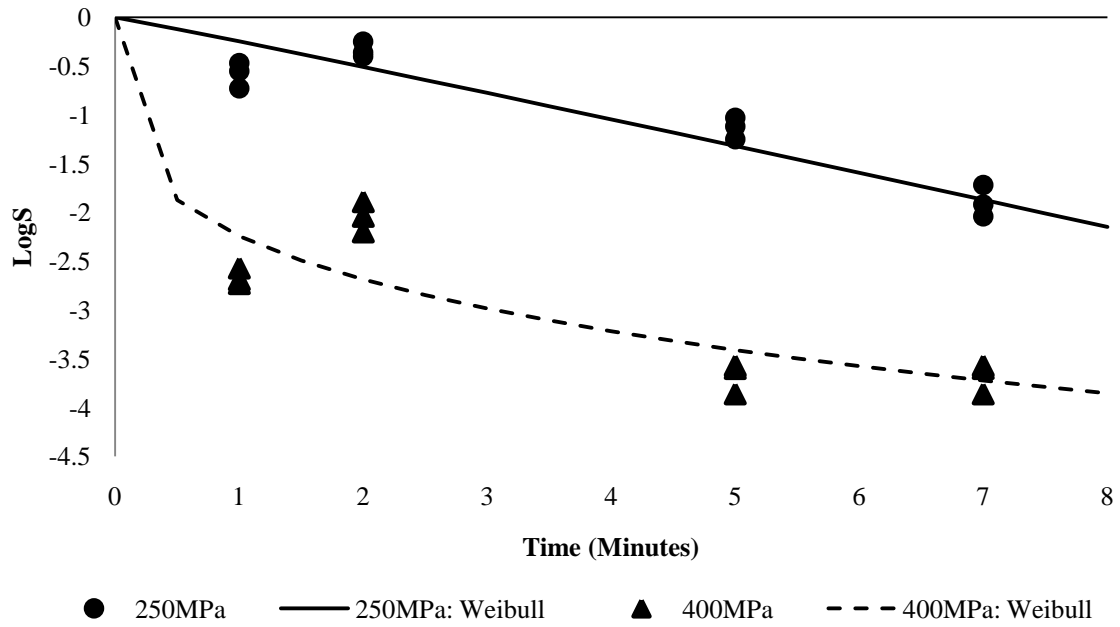
Figure 3.1: *Salmonella* **Survival in 0% Peanut Sauce Fitted with the Weibull Model.** Data are means of each replicate. Values for b and n parameters for 250MPa curve are 0.248 and 1.038, respectively. Values for b and n parameters for 400MPa curve are 2.242 and 0.260, respectively.
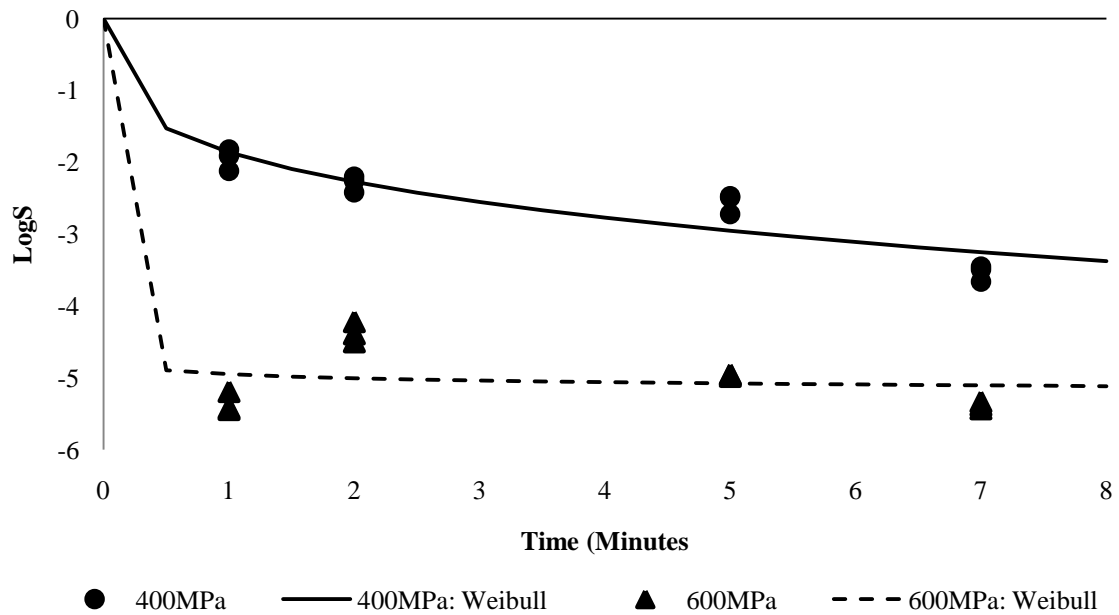
Figure 3.2: *Salmonella* **Survival in 15% Peanut Sauce Fitted with the Weibull Model.** Data are means of each replicate. Values for b and n parameters for 400MPa curve are 1.86 and 0.286, respectively. Values for b and n parameters for 600MPa curve are 4.95 and 0.0158, respectively.
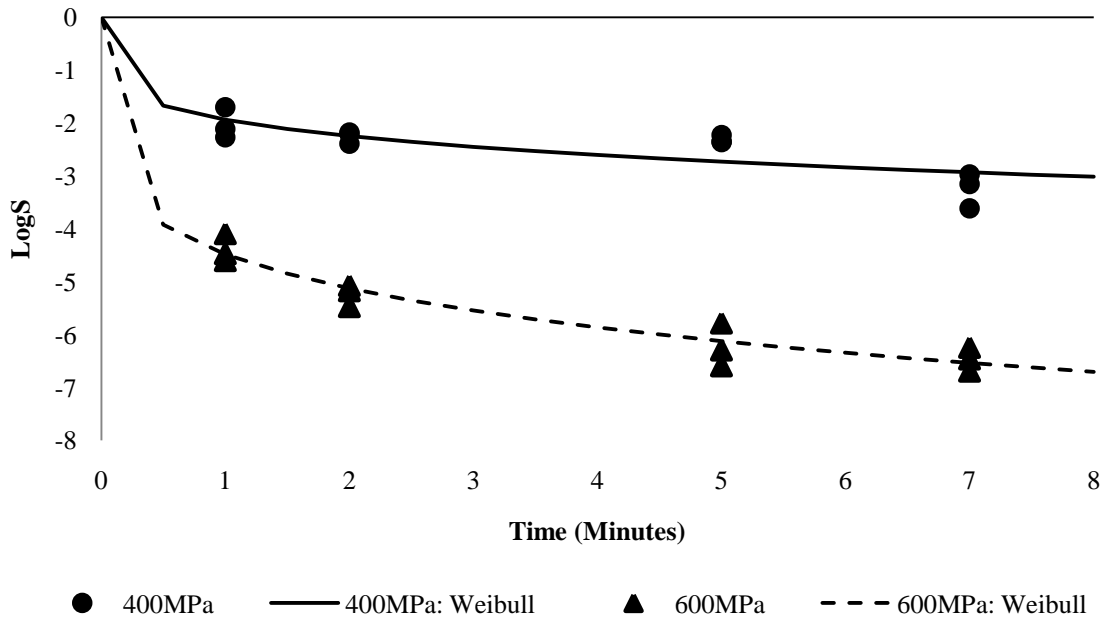
Figure 3.3: *Salmonella* **Survival in 20% Peanut Sauce Fitted with the Weibull Model.** Data are means of each replicate. Values for b and n parameters for 400MPa curve are 1.94 and 0.212, respectively. Values for b and n parameters for 600MPa curve are 4.48 and 0.196, respectively.
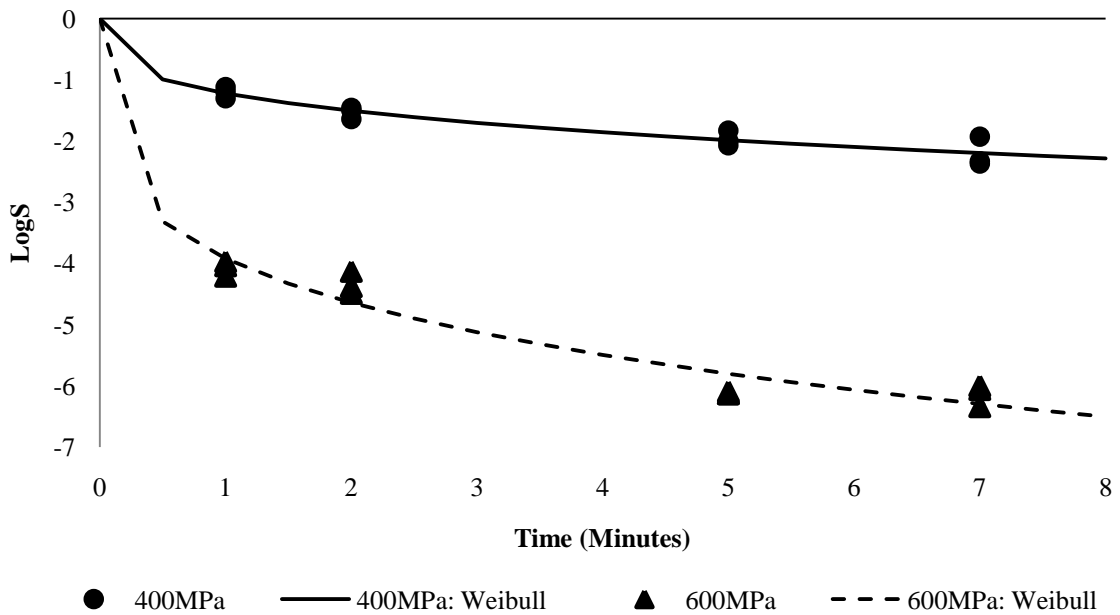
Figure 3.4: *Salmonella* **Survival in 33% Peanut Sauce Fitted with the Weibull Model.** Data are means of each replicate. Values for b and n parameters for 400MPa curve are 1.22 and 0.301, respectively. Values for b and n parameters for 600MPa curve are 3.92 and 0.243, respectively.
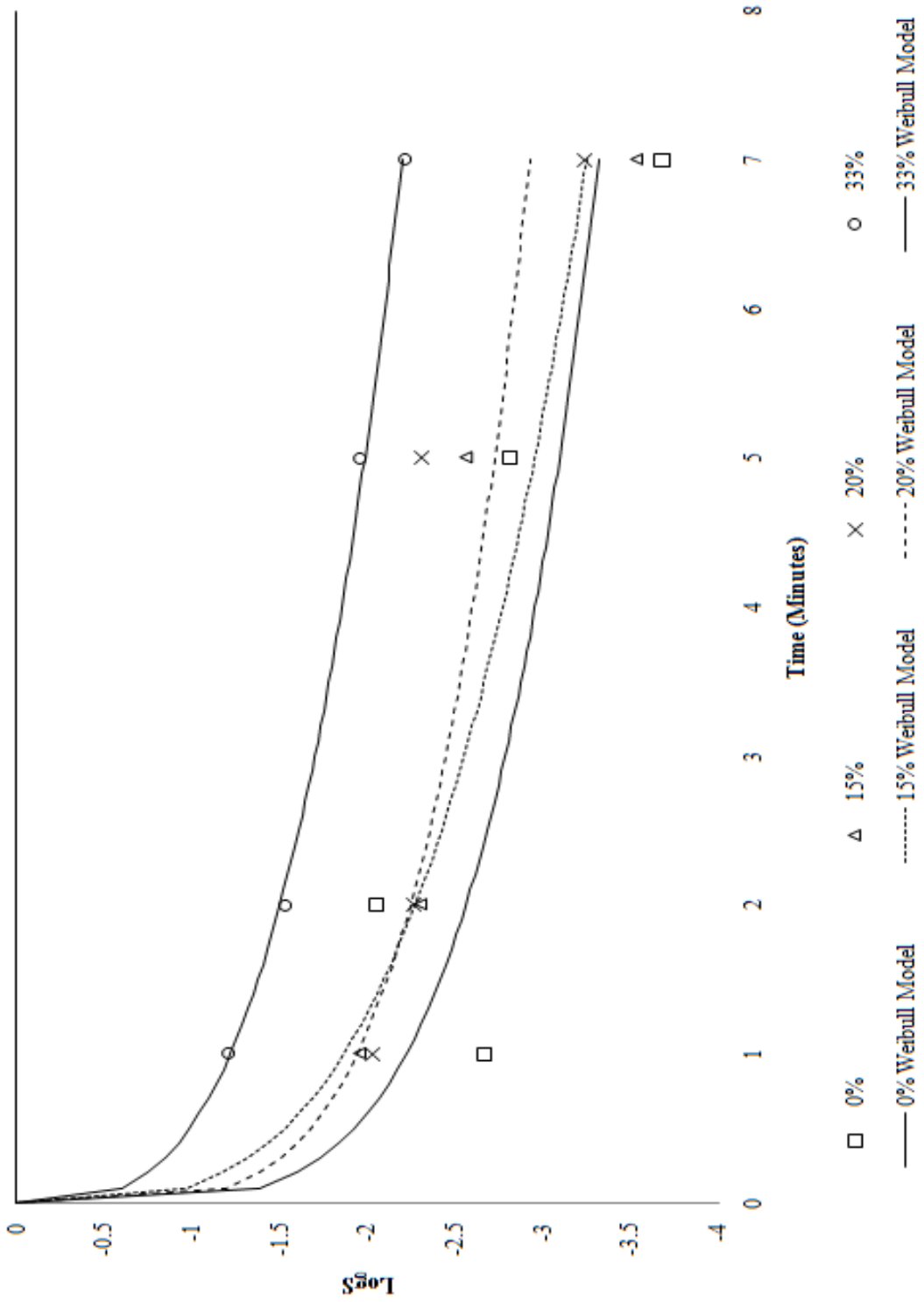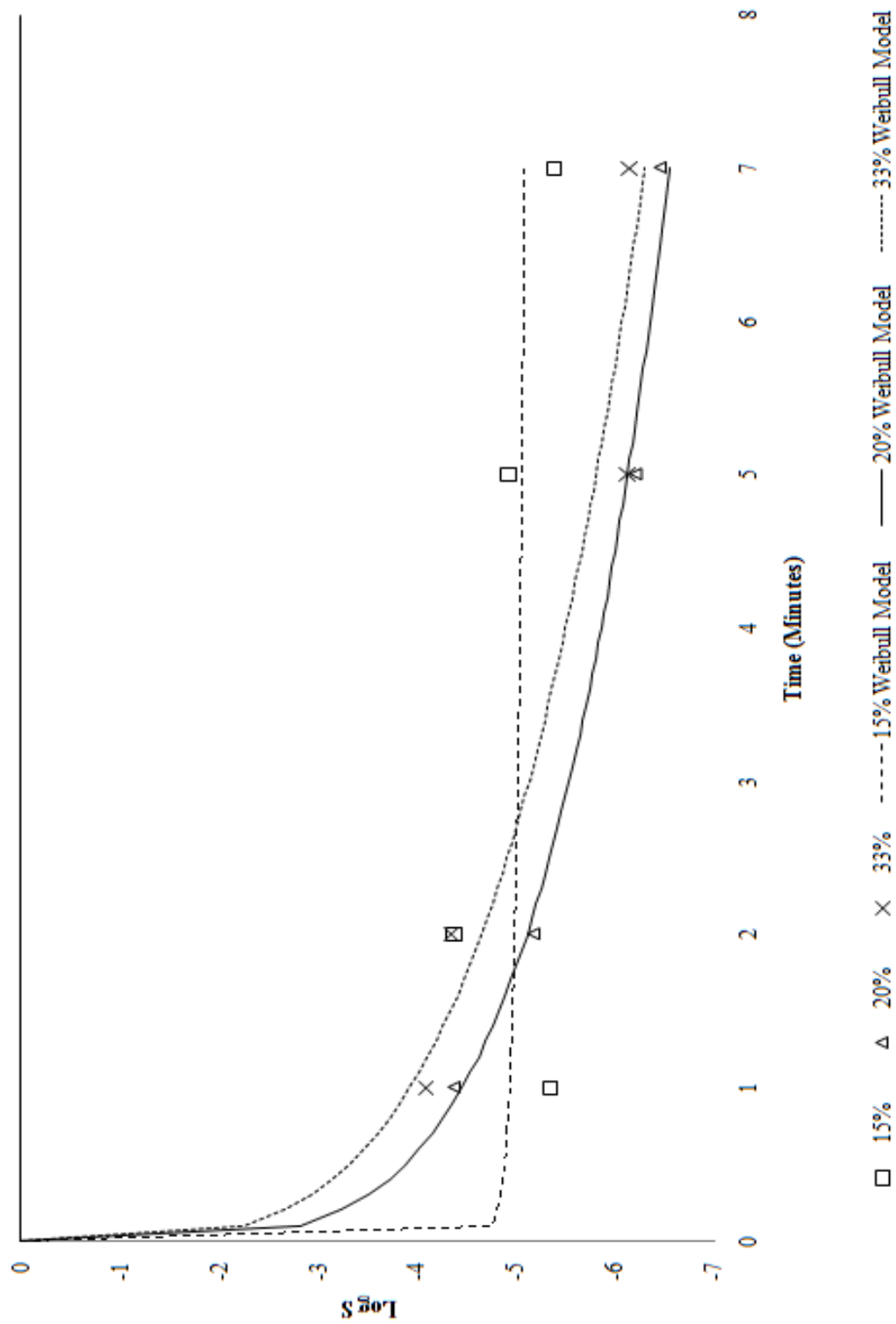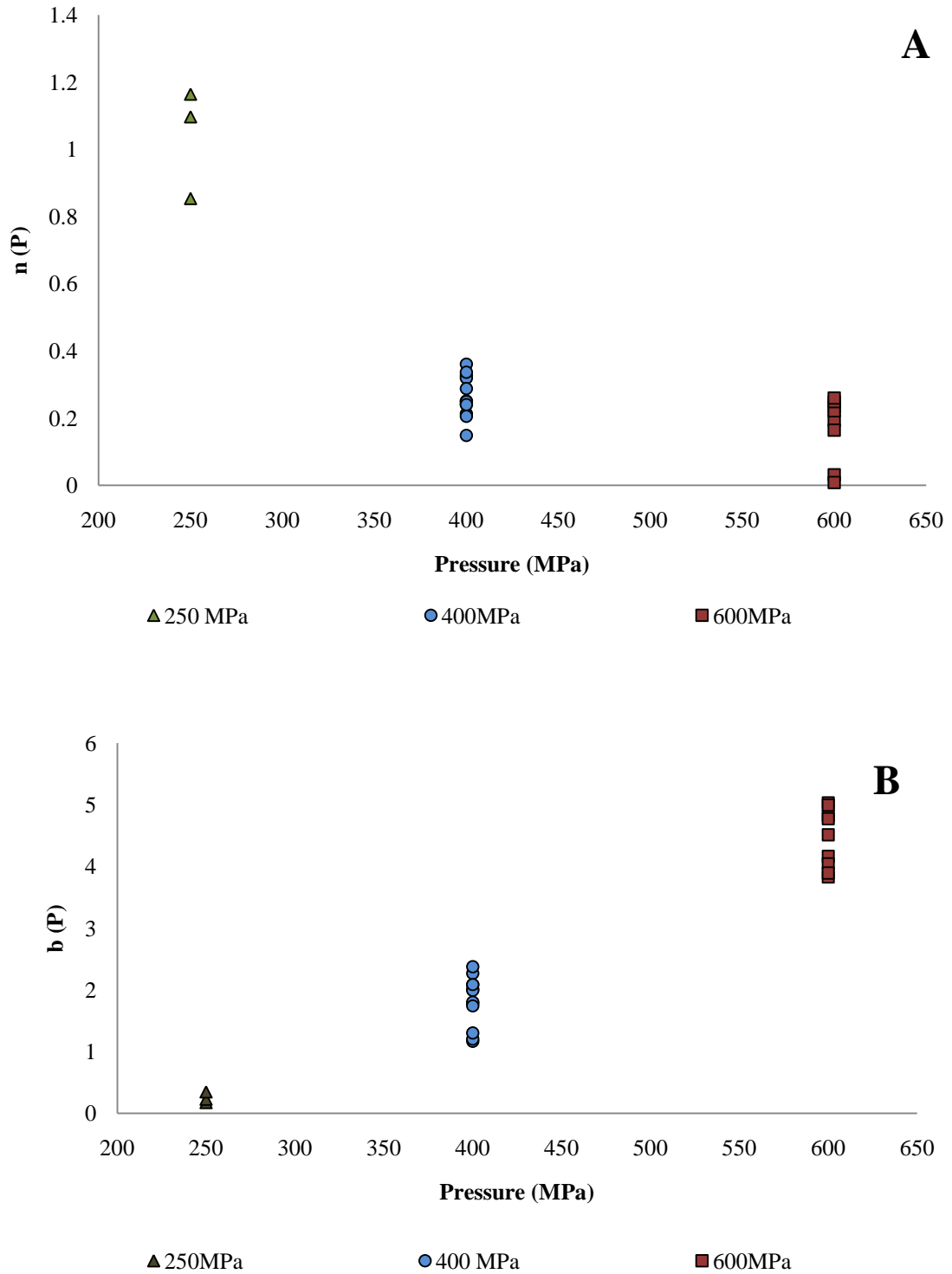
Figure 3.5: *Salmonella* Survival at 400MPa.

Figure 3.6: *Salmonella* Survival at 600MPa.

Figure 3.7:Pressure Dependence of the Weibull Model Parameters.
 Figure A is the pressure dependence on parameter n. Figure B is the pressure
dependence on parameter b.

V. Discussion and Conclusions

The variation in initial population enumerations was due to runs being conducted on different days, without having the ability to maintain the same inoculum from day to day. Hence, this was used as the N_0 such that the initial levels could be accounted for in each instance. The data show final population enumerations to be reduced as pressure or time is increased, which results in smaller LogS values. Reductions in LogS values represent the reductions due to lethal injuries to *Salmonella*. A worst-case scenario was warranted and thus no injury data was collected using selective media. A cocktail of *Salmonella* was used instead of comparing the serotypes individually because it is well known that serotypes vary in their high pressure survivability (Alpas et al., 1999). The goal was to assemble serotypes that represent those found in food borne outbreaks and to determine how they as a collective respond to high pressure processing. Therefore, any conclusions are made on the cocktail and not any specific serotype within the cocktail.

Standard deviations from enumeration data suggest that there was greater variability within the sample population than the control population. However, there were consistent decreases in sample populations after high pressure processing, indicating that peanut sauce did not provide complete protection from the effects of high pressure processing. The high water activities of the peanut sauces also suggest that there was not any filamentation as seen with low water activity products in earlier work. The data were analyzed for outliers, in which two plates were significantly different (p-value <0.05) and excluded from any further analysis, including enumeration averages.

Salmonella reductions and Weibull curves were analyzed by evaluating relationships between the peanut sauce components as well as high pressure parameters. The organic peanut butter percentages and water activities were evaluated for the peanut sauces and the hold times and pressures were evaluated for the high pressure parameters.

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The percentage of organic peanut butter had an effect on *Salmonella* survival. As organic peanut butter percentage increased, the LogS values increased and $\log_{10}$ reductions decreased. This indicated that organic peanut butter had a protective effect on *Salmonella,* which may have been due to an increase in readily available nutrients or an increase in the lipid content. Increased amounts of organic peanut butter may have provided *Salmonella* with greater amounts of readily available nutrients that were not found in lite coconut milk or distilled water that contributed to injury recovery (Considine *et al.,* 2008; Hoover *et al.,* 1989). The availability of calcium and magnesium in particular might have helped stabilize ribosomes after processing, which may have helped *Salmonella* replace damaged or destroyed proteins. This correlation may be changed by using peanut butter formulations with added salts and sugars, as these are known to affect microorganism survival under high pressure (Considine *et al.,* 2008; Hoover *et al.,* 1989). The protective effect may also have been due to increased lipid content. *Salmonella* have increased heat tolerance in lipid-rich matrices, and thus it is reasonable to conclude that increased lipid content may provide a protective effect against pressure and temperature changes due to adiabatic heating (Bell, 2002; Goepfert and Biggie, 1968; Goepfert et al., 1970; Mattick et al., 2000a; Shachar and Yaron, 2006). *Salmonella* are thought to survive heat in lipid matrices by residing in the water-portion of the matrix and it is proposed that the lipids form a micelle-like structure around the water droplets, forming a protective shell that buffer the effects of high temperatures and high pressures (Bell, 2002; Goepfert and Biggie, 1968; Goepfert et al., 1970; Mattick et al., 2000a; Schaschke et al., 2007; Shachar and Yaron, 2006). Studies have shown that lipids crystallize under high pressure, which may also contribute to the rigidity of the micelle-structure and the protective effects against high pressure and temperature (Schaschke et al., 2007). As pressure increases, lipids become more viscous and solidify under relatively low pressures (<100MPa) (Schaschke et al., 2007). However, the interaction between lipid and other components within a food matrix such as proteins and vitamins causes the solidification point to vary (Schaschke et al., 2007). The effects on high pressure on a lipid rich matrices or emulsions have yet to be fully understood and more research is needed, but it is reasonable to conclude that organic peanut butter helped protect *Salmonella* from high pressure processing, either by one of the proposed methods, or another method not mentioned here.

Increasing water activity to ≥0.99 had an effect on the survivability of *Salmonella* such that LogS values decreased to values below -6.00. Water activities for each peanut sauce varied slightly such that peanut sauces with higher percentages of organic peanut butter had slightly decreased water activity values. A study by Setikaite et al. indicated that increasing water activity resulted in larger $\log_{10}$ reductions or lower logS values (2009). That study used *E. coli* as their model organism and subjected it to water activities ranging from 0.90 to 0.99 using various humectants such as glycerol, fructose, sodium chloride and sorbitol (Setikaite et al., 2009). It was concluded that protection to high pressure processing offered by lower water activity matrices mimics other studies that indicated that lower water activities provided increased heat tolerance (Goepfert et al., 1970; Ma et al., 2009; Setikaite et al., 2009; Shachar and Yaron, 2006). Our results were consistent with these in that *Salmonella* had larger logS values in peanut sauces that contained a higher percentage of organic peanut butter in the matrix, which had reduced water activities. The decrease in water activity seen across the peanut sauces was across a narrower range than the Setikaite study, but there was a noticeable trend, nonetheless. Water activity changes do not need to be dramatic for there to be a drastic change in microorganism survival, as shown in a study conducted by Oxen and Knorr. In this study, $\log_{10}$ reductions were >7 at a water activity level of 0.96, but there was no reduction at water activity of 0.91 for *Rhodotorula rubra* (Oxen and Knorr, 1993). Other studies have also indicated that lowered water activities may provide a protective effect, but also inhibits injured microorganisms from recovering (Barbosa-Canovas et al., 1998; Considine et al., 2008; Goodridge et al., 2006; Koseki & Yamamoto, 2007; San Martin et al., 2002; Smelt, 1998). Future studies should investigate the affect of water activity across a broader range for *Salmonella* inactivate rates in peanut sauces.

The ramp rate, depressurization rate and processing temperatures were constant throughout the study. There were changes in processing temperatures that were due to adiabatic heating. The adiabatic heating effect seen when the samples are pressurized (3-9°C increase per 100MPa) is thought to cause lethal injury to microorganisms (Balasubramaniam et al., 2004). The adiabatic heating effect was measured in the processing fluid as the peak temperature, but the actual temperature of the peanut sauce could not be measured accurately. The peanut sauce's thermodynamic properties such as specific heat would help to predict what the actual peak temperature was, but since the temperature change of the processing fluid and

pressure vessel needed to be taken into account, actual temperature change could only be estimated (Otero and Sanz, 2003). Since heat tends to have a radiating effect, the size of the peanut sauce package may also have a compounding effect that must be taken into account for industrial applications where peanut sauce packages are larger (Otero and Sanz, 2003). The sample pouches in this study were small and thus was not considered to be an issue. From measurements taken from the processing fluid thermocouple, it was noticed that temperature changes in the processing fluid may have an effect on LogS values. Data for 15% peanut sauce pressurized at 600MPa had decreasing temperatures as time was increased and thus had lower temperatures at each time point than other sauces. The LogS values were larger than was expected, indicating that the lowered temperatures may have provided some protection. Keeping the vessel the same temperature between runs proved to be exceedingly difficult because temperature readings were taken from the processing fluid within the pressure vessel, which was not always consistent with the temperature of processing fluid in the reservoir tanks. Thus, when the vessel was filled with processing fluid to begin the pressure cycle, temperatures may have changed from what they were originally set. Temperature fluctuation was also affected by the pressure vessel not being insulated, which allowed for a transfer of heat during processing. The temperature change from start to finish also caused the vessel to be cooler after a high pressure run and thus the vessel progressively became colder as more runs were performed.

Increased hold times and/or pressures were consistently related to increased $\log_{10}$ reductions or decreased logS values. Literature on high pressure processing indicate that this relationship holds true due to the isostatic nature of high pressure processing, LeChatlier's principle, and the mechanisms in which high pressure lethally injures microorganisms (Alpas et al., 2000; Caner et al., 2004; Considine et al., 2008; Doona and Feeherry, 2007; Farr, 1990; Patterson, 2005;Pilavtepe-Celik et al., 2008; Rastogi et al., 2007; San Martin et al., 2002; Ting et al., 2002; Westerlund, 1994). Statistical analyses show that there was a significant interaction between pressures and sauce percentages, indicating that there were significant differences between data when one parameter was held constant.

The Weibull curve shape parameters provide a population profile, in which the n parameter describes resistance. Each kill curve replicate was fit with b and n values that had a significant fit (p-value

< 0.05). The Weibull model for each treatment was found by averaging values across each replicate. The value of the n parameter decreased as pressure was increased, indicating that the tailing effect became more pronounced at higher pressures and the resistant population decreased. Other high pressure studies have shown that the n variable decreases as pressure increases (Buzrul et al., 2008)The value of the b parameter increased as pressure was increased, indicating that there was a larger initial reduction in *Salmonella* at high pressures.

The data was also analyzed based on either the b or n variable of the Weibull model for each kill curve using an analysis of variance (ANOVA). The ANOVA based on the b parameter had many more significant differences between least square means (LSMeans) than the ANOVA based on the n parameter. This indicated that the b parameter changed more between pressure/hold time data curves than the n parameter. All curves except one (250MPa, 0%) exhibited similar tailing effects, indicating that most treatments had a resistant sub-population and that they were similar in their logistics. Increasing pressure and/or hold time resulted in a decrease of resistant population but also indicated that there was a resistant population present under all conditions tested. The tailing effect describes a process where a sensitive population is initially affected, leaving a resistant subpopulation (Abee & Wouters, 1999; Alpas et al., 2000; Buzrul et al., 2008). For 0% peanut sauces processed at 250MPa, the relationship was linear (n = 1), but that may be because of the range of hold times that were tested. If the hold time was extended beyond 7 minutes, the Weibull curve may have shown tailing effects (n < 1).

The viscosity of the peanut sauces were affected by high pressure processing in that higher pressures and longer times resulted in more viscous sauces. The sauce characteristics before and after high pressure processing were not measured by any standard methods because preliminary studies indicated that viscosity did not affect *Salmonella*'s survival in peanut sauce. It is assumed that viscosity or density did not affect the isostatic nature of high pressure processing. There have been studies using whey proteins as a synthetic food matrix for high pressure processing in which inactivation kinetics were examined for *E. coli* ATCC 11229. The whey proteins were denatured by high pressure processing and thus formed a gel matrix, but this had no significant protective effect on the microorganisms being studied (Doona et al., 2007).

Thus, despite the detected change in peanut sauce viscosity, it is reasonable to assume that it had no significant affect on the inactivation kinetics of *Salmonella*.

In conclusion, this study showed that high pressure processing may be a viable option for reducing *Salmonella* in peanut sauces. The main reason peanut sauce was a suitable matrix for high pressure processing was the high water activity. The percentage of organic peanut butter within the peanut sauce matrix altered the survivability of *Salmonella* with effects most likely due to nutrient availability or lipid content. High pressure processing parameters also affected *Salmonella,* with increased hold times and pressures related to decreased logS values. All sauce formulations had the lowest logS value at 7 minutes, indicating that this time is the most lethal hold time that was studied. Weibull curves for logS curves indicate that there was a tailing effect in which there was an ubiquitous resistant sub-population. Increased pressures resulted in a decrease in the n variable and an increase in the b variable for the Weibull model. ANOVA data showed that there were more significant differences between b variables between treatments.

Applications for an industrial setting seem possible, but adiabatic heating effects must be considered for larger packages of peanut sauces and log_{10} reduction data must be obtained for *Salmonella* in industry-scaled packages. Larger packages of peanut sauce may have a temperature gradient at the peak temperature, similar to that seen when retorting a can, which must also be taken into account for future work. It is worth noting that peak temperatures seen in this study were lower than expected, most likely due to an un-insulated pressure vessel leading to a loss of adiabatic heating. This difference in peak temperatures must be considered in industrial applications because it would be reasonable to assume that there would be greater reductions of *Salmonella* when using an insulated pressure barrel due to greater adiabatic heating effects. Other factors such as lipid content and additives such as salts, sugars, and spices traditionally found in commercial peanut sauces must be investigated for their effects on the thermophysical properties of the peanut sauces as well as their effect on *Salmonella* survivability.

Future work should include work on water activity, adiabatic heating, lipid content, and new foods made from high fat, low water activity products. Work on water activity should investigate a wider range of water activity values and investigate the use of various sugars and salts to determine which are most lethal

for pathogens such as *Salmonella* for high pressure processing. Adiabatic heating should be investigated on many levels. First is to determine how an insulated pressure barrel would affect *Salmonella*'s survivability in peanut sauces. The pressure barrel used for this study was not insulated and thus allowed dissipation of heat, lowering the expected peak temperatures. Adiabatic heating would also be important for industrial sized packages of peanut sauces because a temperature gradient would occur in larger packages, similar to a temperature gradient for retorting canned goods. Thirdly, thermophysical properties of peanut sauces would change according to sauce formulations. Differences in the oils used for stabilization or spices used would alter how fast the sauces heat under pressure. The lipid content of sauces may also alter kill curve data as well. Various lipid s would also affect the thermophysical properties of the peanut sauces and may affect the emulsion characteristics as well. New foods that could be high pressure processed should also be investigated. The majority of foods that are high pressure processed tend to have a low pH, but it was demonstrated that this does not need to be the case. Products stemming from peanuts or other high fat, low water activity foods should be investigated as well to determine if they are suitable for high pressure processing. Such products may include syrups, soups, or drinks.

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Chapter 4: Pyrosequencing Studies

I. Introduction

High throughput sequencing is used for a wide range of applications relating to bacteria, including identification of viruses, bacteria, and fungi. Identification can be used to determine possible treatments for pathogens, identify genes of interest for metabolite production, or to determine ecological relationships within a niche, among others (Diggle, 2004). A more recent application has been the analysis of food matrices. This approach provides information on the resident flora of a food, which is important for producing high quality, low risk foods (Juste et al., 2008). Many foods also require microorganisms for flavor and development, such as sourdough bread, yogurt, cheese, sausage, and wine, among others (deVos, 2001; Juste et al., 2008; Kuipers, 1999). Ensuring these foods are being developed using optimal cultures is also important for industry to ensure quality and consistency (deVos, 2001; Kuipers, 1999).

High throughput sequencing and other molecular techniques do not require the microorganism of interest to be culturable, so there is little selection on what is being analyzed (Juste et al., 2008). These techniques also eliminate the possibility of not detecting injured microorganisms that may have been damaged by food processing steps (Juste et al., 2008). Analysis at each processing step allows for better contamination risk assessment and control, with the hopes of a reduction in the occurrence of contaminations, recalls, and illnesses associated with food-borne outbreaks.

The general procedure for high throughput sequencing is to first extract DNA from the food matrix (Juste et al., 2008). It is important to remove inhibitors such as proteins and lipids because these can inhibit PCR reactions (Juste et al., 2008). The DNA is then amplified using PCR, in which the primers used target a conserved region, such as the 16s gene (Juste et al., 2008). It is also important for the primers to encompass a variable region of the genome such that the amplified fragment can give information on the species from which it came from. Each sample is "barcoded" by labeling one of the primers with a specific tag that can be read by the sequencer. All sequences with the same barcode come from the same sample, allowing the sequences to be pooled based on which original sample they came from. The amplified DNA

is then sequenced according to pyrosequencing protocols and the data is analyzed using databases that match the variable regions of the DNA to specie information (Juste et al., 2008).

More specifically, Pyrosequencing ^{TM} is a real-time sequence by synthesis method of DNA sequencing that uses luciferase and extremely sensitive cameras to detect light from the incorporation of deoxynucleotides in a DNA sequencing reaction (Diggle, 2004; Rahman et al., 2009; Ramon, 2003, Ronaghi, 1998, Ronaghi, 2003). The apparatus consists of many tiny wells that contain a probe that hybridizes with a sample of single stranded DNA (ssDNA) (Diggle, 2004; Ramon, 2003, Ronaghi, 1998, Ronaghi, 2003). The ssDNA is then used as a template for the addition of complementary nucleotides (Diggle, 2004; Rahman et al., 2009; Ramon, 2003, Ronaghi, 1998, Ronaghi, 2003). Each cycle consists of adding a specific deoxynucleotide along with DNA polymerase, ATP sulfurase, luciferase, and apyrase (Diggle, 2004; Ramon, 2003, Ronaghi, 1998, Ronaghi, 2003; Shendure et al., 2008). During a cycle, if the specific base is incorporated, the PPi remaining after base incorporation is used to make ATP via ATP sulfurase which then drives the luciferase to oxyluciferin reaction, giving off light (Diggle, 2004; Rahman et al., 2009; Ramon, 2003, Ronaghi, 1998, Ronaghi, 2003; Shendure et al., 2008). The magnitude of light emitted is directly proportional to the number of bases incorporated (Diggle, 2004; Ramon, 2003, Ronaghi, 1998, Ronaghi, 2003). If no bases are incorporated, the apyrase degrades the luciferase and the next base cycle is performed (Diggle, 2004; Ramon, 2003, Ronaghi, 1998, Ronaghi, 2003).

Analysis of food products using molecular techniques such as pyrosequencing have included ready to eat (RTE) salads, pork, biofilms in a beer processing plant, and atlantic cod (Rudi et al., 2002). The study conducted on the RTE salads discovered that there was a difference in the flora based on where the salad was grown as well as storage conditions (Rudi et al., 2002). Microorganisms found consisted mostly of plant pathogens from either the genus *Pseudomonas* or the family *Enterobacteriaceae* (Rudi et al., 2002). The study conducted on pork consisted of sampling from fresh pork as well as pork stored at 4°C for 4 days (Olsson et al., 2003). The diversity of genera decreased in the stored sample, perhaps due to exposure to oxygen (Olsson et al., 2003). The genera for both samples consisted mostly of *Acinetobacter, Staphylococcus, Macrococcus,* and *Pseudomonas,* which was consistent with culture-based methods

(Olsson et al., 2003). The prevalence of unknown genera increased with the stored samples (Olsson et al., 2003). The study on the biofilm formation at a beer bottling plant investigated the efficiency of the sanitation protocols at removing biofilms (Timke et al., 2005). This study found a large variety of biofilm-forming genera that were previously not found using culture-based methods, such as *Methylobacterium* (Timke et al., 2005). Some of these genera were capable of breaking down formaldehyde, which was an ingredient in the sanitizers being used (Timke et al., 2005). The study did not find spoilage organisms within their biofilm samples and thus the sanitation protocols were accepted as suitable (Timke et al., 2005). The study on cod dealt with the biofilm formation of bacteria within the epidermal mucous that cod produce to reduce drag as they swim (Wilson et al., 2008). Samples were taken from three different bodies of water at three different seasons to test whether bacterial populations change with location and/or with seasons (Wilson et al., 2008). They found that *γ-proteobacteria* and *Cytophaga-Flavobacter-Bacteroides* species were prominent for all samples, indicating that bacterial populations do not differ significantly according to geographical location (Wilson et al., 2008). The bacterial populations did not change significantly for two of the three locations over time, indicating that the cod maintained bacterial communities through the seasons (Wilson et al., 2008). These studies show the diversity of food samples that have been tested with molecular techniques such as pyrosequencing. They demonstrate the vast diversity that can is not always captured using culture-based techniques, and thus that the use of non-culture based techniques plays a vital role in food microbiology.

II. Materials and Methods

Samples

Samples were taken from a peanut butter production line as well as various peanut butters available to consumers. Samples from the peanut butter production line came from Once Again Nut Butters in New York and include samples taken from raw peanuts, roasted peanuts, peanuts as they were ground in a mill, peanut paste from a filling hopper, and peanut butter after bottled in glass jars. The commercial brands of peanut butter sampled can be found in Appendix H. Pyrosequencing Sample List. Samples were

divided into 5 categories of peanut butter: organic, natural, reduced fat, alternative, and commercial. Each category had multiple brands and types of peanut butter such that there were 13 total samples. The peanut butters and production line samples were held at room temperature prior to sampling and sterile instruments were used to obtain samples. Peanut butters that had oil separation were mixed before sampling.

DNA Extraction

The detailed protocol used for DNA extraction can be found in Appendix I. DNA Extraction Protocol. Briefly, samples were either taken from a wash or directly from the peanut butter. The wash protocol was used for whole peanuts from the peanut production line and direct samples were taken from the peanut pastes and peanut butters from the production line samples as well as commercial peanut butter samples. The direct samples were done in duplicates in which the sample size was either 100mg or 500mg to ensure that all possible organisms were detected. Since peanut butter contains a lot of lipids and proteins, there was a concern that too much peanut butter in a sample would compromise the DNA extraction efficiency. The cells were lysed using the bead beating technique to remove extraction differences between gram positive and gram negative organisms. Reagents used were obtained from the QIAGEN stool lysis kit and the final DNA was extracted using the QIAGEN AE buffer and stored at -80°C (QIAGEN, Hilden, Germany).

Pyrosequencing

DNA extractions were processed by the Center for Applied Genomics and Ecology (University of Nebraska-Lincoln). Their facility amplified the DNA extractions using primers specific for variable regions of the 16s genome such that the amplicons produced would represent those found in soil-like samples. Primer information and specific protocols are available through the CORE facility.

III. Results and Discussion

Results for the pyrosequencing study are pending. There have been recent studies on the microbial quality of ready-to-eat peanuts using traditional culture methods. In this study, *Salmonella, Listeria monocytogenese, Escherichia coli,* and coagulase-positive staphylococci were detected (Eglezos, 2010).

Only 46% of aerobic plate counts yielded growth at or above the detection limit, indicating that traditional plating methods may not encompass the complete microbial load for peanuts (Eglezos, 2010). Thus, it is anticipated that these results will reflect the results from this study and reveal other species which are not captured using culture-based methods alone.

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Appendix

A. High Pressure Processing Machinery Set-Up

Appendix A: High Pressure Processing Machinery Set-Up
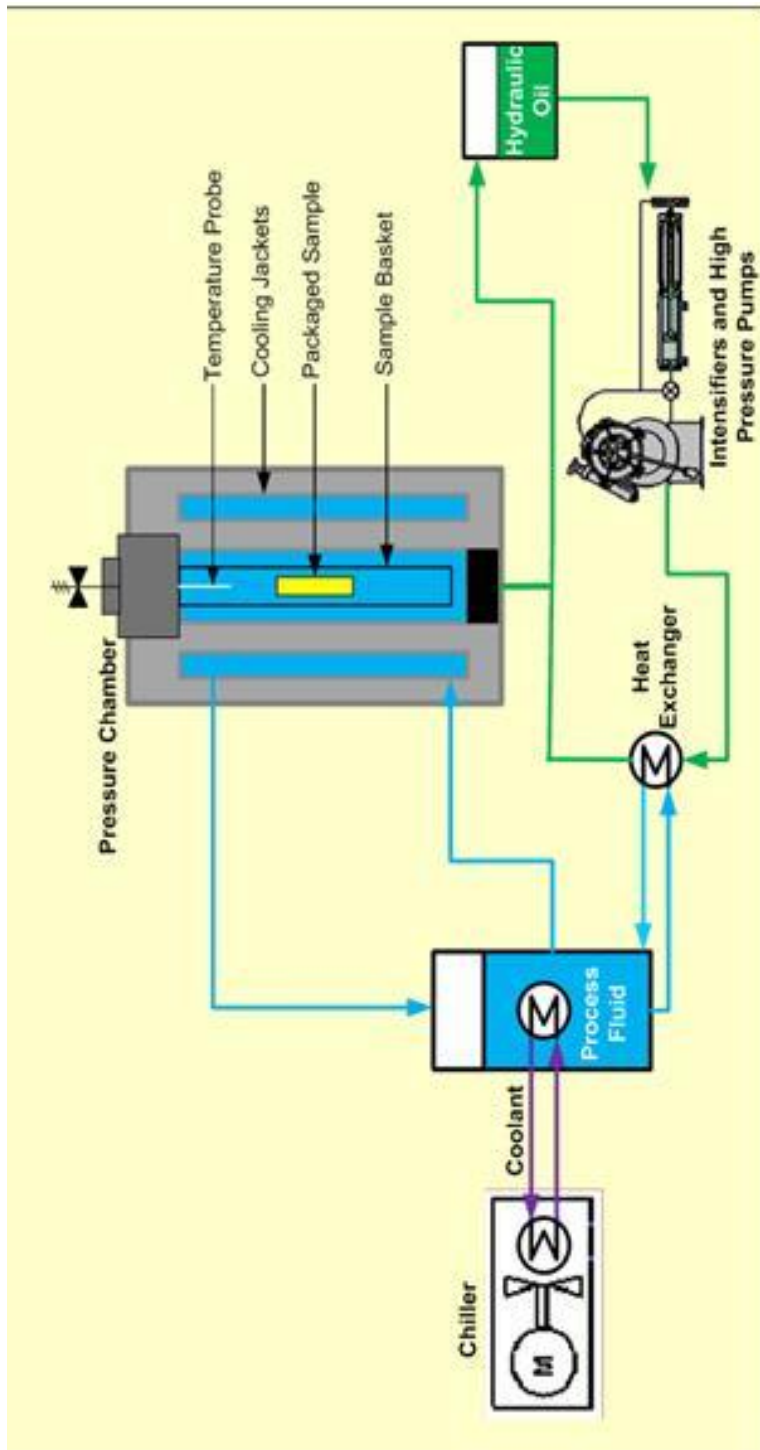
B. SAS Code Used for Statistical Analysis of Outlying Data Points

```
*************************Individual Plates*************************;

Data peanut;

 input day group rep plate cfug;


Datalines;

1     1     1     1     1.03E+03

1     1     1     1     1.40E+03

1     1     1     1     1.92E+02


Etc


;



*This part of the program transformed the data to log;

data peanut2;

  set peanut;

  logcfug = log(cfug);

run;



proc mixed data=peanut2;

class day group rep plate;

model logcfug=day*group*rep*plate/residual outp=res;

run;

data res;set res;

abst=abs(studentresid);

dfe=df-1;

n=1114;

pvalue=2*(1-probt(abst,dfe));
```

```
sig=" ";

if pvalue < .05/n  then sig="*";

run;

proc print data=res; where sig="*";

run;



**************************Average Plates************************;

Data peanut3;

 input day group rep logcfug;



Datalines;

1     1     1     3.16

Etc.,

;



proc mixed data=peanut3;

class day group rep;

model logcfug=day*group*rep/residual outp=res;

run;

data res;set res;

abst=abs(studentresid);

dfe=df-1;

n=557;

pvalue=2*(1-probt(abst,dfe));

sig=" ";

if pvalue < .05/n  then sig="*";
```

```sas
run;

proc print data=res; where sig="*";

run;


***************************Average Reps*************************;

Data peanut4;

  input day group rep avglogcfug;


  datalines;

 1     2     1      8.16

1     1     1      2.79


Etc.,

;

proc mixed data=peanut4;

class day group rep;

model avglogcfug=day*group rep/residual outp=res;

run;

data res;set res;

abst=abs(studentresid);

dfe=df-1;

n=186;

pvalue=2*(1-probt(abst,dfe));

sig=" ";

if pvalue < .05/n   then sig="*";

run;

proc print data=res; where sig="*";

run;
```

C. SAS Code Used for Statistical Analysis of Weibull Model Parameters per Replication

```
Data PB;

Input Rep Pres Perc Time LogS;

Cards;

1      250    0      1       -0.47

2      250    0      1       -0.55

……

;

Proc print;

Proc Sort data=PB; by Pres Perc Rep;

Proc NLIN data=PB; by Pres Perc Rep;

Parms b= 0.2 to 2.4 by 0.2

n=0.2 to 1.2 by 0.2;

Model logS=-b*(time**n);

Run;
```

D. SAS Code Used for Analysis of Variance (ANOVA) of b and n Parameters

```
Data PB;

      Input Pressure Percent b n;

Cards;

250    0      0.1721        1.1641

250    0      0.2267        1.0967

250    0      0.3454        0.8533

400    0      2.0835        0.3269

400    0      2.2678        0.2413

400    0      2.3735        0.2131

400    15     1.779         0.3192

400    15     1.802         0.2878

400    15     2.0085        0.251

600    15     4.8208        0.0312

600    15     5.0317        0.00804

600    15     4.9942        0.0082

400    20     1.74          0.2489

400    20     1.9913        0.2401

400    20     2.085         0.1484

600    20     4.5113        0.193

600    20     4.7726        0.1641

600    20     4.1615        0.232

400    33     1.1656        0.3608

400    33     1.2012        0.3364

400    33     1.3027        0.2054

600    33     4.0407        0.2214

600    33     3.8283        0.2486
```

```
600    33    3.8933        0.26
;

Proc mixed;

      Class Pressure Percent;

      Model b=Pressure|Percent;

      LSmeans Pressure*Percent / Diff Slice=Pressure Slice=Percent;

Run;

Proc Mixed;

      Class Pressure Percent;

      Model n=Pressure|Percent;

      LSmeans Pressure*Percent / Diff Slice=Pressure Slice=Percent;

Run;
```
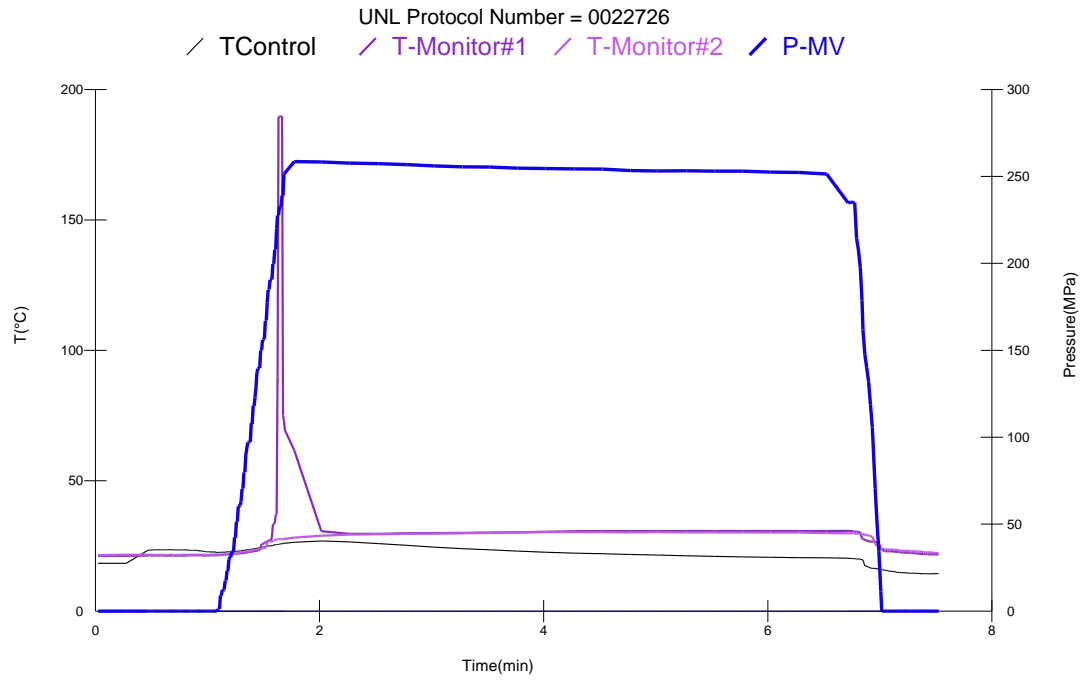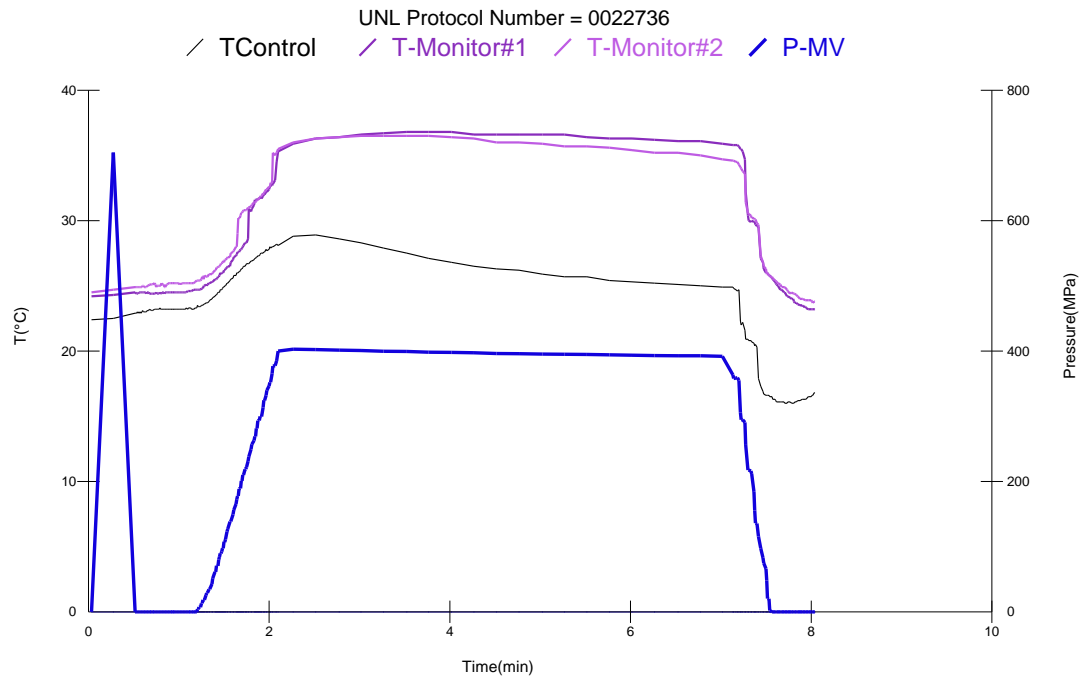
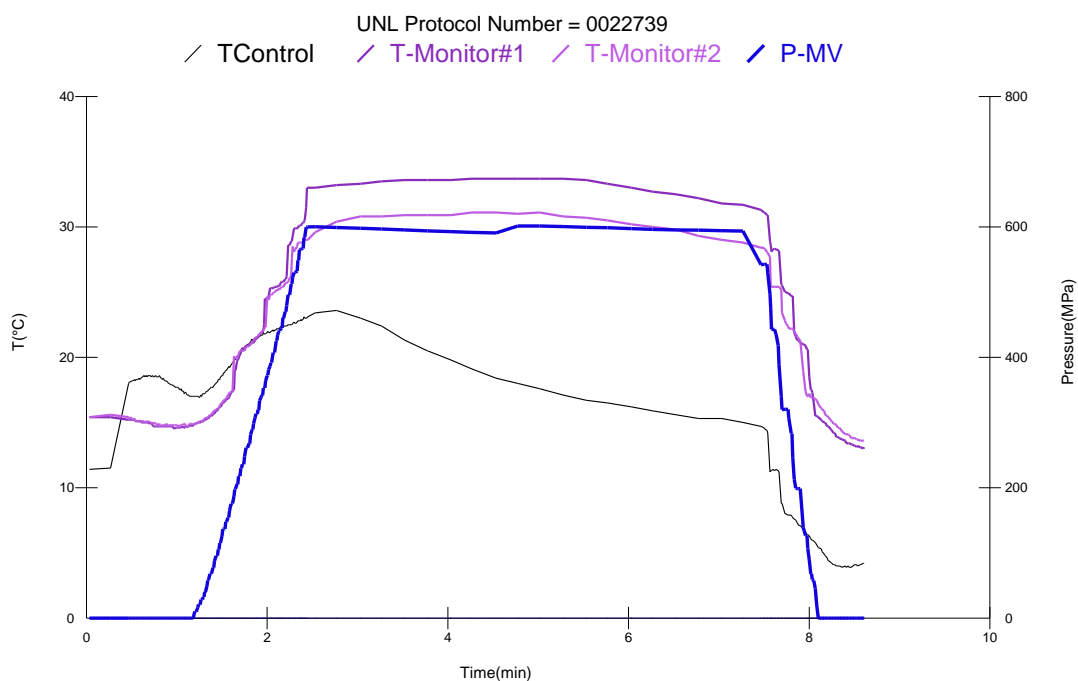**E. High Pressure Print-Out: 250MPa**

UNL Protocol Number = 0022726

╱ TControl   ╱ T-Monitor#1   ╱ T-Monitor#2   ╱ P-MV

F. High Pressure Print-Out: 400MPa

UNL Protocol Number = 0022736

╱ TControl   ╱ T-Monitor#1   ╱ T-Monitor#2   ╱ P-MV

G. High Pressure Print-Out: 600MPa

UNL Protocol Number = 0022739

╱ TControl   ╱ T-Monitor#1   ╱ T-Monitor#2   ╱ P-MV

H. Pyrosequencing Sample List

Organic Peanut Butters:

Once Again Nut Butter, Nunda, NY: Organic Crunchy Peanut Butter, Stabilized; organic peanuts with palm

oil, sugar and salt

Woodstock Farms, Dayville, CT: Organic Peanut Butter, Smooth, Unsalted; Organic unblanched peanuts

Tree of Life, Inc., St. Augustine, FL: Organic Crunchy Peanut Butter, Unsalted; Organic unblanched

peanuts.

Arrowhead Mills, Inc., Melville, NY: Creamy organic Peanut Butter, Unsalted; Organic dry roasted

peanuts.

Natural Peanut Butters:

Earth Balance, GFA Brands, Paramus, NJ: Natural Peanut Butter with flaxseed, crunchy; Peanuts, flaxseed,

agave syrup, palm fruit oil, salt.

Better'n Peanut Butter, Wonder Natural Foods Corp., Watermill, NY: Retorted Peanut Butter; Peanuts
(Peanut defatted flour), water, tapioca syrup, rice syrup, vegetable glycerine, dehydrated cane
juice, tapioca starch, natural colors and flavors, salt, calcium carbonate, lecithin, tocopherol,
sodium ascorbate.

Reduced Fat Peanut Butters:

Jif Reduced Fat Crunchy Peanut Butter, The J.M. Smuckers Corp., Orville, OH: Peanuts, corn syrup solids,
sugar, soy protein. <2% of fully hydrogenated vegetable oils, salt, mono and diglycerides,
molasses, niacinamide, folic acid pyroxidine hydrochloride, magnesium oxide, zinc oxide, ferric
orthophosphate, copper sulfate.

Skippy Reduced Fat Creamy Peanut Butter, Unilever, Englewood Cliffs, NJ: Roasted Peanuts, corn syrup
solids, sugar, soy protein, hydrogenated vegetable oil, mono and diglycerides, palm oil, minerals
(magnesium oxide, zinc oxide, ferric orthophosphate, copper sulfate), vitamins (Niacinamide,
pyridoxine hydrochloride, folic acid)

Alternative Peanut Butters:

Simply Jif Low Sodium Creamy Peanut Butter, The J.M. Smuckers Corp., Orville, OH: Roasted peanuts,
2% or less of vegetable oil, mono and diglycerides, molasses, sugar, salt.

Jif with Omega-3 DHA and EPA creamy Peanut Butter, The J.M. Smuckers Corp, Orville, OH: Roasted
peanuts, sugar, 2% or less of molasses, fully hydrogenated vegetable oils, mono and diglycerides,
salt, anchovy, sardine oil, tilapia gelatin, tocopherols and citric acid.

Commercial Peanut Butters:

Peter Pan Crunchy, ConAgra Foods, NE: Roasted Peanuts, sugar. Less than 2% of hydrogenated vegetable
oil, salt, partially hydrogenated cottonseed oil

Jif, Creamy, The J.M. Smuckers Corp., Orville, OH: Roasted Peanuts, sugar. Less than 2% of fully
hydrogenated vegetable oils, mono and diglycerides, salt

Midwest Country Fare Creamy Peanut Butter, HyVee, IA: Peanuts, dextrose, hydrogenated vegetable oil,
salt

Plant Samples:

OV261 Pre-roast 6/7/10

OV261 Post-roast 6/7/10

OV261 Mill Sample 6/7/10

OV261 Filler Sample 6/7/10

OV261 Post Cap (2 samples)

I. DNA Extraction Protocol

Sampling

- For peanut butter samples: prepare two samples per peanut butter

    o Aseptically weight 0.1g into a 2mL tube containing zirconium beads

    o Aseptically weigh 0.5g into a 2mL tube containing zirconium beads

    o Spin tubes at full speed for 1 minute to ensure sample is at bottom of tube.

- For all other samples:

    o Weight 10g of sample into a 50mL Falcon tube. Add 10mL of 1xDNA grade PBS and shake on titer plate shaker for 1 hour. Transfer 0.1mL of liquid to 2mL tube containing zirconium beads

Lysis

- Add 100 ul **Lysis buffer** (with **lysozyme**) to tube containing 300 mg of zirconium beads (0.1 mm, BioSpec Products)
    o Prepare Lysis buffer by adding 20mg/mL of Lyzozyme into the lysis buffer. Use a 10mL conical tube.
- Incubate at 37°C for 30 min

Proceed as described in Stool Kit protocol (page 19 + include a bead beating step) or follow the steps here described

- Add 1.7 mL **Buffer ASL** (make sure dissolved) to each stool tube. Vortex for 1 min or until the stool sample is thoroughly homogenized
- **Bead beat** the sample for 2 min at max speed – place samples in ice for cooling after beating
    o **During this time prepare the 95°C water bath**
- Pipet 1.6 mL of the stool lysate into a **labeled 2 mL** centrifuge tube
- Heat the suspension for 5 min at 95°C
- Vortex for 15 sec and centrifuge sample at full speed for 1 min to pellet stool particles

- Pipet 1.2 mL of the supernatant in a **new labeled 2 mL** microcentrifuge tube and discard the pellet
- Add 1 **InhibitEX Tablet** to each sample and vortex immediately and continuously for 1 min or until the tablet is completely suspended. Incubate suspension for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix
- Centrifuge sample at full speed for 3 min to pellet stool particles and inhibitors bound to the InhibitEX matrix
- Pipet all the supernatant into a **new labeled 1.5 ml** microcentrifuge tube (not provided) and discard the pellet. Centrifuge the sample at full speed for 3 min.
- Pipet 15 ul **proteinase K** into a **new labeled 1.5 mL** microcentrifuge tube
- Pipet 200 ul supernatant from the sample tube into the 1.5 mL microcentrifuge tube containing proteinase K
- Add 200 ul **Buffer AL** and vortex for 15 sec (Do not add proteinase K directly to buffer AL)
- Incubate at 70°C for 10 min
- Add 200 ul of **ethanol** (96-100%) to the lysate, and mix by vortexing
- Carefully apply the complete lysate to the **QIAamp spin** column (placed in a 2 mL collection tube) without moistening the rim. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2mL collection tube and discard the tube containing the filtrate. (If the lysate has not completely passed through the column after centrifugation, centrifuge again until the QIAamp spin column is empty)
- Carelly open the the QIAamp spin column and add 500 ul **Buffer AW1**. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 mL collection tube and discard the collection tube containing the filtrate.
- Carelly open the the QIAamp spin column and add 500 ul **Buffer AW2**. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 mL collection tube and discard the collection tube containing the filtrate.
- Place the QIAamp spin column in a new 2 mL collection tube and discard the old collection tube with the filtrate. Centrifuge al full speed for 1 min
- Transfer the QIAamp spin column into a **new, labeled 1.5 ml** microcentrifuge tube. Carefully open the QIAamp spin column and pipet 200 ul **Buffer AE** directly onto the QIAamp membrane. Close the cap and incubate for 1 min at room temperature, the centrifuge at full speed for 1 min to elute DNA.
- Keep the 1.5mL mirocentrifuge tube and throw away the QIAamp spin column. Store the DNA at -20°C.

J. SOP HPP 1: High Pressure Processing Laboratory

High Pressure Processing System	Facility: **High Pressure Processing Laboratory**		SOP Reference Number: SOP- HPP 1	Rev.: *0*
TITLE: Packaging of Product Inoculated with Foodborne Pathogens and Sanitizing the Surfaces of Packages for use in the High Pressure Processing System (Rm. of Food Industry Complex)				Page #: *1 of 2*
Written by: Harshavardhan Thippareddi Edited by: _____ Effective Date: September 3, 2009		Authorization signature: _____ Date: _____		Replaces: None

PURPOSE

This SOP explains the protocol for packaging food products inoculated with foodborne pathogens for surface disinfection of the packages for use in the High Pressure Processing System (HPP Unit) located in of the Food Industry Complex.

SCOPE

This SOP describes how to package products inoculated with foodborne pathogens for HPP treatment in the HPP unit located in Rm. of the Food Industry Complex and for surface disinfection of the packages before transfer to the processing room. The HPP unit is used to reduce foodborne pathogens in food products for research purposes. The HPP unit can also be used for improving quality and other research activities/investigations as well.

RESPONSIBILITY

1. The HPP unit supervisor will be responsible for training the personnel on proper packaging of the inoculated material and implementing the protocol/procedure.
2. The HPP unit supervisor will maintain a log of the trained personnel on the inoculated food product packaging requirements and the usage by trained personnel.

MATERIALS REQUIRED

1. HPP unit in Rm in the Food Industry Complex
2. Packaging material (3 mil thickness; Prime Source, Bunzl Distribution USA, Inc., St. Louis, MO) or similar material of equivalent strength
3. Vacuum packaging machine or a heat sealing system to exclude pathogen transfer/contamination of the HPP unit and the HPP unit room
4. Ethanol solution (70% strength) or a sanitizer of equivalent sanitizer efficacy

PROCEDURE

1. Aseptically transfer small volumes of the inoculated product into a polyethylene or similar bag (as mentioned in the materials section) in a biosafety cabinet.
2. Vacuum package the material or heat seal the package (Bag 1).
3. Thoroughly sanitize the outer surface of the package with 70% ethanol solution or a disinfectant with equivalent efficacy.
4. Place the Bag 1 in an additional polyethylene or similar bag and package as described in step 3 of the Procedure (Step 1 of this section).
5. Vacuum package the material or heat seal the package (Bag 2) as described in Step 2 of this section.
6. Thoroughly sanitize the outer surface of Bag 2 using 70% ethanol solution or a disinfectant with equivalent efficacy.
7. Transport the packaged bags (Bag 2) to the HPP processing unit (Rm.).
8. Re-package the Bag 2 for each treatment in an additional bag of similar material as described in step 2 of the procedure in the HPP processing room.

PROCEDURE: FINAL STEPS AND REPORTING

1. Record the type of product used in the experiment and also the type of bacterium used in the log book in the HPP unit Rm.

High Pressure Processing System	Facility: **High Pressure Processing Laboratory**	SOP Reference Number: SOP- HPP 1	Rev.: *0*
TITLE: Packaging of Product Inoculated with Foodborne Pathogens and Sanitizing the Surfaces of Packages for use in the High Pressure Processing System (Rm. of Food Industry Complex)		Page #: *2 of 2*	
Written by: Harshavardhan Thippareddi Edited by: _____	Authorization signature: _____		
Effective Date: September 3, 2009	Date: _____		Replaces: None

2. Record any issues with processing (tear in the bag, leakage in the bag, etc.) in the log book.
3. In case of a leak in the bag and potential contamination of the HPP unit, notify the HPP unit supervisor immediately by telephone call/message and also by email.
4. The HPP unit supervisor will take immediate corrective actions for decontamination of the system in case of potential contamination.
5. The HPP unit supervisor will verify the efficacy of the decontamination procedure through appropriate microbial testing to assure the decontamination of the system.
6. The HPP unit supervisor will record the corrective actions and the verification results in the log book.

REVISION HISTORY

Revision 0.