EFFECT OF TEMPERATURE, pH AND BACTERIOCINS ON THE VIABILITY OF SELECTED FOOD PATHOGENS IN FOOD SYSTEMS DURING HIGH HYDROSTATIC PRESSURE (HHP) PROCESSING

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ABSTRACT

EFFECT OF TEMPERATURE, pH AND BACTERIOCINS ON THE VIABILITY OF SELECTED FOOD PATHOGENS IN FOOD SYSTEMS DURING HIGH HYDROSTATIC PRESSURE (HHP) PROCESSING

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The main objective of this study was to analyze the effect of temperature, pH and bacteriocins on the viability of 28 different strains of *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli* O157:H7 and *Salmonella* in peptone water and in food systems during High Hydrostatic Pressure (HHP) processing.

The results showed that strains of foodborne pathogens differ in resistance to HHP (345 MPa) at 25°C, but this difference is greatly reduced at 50°C. Thus, pressurization at 50°C, in place of 25°C, will ensure greater safety of foods.

Among the three factors (pressure, time and temperature), pressurization temperature induced highest microbial viability loss, especially above 35°C. Also, pressurization in the presence of either citric or lactic acid in peptone water increased viability loss as well as injury among the survivors than pressurization alone.

Without the addition of a bacteriocin-based biopreservative (BP_1), 5.5 log cycle reduction was obtained for the most pressure-resistant strain, *S. aureus* 485, in milk whereas more than 8 log cycle reduction was achieved for all the other strains studied. For orange juice samples, more than 8 log cycle reduction was achieved for all the bacterial species studied.

When BP_1 was combined with HHP, more than 8 log-cycle reduction in cell population of the resistant strains of *S. aureus* and *L. monocytogenes* were achieved in milk after pressurization. Milk samples were stored at 25°C up to 30 day to test the effect of treatment and samples showed no growth where as all the controls were positive. The synergistic effect of HHP together with BP_1 was successful to inactivate the Gram-positive bacterial strains in cream of chicken

soup up to 3 days of storage at 25°C with a rapid increase in cell number thereafter.

It can be concluded that, high hydrostatic pressure (345 MPa, 50°C, 5 min) applied to high acid foods and for low acid foods (when combined with bacteriocins) can be successfully applied to obtain high levels of microbial destruction with respect to the inoculated foodborne pathogens. The recommended shelf life of these products can also be extended even at elevated storage temperatures.

Key words: High Hydrostatic Pressure, Foodborne Pathogens, Bacteriocins, Milk, Orange Juice, Cream of Chicken Soup.

ÖZ

SICAKLIK, pH VE BAKTERİOSİNLERİN YÜKSEK HİDROSTATİK BASINÇ (YHB) İŞLEMİ SIRASINDA GIDA SİSTEMLERİ İÇİNDEKİ SEÇİLMİŞ GIDA PATOJENLERİNİN YAŞAMLARINA ETKİSİ

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Bu çalışmanın ana amacı, sıcaklık, pH ve bakteriosinlerin Yüksek Hidrostatik Basınç (YHB) işlemi sırasında peptonlu su ve gıda sistemleri içinde bulunan *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli* O157:H7 ve *Salmonella* patojenlerinin toplam 28 değişik suşu üzerindeki etkisini analiz etmektir.

Sonuçlar, 25°C'de patojen suşlarının YHB'a karşı değişik oranda direnç gösterdiklerini, fakat bu direnç varyasyonunun 50°C'de büyük ölçüde azaldığını göstermiştir. Bu yüzden gıdaların 25°C yerine 50°C'de basınçlanmaları daha fazla mikrobiyel güvenlik sağlayacaktır.

Basınç, zaman ve sıcaklık faktörleri arasında, özellikle 35°C'nin üzerindeki sıcaklıklarda basınçlamanın daha yüksek mikrobiyel yaşam kaybına yolaçtığı gözlenmiştir. Bununla birlikte sitrik ve laktik asit solüsyonları içinde basınçlama sadece peptonlu su kullanarak basınçlamaya göre mikrobiyel yaşam kaybını ve hücre yaralanmasını arttırmıştır.

Pastörize süt, bakteriosin-bazlı biokoruyucu (BP_1) kullanılmadan basınçlandığında; çalışılan suşlar içinde basınca karşı en dirençli olan *S. aureus* 485 suşu dışındaki tüm suşlarda 8 logaritmik değerden fazla yaşam kaybı sağlanmıştır. Bu suş içinse yaşam kaybı 5,5 logaritmik değerdir. Pastörize portakal suyunda ise yaşam kaybı çalışılan tüm suşlar için 8 logaritmik değerden fazladır.

YHB ile BP_1 birarada kullanıldığında ise, pastörize sütte *S. aureus* ve *L. monocytogenes* basınç-dirençli suşlarının hücre popülasyonlarında 8 logaritmik değerden fazla azalma sağlanmıştır. YHB ve BP_1 kombinasyonunun etkisini ölçmek için süt örnekleri 25°C'de 30 güne kadar saklanmış ve örneklerde mikrobiyel büyüme görülmemiştir. YHB ve BP_1'in birarada kullanımının

sağladığı bütünsel etki kremalı tavuk çorbasına inoküle edilmiş Gram-positif bakteri suşlarının 25°C'de büyümesini ancak 3 güne kadar geciktirebilmiştir.

YHB (345 MPa, 50°C, 5 dakika) uygulamasının yüksek asitli gıdalarda ve ancak bakteriosinlerle beraber kullanıldığında düşük asitli gıdalarda, çalışılan patojen suşları için, yüksek oranda mikrobiyel yıkıma yolaçtığı gözlenmiştir. Böyle bir uygulama bu tip gıdaların tavsiye edilen raf ömürlerinin yüksek saklama sıcaklıklarında bile uzatılabileceğini göstermiştir.

Anahtar kelimeler: Yüksek Hidrostatik Basınç, Patojen, Bakteriosin, Süt, Portakal Suyu, Kremalı Tavuk Çorbası.

To the honourable memory of Tezer GÜRSEL…

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CHAPTER I

INTRODUCTION

Since 1980's health conscious consumers have been concerned about the possible adverse effects of the "harsh processing" and "harsh non-food chemicals" might have on their health. Many of the food preservation methods that are used at present have several drawbacks. High heat treatment used for canned foods destroys the heat labile vitamins and adversely effects the natural color, texture and flavor of foods. Low heat-treated pasteurized foods need to be stored at refrigerated temperature and have a limited shelf life. Drying and freezing with time, also reduce nutritional and acceptance qualities. Many of the chemical preservatives used are of non-food origin and have limited efficiency.

Consumers have a concern about the possible adverse effects that non-food preservatives may have on their health and the health of future generations (Miller 1977). Some of the non-food additives that were allowed to be used before, but later found to be harmful, have shattered consumer confidence. In many developed countries the philosophy of health conscious food consumers

has currently changed from "how long will I live?" to "how well shall I live?". This concern has shifted the desire of the consumers for nutritious and "natural" foods that are not processed by "harsh techniques" and preserved by "harsh chemicals". Due to changes in socio-economic patterns, the consumers are also interested in foods that have long shelf life and take very little time to prepare.

In order to satisfy these needs food processors have started producing many refrigerated foods that are given very little processing treatment, contain very little or no preservatives, have long shelf-life and need very little effort to prepare (Swientek 1988). However, these "ready-to-eat" "heat (microwave) and eat" food invariably harbor spoilage bacteria and can contain pathogenic bacteria which grow at refrigerated temperature. From an initial low level they can reach a high level during long storage and reduce the safety and shelf life of the foods (Ray 1992a). Some of these foods have been implicated in foodborne disease outbreaks in recent years. Regulatory agencies are concerned about the safety of these products and food advisory committees have advocated use of proper processing and preservation techniques to ensure safety of consumers.

To satisfy these needs, several non-traditional food processing and preservation techniques are being studied. The efficiency of some of non-thermal processing methods being studied are ultra high pressure, high electric pulses, oscillating magnetic pulses and intense light pulses. Similarly, several biopreservatives such as antibacterial peptides from food grade safe bacteria,

lysozyme from egg whites, chitosan from crab and shrimp shells are being tested as alternatives to non-food chemical preservatives.

Among these new processing technologies, High Hydrostatic Pressure (HHP) technology has generated international research and development activity and within a short time produced several commercial products in the market. HHP technology has been used to produce ceramic materials and synthetic quartz for the last 35 years. Recently, it has been refined for application in food preservation.

CHAPTER II

LITERATURE SURVEY

2.1. History of Hydrostatic Processing:

The concept of using high hydrostatic pressure as a method of food processing is not new. Bert Hite, a chemist at the Agricultural Experiment Station in Morgantown, W.Va., explored the possibilities of adopting high hydrostatic pressure as a food processing method about a century ago. Before the turn of the century, he assembled a machine that could reach pressures in excess of 690 MPa. He and his coworkers (Hite 1899; Hite et al., 1914) examined a wide range of foods and beverages for the potential use of high pressure processing and reported that 5-6 log reduction in the number of microorganisms when milk was treated at 680 MPa for 10 min at room temperature. They also studied pressure inactivation of viruses (Giddings et al., 1929). The level of sophistication that was accomplished is remarkable, given the technological disadvantages of that time period regarding the processing units and packaging materials.

Cruess referred to Hite's experiments (Hite et al., 1914) in his textbook on commercial fruit and vegetable products (Cruess 1924) and stated that high

pressure could become a means of food processing fruit juices. Considering the benefits of hydrostatic pressure, it is somewhat surprising that it took almost 80 years until Cruess' prediction became true and the first high-pressure-treated fruit products reached the Japanese market in 1991.

Until the 1980's, few attempts were made to investigate the relationship between high hydrostatic pressure and foods. There were notable papers during the interim such as; Bridgman's (1914) work on pressure denaturation of egg-white proteins, Timson and Short's (1965) work on pressure effects on microorganisms in raw milk, Wilson's (1974) work on sterilization of low-acid foods using pressure and pasteurization temperatures, and Elgasim and Kennick's (1980) work on effects of pressurization on the protein quality of beef. Charm et al., (1977) suggested the use of hydrostatic pressures of about 20 MPa for long term-refrigerated storage of foods.

According to the Pascal principle, high hydrostatic pressure acts without time delay and is independent of product size and geometry. It is applied via a pressure-transferring medium (most often water) and can be effective at ambient temperatures, thus reducing the amount of thermal energy needed to be supplied to food products during conventional processing. Key effects of high pressure include:

i) inactivation of microorganisms (Hoover et al.,1989),

ii) modification of biopolymers, such as protein denaturation (Heremans 1982), enzyme inactivation or activation (Morild 1981), gel formation (Cheftel 1991), influence on degradation (Okatomo et al., 1991), or extraction (Kuribayashi and Hayashi, 1991),

iii) quality retention (especially flavor and color), due to the fact that only noncovalent bonds are effected by pressure (Hayashi 1989),

iv) product functionality, as exemplified by density changes, freezing and melting temperatures or textural attributes (Farr, 1990; Deuchi and Hayashi, 1991).

High pressure treatment of food is usually carried out in the range of 300-1000 MPa at room temperature or a little higher; the pressure itself causes only a slight rise in the temperature of the food. Process times are short, usually between 2 and 30 min.

The better retention of sensory and nutritional qualities is partly attributable to the low temperature and partly to the even and instant distribution of the pressure throughout the food, regardless of shape and volume. The latter contrasts sharply with conduction heating, where the effects on different parts of the food can be markedly different (Galazka and Ledward 1995; Vardag et al., 1995; Hill 1997).

The major advantages of high pressure for the processing and preservation of foods are (Eley 1992; Ogawa et al., 1992; Zimmerman and Bergman 1993; Langley-Danysz 1993):

i) elimination or significant reduction of heating, thus avoiding thermal degradation of food components,

ii) high retention of flavor, color and nutritional value,

iii) uniform penetration of product by the pressure; thus food is treated evenly throughout and no particle can escape from the treatment,

iv) energy efficient,

v) reduced requirement for chemical additives,

vi) potential for new food product designs due to the creation of new textures, tastes and functional properties,

vii) improved water-binding and emulsifying capacity, stability, gelling and foaming of proteins through unfolding of their tertiary and quaternary structures,

viii) ability to process in semi-continuous and continuous (liquid foods) modes,

ix) lack of time/mass dependence, thus reducing processing times,

x) potential for control of enzymatic reactions,

xi) increase in the bioavailability of some components.

2.2. High Pressure Equipment and Operation:

A typical high pressure system consists of four main parts:

i) A high pressure vessel and its closure,

ii) A pressure generation system,

iii) A temperature control device,

iv) A material handling system.

The most important part is the pressure vessel, which is generally constructed of forged steel or low-alloy steel of high tensile strength, or reinforced with tensioned wire windings (Mertens and Deplace 1993).

Pressure vessels for retaining high-pressure fluids have been in use for many decades. Cold isostatic pressing of metals and ceramics is one of many commercialized processes. Food processing is similar in concept to cold isostatic pressing except that it demands higher pressures and faster cycling. This poses a real challenge to engineers for delivering safe and reliable equipment. The equipment must be able to generate the desired pressure for the desired time period.

Non-liquid products are usually sealed in flexible packages before being placed into the pressure vessel filled with a pressure-transmitting medium. In most isostatic pressure applications, this is simply water mixed with a small percentage of soluble oil for lubrication and anti-corrosion purposes. Liquid food

can be compressed directly in the pressure vessel, eliminating the use of a pressure-transmitting medium. After all the air has been removed from the vessel, high pressure is generated by direct compression, indirect compression or heating of the pressure medium (Earnshaw 1996). Since oxygen dissolved in products can drastically shorten shelf-life, it is generally removed from the product before pressurization (Manvell 1997). Pressure vessels are generally temperature controlled to compensate for the rise in temperature during the pressure treatment. Most pressure applications are carried out at ambient temperature or a little higher, but there is increased interest in combined pressure-heat treatments.

The packaging used for solid food subjected to high pressure processing should be sufficiently flexible to compensate for the complete compression of the head space, as well as the limited reduction in the product volume. Only one part of the package has to deform, due to limited volume reduction of the food (12% volume reduction of water at 500 MPa). Therefore, rigid containers with flexible laminate or coated aluminum foil lids can be used. A Japanese company is making high pressure processed jam in a cup shaped glass bowl (Cole 1997). Multi layer plastic and aluminum foil packaging is generally used in high pressure processing (Mertens 1993). No migration of chemicals to the food from the plastic containers used has been detected (Mertens 1993; Cole 1997).

Three kinds of pressure treatment are used in food processing:

i) batch operation

ii) semi continuous operation

iii) continuous operation

Batch processing has been viewed as the preferred method for high-pressure treatments, mainly for cleanliness, flexibility, and technical reasons. The food is prepared and aseptically filled/sealed in plastic containers, then placed in a pressure chamber for pressurizing, using pure water as the pressure-transmitting fluid. The holding time depends on the food and the processing temperature. Typical cycle times vary from one min to several minutes (Mertens and Deplace 1993). The chamber is then decompressed, and the cycle begins again. Batch processing eliminates any risks that the food may be contaminated by lubricants or wear particles from machinery, and the equipment does not need cleaning between product changes, thus eliminating any danger of cross-contamination by food particles.

In semi continuous processing, several vessels are connected in series; while some are under constant pressure, others are being pressurized, unloaded or loaded. This minimizes the operation time and allows a portion of the energy contained in the vessel under pressure to be used to pressurize another vessel, thus reducing operating costs (Vardag et al., 1995).

Continuous operation is suitable for liquid food such as milk (Itoh et al., 1996). However, it has been reported that cross contamination and corrosion can take place in continuous systems (Mermelstein 1998).

Preliminary economic analysis indicates that the cost for high pressure batch processing of liquid foods can be less than 10 cents/L, considering capital investment, operational and service-utility factors (Zimmerman and Bergman, 1993; Westerlund 1994).

2.3. Isostatic Pressing of Food:

Isostatic pressure has been used for more than three decades to produce innovative components from such materials as metals, ceramics, and composites. The same technology is now being tested for use in food preservation.

Since the turn of the century, scientists have known that extremely high pressures can be used to destroy microorganisms that spoil food (Hoover et al., 1989). It was not until the late 1980's, however that researchers began investigating ways to commercialize high-pressure treatment of foods (Hayashi, 1989).

While thermal treatment of food effectively controls microorganisms, it can alter a food's taste and destroy vitamins. Research has shown that hydrostatic pressure treatment of foods at 400-900 MPa inactivates enzymes and

bacteria but preserves the taste and nutrient content. The method did not adversely effect the food quality but retained the heat labile natural flavors, vitamins and other essential nutrients in various foods (jams, jellies, surimi, liquid egg, fruit juices, meat and fish products). Pressure-treated citrus juices, not only retain their just-squeezed taste but also show no loss of vitamin C through extended shelf-life up to 17 months after hydrostatic pressure treatment (Farr, 1990). Food researchers are currently exploring high-pressure processing to determine the ideal process parameters for a variety of foods, such as citrus juices, dairy products, jams, meat, fish, and eggs (Zimmerman and Bergman 1993).

Since pressure is uniform throughout the food (i.e. isostatic), the food is preserved evenly throughout, without any particle escaping preservation. Unlike thermal processing, isopressing is not time and mass dependent, thus reducing the processing time. Some foods are treatable at moderate temperatures up to 80°C (Eley, 1992).

2.3.1. Uses in Food Science:

2.3.1.1. Effect of High Hydrostatic Pressure on Microorganisms:

A major function of high pressure processing of food is destruction of microorganisms. The system is also energy efficient and the antimicrobial effect is isostatic and instantaneous (Hoover et al., 1989; Hoover 1993; Knorr 1993).

When a microbial cell is subjected to high pressure, several changes have been reported to take place which are detrimental to the cell:

i) cell membranes are destroyed with irreversible changes to the structure of the membrane macromolecules, particularly proteins (Chong and Cossins 1983),

ii) the homogeneity of the intermediate layer between the cell wall and the cytoplasmic membrane is disrupted and membrane ATPase is inactivated (Kriss et al., 1969; Smelt 1995),

iii) the nucleic acids and ribosomes involved in the synthesis of proteins are disrupted (Landau 1967).

The result is permeabilisation of the membranes and concomitant leakage of the contents of the cells and organelles (Hayashi 1988; Smelt 1998). It has been reported that bacteria are more resistant in the stationary phase than in the early log phase of growth (Zobell 1970). Gram-negative bacteria are inactivated at a lower pressure than Gram-positive bacteria, with the pressure sensitivities of yeast reported to be intermediate between these two bacterial groups (Hoover et al., 1989; Shigehisa et al., 1991; Cheftel 1995; Mackey et al., 1995; Anonymous, 1997). The lower resistance of Gram-negative bacteria compared with Gram-positives has attributed to their lack of teichoic acid, which is responsible for the rigidity of the cell wall of Gram-positive bacteria (Elaamadi et al., 1996). Bacterial cells are relatively less sensitive to hydrostatic pressure at 20 to 35°C but become more sensitive to pressurization above 35°C, due to phase transition of

membrane lipids (Ludwig et al., 1992; Kalchayanand et al., 1998a; Kalchayanand et al., 1998b). Microbial cells surviving pressurization also became sublethally injured and developed sensitivity to physical and chemical environments to which the normal cells were resistant (Kalchayanand et al., 1995; Hauben et al., 1996; Kalchayanand et al., 1998a). It is well established now that a species of foodborne pathogen contain strains that are relatively resistant to pressure in comparison to other strains of the species (Styles et al., 1991; Patterson et al., 1995; Hauben et al., 1997; Alpas et al., 1999; Benito et al., 1999). This could be the reason for variation in results obtained by researchers using different strains of the same species.

Substantial momentum has been generated in the study of the inactivation of groups or strains of microorganisms in pressure treated foods. Shigehisa et al., (1991) inoculated pork slurries with a range of microorganisms before subjecting them to pressures of 100-600 MPa for 10 min at 25°C. The microorganisms were *Bacillus cereus, Campylobacter jejuni, Candida utilis, Escherichia coli, Micrococcus luteus, Pseudomonas aeruginosa, Saccharomyces cerevisiae, Salmonella typhimurium, Staphylococcus aureus, Streptococcus faecalis,* and *Yersina enterocolitica.* Treatments above 300 MPa resulted in coagulation and discoloration of the pork slurries. With the exception of the spore former, *Bacillus cereus*, all of the tested microorganisms (initial population approximately 10^6 cfu/g) were killed at pressure treatments of 300-600 MPa.

Tanaka and Hatanaka (1992) found pressure treatment effective in the prevention of high acid yogurt, caused when the yogurt cultures continue fermentation during storage and results in excess tartness. A treatment of 200-300 MPa for 10 min at 10°C to yogurt prevented after-acidification, but maintained the initial number of lactic acid bacteria and the desired texture of the yogurt.

Horie et al., (1992) isolated a pressure-resistant strain of *Candida parapsilopsis* from fresh strawberries. *C. parapsilopsis* and *Candida tropicalis* were significantly more pressure resistant than *S .cerevisiae* in strawberry jam. Elevated temperatures of 34-54°C with pressure co-treatment sterilized products containing 10^6 cfu/g of *C. parapsilopsis* more quickly than pressure treatment alone. For *S. cerevisiae*, the inactivation rate in sucrose solutions was independent of pH over a pH range of 3.5-6.5.

Ogawa et al., (1990) also found the pressure inactivation of indigenous fungi in Satsuma mandarin orange juice to be independent of pH in the pH range of 2.5-4.5. Using acid-free juice with individual acids added, they found that the type of organic acid was not an influencing factor. Citric, lactic, tartaric, and acetic acids were evaluated. To reduce the number of viable microorganisms by five log cycles, pressure treatments of 355 MPa for 30 min or 400 MPa for 5 min were required.

The effectiveness of several variables on hydrostatic pressure inducing death of microbial cells has been reported. These include: magnitude of pressure, pressurization time and temperature, microbial types, cell growth phase, suspending media, and the presence of antimicrobial substances (Metrick et al., 1989; Shigehisa et al., 1991; Styles et al., 1991; Carlez et al., 1992; Mackey et al., 1995; Patterson et al., 1995; Kalchayanand et al., 1998a; Kalchayanand et al., 1998b; Benito et al., 1999). The efficiency of cell destruction is also increased by the combined action of pressure, temperature and other conditions, such as ultrasonic waves, shear, electromagnetic fields or high-voltage pulses (Williams 1994). Ethanol, lysozyme, chitosan, organic acids and bacteriocins of food grade safe bacteria enhance the effect of pressure on microorganisms, thus permitting lower pressures, lower temperatures or shorter application times to be used to achieve inactivation (Mozhaev et al., 1994). The combined application of these variables to processing or preservation of food often allows for the development of foods with less product damage and greater consumer appeal.

The bactericidal effectiveness varies with the medium containing the bacteria. For example, *Listeria monocytogenes* is more susceptible in buffer solutions than in milk, as proteins, carbohydrate and fat protects the bacterial cells against pressure (Styles et al., 1991; Patterson et al., 1995; Simpson 1995). The protective effect of different carbohydrates on the cell membrane during pressurizing is in the order of glycerol < fructose < glucose < sucrose < trehalose (Smelt et al., 1997). In real food situations, two effects always determine

microbiological safety and stability: the effect of the food during treatment and the effect after treatment during recovery of the microorganism.

In general, bacterial spores are more resistant to high hydrostatic pressure then vegetative cells (Sale et al., 1970; Hoover et al., 1989; Knorr 1993; Smelt 1998). This has been attributed to the protection afforded by dipicolinic acid of the spore proteins against solvation and excessive ionization that are responsible for cell death (Timson and Short 1965). However, high pressure can stimulate germination of bacterial spores and then destroy the resulting vegetative form (Timson and Short 1965). Germination can be markedly increased to 95-99 % when spores are treated in the presence of L-alanine (Gould and Sale 1970). The endospores of the Gram-positive bacteria *Bacillus* and *Clostridium* are not extensively inactivated by hydrostatic pressure. This was evident from the very first experiments with pressure effects on food microorganisms (Hite, 1899; Hite et al., 1914). Spore counts can be lowered by exposure to high pressures, but it appears that neutral-pH foods can not rely on pressure treatment alone as a sterilization process, or even as a means for substantial reduction of viable spore counts.

2.3.1.2 Effect of High Hydrostatic Pressure on Enzymes and Proteins:

In recent years, most of the attention to the adaptation of pressure treatment to food preservation has been toward the inactivation of

microorganisms. The response of food enzymes to hydrostatic pressure is now beginning to receive equitable study. Contrary to what is usually found with the application of high hydrostatic pressure to microorganisms, enzymes may display activation or enhancement of activity, in addition to possible inactivation.

In general, enzymes are much less affected by high pressure treatment than by heat. Some are inactivated, either partially or completely. For example, aminopeptidase and carboxypeptidase in beef are inactivated at 507 MPa/10 min and 405 MPa/10 min at 25°C, respectively (Ohmori et al., 1991), pectinesterase activity in Satsuma mandarin orange juice decreases significantly at 304 MPa (Ogawa et al., 1990), but acid protease and neutral protease in beef are only slightly inactivated at 405 MPa (Ohmori et al., 1991). A combination of pressure and temperature increases the degree of enzyme inactivation (Cano et al., 1997).

High pressure can also protect enzymes against heat inactivation. It was reported that the maximum activity of cathepsin B1, an endogenous muscle enzyme which degrades structural proteins and improves meat tenderness, is observed at 60°C and 150 MPa and activity increases 12 times that observed at 60°C and atmospheric pressure (Kurth 1986).

Food ingredients have a protective effect on most enzymes from inactivation by pressure treatment. For example, sucrose protects pectinesterase from inactivation, while lactoperoxidase and phosphatase are less susceptible to pressure in milk than in buffer (Seyderhelm et al., 1996).

In addition to the effect on the enzyme molecule itself, high pressure can also have a significant effect on the rates of enzyme-catalyzed reactions. For example, the rates of trypsin and carboxypeptidase Y catalyzed reactions are reduced by high pressure (Kunugi et al., 1982; Fukuda and Kunugi 1985), while reactions catalyzed by thermolysin and cellulase are stimulated (Fukuda and Kunugi 1984; Murao et al., 1992). Pepsin, which is unable to hydrolyze β-lactoglobulin (BLG) at atmospheric pressure, completely hydrolyses it in less than 40 min at 300 MPa. In fact, the hydrolysis rate at pH 3.0 increases 270 fold between 0.1 and 300 MPa (Dufour et al., 1995). This has been explained in terms of a decrease in reaction volume (∇V = -44.8 mL/mol) during the hydrolysis, an example of the application of Le Chatelier's principle.

Moderate pressures (<150 MPa) favor dissociation of oligomeric proteins, while pressure higher than 150-200 MPa induce unfolding of proteins and reassociation of subunits from dissociated oligomers. For example, below 150 MPa, β-casein is reversible depolymerised, whereas at higher pressures a temperature-dependent reversible reassociation occurs. Significant tertiary structure changes are observed beyond 200 MPa. However, reversible unfolding of small proteins occur at higher pressures (400 MPa to 800 MPa) (Balny and Masson 1993).

Secondary structure changes take place at very high pressures (above 700 MPa) and lead to non-reversible denaturation. No changes in covalent bonding

including S-S bridges have yet been observed in the pressure range studied (Datta and Deeth, 1999).

2.3.1.3 Effect of High Hydrostatic Pressure on Water:

Pressure causes changes in water, which have direct and indirect relevance to foods. First, application of pressure to water results in a reduction of volume, which at high pressure can be substantial, e.g. 15% at 600 MPa. This had a major influence on the chemical changes in foods, since according to Le Chatelier's principle, any reaction that results in a decrease in volume will be favored during high pressure treatment.

Second, pressure changes the freezing point of water. At 50 MPa, the freezing point is reduced to –4°C, at 100 MPa to –8°C and at 210 MPa to –22°C. Thus water at 210 MPa remains liquid down to –22°C. This phenomenon gives rise to interesting effects including sub-zero food storage without ice crystal formation, rapid thawing of conventionally frozen foods and pressure-shift crystallization. Cooling to sub-zero temperatures under pressure and then suddenly releasing the pressure effects crystallization. This results in frozen products containing very small ice crystals and hence improved quality (Kalichevsky et al., 1995). At increasing pressures above 210 MPa, the freezing point increases again. At 1000 MPa, the upper end of the range for high pressure processing, water freezes at approximately 20°C producing an unusual form of high density "warm ice".

Third, increasing the pressure on water from 100 MPa to 1000 MPa causes a decrease in the pH value of about one unit, due to dissociation of water molecules, to produce an increase in hydrogen ion concentration. This can have significant effects on food products and also contributes to the microcidal effect of high pressure treatment (Hinrichs et al., 1996).

2.4. Antimicrobial Agents:

2.4.1. Bacteriocins of Lactic Acid Bacteria (LAB):

Current consumer interest for commercially processed foods that are not processed with non-food preservatives has provided an incentive to search for safe and food-grade preservatives (biopreservatives) of biological origin (Miller 1977; Smith et al., 1981; Parente and Hill 1992; Ray 1992a; Miller 1993). Antibacterial peptides or bacteriocins produced by many strains of lactic acid bacteria (LAB) have generated interest as potential food biopreservatives (Daeschel 1990; Lücke 1992; Ray 1992b). Bacteriocins are small hydrophobic, cationic proteins, which produce bactericidal effect by functional destabilization of cytoplasmic membranes of sensitive Gram positive bacteria. Their bactericidal properties are retained after high heat treatment (121^{o}C for 10 to 15 min), at wide pH (4 to 8) during storage and in the food systems studied but they can be destroyed by some proteolytic enzymes (Jack et al., 1995). Thus, there is a potential for use of some bacteriocins as suitable food biopreservatives (Ray, 1993). Many LAB have important roles in the production of fermented foods,

and some of these bacteria have been shown to be capable of inhibiting the growth of a wide variety of food spoilage organisms (Stiles and Hastings 1991; Ray and Daeschel 1992). Humans have consumed cells of LAB and their metabolites, including the bacteriocins, for thousands of years through fermented foods without any known adverse effect.

The bacteriocins, especially those with broad antibacterial spectrum, are bactericidal or can be made bactericidal to many spoilage and pathogenic bacteria important in food (Kalchayanand et al., 1992; Ray 1992b; Ray 1992c; Ray 1992d; Ray 1993). Before a bacteriocin is considered for application in food, information on its antibacterial spectrum, biochemical and genetic characteristics, effectiveness in food systems, and regulatory implications should be known. Among the well-characterized bacteriocins of LAB with a wide antibacterial spectrum, limited information in these areas is available except for nisin and pediocin AcH (Ray 1992c; Ray 1992d). Another important factor to be considered would be the economical aspects or cost of using a bacteriocin in foods. One way to reduce the cost is to determine the optimum parameters for the production of a bacteriocin.

The classic example of a commercially successful naturally produced inhibitory agent is nisin. It has been known since 1928 (Rogers 1928) to be produced by some *Lactococcus lactis* isolates and structurally characterized in 1971 as a lanthionine-containing peptide (Gross and Morell 1971). Nisin and nisin producing strains have had a long history of application in food

preservation, especially of dairy products (Hurst 1981; Hugenholtz and de Veer 1991; Molitor and Sahl 1991). Recognition that nisin may be produced by *L. lactis* strains while they are naturally associated with certain foods during processing and that it has no apparent adverse effects when ingested has led the US Food and Drug Administration to accord GRAS (generally recognized as safe) status to nisin (Federal Register 1988). It is active against a range of Gram-positive bacteria but its principal commercial applications are in canned foods and processed cheeses to inhibit the outgrowth of spores which show particular sensitivity (Hurst, 1981; Delves-Broughton, 1990).

The influence of different parameters on the production of nisin and pediocin AcH in simple media containing food grade ingredients or in complex media has been reported (Hirsch, 1951; Biswas et al., 1991; Ray 1992c; Ray 1992d). Commercial dairy-based preparations containing nisin and pediocin are now available but the methods of these preparations are not made public (Federal Register, 1988; Pucci et al., 1988). Nisin production by some *Lactococcus lactis* ssp. *Lactis* strains in complex bacteriological broth have been studied (Hirsch, 1951; Egorov et al., 1971; De Vuyst and Vandamme, 1992; Meghrous et al., 1992; Ray 1992c). Besides nisin, pediocin AcH is the most thoroughly studied bacteriocin of lactic acid bacteria. Influence of growth conditions on the production of pediocin AcH by a *Pediococcus acidilactici* strain in a relatively simple medium containing food-grade ingredients have been reported (Biswas et al., 1991; Ray 1992d). These studies showed the need of a low final pH of the culture broth for high pediocin AcH production. However, it has also been

reported that these bacteriocins could be produced in high concentrations in relatively simple broths (Yang et al., 1992).

Bacteriocins as food biopreservatives have several limitations (Ray and Daschel, 1992; Jack et al., 1995; Kalchayanand et al., 1995):

i) they differ greatly in antibacterial spectrum,

ii) even those with wider spectrum, such as nisin (A and Z) and pediocin (AcH or PA1), are not bactericidal against many Gram-positive pathogenic and spoilage strains,

iii) a bacteriocin does not kill all cells present normally in a population of a sensitive Gram-positive strain, and the survivors can multiply in the presence of the bacteriocin,

iv) they are not normally bactericidal to Gram-negative bacterial cells, bacterial spores, yeasts and molds in a food,

v) they may not be useful in raw foods as many proteolytic enzymes destroy their bactericidal property,

vi) except for nisin and pediocin AcH complete information that is necessary for their use in food systems is not available.

To overcome these problems, it has been suggested that:

i) bacteriocins in combination can be bactericidal against more Gram-positive strains and more cells in a sensitive population (Hanlin et al., 1993) and

ii) sublethal stress can make both resistant Gram-positive and Gram-negative bacterial cells sensitive to a bacteriocin (Kalchayanand et al., 1994).

Once the various ethical and regulatory hurdles are crossed, the path should be clear for commercial applications of starter cultures that produce a wide range of antibacterial peptides, and this in turn should bring about a significant reduction in the usage of potentially toxic nitrites in food preservation and should improve the safety of fermented foods (Jack et al., 1995).

2.4.2. Organic Acids and Lysozyme:

Lactic and citric acids produced as metabolic end products of food grade starter culture bacteria have been well studied for both their bactericidal and bacteriostatic effects; some also have an antifungal effect (Ray and Sandine 1992). Their antimicrobial action is produced both by the undissociated molecules and dissociated ions. Lactic acid produces an inhibitory effect mainly by neutralizing the membrane proton gradient whereas the antibacterial effect of citric acid is partially due to its ability to chelate divalent cations. L(+)-lactic acid is preferred over D(-)-lactic acid as a food preservative.

The effectiveness of both acids was tested in different types of foods and some have been approved for use in food systems (Ray and Sandine 1992). Bacteriocins in combination with lactic and other organic acids were found to

produce bactericidal action against resistant Gram-positive and Gram-negative bacteria (Ray, 1992c; Ray, 1992d; Kalchayanand et al., 1992).

The enzyme lysozyme is present in large quantities in some foods such as egg white and shellfish (oysters and clams), and in small amounts in milk and some plant tissues. It is a white odorless powder generally prepared and used in hydrochloride form. It has a somewhat sweet taste. Lysozyme produces antimicrobial effects by lytic action of the mucopeptide layer present in the cell wall of Gram-positive bacteria and in the middle membrane of Gram-negative bacteria (Ray, 1996), including those bacteria associated with food spoilage and food-borne diseases (Hughey and Johnson, 1987). However, Gram-negative bacteria become sensitive to the lysozyme effect only after the barrier function of the outer membrane is destabilized by chemical and physical stresses. The antimicrobial effect is manifested by the lysis of the cells. Lysozyme is most effective at pH 6.0 to 7.0 and at concentrations of about 0.01 to 0.1%. It can be used directly to control Gram-negative bacteria, though Gram-negative bacteria are not normally sensitive, they become highly sensitive to lysozyme following sublethal stresses (Ray, 1989).

2.5. Metabolically Injured Microorganisms:

When microorganisms are subjected to environmental stresses many of the individual cells undergo metabolic injury resulting in their inability to form colonies on selective media that injured cells can tolerate. Whether or not a

culture has suffered metabolic injury can be determined by plating aliquots separately on a nonselective and a selective medium and enumerating the colonies that develop after suitable incubation. The colonies that develop on the nonselective medium represent both injured and uninjured cells, while only the uninjured cells develop on the selective medium. The difference between the number of colonies on the two media is a measure of injured cells in the original culture or population.

The existence of metabolically injured cells in foods and their recovery during culturing procedures is obviously of great importance not only from the standpoint of pathogenic organisms but for spoilage organisms as well. Injury of food-borne microorganisms has been shown by a large number of investigators to be induced not only by sublethal heat and freezing but also by freeze drying, irradiation, heavy metals, dyes, salts, antibiotics and other chemicals such as EDTA and sanitizing compounds. It has also been shown that bacterial cells surviving high hydrostatic pressurization become sublethally injured (Kalchayanand et al., 1994; Ray 1995; Kalchayanand et al., 1996).

2.5.1. Mechanism and Recovery of Injured Microorganisms:

A large number of investigators have found out that metabolic injury is accompanied by damage to cell membranes, ribosomes, DNA, or enzymes. The cell membrane appears to be the most universally affected (Ray 1989). The lipid components of the membrane are the most likely targets, especially for sublethal

heat injuries. Ribosomal damage is believed to result from the loss of Mg^{2+} and not to heat effects per se (Hurst and Hughes 1978).

Metabolically injured cells can recover and the cells may not recover to the same degree but that the process may take place in a step-wise manner. Also, all cells in a population may not suffer the same degree of injury. It is well established that injury repair occurs in the general absence of cell wall and protein synthesis. The repair of cell ribosomes and membrane appears to be essential for recovery.

Complex media and certain specific components favor the protection of cells from heat and freeze injury. It has been shown long before that some foods like milk provides more protection than saline or mixtures of amino acids (Janssen and Busta, 1973).

2.6. Control by Combination of Methods "Hurdle Concept":

Processing of foods with high hydrostatic pressure (HHP) rather than by traditional heating technologies offers unique advantages to consumers and the food industry. This technology can destroy microorganisms without causing significant changes to the sensory and nutritional attributions of the processed foods. Accordingly, it can produce some interesting effects and food products that are not possible with other preservation technologies.

HHP treatment not only kills bacterial cells but inflicts injury to cells surviving pressure treatment. The injured cells become susceptible to

bacteriocins of lactic acid bacteria including species that are normally resistant to those bacteriocins. If bacteriocins are to be used along with hydrostatic pressure to preserve foods, it is important to know the effect of hydrostatic pressure on the bactericidal property of bacteriocins. This is important as bacteriocins are proteins and hydrostatic pressure is known to cause changes in the properties of proteins by destabilizing ionic and hydrogen bonds. It has been reported that the potency of the bacteriocin remained unchanged up to 5 months at 25°C after pressurizing at 345 MPa for 30 min (Kalchayanand et al., 1995).

Influence of several factors on the viability loss of microorganisms by hydrostatic factors has been studied. These include, magnitude of pressure, pressurization time and temperature, exponential and stationary phase cells, and suspending medium (phosphate buffer versus food systems). Recently, there have been some suggestions about the variation in high hydrostatic pressure sensitivity among strains of foodborne pathogens. In general, the magnitude of death was directly proportional to pressure and time of pressurization, but for the destruction of more than 8 log cycles of some pathogens, namely *Escherichia coli* O157:H7 and *Staphylococcus aureus* in phosphate buffer, pressurization at 20°C at 700 MPa for 15 min was necessary (Patterson et al., 1995). Although this is technically possible, it is not commercially profitable because of the very high pressure and the correspondingly high cost of the equipment required. Also, such a high pressure to obtain a desirable kill of pathogens can not be used to preserve foods without altering their texture and color. Thus, it will be necessary in a commercially successful pressure pasteurization to employ lower pressure yet to

obtain high levels of destruction of foodborne pathogens (probably more than 8 log cycles).

As a result, considering the various factors that influence the microbial resistance, a "Hurdle Concept" in which high hydrostatic pressure (HHP) technique is combined with one or more suitable antimicrobial factors such as, bacteriocins, lysozyme and organic acids to obtain an additive or synergistic effect, will be more of a rational approach to produce shelf-stable, minimally processed foods that have good acceptance and nutritional qualities with extended shelf-life. This system can be uniquely used as a four-dimensional process that includes: pressure-time-temperature and an antimicrobial factor. This study therefore investigated the simultaneous use of these four hurdles for the inactivation of 28 different strains of four foodborne pathogens; *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* in phosphate buffer and in different food systems. The microbial growth of the pressure-sensitive and pressure-resistant strains of these pathogens in pasteurized milk, orange juice and cream of chicken soup during storage at room and elevated temperatures were also investigated.

The data of the proposed research will provide information on the antibacterial effectiveness of HHP technique in combination with bacteriocins and other antibacterial agents. Most importantly, the data will provide information on the possibility of reducing (by at least 5 logs) bacterial populations to desired levels by combining several novel hurdles. Results will

also provide information about the capability of HHP techniques, in combination with other parameters, to reduce bacterial populations to the same levels in food systems (in selected food products) or if any modification is necessary to achieve this goal. In addition results will also provide information on the effect of these treatments on the quality of selected foods. This information will be helpful in designing and developing preservation methods of future foods by using these alternate techniques.

2.7. Commercial Application of High Pressure Technology:

Food processing and preservation by High Hydrostatic Pressure (HHP) technique is being vigorously studied in Japan, U.S. and in several European countries.

In 1989, 21 large industries in Japan have, under the Ministry of Agriculture, formed a consortium to conduct processing and preservation research in many foods including studies on fruits, vegetables, dairy, meat and fishery products. Since 1991, companies have started commercial marketing of fruit juices, jelly, yogurt, sauces, salad dressings and jams, which have received good consumer acceptance.

In U.S., The U.S. Army is interested in the development of storage-stable, acceptable, nutritious and convenient foods. The U.S. Department of Defense has been sponsoring contract research of the 'DoD Combat Feeding

Program' in several universities including The University of Wyoming to produce combat rations with High Hydrostatic Pressure over the last 4 years.

This new technique is also attracted attention of Institute of Food Technologists (IFT) Members. As a result, Nonthermal Processing Division (NPD) was formed in December 1998 in order to bring in new disciplines including HHP to advance food processing technologies in the 21st century.

Eight academic laboratories in Germany, France, Belgium, U.K. and Spain and four private companies have formed a consortium for commercial production of HHP treated foods. Private industries in several European countries are expected to market HHP treated products within a short period. Recently, HHP treated French pate have been marketed in France.

The first commercial high pressure processed food products (high acid jams from strawberry, raspberry, kiwi fruit and apple) were introduced to the Japanese market in April 1990 (Selman 1992; Galazka and Ledward 1995). Other potential applications in the food industry include the production of a range of novel meat, poultry, fish, dairy products, yogurt, spreads, fruit and fruit juices, oyster and salmon (Mermelstein, 1998). The jams which had been treated in their retail containers at pressures up to 500 MPa, had a shelf life of two months when refrigerated. Unlike heat processed jams, they retained the flavor and color attributes associated with the fresh fruit. The range of high pressure treated products was later extended to include yogurts, fruit jellies, salad dressings and

fruit sauces with shelf lives of 60 days at chilled temperature. Semi-continuous, directly pressurized systems with capacities up to 6000 L/h have been established for treatment of citrus juices at 400-500 MPa at ambient temperature with holding time of 1 to 5 min. Other high pressure products include camembert cheese made from raw milk and cream-based emulsions (Vardag et al., 1995).

The first commercially pressure processed product on the market in the US was a guacamole product produced in Mexico (Mermelstein 1997), with first products processed in US being marketed in 1998 (Mermelstein 1998).

2.8. Future Implications:

As high-pressure technology develops in the food processing area, new products and applications will be discovered and investigated. In many ways, high-pressure food processing is now following the same sequence of events that food irradiation was in the late 1950's. However, without the stigma associated with irradiation, the rather simple mechanical aspects of pressure use should prove easier to accept by both the consumer and regulatory agencies. Of course, both the economics and safety of pressure-processed products must be in agreement, but the prognosis seems to be very good.

2.9. Drawbacks:

High pressure technology offers the food industry a unique opportunity to develop new foods with high nutritional and sensory quality, novel texture, more convenience, higher safety, and increased shelf life. The status of technology today is such that capacity, operating, process control, and safety requirements for high-pressure food processing can readily be met.

Commercial high-pressure food processing, however, poses specific requirements in relation to sanitation and cleaning, material handling, package design, and operational cost.

The most difficult challenge lies in the uncertain field of marketing, i.e., identifying those niche applications for which the higher cost for high-pressure processing is justified by superior or unique product properties.

CHAPTER III

MATERIALS AND METHODS

3.1. Bacterial Species:

A total of 28 different strains of four foodborne bacterial pathogens were used in the experiments. These include: *Listeria monocytogenes* CA, $Ohio_2$, Scott A, 117, 35091, 103M, V7, $Camp^+/Beta^+$ and SLR1; *Staphylococcus aureus* strains 315, 485, 565, 582, 743, 765 and 778; *Escherichia coli* O157:H7 strains 932, 933, C7927, EDL 931, 35748-88 and SLR 503; *Salmonella enteritidis* VL and FDA; *Salmonella typhimurium* ATCC 14028 and E 21274; *Salmonella choleraesuis* subsp. *choleraesuis* ATCC 10708 and *Salmonella choleraesuis* subsp *choleraesuis* serotype typhi ATCC 6539.

The strains were obtained from the frozen stock at Food Microbiology Laboratory, University of Wyoming. They were grown in Tryptic soy broth (Difco, Detroit, MI) supplemented with 0.6% yeast extract (Difco; TSBY). The

cultures were grown at 37°C, transferred to fresh broth three times every week and kept at room temperature.

3.2. Determination of Early Stationary Phase of Growth for Each Strain:

Each strain of pathogens was grown in TSBY at 37°C overnight and inoculated at 5% level into TSBY tubes in triplicate. Tubes were incubated at 37°C up to 12h. Duplicate samples of each culture broth were taken from the incubator at 37°C every 2h and the Optical Density at 600 nm ($OD_{600\ nm}$) was measured using a spectrophotometer (Beckman DU-50, Fullerton, CA). This procedure was repeated twice and the average OD was calculated for each strain. A growth curve was plotted for each strain from average OD data as $OD_{600\ nm}$ versus time of incubation, and the time of early stage stationary phase for each strain was determined. This information was used in the subsequent studies to obtain cells at the early stationary phase of growth before high hydrostatic pressurization.

3.3. Growth and Preparation of Cell Suspensions for Pressurization:

Bacterial cell suspensions grown in TSBY were kept at 37°C for the desired time period specific for each strain in order to obtain cells at early stationary phase. Final OD was measured and compared with the growth curve data for each strain.

Following growth of bacterial cells at early stationary phase, a culture broth was diluted with 1% peptone (Becton Dickinson and Co., Cockeysville, MD) solution to give a population of about 10^8 to 10^9 colony forming units (cfu)/ml. The cell suspensions were dispensed in 2 ml portions in sterile plastic cryovials with silicone threads (2 ml capacity, Simport Plastic, Canada) in triplicate. Air bubbles were avoided. The cryovials were individually vacuum-sealed in sterile plastic bags (Fisher Scientific, Pittsburgh, PA) for pressurization. The cryovials in sterile plastic bags were kept at 4°C before and after pressurization that did not exceed 1h. Controls were not pressurized.

3.4. High Hydrostatic Pressurization Equipment:

A high hydrostatic pressurization unit (Engineered Pressure Systems, Wilmington, MA) capable of operating up to 690 MPa between 22 to 95°C was used throughout the experiments (Figure 3.1).

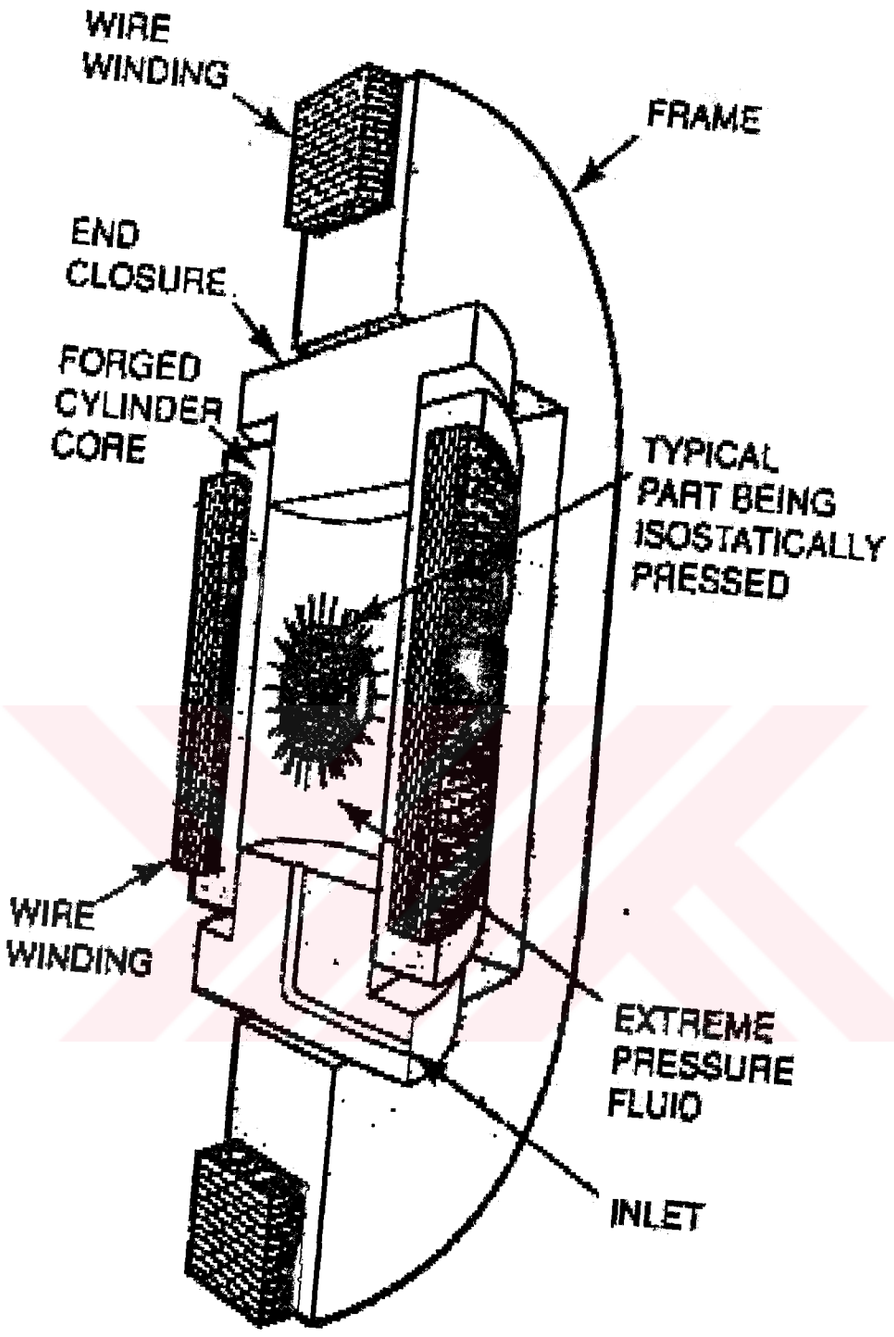

Figure 3.1. High hydrostatic pressurization equipment

The pressurization unit has a reservoir tank, a high-pressure intensifier, a pressure chamber, a movable frame around the chamber and a temperature control device. The pressure chamber (10-cm internal diameter by 45-cm length) was filled with pressure medium, which was a mixture of deionized water with 5% soluble oil (Hydrolubric 2, Houghton International, Valleytorge, PA). A high-pressure intensifier was used to pump the pressure medium from the reservoir tank into the pressure chamber until the desired pressure is reached. The liquid inside the pressure chamber can be heated to a desired temperature prior to pressurization by an electric heating system surrounding the pressure chamber. The rate of pressure increase of the unit was about 140 MPa/min and the pressure release time was about 2 min. The pressure release was performed automatically by the hydrostatic pressurization unit after each pressurization cycle. Pressurization times reported in this study did not include the pressure increase and release times. The pressure level and time and temperature of pressurization were set by an automatic device, which also recorded all the parameters during the pressurization cycle.

3.5. Determination of Temperature and Time Relations for Equilibration:

As the cell suspensions were prepared at room temperature, it was necessary to determine the time of preheating necessary for the cell suspensions in cryovials to equilibrate with the liquid temperature in the pressure chamber before pressurization at different temperatures. For this purpose, a digital

temperature sensor (Beckman Instrument Inc., Fullerton, CA) was placed in the center of a cryovial filled with cell suspension and was placed in a water bath. The water bath temperature was set to 37, 47, 52 and 62°C and the time necessary for the temperature in the cryovial to reach to 35, 45, 50 and 62°C respectively, were recorded. It was determined that keeping the cryovials for 4 min at 37°C for 35°C, 5 min at 47°C for 45°C, 6 min at 52°C and 62°C for 50°C and 60°C treatments respectively, was necessary for temperature equilibration.

3.6. High Hydrostatic Pressurization of Cell Suspensions in Cryovials:

In the first part of the experiments, the cryovials containing cell suspensions for each strain in vacuum-sealed plastic bags were placed in a stainless steel wire basket and submerged in the liquid at 25 or 50°C in the pressure chamber. This study was performed to determine the variation in strain resistance to high hydrostatic pressure. After the chamber was closed, the cell suspensions were kept for 5 min at 25°C and for 6 min at 52°C for temperature equilibration. The cell suspensions were pressurized at 345 MPa either at 25°C for 5 min or at 50°C for 5, 10, and 15 min.

Two strains, one resistant and one sensitive, from each species were used in the second part of the study. The cryovials containing cell suspensions for each strain in vacuum-sealed plastic bags were exposed to 207, 276 or 345 MPa pressure for 5 to 10 min at 25, 35, 45 or 50°C to determine the viability loss and

injury among the survivors. The cryovials for each strain were heated for 4, 5 and 6 min for 35, 45 and 50°C treatments respectively, before pressurization.

Duplicate cryovials were used for each treatment. Immediately after pressurization the cryovials were removed, cooled in an ice bath and stored at 4°C prior to enumeration of cfu per milliliter (within 1h). Unpressurized cell suspensions were enumerated as controls.

3.7. Thermal Inactivation of Bacterial Cell Suspensions:

Two strains, one resistant and one sensitive, from each species were used in this study. Cells from the early stationary phase of growth, diluted in 1% peptone solution to 10^8 to 10^9 cfu/ml, were used. Duplicate cryovials containing the cell suspensions for each strain in vacuum-sealed plastic bags were placed in water bath that was set to 52°C for inactivation at 50°C and to 62°C for inactivation at 60°C. The cryovials were kept for 5 and 6 min, respectively, for temperature equilibration as described previously. Duplicate cryovials were removed after 5, 10, and 15 min from the water bath and then cooled in an ice bath and stored at 4°C prior to enumeration of cfu/ml within 30 min. The cells in unheated suspensions were enumerated as controls.

3.8. Organic Acid Treatment of the Bacterial Cell Suspensions:

Two strains, one resistant and one sensitive, from each species were used in this study. 1% DL-lactic acid (Sigma, St. Louis, MO) and 2.1 % monohydrate citric acid (Sigma, St. Louis, MO) were used for pH treatment of the bacterial strains. 100-mM solutions of both of the organic acids were prepared in 1% peptone (Becton Dickinson and Co., Cockeysville, MD) solution and the pH was adjusted to 4.5, 5.5 or 6.5 by adding 10 N NaOH on a pH meter (# 71 pH meter, Beckman Instrument Inc., Fullerton, CA). Bacterial cells at early stationary phase were suspended at the final concentration of 10^8 cfu/ml for each organic acid treatment at pH 4.5, 5.5 or 6.5. The cryovials in duplicate, containing cell suspensions for each bacterial strain were pressurized at 345 MPa for 5 min at 35°C for Gram-positive bacterial strains and at 25°C for Gram-negative bacterial strains. Immediately after pressurization the cryovials were removed, cooled in an ice bath and stored at 4°C prior to enumeration for cfu per milliliter (within 1h) for viable and injured cells. Unpressurized cell suspensions were enumerated as controls.

3.9. Enumeration of Viable and Injured Cells in Pure Cultures:

Pressurized, thermally inactivated, pH treated and control cell suspensions in cryovials were serially diluted in 0.1% sterile peptone (Becton Dickinson and Co., Cockeysville, MD) solution. From the selected dilutions, 0.1 ml-portions were surface plated in duplicate plates on prepoured non-selective and selective agar media plates specific for each species. With samples containing less than 30 cfu/ml

in a 1:10 dilution, 1 ml of undiluted cell suspension was plated in three plates (0.3, 0.3, and 0.4 ml). The non-selective agar medium for all the pathogens was Tryptic Soy Agar (TSA; Difco, Detroit, MI) supplemented with 0.6% yeast extract (TSAY; Difco, Detroit, MI). TSA was supplemented with 0 to 5% w/v NaCl (TSAN; Fisher Scientific, Fair Lawn, NJ) to determine the optimal salt level to distinguish injury. The criterion for selection of the optimal salt level was to define the salt concentration that suppressed the nonpressurized cells minimally while suppressing the pressurized cells maximally, relative to the nonsupplemented TSA (Stewart et al., 1997; Crawford et al., 1988). The concentration of NaCl with the greatest degree of injury and with little effect on pressurized cells were observed with TSA supplemented with: 5% NaCl for *Staphylococcus aureus* 485 and 765, *Listeria monocytogenes* CA and OH_2; 2.5% NaCl for *Salmonella typhimurium* E 21274, *E.coli* O157:H7 931 and 933; 1.5% NaCl for *Salmonella enteritidis* FDA. The total of four plates (2 cryovials x 2 plates) was incubated at 37°C for 2 days and plates containing 25 to 250 cfu/ml were selected for enumeration. The difference between the viable (cfu in TSAY) and non-injured cells (corresponding cfu in TSAN) was used to estimate injured survivors (Ray 1996). Each experiment, with duplicate cryovials for each strain, was performed twice on separate days, and the average results were presented.

3.9.1. Statistical Analysis:

Analyses of variance (ANOVA) of the data was carried out on the mean logarithm to the base 10 cfu/ml reduction at each pressure-temperature-time combination and for pH treatment for each strain.

3.10. Application of High Hydrostatic Pressure to Food Systems:

3.10.1. Preparation and Inoculation of Food Samples with Bacteria:

Grade A pasteurized milk (Dairy Gold Foods, CO), pasteurized orange juice (Minute Maid, TX), and cream of chicken soup (Safeway Inc., CA) were obtained from a local market, autoclaved for 5 min and stored at 4°C until use. Two strains, one resistant and one sensitive, from each species were used. After a bacterial strain had reached to early stationary phase, the food samples were inoculated with bacteria to obtain about 10^8 colony forming units (cfu)/ml food sample and dispensed in 2 ml portions in sterile plastic cryovials (Simport Plastic, Quebec, Canada) in duplicate. Air bubbles were avoided. The cryovials were vortexed for 2 min and vacuum-sealed in sterile plastic bags (Fisher Scientific, PA) and kept at 4°C prior to pressurization that did not exceed 1h.

3.10.2. High Hydrostatic Pressurization of Food Samples:

High hydrostatic pressurization of pasteurized milk, pasteurized orange juice and cream of chicken soup was carried out at 345 MPa for 5 min at 50°C excluding the pressure increase and release times. Pressurization of food samples in cryovials was performed in the high hydrostatic pressure unit as described before.

3.10.3. Shelf-Life Study of Food Samples at Elevated Temperatures:

Pressurized milk and orange juice were stored at 4°C for 24h to see the effect of chilled storage and then at 37°C for an additional 48h to satisfy the optimum growth condition for the pathogens studied. Although, this storage regime is not likely to be seen in a food-processing environment, it has been used to see the effectiveness of high hydrostatic pressure treatment on destruction of the pressure-resistant strains of the pathogens considered. Pressurized cream of chicken soup was stored at 25°C up to one week.

Bacteriocin (BP_1) treatment with pressurization was applied to milk and cream of chicken soup samples at a final level of 5000 AU/ml food sample. BP_1 treated milk samples were stored at 25°C up to 30d (4 times the recommended shelf life at chilled storage). The storage data can be related to chill storage conditions, as any inhibition of growth at 25°C would also hold true for ant temperature below this. The samples were taken at 2 day interval up to two

weeks and 5 day interval thereafter. BP_1 treated cream of chicken soup samples were stored at 25°C up to 9 days and samples were taken at 2 day interval. New cryovials were opened each time. Controls were not pressurized.

3.10.4. Enumeration of Bacteria in Food Samples:

After pressure treatment, appropriate serial dilutions were performed in sterile dilution fluid (0.1% peptone solution). In all cases duplicate 0.1-ml volumes of food samples were surface plated onto Tryptic Soy Agar (TSA; Difco, Detroit, MI), supplemented with 0.6% yeast agar (TSAY; Difco, Detroit, MI) plates. 1 ml of 0.5% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO) per 100 ml of melted agar was added to distinguish the colonies easily for accurate counting (Vanderzant and Splittstoesser, 1992). The number of surviving organisms was enumerated as cfu after incubation at 37°C for 48h.

For shelf-life studies, duplicate cryovials were opened and streak plated for milk and orange juice samples and surface plated for cream of chicken soup samples on duplicate plates on prepoured non-selective and selective agar media plates specific for each species. The non-selective agar medium was TSAY and selective agar media were: TSAY supplemented with 10% NaCl (Fisher Scientific, Fair Lawn, NJ) for *Staphylococcus aureus* strains, Modified Oxford (MOX; Difco, Detroit, MI) agar for *Listeria monocytogenes* strains, Violet Red Bile Agar (VRBA; Difco, Detroit, MI) for *Escherichia coli* O157:H7 strains and Xylose Lysine Desoxycholate (XLD; Difco, Detroit, MI) agar for *Salmonella*

strains. The total of four plates (2 cryovials x 2 plates) were incubated at 37°C for 48h and examined for presence or absence of pathogens. Each experiment, with duplicate cryovials for each strain, was performed twice on separate days for each food sample, and the average results were presented

3.11. Preparation of Bacteriocin (BP_1) from Nisin and Pediocin AcH:

Lactococcus lactis ATCC 11454 for nisin (Ray 1992a) and *Pediococcus acidilactici* LB 42-923 for pediocin AcH (Bhunia et al., 1988; Ray 1992b) were grown in Tryptic Glucose Yeast Extract (TGE; Table A.1) broth at 30°C for 24h and 37°C for 18h, respectively. The two bacteriocins were prepared from cultures by the method developed in Food Microbiology Laboratory, Animal Science Department, University of Wyoming (Yang et al., 1992; Yang and Ray 1994). In brief, the bacteriocins were partially purified from the culture broth by the method of pH-dependent adsorption and desorption of the molecules on and from the cell surface of the producer strain (Yang et al., 1992). The freeze-dried preparation was assayed for activity units (AU)/mg of powder against a lawn of *Lactobacillus plantarum* NCDO 955 (Biswas et al., 1991; Ray 1992b) and stored at –10°C. Nisin and pediocin AcH were used in combination (BP_1) to increase antibacterial activity (Hanlin et al., 1993). The required amount of the freeze-dried powder was dissolved in sterile deionized water, assayed for AU/ml, and added to the cell suspensions at a final level of 5000 AU/ml food sample before dispensing cells into the cryovials prior to pressurization.

3.11.1. Bacteriocin (BP_1) Production:

3.11.1.1. Nisin Production:

The bacteriocin, nisin, was produced with the producer strain *Lactococcus lactis* ATCC 11454. *L. lactis* ATCC 11454 stock culture was obtained from the University of Wyoming Food Microbiology Laboratory freeze stock and maintained in TGE maintenance broth with buffer (0.2% sodium acetate, 0.2% sodium citrate and 0.1% sodium phosphate; Difco, Detroit, MI) at 30°C. Nisin was produced by batch fermentation of *L. lactis* ATCC 11454 in TGE broth for nisin production on a continuous fermentor (Biostat M, B. Braun, Germany).

Initial pH of the TGE broth was adjusted to pH 7.0 using sterile 5 N NaOH, temperature of the broth was equilibrated to 30°C and the fermentor agitator was set at 100 RPM. The TGE broth was then inoculated with 1% (v/v) of an overnight culture of *L. lactis* ATCC 11454 and allowed to ferment for 16 to 18h. The pH was maintained at 5.8 to 6.0 throughout the entire fermentation by addition of sterile 5 N NaOH. Final pH of the culture broth was double-checked on a pH meter (# 71 pH meter, Beckman Instrument Inc., Fullerton, CA).

After fermentation, the culture broth was aseptically transferred to sterile bottles and heat treated in a water bath at 71.5°C for 30 minutes to kill the bacterial cells. 1 ml culture broth was removed and assayed for AU/ml. The

culture broth was cooled in an ice bath and then allowed to equilibrate to room temperature. The pH of the culture broth was adjusted to 6.5 using sterile 5 and/or 10 N NaOH and let stand overnight at 4°C allowing the bacteriocin peptide to reattach to the bacterial cell wall.

Following attachment to the cell walls, the culture broth was centrifuged (J2-21M Induction Drive Centrifuge, Beckman Instruments Inc., Fullerton, CA) at 11500 RPM for 15 min at 4°C in sterile centrifuge tubes. The supernatant was taken off and assayed for activity. The pellet was resuspended in sterile deionized water (5% v/v of the original culture broth volume). The cell suspension was then frozen (-40°C) and subsequently freeze-dried (Freeze Mobile 6, The Virtis Company Inc., Gardiner, NY) to obtain a preparation of bacteriocin positive bacterial cells in powder form. The freeze-dried preparation was stored at –10°C until assayed for AU/ml.

3.11.1.2. Pediocin AcH Production:

The bacteriocin Pediocin AcH was produced with the producer strain *Pediococcus acidilactici* LB42-923. *Pediococcus acidilactici* LB42-923 stock culture was obtained from the University of Wyoming Food Microbiology Laboratory freeze stock and maintained in TGE maintenance broth (Table A.1) at 37°C. Pediocin AcH was produced by batch fermentation of *Pediococcus acidilactici* LB42-923 in TGE broth for pediocin production.

TGE broth for pediocin production (800 ml) was inoculated with a 1% (v/v) inoculum of an overnight maintenance culture of *Pediococcus acidilactici* LB42-923. The culture was then incubated at 37°C for 16 to 18 hours until a final pH of 3.6-3.9. The pH was controlled by a pH meter (# 71 pH meter, Beckman Instrument Inc., Fullerton, CA).

After fermentation, the culture broth was aseptically transferred to sterile bottles and heat treated in a water bath at 71.5°C for 30 minutes to kill the bacterial cells. 1 ml culture broth was then removed and assayed for AU/ml. The culture broth was then cooled in an ice bath and then allowed to equilibrate to room temperature. The pH of the culture broth was then adjusted to 6.5 using sterile 5 and/or 10 N NaOH and let stand overnight at 4°C to allow the bacteriocin peptide to reattach to the bacteriocin cell wall.

Following reattachment to the cell walls, the culture broth was centrifuged (J2-21M Induction Drive Centrifuge, Beckman Instruments Inc., Fullerton, CA) at 11500 RPM for 15 min at 4°C in sterile centrifuge tubes. The supernatant was taken off and assayed for bacteriocin activity. The pellet was resuspended in sterile deionized water (5% v/v of the original culture broth volume). The cell suspension was frozen (-40°C) and subsequently freeze-dried (Freeze Mobile 6, The Virtis Company Inc., Gardiner, NY) to obtain a preparation of bacteriocin positive bacterial cells in powder form. The freeze-dried preparation was stored at –10°C until assayed for AU/ml.

3.11.1.3.Standardization of Activity Units (AU):

After preparation of freeze dried bacterial cell preparations containing either pediocin AcH or nisin, the activity units per gram (AU/g) of the cell preparations was assayed by serial dilution and spotting over a bacterial lawn of a bacteriocin sensitive strain indicator organism, *Lactobacillus plantarum* NCDO 955. *Lactobacillus plantarum* NCDO 955 was grown and maintained in Trypticase Glucose Yeast Extract (TGE) broth for culture maintenance (Table A.1) at 30°C. 5 µl of fresh overnight indicator culture broth was inoculated into 5 ml of melted TGE soft agar (Table A.1) and overlaid on TGE plating agar (Table A.1).

After preparation of the TGE plates overrlaid with the indicator bacterial lawn, 3 to 4 mg of freeze-dried cell preparation was dissolved in 1 ml of sterile deionized water. This stock solution was then serially diluted (1:10, 1:20, 1:30 to 1:300) by adding 10 µl aliquots of stock solution to the appropriate volume of sterile deionized water. Duplicate 5 µl portions from each dilution were spotted directly on the lawn seeded with indicator microorganism. The plates were incubated at 30°C for 16 to 18h and then examined to determine the highest dilution which exhibited a clear zone in the lawn of indicator microorganism. A zone of 2 mm or more in diameter was considered positive result for bacteriocin activity at that dilution. The same procedure was also followed to determine the AU/ml of bacteriocin positive culture broth before the cells were harvested and

freeze dried. The AU/g of freeze-dried cell preparations were then calculated in the following manner:

$$\frac{\text{g of freeze dried cells in 1 ml stock solution}}{1000\ \mu l} \times 10\mu l = \frac{\text{g of freeze dried cell prep.}}{\text{total } \mu l \text{ in final dilution}}$$

$$1\ \text{AU} = \frac{\text{g of freeze dried cell prep.}}{\text{total } \mu l \text{ in final dilution}} \times 5\mu l$$

$$\frac{1}{1\,\text{AU}} = \frac{\text{AU}}{\text{g freeze dried cell preparation}}$$

CHAPTER IV

RESULTS

4.1. Growth of the Foodborne Pathogens:

Data for nine strains of *Listeria monocytogenes*, seven strains of *Staphylococcus aureus*, six strains of each of *Escherichia coli* O157:H7 and *Salmonella* species are given in Figure 4.1. Average results are given in Table A.2. All the *Listeria monocytogenes* and *Staphylococcus aureus* strains had reached the early stationary phase of growth after 6h of incubation at 37°C. The time to reach the early stationary phase for six strains of each of *Escherichia coli* O157:H7 strains and *Salmonella* species was 8h at 37°C. Individual average results of OD 600 nm readings for *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *Salmonella* serotypes are given in Figure A.3.A through A.3.H, respectively.

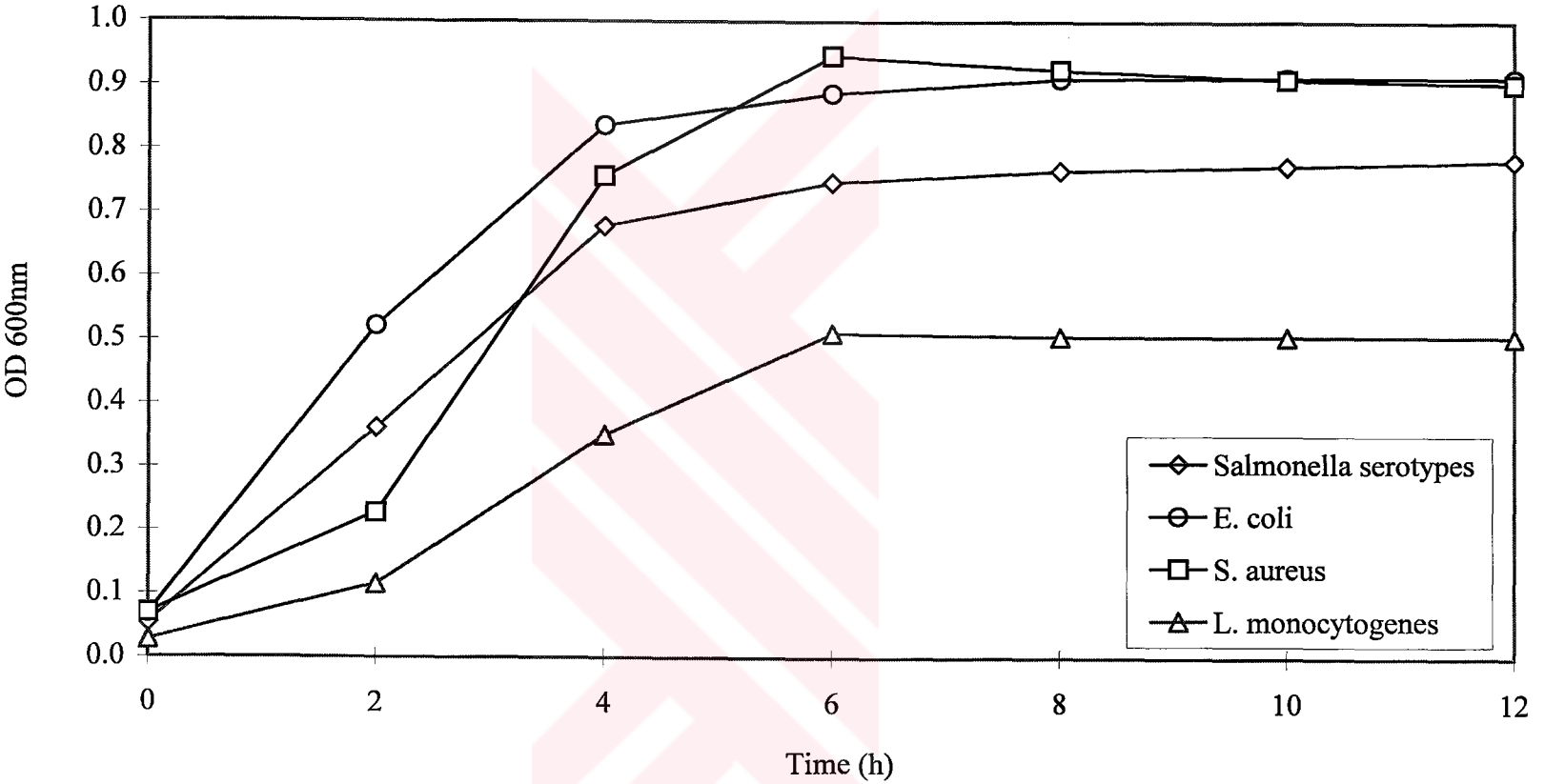

Figure 4.1. Growth of four foodborne pathogens at 37°C in TSBY.

4.2. Variation in Pressure Resistance among Strains of Foodborne Pathogens at 25°C:

The cells from the early stationary phase of growth for each pathogen were used for pressurization. The cell suspensions in cryovials were exposed to 345 MPa hydrostatic pressure for 5 min at 25°C. Viability loss for each strain was determined from the difference in cfu between the control and pressure treated samples.

Among the nine *L. monocytogenes* strains, the viability loss ranged between 0.92 to 3.53 log cycles (Figure 4.2.A). The standard deviation of the plate counts was between 0.051 and 0.243. The most resistant (strain CA) and most sensitive (strain SLR1) differed in viability loss by a factor of about 4.0. Strain ScottA, which has been used in many *L. monocytogenes* studies (Styles et al., 1991; Kalchayanand et al., 1994; Kalchayanand et al., 1998a; Kalchayanand et al., 1998b; Patterson and Kilpatrick, 1998), was also quite resistant to pressure.

Viability loss among seven *S. aureus* strains ranged from 0.70 to 7.80 log cycles (Figure 4.2.B), about 11-fold difference between the most resistant (strain 485) and most sensitive (strain 582) strains. The standard deviation of the individual plate counts was between 0.048 and 0.130.

For *E. coli* O157:H7 strains, viability loss ranged from 2.80 to 5.64 log cycles, which is about a two fold difference between the most resistant (strain 933) and most sensitive (strain 932) strains (Figure 4.2.C.). The standard deviation of the individual plate counts was between 0.037 and 0.131.

Among the six *Salmonella* strains, the smallest viability loss, of 5.45 log cycles, was for the strain *S. enteritidis* FDA, and the highest, of 8.34 log cycles, was for the strain *S. choleraesuis* subsp. *choleraesuis* ATCC 10708; this is about a 1.5-fold difference (Figure 4.2.D.). For the two strains of either *S. typhimurium* or *S. enteritidis*, viability loss differed by about 1.5 to 2.0 log cycles. The standard deviation of the plate counts was between 0.061 and 0.160 for the *Salmonella* strains.

The individual plate counts and their standard deviation for each strain of *L. monocytogenes*, *S. aureus*, *E. coli*O157:H7 and *Salmonella* are given in Table A.4.A through A.4.D.

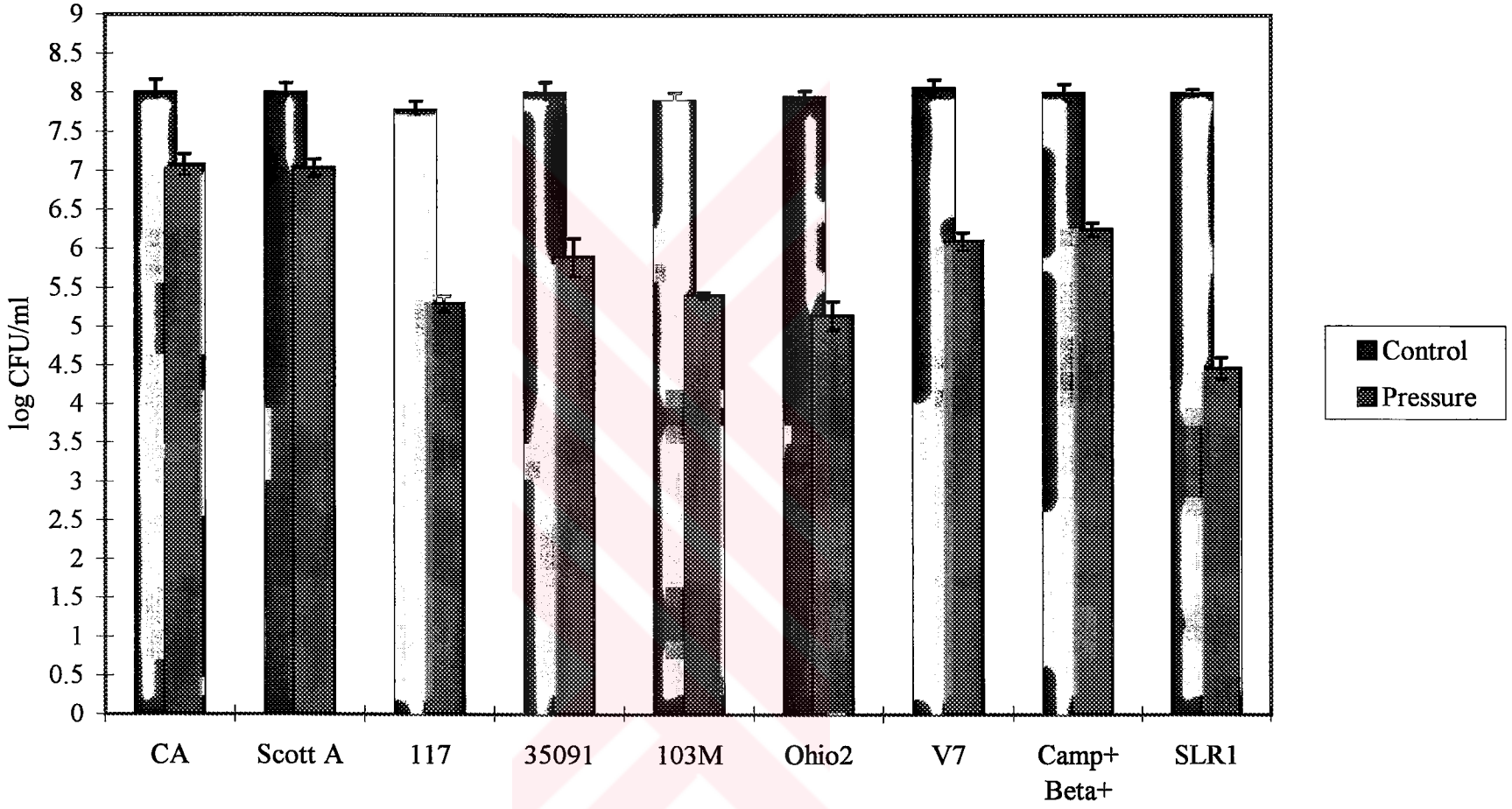

Figure 4.2.A. Viability loss of *Listeria monocytogenes* strains following pressurization at 345 MPa for 5 min at 25°C (pH=7.0).

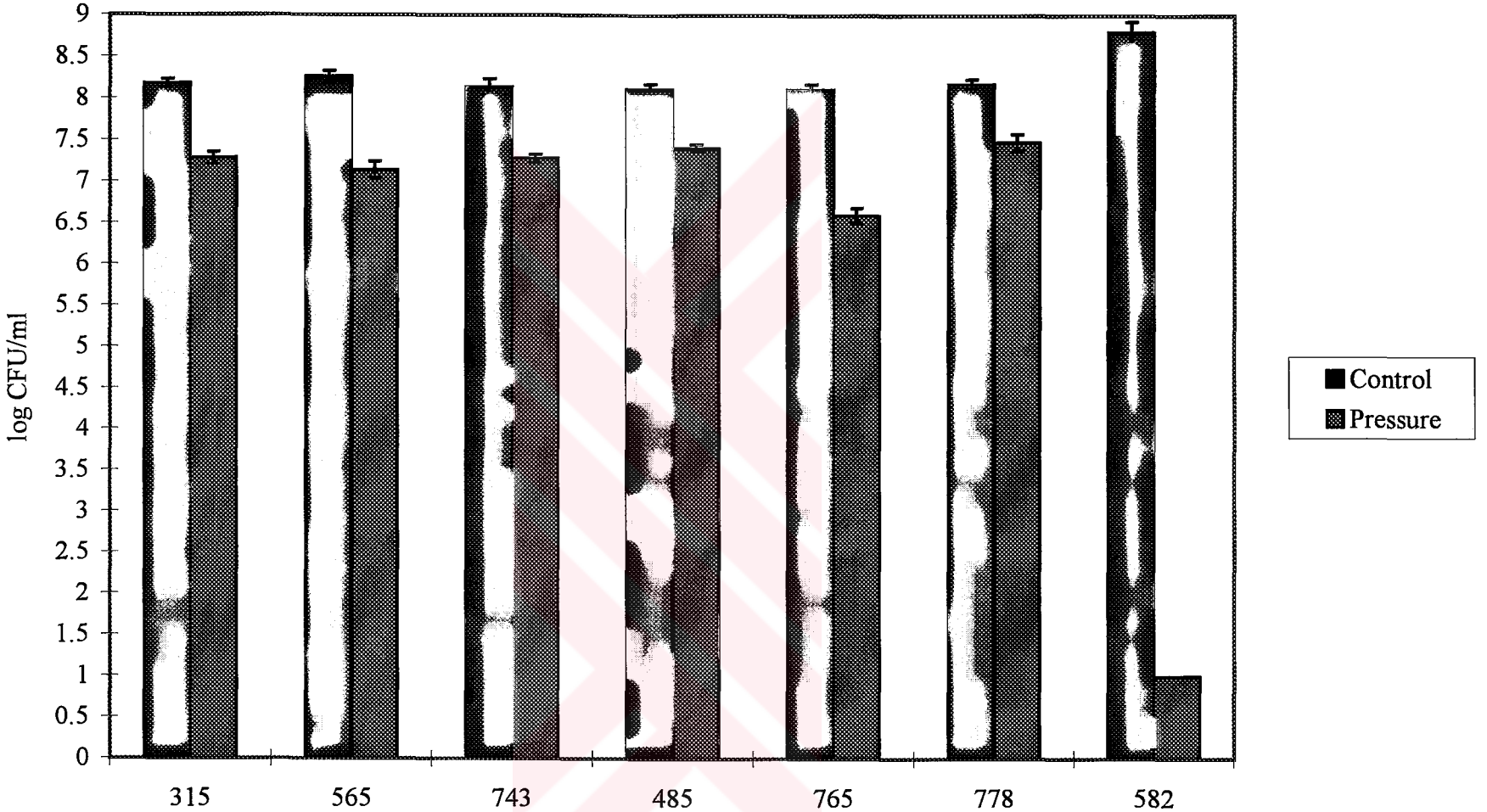

Figure 4.2.B. Viability loss of *Staphylococcus aureus* strains following pressurization at 345 MPa for 5 min at 25°C (pH=7.0).

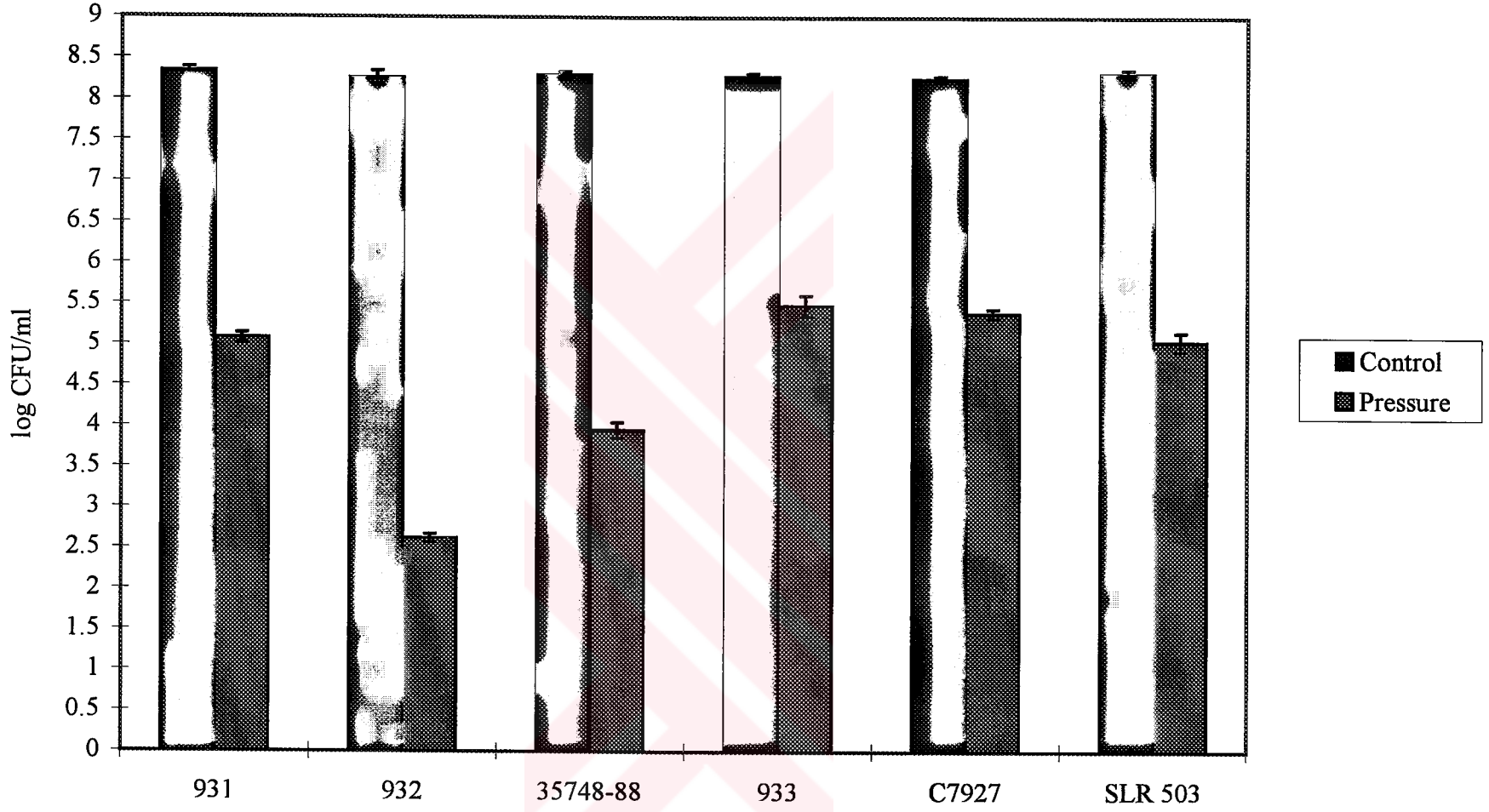

Figure 4.2.C. Viability loss of *Escherichia coli* O157:H7 strains following pressurization at 345 MPa for 5 min at 25°C (pH=7.0).

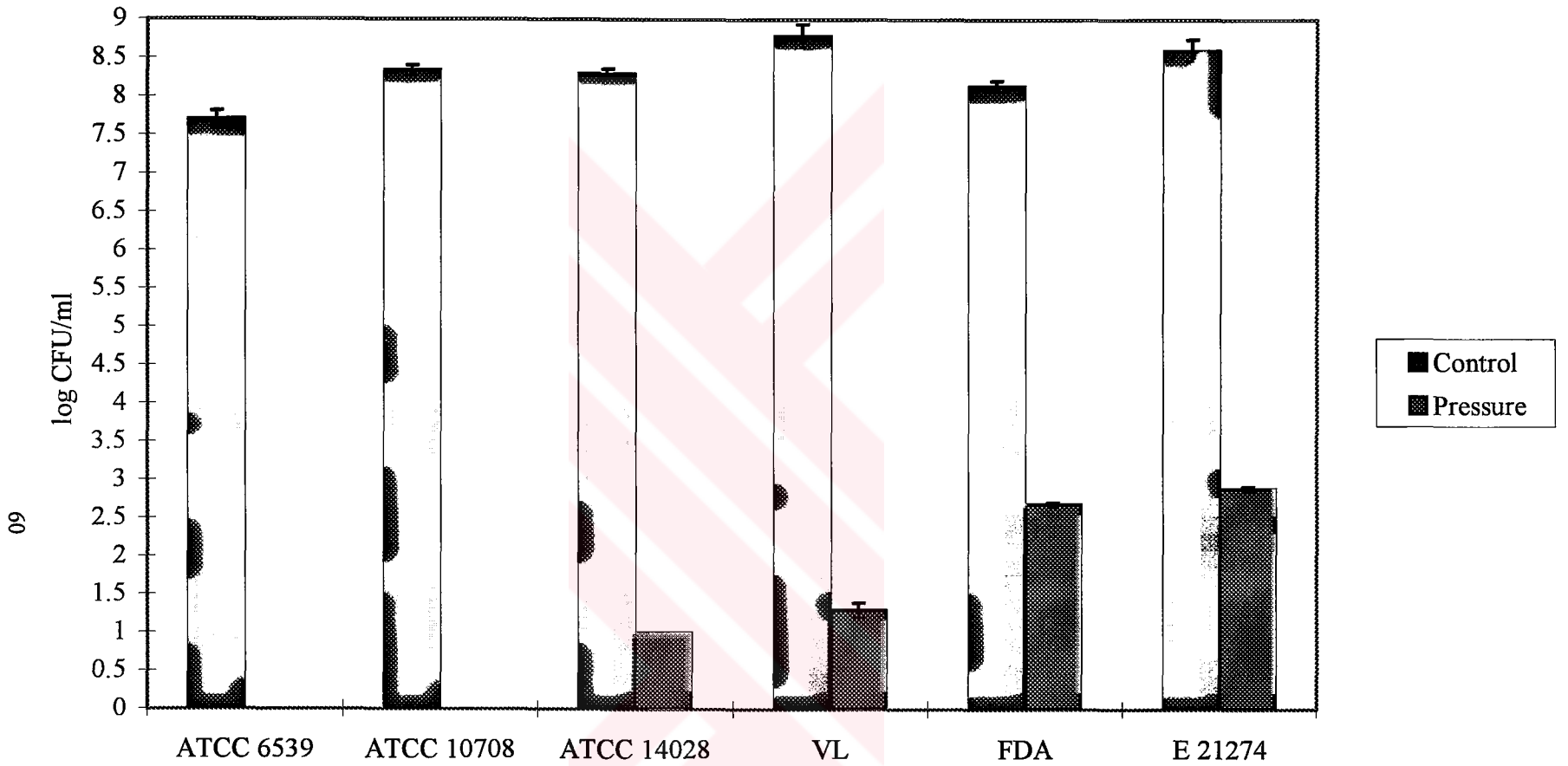

Figure 4.2.D. Viability loss of *Salmonella* strains following pressurization at 345 MPa for 5 min at 25°C (pH=7.0).

4.3. Sensitivities of Pressure-resistant and Pressure-sensitive Strains to Pressure at 50°C:

From the studies described above, two strains from each species were selected for this study. The most pressure-resistant and one relatively less resistant strains were used in the following pressurization studies (Table 4.1).

Table 4.1. Selected pressure-resistant and pressure-sensitive strains.

Bacteria	**Pressure-resistant strain**	**Pressure-sensitive strain**
L. monocytogenes	Ca	$Ohio_2$
S. aureus	485	765
***E. coli* O157:H7**	933	931
Salmonella	*enteritidis* FDA	*typhimurium* E 21274

The cell suspensions were subjected to 345 MPa at 50°C for 5, 10, or 15 min, and survivors were enumerated as described. The results are depicted in Table 4.2. Individual plate counts are given in Table A.5.A through A.5.D.

Table 4.2. Viability loss of strains of foodborne pathogens by pressurization at 345 MPa and 50°C [a].

Bacterial Strains	Control	Log_{10} cfu/ml after pressurization for 5 min	10 min	15 min
***Listeria monocytogenes*:**				
CA	8.11 ± 0.03	ND	ND	ND
$Ohio_2$	8.00 ± 0.08	ND	ND	ND
***Staphylococcus aureus*:**				
485	8.08 ± 0.09	2.70 ± 0.05	2.00 ± 0.07	1.78 ± 0.06
765	8.11 ± 0.11	ND	ND	ND
***Escherichia coli* O157:H7:**				
933	8.30 ± 0.04	ND	ND	ND
931	8.18 ± 0.07	ND	ND	ND
***Salmonella* spp.:**				
S. enteritidis **FDA**	8.18 ± 0.08	ND	ND	ND
S. typhimurium **E 21274**	8.39 ± 0.06	ND	ND	ND

[a] ND, no cfu was detected in 1 ml of cell suspension from each of the samples tested.

No survivors were detected for seven of eight strains after 5 min of pressurization at 50°C; a viability loss of more than 8 log cycles occurred in all seven strains. Among the different pathogens studied, highest pressure-resistance was observed for *S. aureus* 485. The viability losses in this pressure-resistant strain were 5.38, 6.08, and 6.30 log cycles, respectively, after pressurization for 5, 10, and 15 min. In comparison, viability loss due to thermal inactivation alone at 50°C was less than 2 log cycles after 15 min in all eight strains (Table 4.3).

In all four genera, the pressure-resistant strains were also found to be comparably resistant to thermal treatment. Individual plate counts for thermal treatment alone are given in Table A.6.A through A.6.D.

Table 4.3. Viability loss of strains of foodborne pathogens by thermal inactivation at 50^0C.

Bacterial Strains	Control	Log_{10} cfu/ml after thermal inactivation for 5 min	10 min	15 min
***Listeria monocytogenes*:**				
CA	8.30 ± 0.09	7.60 ± 0.11	7.23 ± 0.11	7.00 ± 0.10
$Ohio_2$	8.14 ± 0.10	7.04 ± 0.10	6.90 ± 0.12	6.70 ± 0.19
***Staphylococcus aureus*:**				
485	8.11 ± 0.15	8.08 ± 0.10	7.60 ± 0.08	7.48 ± 0.08
765	8.08 ± 0.11	7.46 ± 0.05	7.15 ± 0.14	7.04 ± 0.10
***Escherichia coli* O157:H7:**				
933	8.04 ± 0.08	7.30 ± 0.11	6.95 ± 0.16	6.48 ± 0.15
931	8.11 ± 0.12	6.84 ± 0.21	6.60 ± 0.14	6.30 ± 0.12
***Salmonella* spp.:**				
S. enteritidis **FDA**	8.32 ± 0.11	7.26 ± 0.14	7.08 ± 0.10	6.70 ± 0.15
S. typhimurium **E 21274**	8.08 ± 0.09	6.60 ± 0.13	6.48 ± 0.22	6.00 ± 0.08

4.4. Decimal Reduction (D) Values:

From the viability loss data given in Table 4.2 and Table 4.3, decimal reduction times (D values) were calculated for pressurization and thermal treatment at 50°C (Bradshaw et al., 1987; Kalchayanand et al., 1998b). Where no survivors were detected after 5 min, D values were estimated by dividing the initial $\log_{10}$ cfu/ml by 5. The D values of pressurization for seven strains were ≤0.62 min except for *S. aureus* 485 (2.55 min) (Table 4.4). The D value for thermal treatment was much higher, at both 50 and 60°C.

4.5. Effect of High Hydrostatic Pressure, Time and Temperature on Pressure-resistant and Pressure-sensitive Strains of Foodborne Pathogens:

Pressure-resistant and pressure-sensitive strain indicated at Table 4.1 were used in this study. Cell suspensions were exposed to 207, 276 or 345 MPa pressure for 5 to 10 min at 25, 35, 45 or 50°C to determine viability loss and degree of injury.

Table 4.4. Estimated decimal reduction time (D)[a] of pressure resistant and pressure sensitive strains of four pathogens following pressurization at 345 MPa 50°C and thermal inactivation at 50°C and 60°C.

Bacterial Species	345 MPa at 50°C	50°C	60°C
Listeria monocytogenes:			
CA	≤0.62	11.71	7.12
Ohio$_2$	≤0.62	11.21	6.56
Staphylococcus aureus:			
485	2.55	21.10	6.91
765	≤0.62	14.58	6.33
***Escherichia coli* O157:H7:**			
933	≤0.60	9.94	4.96
931	≤0.61	8.82	4.78
***Salmonella* spp.:**			
S. enteritidis			
FDA	≤0.61	9.92	3.68
S. typhimurium			
E 21274	≤0.60	7.86	1.96

[a] D-value was calculated from the absolute value of the inverse of the slope from linear regression between logarithm of survivors and times (Bradshaw et al., 1987). In the absence of any survivors, D value was estimated by dividing the initial population (in $\log_{10}$) by time (min). Each value is the mean of eight counts.

4.5.1. Viability Loss:

The viability loss data for pressure-resistant and pressure-sensitive strains of Gram-positive and Gram-negative pathogens are given in Table 4.5 and Table 4.6, respectively. Increasing the pressurization time from 5 to 10 min had a significant effect only at 207 MPa of pressurization ($P < 0.05$). Increasing the pressurization temperature from 25 to 35 and to 45 and then to 50°C had a significant effect on the viability loss of all the strains ($P < 0.01$) within the pressure range of the study.

At 207 MPa the reduction in population of all strains, except *Salmonella* strains, was less than 1.20 log cycle at both 25°C and 35°C even after 10 min of pressurization. At 45°C after 10 min of pressurization at 207 MPa, the relatively sensitive *E. coli* O157:H7 931 and both *Salmonella* strains showed 3.71 and 4.30 log cycles reduction, respectively, while the populations of the other strains were reduced by 0.74 to 3.40 log cycles. At 50°C and 207 MPa, the viability loss of the relatively sensitive strains was between 3.08 and 5.70 log cycles with *Salmonella typhimurium* E21274 being the most sensitive; the viability loss of relatively resistant strains was between 2.05 and 5.46 log cycles; *S. aureus* 485 being the most resistant.

The viability loss of the Gram-positive strains at 276 MPa and 25°C and 35°C remained below 1.90 log cycles after 10 min and increased to about 3.28 to 4.73 log cycles after 10 min as the temperature was raised to 45°C (Table 4.5). However at 50°C, the cell population of all the strains of the pathogens was reduced

by at least 5.82 log cycles after 10 min. The cell populations of pressure-sensitive *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and both *Salmonella* strains was reduced by more than 8 log cycles within 5 min of pressurization at this temperature.

Pressurization at 345 MPa for 5 min at 35°C decreased the cell populations of *L. monocytogenes* CA and $Ohio_2$ by 5.0 and 5.40 log units, respectively (Table 4.5); however, the cell populations of four different strains of Gram-negative bacteria were completely destroyed (Table 4.6). When the temperature was increased to 50°C at this pressure range except strain *S. aureus* 485, more than 8 log cycle reduction for all strains was observed within 5 min.

S. aureus 485 seemed to be relatively the most resistant strain among all the strains within the conditions of the study. The death rate for this strain did not follow first order kinetics within time limits of the study. It was exponential initially, followed by a tailing effect, indicating that the rate of viability loss was at its maximum during the initial period of pressurization (Figure 4.3). This was especially evident when the cells were pressurized at and above 276 MPa at 45°C and 50°C or below 207 MPa at 50°C.

Table 4.5. Viability loss of pressure-resistant and pressure-sensitive strains of Gram-positive bacteria following combined treatment of hydrostatic pressure, time and temperature (pH=7.0).

		Mean Log_{10} cfu/ml[a] following pressurization at					
		207 MPa for		276 MPa for		345 MPa for	
	Temperature	5	10	5	10	5	10
Bacterial Species	°C	min		min		min	
Listeria monocytogenes							
CA		(8.17)[b]		(8.12)		(8.00)	
	25	0.47	0.69	0.64	0.78	0.86	2.40
	35	0.79	1.06	1.64	1.84	4.72	5.00
	45	1.03	1.33	2.84	3.28	5.70	6.10
	50	3.03	3.13	6.52	6.82	8.00	8.00
$Ohio_2$		(8.09)		(8.08)		(8.00)	
	25	0.63	0.79	0.72	0.85	2.64	3.05
	35	1.01	1.14	1.78	1.90	5.05	5.40
	45	1.25	1.49	3.18	3.60	8.00	8.00
	50	3.14	3.25	8.08	8.08	8.00	8.00
Staphylococcus aureus							
485		(8.00)		(8.00)		(8.03)	
	25	0.40	0.52	0.60	0.70	0.92	1.08
	35	0.50	0.64	0.80	1.10	2.03	2.43
	45	0.60	0.74	3.70	3.89	3.73	3.99
	50	1.77	2.05	5.22	5.82	5.33	6.03
765		(8.03)		(8.03)		(8.04)	
	25	0.59	0.65	0.80	0.95	1.96	2.09
	35	0.63	0.77	1.33	1.89	3.56	4.00
	45	0.71	1.08	3.83	4.73	5.20	5.64
	50	2.65	3.08	8.03	8.03	8.04	8.04

[a] n = 8.

[b] Values in parantheses represent the log_{10} cfu/ml of unpressurized control samples for a data set of time and temperature at one pressure.

Table 4.6. Viability loss of pressure-resistant and pressure-sensitive strains of Gram-negative bacteria following combined treatment of hydrostatic pressure, time and temperature (pH=7.0).

		Mean Log_{10} cfu/ml[a] following pressurization at					
		207 MPa for		276 MPa for		345 MPa for	
	Temperature	5	10	5	10	5	10
Bacterial Species	°C	min		min		min	
***Escherichia coli* O157:H7**							
933		(8.18)[b]		(8.17)		(8.22)	
	25	0.58	0.70	1.39	1.47	2.52	2.74
	35	0.78	0.88	1.87	2.39	8.22	8.22
	45	2.48	3.28	5.69	6.39	8.22	8.22
	50	3.86	4.00	5.85	6.47	8.22	8.22
931		(8.19)		(8.16)		(8.14)	
	25	0.79	0.85	1.46	1.56	3.66	4.00
	35	0.89	0.99	3.38	3.96	8.14	8.14
	45	2.71	3.71	5.88	6.56	8.14	8.14
	50	3.99	4.15	8.16	8.16	8.14	8.14
***Salmonella* spp.**							
***S. enteritidis* FDA**		(8.24)		(8.19)		(8.22)	
	25	0.98	1.04	2.24	2.41	4.12	4.92
	35	1.84	1.98	4.41	5.41	8.22	8.22
	45	3.24	3.40	5.71	6.05	8.22	8.22
	50	5.20	5.46	8.19	8.19	8.22	8.22
***S. typhimurium* E21274**		(8.74)		(8.64)		(8.51)	
	25	1.54	1.60	3.56	3.86	5.01	5.51
	35	2.14	2.31	4.46	5.86	8.51	8.51
	45	4.14	4.30	7.64	8.64	8.51	8.51
	50	5.60	5.70	8.64	8.64	8.51	8.51

[a] n = 8.

[b] Values in parantheses represent the log_{10} cfu/ml of unpressurized control samples for a data set of time and temperature at one pressure.

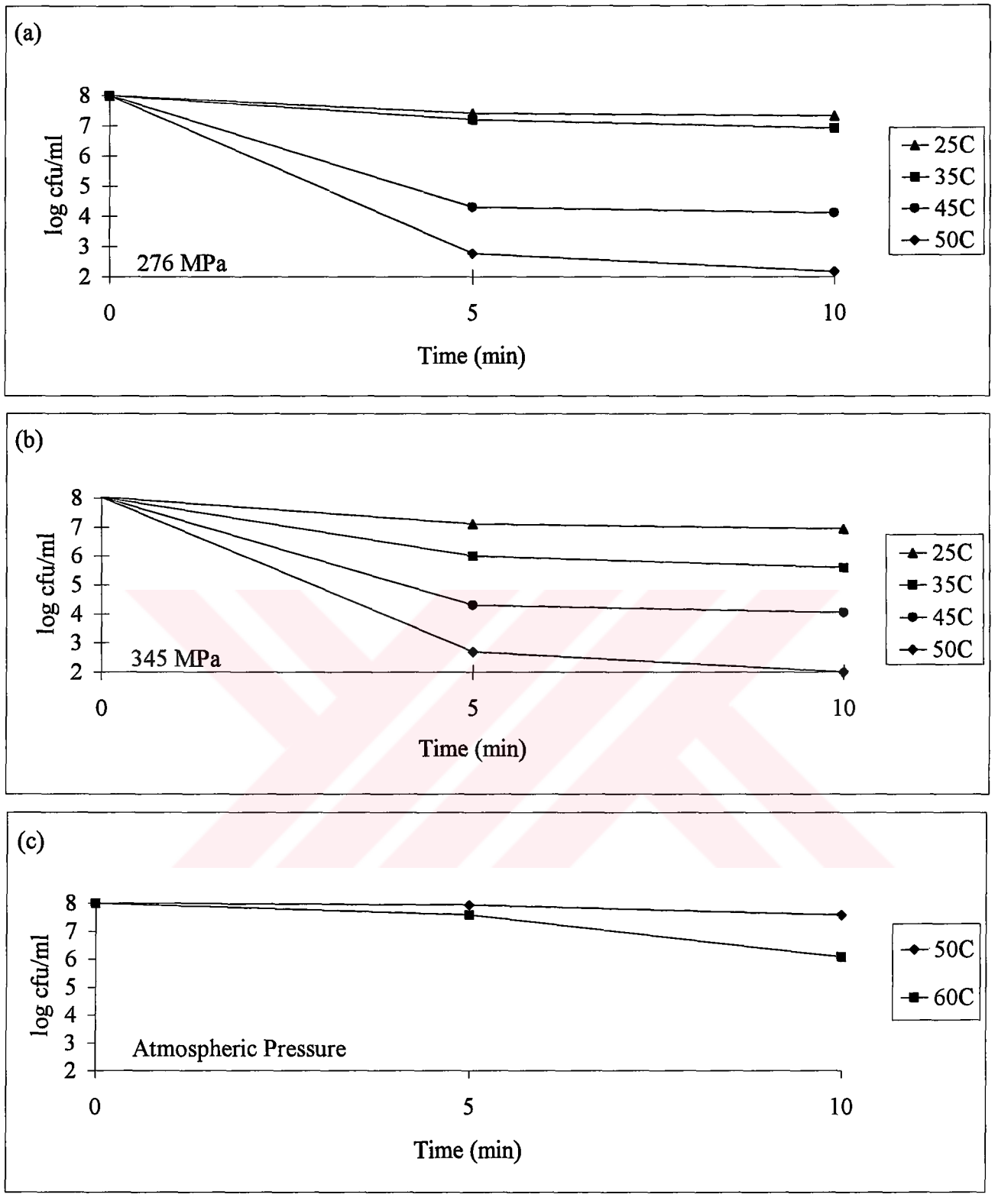

Figure 4.3. Viability loss of *S. aureus* 485 at (a) 276 MPa, (b) 345 MPa and (c) atmospheric pressure.

4.5.2. Degree of Sublethal Injury:

The level of sublethal injury, induced by high hydrostatic pressure, calculated by the difference in viable (cfu in TSAY) and non-injured cells (corresponding cfu in TSAN), for Gram-positive and Gram-negative strains is given in Tables 4.7 and 4.8, respectively. The percent of sublethally injured cells increased as the combinations of pressure, time and temperature were increased.

At 207 MPa and 25°C, the level of sublethally injured cells were 2% at the maximum even after 10 min of pressurization. The level of sublethally injured cells among the survivors varied between 4% to 57% at 35°C and 6% to 64% at 45°C after 10 min of pressurization and finally reached to 100% at 50°C after 10 min, except for *S. aureus* strains.

At 276 MPa, the extent of sublethal injury was less than 30% for the two *S. aureus* strains after 5 min of pressurization even at 45°C. However, at 50°C all the survivors of the species were totally injured after 10 min of pressurization.

At 345 MPa and 25°C, the survivors of all species were totally sublethally injured even after 5 min of pressurization except, *S. aureus* 765 and 485 (Table 4.7), and *E. coli* O157:H7 933 (Table 4.8). These species were totally injured after 5 min of pressurization at and above 35°C.

Table 4.7. Percent sublethal injury of pressure-resistant and pressure-sensitive strains of Gram-positive bacteria following combined treatment of hydrostatic pressure, time and temperature (pH=7.0).

		Percent Injury[a] (%) following pressurization at					
		207 MPa for		276 MPa for		345 MPa for	
	Temperature	5	10	5	10	5	10
Bacterial Species	°C	min		min		min	
Listeria monocytogenes							
CA	25	0	0	5	6	100	100
	35	27	42	53	68	100	100
	45	42	58	100	100	100	100
	50	75	100	100	100	ND[b]	ND
$Ohio_2$	25	1	1	6	7	100	100
	35	44	57	61	100	100	100
	45	54	64	100	100	ND	ND
	50	100	100	ND	ND	ND	ND
Staphylococcus aureus							
485	25	0	1	1	1	15	17
	35	2	4	14	16	33	41
	45	3	6	25	32	57	100
	50	13	17	42	100	100	100
765	25	1	2	2	3	43	45
	35	1	4	18	20	49	60
	45	6	10	30	39	100	100
	50	18	21	ND	ND	ND	ND

[a] n = 8.

[b] ND, not determined as no survivors were detected at the corresponding treatment.

Table 4.8. Percent sublethal injury of pressure-resistant and pressure-sensitive strains of Gram-negative bacteria following combined treatment of hydrostatic pressure, time and temperature (pH=7.0).

		Percent Injury[a] (%) following pressurization at					
		207 MPa for		276 MPa for		345 MPa for	
	Temperature	5	10	5	10	5	10
Bacterial Species	°C	min		min		min	
***Escherichia coli* O157:H7**							
933	25	0	1	13	16	37	42
	35	5	11	27	33	ND[b]	ND
	45	29	35	100	100	ND	ND
	50	50	100	100	100	ND	ND
931	25	2	2	15	20	100	100
	35	5	13	42	55	ND	ND
	45	36	49	100	100	ND	ND
	50	100	100	ND	ND	ND	ND
***Salmonella* spp.**							
***S. enteritidis* FDA**	25	0	1	6	10	100	100
	35	3	9	26	42	ND	ND
	45	11	21	48	100	ND	ND
	50	100	100	ND	ND	ND	ND
***S. typhimurium* E 21274**							
	25	1	2	9	13	100	100
	35	3	11	100	100	ND	ND
	45	14	30	100	ND	ND	ND
	50	100	100	ND	ND	ND	ND

[a] n = 8.

[b] ND, not determined as no survivors were detected at the corresponding treatment.

4.6. Effect of High Hydrostatic Pressure, Time, Temperature and Organic Acids on Pressure-resistant and Pressure-sensitive Strains of Foodborne Pathogens:

1.0 % DL-lactic acid (Sigma, St. Louis, MO) and monohydrate 2.1 % citric acid (Sigma, St. Louis, MO) were used in pressure studies. These organic acids were selected to represent the organic acids found in milk and orange juice, respectively. In general, the lethality of all the bacterial strains for both organic acid treatments increased significantly as the pH of the pressure medium decreased from 6.5 to 5.5 and to 4.5 ($P<0.01$).

4.6.1. Viability Loss:

The viability loss data for pressure-resistant and pressure-sensitive strains of Gram-positive and Gram-negative bacteria in citric acid and lactic acid are given in Table 4.9 and Table 4.10, respectively.

It was observed that pressurization (345 MPa for 5 min) in lactic acid solution caused more viability loss than pressurization in citric acid solution. The reduction in cell populations for different Gram-positive strains ranged from (in log cycles): 1.58 to 4.91, 1.78 to 5.10 and 3.47 to 8.14 at pH 6.5, 5.5 and 4.5 for both acids, respectively. The reduction in cell population for different Gram-negative strains were between (in log cycles): 1.86 to 5.79, 2.30 to 7.06 and 3.82 to 8.95 at pH 6.5, 5.5 and 4.5 for both acids, respectively. A much higher reduction was

observed with Gram-negative strains although they were pressurized at a lower temperature than Gram-positive strains (25°C vs. 35°C). *S. aureus* 485 was still the most resistant strain with only 4.22 log cycles reduction even at pH 4.5 in lactic acid solution.

The additional increase in viability loss of pressure-resistant strains varied between 0.24 to 1.44 log cycles in citric acid solution and 1.44 to 3.42 log cycles in lactic acid solution after pressurization at pH 4.5 at 345 MPa for 5 min as compared to pressurization without organic acids. The additional increase in viability loss of pressure-sensitive strains were between 1.30 to 3.94 log cycles in citric acid solution and 1.24 to 3.89 log cycles in lactic acid solution (Table 4.9 and 4.10).

4.6.2. Degree of Sublethal Injury:

Pressurization of pressure-resistant and pressure-sensitive strains of foodborne pathogens in the presence of both organic acids had a significant effect on the percentage increase of sublethally injured cells ($P<0.05$). 100% sublethal injury was obtained for all the strains even at pH 6.5 in both citric and lactic acid solutions, except for *S. aureus* strains and *E. coli* O157:H7 933 (Table 4.11 and Table 4.12). The percentage of injured cells increased from; 33% to 54% for *S. aureus* 485, 49% to 66% for *S. aureus* 765 and 37% to 100% for *E. coli* O157:H7 933 with the concurrent application of pressure, temperature and lactic acid at pH 4.5 for 5 min.

Table 4.9. Viability loss of pressure-resistant and pressure-sensitive strains of four foodborne pathogens following combined treatment of hydrostatic pressure, time, temperature and low pH in citric Acid.

Bacterial species	Temperature (°C)	Mean $\log_{10}$ cfu/ml[a] at 345 MPa for 5 min at pH 4.5	5.5	6.5
Listeria monocytogenes				
CA	35	4.96 (8.14)[b]	4.49 (8.20)	3.90 (8.08)
$Ohio_2$	35	5.10 (8.00)	4.70 (8.00)	4.52 (8.00)
Staphylococcus aureus				
485	35	3.47 (7.95)[a]	1.78 (8.04)	1.58 (8.04)
765	35	4.00 (7.95)	2.97 (8.08)	2.16 (8.00)
***Escherichia coli* O157:H7**				
933	25	3.82 (8.08)	2.30 (8.00)	1.86 (8.00)
931	25	4.82 (8.08)	2.60 (8.20)	2.13 (8.08)
***Salmonella* spp.**				
***S. enteritidis* FDA**	25	5.67 (8.41)	5.35 (8.46)	4.00 (8.30)
S. typhimurium				
E 21274	25	8.95 (8.95)	6.82 (8.90)	5.49 (8.90)

[a] n = 8.

[b] Values in parantheses represent the $\log_{10}$ cfu/ml of unpressurized control samples.

Table 4.10. Viability loss of pressure-resistant and pressure-sensitive strains of four foodborne pathogens following combined treatment of hydrostatic pressure, time, temperature and low pH in lactic Acid.

Bacterial species	Temperature	Mean $\log_{10}$ cfu/ml[a] at 345 MPa for 5 min at pH		
	(°C)	4.5	5.5	6.5
Listeria monocytogenes				
CA	35	8.14 (8.14)[b]	4.98 (8.18)	4.84 (8.14)
Ohio$_2$	35	8.00 (8.00)	5.10 (8.00)	4.91 (7.95)
Staphylococcus aureus				
485	35	4.22 (7.90)	2.16 (8.00)	1.70 (8.11)
765	35	5.00 (7.90)	3.10 (8.00)	2.40 (8.00)
***Escherichia coli* O157:H7**				
933	25	3.88 (8.14)	3.54 (8.14)	2.41 (8.11)
931	25	4.90 (8.20)	4.04 (8.18)	2.71 (8.11)
***Salmonella* spp.**				
***S. enteritidis* FDA**	25	5.96 (8.34)	5.60 (8.38)	5.01 (8.41)
S. typhimurium				
E 21274	25	8.90 (8.90)	7.06 (8.84)	5.79 (8.90)

[a] n = 8.

[b] Values in parantheses represent the $\log_{10}$ cfu/ml of unpressurized control samples.

Table 4.11. Percent sublethal injury of pressure-resistant and pressure-sensitive strains of four foodborne pathogens following combined treatment of hydrostatic pressure, time, temperature and low pH in citric Acid.

Bacterial species	Temperature	Percent Injury[a] (%) at pH		
	(°C)	4.5	5.5	6.5
Listeria monocytogenes				
CA	35	100	100	100
$Ohio_2$	35	100	100	100
Staphylococcus aureus				
485	35	47	40	34
765	35	63	55	50
***Escherichia coli* O157:H7**				
933	25	100	100	45
931	25	100	100	100
***Salmonella* spp.**				
***S. enteritidis* FDA**	25	100	100	100
S. typhimurium				
E 21274	25	ND[b]	100	100

[a] n = 8.

[b] ND, not determined as no survivors were detected at the corresponding treatment.

Table 4.12. Percent sublethal injury of pressure-resistant and pressure-sensitive strains of four foodborne pathogens following combined treatment of hydrostatic pressure, time, temperature and low pH in lactic Acid.

Bacterial species	Temperature	Percent Injury[a] (%) at pH		
	(°C)	4.5	5.5	6.5
Listeria monocytogenes				
CA	35	ND[b]	100	100
Ohio$_2$	35	ND	100	100
Staphylococcus aureus				
485	35	54	45	41
765	35	66	60	56
***Escherichia coli* O157:H7**				
933	25	100	100	64
931	25	100	100	100
***Salmonella* spp.**				
***S. enteritidis* FDA**	25	100	100	100
S. typhimurium				
E 21274	25	ND	100	100

[a] n = 8.

[b] ND, not determined as no survivors were detected at the corresponding treatment.

4.7. Effect of High Hydrostatic Pressure on Pressure-resistant and Pressure-sensitive Strains of Foodborne Pathogens in Food Samples:

Grade A pasteurized milk (Dairy Gold Foods, CO), pasteurized orange juice (Minute Maid, TX), and cream of chicken soup (Safeway Inc., CA) were inoculated with *L. monocytogenes* Ca and $Ohio_2$, *S. aureus* 485 and 765, *E. coli* O157:H7 933 and 931 and *Salmonella enteritidis* FDA and *Salmonella typhimurium* E21274 as our previous studies indicated that they were among the most pressure-resistant strains of these vegetative pathogens. The food samples were pressurized at 345 MPa at 50°C only for 5 min. It was hypothesized that the treatment conditions used to inactivate these organisms would be sufficient to kill other, less resistant pathogens.

4.7.1. Pasteurized Orange Juice:

The effect of high hydrostatic pressure (345 MPa, 50°C, 5 min) on viability loss of pressure-resistant and pressure-sensitive strains of foodborne pathogens in pasteurized orange juice is given in Table 4.13. The low pH (3.76) of the orange juice modulated the inactivation of the pathogens studied. The population of all the bacterial species was reduced by more than 8 log-cycles. No growth was observed for these species on their respective selective media right after pressurization. The orange juice samples were stored at 4°C for 24h and even at 37°C for an additional 48h and again no growth was observed on respective selective media.

Table 4.13. Viability loss[a] of pressure-resistant and pressure-sensitive strains of foodborne pathogens in orange juice (pH=3.76) (345 MPa, 50°C, 5 min).

	Pressurization	24 h at 4°C	24 h at 37°C	48 h at 37°C
***L. monocytogenes*:**				
CA (8.30)[d]	ND[b]	-[c]	-	-
Ohio$_2$ (8.32)	ND	-	-	-
***S. aureus*:**				
485 (8.34)	ND	-	-	-
765 (8.30)	ND	-	-	-
***E. coli* O157:H7:**				
933 (8.30)	ND	-	-	-
931 (8.41)	ND	-	-	-
***Salmonella* spp.:**				
***S. enteritidis* FDA** (8.48)	ND	-	-	-
***S. typhi.* E 21274** (8.95)	ND	-	-	-

[a] Mean (n=8) $\log_{10}$ cfu/ml.

[b] ND, no cfu was detected in 1 ml of cell suspension from each of the samples tested.

[c] Food samples were incubated at 4°C for 24 h and then at 37°C for an additional 24 and 48 h and streak plated for Presence (+) or Absence (-) on respective selective media.

[d] Values represent mean (n=8) $\log_{10}$ cfu/ml of unpressurized control samples.

4.7.2. Pasteurized Milk:

The effect of high hydrostatic pressure (345 MPa, 50°C, 5 min) on viability loss of pressure-resistant and pressure-sensitive strains of foodborne pathogens in Grade A pasteurized milk is given in Table 4.14. Only *S. aureus* 485 showed growth on selective media right after pressurization with 5.50 log cycle reduction in cell population. After storage at 4°C for 24h, both *S. aureus* strains showed growth on selective media, where all the other strains showed no growth. However, when milk samples were stored for an additional 48h at an elevated temperature of 37°C, the strains of other Gram-positive bacteria, *L. monocytogenes* CA and $Ohio_2$, also showed growth on selective media plates whereas all the other Gram-negative strains were still negative. This indicates the presence of injured cells and possibly their recovery at an elevated storage temperature.

The pressure-temperature-time combination (345 MPa, 50°C, 5 min) used in this study caused more than 8 log-cycle reduction of the *E. coli* O157:H7 and *Salmonella* species in Grade A pasteurized milk. These strains were also not able to show growth during storage at refrigeration (4°C) and even at elevated (37°C) temperatures.

Table 4.14. Viability loss[a] of pressure-resistant and pressure-sensitive strains of foodborne pathogens in Pasteurized Milk (pH=6.65) (345 MPa, 50°C, 5 min).

	Pressurization	24 h at 4°C	24 h at 37°C	48 h at 37°C
***L. monocytogenes*:**				
CA (8.30)[d]	ND[b]	-[c]	+	+
Ohio$_2$ (8.32)	ND	-	+	+
***S. aureus*:**				
485 (8.34)	2.84	+	+	+
765 (8.30)	ND	+	+	+
***E. coli* O157:H7:**				
933 (8.30)	ND	-	-	-
931 (8.41)	ND	-	-	-
***Salmonella* spp.:**				
***S. enteritidis* FDA** (8.48)	ND	-	-	-
***S. typhi.* E 21274** (8.95)	ND	-	-	-

[a] Mean (n=8) $\log_{10}$ cfu/ml.

[b] ND, no cfu was detected in 1 ml of cell suspension from each of the samples tested.

[c] Pressurized milk samples were incubated at 4°C for 24 h and then at 37°C for an additional 24 and 48 h and streak plated for Presence (+) or Absence (-) on respective selective media.

[d] Values represent mean (n=8) $\log_{10}$ cfu/ml of unpressurized control samples.

4.7.3. Cream of Chicken Soup:

The effect of high hydrostatic pressure (345 MPa, 50°C, 5 min) on viability loss of pressure-resistant and pressure-sensitive strains of foodborne pathogens in cream of chicken soup is given in Table 4.15. No growth was observed for all the bacterial species on their respective selective media upon pressurization except *S. aureus* 485. The population of all the bacterial species reached their initial value within a week.

4.8. Bacteriocin (BP_1) Treatment of Food Samples:

In the second part of the study with food samples, a bacteriocin-based biopreservative (BP_1) was added to the pressure-time-temperature combination as a fourth parameter. This combination was used for inhibition of bacterial species in Grade A pasteurized milk and cream of chicken soup surviving after HHP. The results are given in Table 4.16 and Table 4.17, respectively.

The results revealed that when pressure treatment was used in combination with a bacteriocin-based biopreservative (BP_1), more than 8 log-cycle reduction in cell population of *S. aureus* and *L. monocytogenes* strains was achieved right after pressurization in Grade A pasteurized milk. Milk samples were also stored at 25°C to test the effect of temperature on microbial growth and the samples with bacteriocin-based biopreservative showed no growth up to 30d where as all the controls were positive.

In cream of chicken soup samples no growth was observed for all the bacterial species on their respective selective media right after pressurization and up to 5 days upon storage at 25°C. However, after 7 days, cell population of the resistant Gram-positive strains was increased to 5.08 log cycles for *L. monocytogenes* Ca and 6.90 log cycles for *S. aureus* 485. The cell population of Gram-negative strains was reached at least to 1.70 log cycles (*S. typhimurium* E 21274). Finally, the microbial growth reached the initial load for each strain after 9 days of storage at 25°C.

Table 4.15. The combined effect of high hydrostatic pressure (345 MPa, 50°C, 5 min) on microbial counts[a] of cream of chicken soup (pH=5.96) and growth during storage at 25°C.

	Pressurization	1 d	3 d	5 d	7 d
***L. monocytogenes*:**					
CA	(8.26)[b]	ND[c]	2.00	6.48	8.60	NP[d]
Ohio$_2$	(8.28)	ND	1.70	6.30	8.44	NP
***S. aureus*:**					
485	(8.30)	2.60	4.00	7.00	8.69	NP
765	(8.20)	ND	2.84	6.90	8.48	NP
***E. coli* O157:H7:**					
933	(8.30)	ND	1.60	4.48	8.60	NP
931	(8.26)	ND	ND	4.30	8.43	NP
***Salmonella* spp.:**					
S. enteritidis					
FDA	(8.30)	ND	ND	2.00	8.48	NP
S .typhimurium					
E 21274	(8.70)	ND	ND	ND	5.20	8.78

[a] Mean (n=8) $\log_{10}$ cfu/ml.

[b] Values represent mean (n=8) $\log_{10}$ cfu/ml of unpressurized control samples.

[c] ND, no cfu was detected in 1 ml of cell suspension from each of the samples tested.

[d] NP, not performed as the microbial count exceeded the initial microbial load.

Table 4.16. The combined effect of high hydrostatic pressure (345 MPa, 50°C, 5 min) and bacteriocin based biopreservative (BP_1) on microbial counts of Grade A pasteurized milk (pH=6.65) and growth during storage at 25°C.

	Pressurization		1 d	3 d	5 d	7 d	9 d	11 d	13 d	15 d	20 d	25 d	30 d
***L. monocytogenes*:**													
CA	(8.30)[a]	ND[b]	-[c]	-	-	-	-	-	-	-	-	-	-
$Ohio_2$	(8.32)	ND	-	-	-	-	-	-	-	-	-	-	-
***S. aureus*:**													
485	(8.34)	ND	-	-	-	-	-	-	-	-	-	-	-
765	(8.30)	ND	-	-	-	-	-	-	-	-	-	-	-

[a] Values represent mean (n=8) $\log_{10}$ cfu/ml of unpressurized control samples.

[b] ND, no cfu was detected in 1 ml of cell suspension from each of the samples tested.

[c] The milk samples were streak plated for Presence (+) or Absence (-) on respective selective media. Samples after 30 d of storage were pour plated.

Table 4.17. The combined effect of high hydrostatic pressure (345 MPa, 50°C, 5 min) and bacteriocin based biopreservative (BP_1) on microbial counts[a] of cream of chicken soup (pH=5.96) and growth during storage at 25°C.

	Pressurization		1 d	3 d	5 d	7 d	9 d
***L. monocytogenes*:**							
CA	(8.26)[b]	ND[c]	ND	NC	2.00	5.08	8.30
$Ohio_2$	(8.28)	ND	ND	ND	1.70	4.48	8.40
***S. aureus*:**							
485	(8.30)	ND	ND	ND	2.48	6.90	8.70
765	(8.20)	ND	ND	ND	2.30	6.40	8.34
***E. coli* O157:H7:**							
933	(8.30)	ND	ND	ND	1.00	2.48	8.34
931	(8.26)	ND	ND	ND	ND	2.00	8.41
***Salmonella* spp.:**							
S. enteritidis							
FDA	(8.30)	ND	ND	ND	ND	1.84	8.40
S .typhimurium							
E 21274	(8.70)	ND	ND	ND	ND	1.70	8.78

[a] Mean (n=8) $\log_{10}$ cfu/ml.

[b] Values represent mean (n=8) $\log_{10}$ cfu/ml of unpressurized control samples.

[c] ND, no cfu was detected in 1 ml of cell suspension from each of the samples tested.

CHAPTER V

DISCUSSION

Non-thermal technologies for preservation and modification of foods and food constituents such as high hydrostatic pressure (HHP) have been re-emerging world wide during the last 15 years. Considering the time span of almost 200 years available for thermal processes to be refined and optimized, substantial progress has been made in the field of emerging technologies within this short time period. Such achievement has been made especially regarding the increase in scientific knowledge and the development of new processes.

HHP is a new and emerging technique. It does not have the same level of research background as the other conventional techniques such as thermal processing for process establishment and demonstration of product safety. Hence studies related to process establishment and verification play a key role for the success of the high pressure process in the marketplace.

HHP processing of foods is essentially a batch process and a short processing time will be advantageous to make it commercially competitive and feasible. Up to date several low pH low protein fruit products processed by high

hydrostatic pressure have been commercially marketed (Selman 1992; Galazka and Ledward 1995; Mermelstein, 1998). There is a growing interest of producing high hydrostatic pressure preserved foods of both animal and plant sources that are high in pH and protein. To retain acceptance qualities as well as to enhance safety and shelf-life of these products and to make the process economically feasible it might be advantageous to use high hydrostatic pressure at a moderate range (300-400 MPa). However, it is necessary to generate information on the response of foodborne pathogens to moderate hydrostatic pressure before such a method is used to process foods and limited data are available on the impact of processing, distribution and storage on microbial cell viability. For this purpose, parameters for hydrostatic pressure pasteurization of food have to be developed to reduce populations of vegetative cells of food-borne pathogens probably by more than 6 log cycles. At ambient temperature (25 to 30°C), viability loss of this magnitude may be achieved either at a pressure range of 600 to 700 MPa, in 15 min (Patterson et al., 1995) or over 40 min at 350 MPa (Metrick et al., 1989). The quality of many protein-rich foods could be adversely affected by processing at such ultra high pressure or for a prolonged period (Shimada et al., 1990; Shigehisa et al., 1991; Cheftel 1992; Hayashi 1992).

Ultra high pressure and long pressurization time are also not economical and may not be commercially acceptable (Hoover et al., 1989; Mertens and Deplace 1993; Kalchayanand et al., 1998b). Elevated temperature, lower pH, and antimicrobial compounds, such as bacteriocins, lysozyme, and chitosan, were found to considerably enhance bactericidal efficiency of high hydrostatic

pressure (Knorr, 1993; Kalchayanand et al., 1995; Hauben et al., 1996; Kalchayanand et al.,1998b).

Many researchers have studied destruction of foodborne bacteria at a wide range of high hydrostatic pressure (Patterson et al., 1995; Shigehisa et al., 1991; Styles et al., 1991; Kalchayanand et al., 1998a; Kalchayanand et al., 1998b). However, the resistance among different strains of foodborne pathogens to moderate hydrostatic pressure has not been studied (Anonymous, 1997). Also at a moderate pressure range, a "Hurdle Concept" in which high hydrostatic pressure technique is combined with one or more suitable antimicrobial factors to have an additive or synergistic effect, might be necessary to have a desirable level of microbial destruction. Although high pressure technique is gaining more and more interest everyday, very limited scientific information is available in this area and documentation of HHP resistance of microorganisms in food systems is required to validate adequate processes and ensure microbiological safety (Kalchayanand et al., 1998a and Kalchayanand et al., 1998b; Sirichote et al., 2000). Based on these facts, the objectives of this study were to determine the combined effect of moderate temperature (25 to 50°C) and pressure (276 to 345 MPa) on:

i) the variation of pressure-resistance among 28 different strains of four foodborne pathogens and how this variation is modified by moderate pressurization at 50°C,

ii) D-values of the pressure-resistant strains of the four pathogens to have about 8 log cycle viability loss,

iii) the viability loss and injury of pressure-resistant strains of these pathogens in 1% peptone solution,

iv) the viability loss and injury of pressure-resistant strains of these pathogens in 1% peptone solution supplemented with organic acids at varying pH (lactic and citric acid at pH 4.5, 5.5 and 6.5),

v) the viability loss and injury of pressure-resistant strains of these pathogens in pasteurized orange juice, pasteurized milk and cream of chicken soup and their respective shelf-life,

vi) the viability loss of pressure-resistant strains of these pathogens in pasteurized milk and cream of chicken soup supplemented with a bacteriocin based biopreservative (BP_1) and their respective shelf life.

5.1. Variation in Strain Resistance to HHP:

In general, the results of the strain variation study confirmed the results of other researchers (Styles et al., 1991; Patterson et al., 1995; Patterson and Kilpatrick, 1998; Benito et al., 1999) showing that at a lower temperature of pressurization, the strains of a species varied in pressure resistance. However, this difference in pressure resistance was greatly eliminated by pressurizing cells at 50°C, even for 5 min. Thus, a combination of moderate hydrostatic pressure (such as 345 MPa) and a temperature of 50°C can be used to obtain a viability loss of pathogens of more than 6 log cycles.

The death rate kinetics of the strains of four pathogens studied is similar to those reported by other researchers (Kalchayanand et al., 1998; Patterson et al., 1995; Metrick et al., 1989; Shigehisa et al., 1991 and Styles et al., 1991). At 345 MPa and 50°C the death rate followed first order kinetics for the strains studied and produced over 8 log cycle decrease in cell population within 5 min for all the strains except *S. aureus* 485. An initial rapid decrease in cell numbers during the first 5 min of treatment followed by a "tail", indicated that a small fraction of the cell population was more pressure resistant. Tailing effects have also been reported for another pressure-resistant *S. aureus* strain, NCTC 10652 pressurized at 350 to 700 MPa in phosphate buffer at 20°C (Patterson et al., 1995). The increase in death rate by pressurization above 35°C was possibly due to a phase transition of bacterial cell membrane lipids as indicated by other researchers (Ludwig et al., 1992; Yano et al., 1998).

Reduced inactivation was observed at 25°C, at 345 MPa after 5 min especially for *L. monocytogenes* and *S. aureus* strains compared to literature data. Stewart et al. (1997) and Styles et al. (1991) reported a 7 log cycle reduction in *L. monocytogenes* CA after exposing the cell suspension to 345 MPa at 23°C for 5 min. A reduction of less than 1 log cycle was obtained under the same conditions, for cells from early stationary phase, after 5 min in our study. This could be due to the difference in the age of the cells used in two studies.

Increasing the temperature during pressurization enhanced the lethal effect on *L. monocytogenes* CA and $Ohio_2$. At 50°C and 345 MPa over 8 log reduction was achieved in 5 min for these strains. However, *S. aureus* 485 was still the most pressure-resistant strain among the strains considered. The spherical morphology and cell wall composition of *S. aureus* could enhance its resistance to pressure but real causes of cellular damage are not well known. All the studies of HHP effects on *Staphylococcus* spp. agree that inactivation rates increase linearly with pressure and exposure time (Gervilla et al., 1999). The response of *S. aureus* to HHP depends on the temperature of the treatment as well, but *S. aureus* showed different behavior at low to ambient temperatures and at medium to high temperatures. In the first interval (low to ambient), *S. aureus* response to HHP was not affected by temperatures between –20 to 25°C; in the second interval, at 40 to 50°C and higher, *S. aureus* was much more sensitive to HHP as temperature increased (Takahashi, 1992; Patterson et al., 1995). Although no inactivation effect could be attributed to the temperature of the treatment per se between 1 to 40°C, temperatures over 40°C would seem to enhance the destructive effect of pressure on *S. aureus* (Butz and Ludwig, 1986). Patterson et al. (1995) reported *S. aureus* NCTC 12079 to be more resistant to pressure treatment at 20°C up to 700 MPa than *E. coli* O157:H7 NCTC 12079, *S. typhimurium*, *S. enteritidis*, *L. monocytogenes* strains NCTC 11994, ScottA and 2433.

The *Salmonella* strains were the most pressure sensitive among the strains used in this study. The complete destruction of *E.coli* O157:H7 933 and

931 cells (>8 log cycle) in this study was also confirmed with *E. coli* O157:H7 932 (Kalchayanand et al., 1998a). This may be explained by the relatively greater sensitivity of the Gram-negative bacteria to HHP, which is also reported by other researchers (Styles et al., 1991; Shigehisa et al., 1991; Patterson et al., 1995; Gervilla et al., 1997; Lopez-Caballero et al., 1999).

One of the objectives of this study was to determine a pressure and temperature combination that would cause about 8-log cycle viability loss of the eight resistant strains of the four pathogens in 5 min. From the D-values at 345 MPa and 50°C, it became evident that this could be achieved for all strains except for *S. aureus* 485, which has a D-value of 2.55 min. The results also indicate that the D value of a bacterial species or strain due to thermal treatment at a lower temperature range (50 or 60°C) can be greatly reduced by combining thermal treatment with pressurization even at a moderate pressure range of 345 MPa. For such resistant strains of pathogens, a suitable antibacterial preservative, such as a bacteriocin, could be incorporated along with pressure, temperature and time, as a fourth dimension of treatment to enhance viability loss of Gram positive and Gram negative bacteria which are important in food (Kalchayanand et al., 1994).

Considerable variations in pressure-resistance between species and between strains of the same species were observed in this part of the study. This fact must be considered when making recommendations for improving the safety of pressure treated foods. It would be desirable to have recommendations on

pressure processing methods based on information from the most resistant strains relevant to the food of interest.

5.2. Interaction of HHP, Time, Temperature and Organic Acids on Inactivation and Injury of Pressure-resistant and Pressure-sensitive Strains of Foodborne Bacteria:

Previous studies showed that; at 207 MPa in 5 min, the viability loss of four foodborne pathogens and four spoilage bacteria increased from 1 log cycle at 25°C to 6 to 7 log cycles at 50°C (Kalchayanand et al., 1998b). The viability loss of these pathogens due to thermal inactivation alone at 50°C was less than 2 log cycles after 15 min (Alpas et al., 1999). Limited information is available in literature with pressure-resistant strains of foodborne pathogens and effect of pH (Metrick et al., 1989; Shigehisa et al., 1991; Patterson et al., 1995; Hauben et al., 1997, Alpas et al., 1999; Benito et al., 1999; Alpas et al., 2000).

The pressure resistant-strains of each species had more survivors than the respective pressure-sensitive strains. Among the three factors, pressurization temperature induced highest viability loss, especially above 35°C. This is probably related to a phase transition of bacterial cell membrane lipids (Ludwig et al., 1992; Smelt et al., 1994; Kalchayanand et al., 1998a; Kalchayanand et al., 1998b). Increasing the pressurization time from 5 to 10 min resulted in a maximum of 1.54 log cycle additional reduction of microbial population in all strains. The results of other studies have also shown that combinations of

efficient temperatures and pressures are more effective than extending exposure time at lower pressures (Metrick et al., 1989; Styles et al., 1991; Patterson et al., 1995; Kalchayanand et al., 1998a; Kalchayanand et al., 1998b; Gervilla et al., 1999; Prestamo et al., 1999). It appears that pressurization for a longer time at lower pressure range (to minimize adverse effects on food texture and color) may not be of great advantage for microbial inactivation. Pressurization temperature, especially beyond 35°C at 345 MPa, reduced viability by 4 to 8 log cycles. At 345 MPa and 50°C, the death rate did not follow first-order kinetics for *S. aureus* 485. A similar trend for this pathogen was reported by Kalchayanand et al. (1998b). However, 100% of the survivors for this strain were sublethally injured as they failed to form colonies on TSAN plates with 5% NaCl.

When the cells were suspended in 100 mM of citric or lactic acid solution, Gram-negative strains were more susceptible to pressurization than Gram-positive strains. Moreover, the pressure-resistant strains were also more resistant to acid than the respective sensitive strains. Lactic acid at pH 4.5 was more bactericidal than citric acid at pH 5.5 or 6.5 ($P < 0.01$). Lactic acid was reported to be more active than EDTA or HCL in liberating lipopolysaccharide from the outer membrane (Alakomi et al., 2000). Thus, lactic acid, in addition to its antimicrobial property due to lowering of the pH, also functions as a permeabilizer of the bacterial outer membrane and may act as a potentiator of the effects of other antimicrobial substances. Stewart et al. (1997) reported an additional 3 log cycle reduction in *L. monocytogenes* CA when pressurized in buffer at pH 4.0 as compared with pH 6.0 at 353 MPa, 45°C for 10 min.

However, in our study, a higher reduction in cell population was observed for this strain at pH 4.5 as compared with pH 6.5 in lactic acid at a lower pressure, temperature and time combination (345 MPa, 35°C for 5 min.). The additional reduction in cell population in the presence of lactic acid may be explained by the enhanced activity of the undissociated form (which gave organic acids the antimicrobial activity) under pressure (Ray 1996; Smelt 1998).

The increased injury among survivors of the strains with elevated temperature gave the possibility of increasing cell death at a lower pressure range by raising temperature above 35°C. In fact, raising the pressurization temperature to 50°C at 207 MPa for 10 min was enough to achieve complete injury of *L. monocytogenes* CA from an initial concentration of $>10^8$ cfu/ml in this study. Stewart et al (1997) reported the same level of injury after 30 min at 404 MPa and 45°C. *L. monocytogenes* cells treated at 345 MPa with a pH below 6.5 were incapable of showing growth on TSAN. Baily et al. (1990) reported that heat injured *L. monocytogenes* was incapable of growth in acidified media of pH lower than 5.2. Also, 0.5 to 3.0 fold increase in the percentage of injured cells was observed for both *S. aureus* strains at 35°C and for the resistant *E. coli* O157:H7 933 at 25°C when lactic acid was combined with pressurization at 345 MPa for 5 min.

The injury data obtained in this study are applicable to the food industry as it considers the use of hydrostatic pressure as an industrial method of food processing. Foods harboring the pathogens studied here could receive a high

level of microbial inactivation with minimal process severity to the food by the application of high hydrostatic pressure with mild heat.

5.3. Application of HHP to Food Systems:

The objective of this part of the study was to evaluate the development and verification of high hydrostatic pressure processes for orange juice, milk and cream of chicken soup based on the gathered data on pressure destruction kinetics of pressure-resistant strains of pathogenic microorganisms. It is well known that the nature of the food itself can influence the pressure resistance of the organism. Pressure treating in UHT milk gave more protection than treatment in poultry meat to *L. monocytogenes*, *E. coli* O157:H7 and *S. aureus* (Patterson et al., 1995). *L. innocua* was more pressure resistant in liquid UHT dairy cream than in minced beef (Carlez et al., 1993; Raffalli et al., 1994). *S. typhimurium* and *L. monocytogenes* were more pressure sensitive in buffer than in strained chicken or UHT milk respectively (Metrick et al., 1989; Styles et al., 1991). Thus it is apparent that certain foods protect microorganisms from HHP inactivation. Although further work is required to determine the nature of these protective mechanisms, it was hypothesized that the treatment conditions used to inactivate the organisms studied would be sufficient to kill other, less resistant pathogens.

5.3.1. Effect of HHP Treatment on Pasteurized Orange Juice:

The low pH (3.76) of the orange juice modulated the inactivation of the pathogens studied. The viability of all the bacterial species were reduced by more than 8 log-cycles. Among the four foodborne pathogens studied, the prevalence of *E. coli* O157:H7 in foods is enhanced by its ability to survive at low pH (Arnold and Kaspar 1995). This microorganism has been shown to survive for 31 days at pH 3.7 in apple cider stored at 8°C (Zhao et al., 1993). Linton et al. (1999a) reported 6 log cycle reduction of *E. coli* O157:H7 in orange juice after pressurizing at 550 MPa for 5 min at 20°C from pH 3.4 to 4.5. Our results revealed a higher reduction at a much lower pressure (345 MPa) at 50°C at pH 3.76. The pH of aqueous systems has been reported to decrease during pressurizing due to electrostriction (Heremans 1995) but any change in the pH of foods during pressure treatment is very difficult to measure (Patterson et al., 1995). It could be postulated that the buffering capacity of foods might influence the extent to which the bacteria are exposed to a pH change and hence could affect their viability.

The shelf life of freshly squeezed orange juice varies depending on initial microbiological quality and pH, but it is usually 10 to 14 days at 4.4°C or 5 to 8 days at 7.8°C (Eleftheriadou et al., 1998). Thus, there would be a risk of food poisoning if orange juice become contaminated with *E. coli* O157:H7 and did not receive treatment before consumption, particularly since the growth of *E. coli*

O157:H7 cells in freshly pressed orange juice is possible during the time required for spoilage to make the juice unacceptable (Linton et al., 1999b).

No growth was seen for these species on their respective selective media agar plates after storage at 4°C for 24h and even at 37°C for an additional 48h. Linton et al. (1999b) reported the presence of *E. coli* O157:H7 NCTC 12079 after pressurization at 400 MPa for 1 min at 10°C upon storage at 3°C after 10 to 13 days of storage. It is evident that high hydrostatic pressure processing of orange juice to increase the juice's shelf-life and to inactivate pathogens has an added advantage that it sensitizes the pathogens to the high acid conditions found in orange juice, which results in more than 8 log cycle reduction in viability of the pathogens studied.

5.3.2. Effect of HHP Treatment on Pasteurized Milk:

Only *S. aureus* 485 survived right after pressurization with a 5.5 log cycle reduction in cell population after HHP treatment of foodborne pathogenic strains in Grade A pasteurized milk. 6.0 and 7.3 log cycle reductions in cell populations of *S. aureus* NCTC 10652 in UHT and *S. aureus* CECT 534 in ovine milk samples were reported by Patterson and Kilpatrick (1998) and Gervilla et al. (1999) after 15 min treatment of 500 MPa at 50°C, respectively. *S aureus* was still the most pressure resistant pathogen within the conditions of the study. This pathogen was also reported to be the most high hydrostatic pressure resistant vegetative pathogen studied in milk of the pathogens tested under the same

conditions and pressurization medium (Datta and Deeth, 1999; Gervilla et al., 1999). The pressure-temperature-time combination used in this study caused more than 8 log-cycle reduction of the Gram-negative bacterial species in Grade A pasteurized milk. For *E. coli* O157:H7, a 15 min treatment of 400 MPa at 50°C for a 5 log cycle reduction in UHT milk was reported (Patterson and Kilpatrick, 1998). A baroprotective effect of fat that has been suggested for *E. coli* and *Listeria* species by various authors (Styles et al., 1991; Gervilla et al., 1997; Garcia-Risco et al., 1998; Patterson and Kilpatrick, 1998; Garcia-Graells et al., 1999) was not observed in the present study. This may be due to the lower fat levels of milk used in our study.

5.3.3. Effect of HHP Treatment on Cream of Chicken Soup:

A comparison of the inactivation data obtained for the different strains of pathogens in Grade A pasteurized milk and cream of chicken soup reveals a dramatically increased ability of all the strains to survive HHP treatment in cream of chicken soup. The difference is particularly evident even for Gram-negative strains, because these strains were relatively sensitive in Grade A pasteurized milk. A protective effect of different vegetative bacteria from HHP inactivation has been reported by several authors, in complex, high-water-activity, low-acidity foods (Knorr et al., 1992; Carlez et al., 1993; Maggi et al., 1994; Raffalli et al., 1994; Carballo et al., 1997; Gervilla et al., 1999). This effect is usually explained by the presence of nutrients essential for repair of

sublethally injured cells (Metrick et al., 1989; Cheftel 1991; Gervilla et al., 1999).

5.4. Effect of HHP and BP_1 on Pasteurized Milk and Cream of ChickenSoup:

In the final part of the study, a bacteriocin-based biopreservative (BP_1) was added to the pressure-time-temperature combination as a fourth parameter and this combination was used to achieve a higher level of microbial destruction of surviving Gram-positive bacterial species in Grade A pasteurized milk and cream of chicken soup.

The results revealed that when pressure treatment was used in combination with BP_1, more than 8 log-cycle reduction in cell population was achieved for the resistant strains of *S. aureus* and *L. monocytogenes* right after pressurization in milk. The ability of *L. monocytogenes* to develop resistance to nisin in phosphate broth (Davies and Adams, 1994) was overwhelmed probably by using pediocin AcH and nisin in combination.

Milk samples were also stored at 25°C to test the effect of temperature on microbial growth and the samples treated with bacteriocin-based biopreservative (BP_1) showed no growth up to 30d where as all the controls were positive (data not shown). This shows the continued effect of the combination of high hydrostatic pressurization with BP_1 on both bacteria during storage at 25°C. In

this respect, our results differ partially from those reported in liquid whole egg, in which nisin had a continued effect on *E. coli* during storage after pressurization at 4°C but not at 20°C (Ponce et al., 1998).

The synergistic effect of HHP together with BP_1 was successful to inactivate the Gram-positive bacterial strains in cream of chicken soup up to 3 days of storage at 25°C with a rapid increase in cell number thereafter. This may be attributed to the reduction in bactericidal efficiency of antimicrobial peptides in complex food substrates that is probably caused by adsorption of the active compounds to food hydrocolloids or fat (Scott and Taylor, 1981; Jung et al., 1992).

CHAPTER VI

CONCLUSIONS

The results presented in this study indicate that, high hydrostatic pressure processing has the potential to improve the safety of food products. However, there are considerable variations in the inactivation achieved with different species and strains of the same species. Also, a similar variation should be expected with food systems. These variations must be taken into account when recommendations for commercial processing of foods are being considered as sublethal injury of bacterial cells can occur at lower pressure range than that is required for their inactivation.

This study has also indicated that high hydrostatic pressure applied in conjunction with mild heat and acidity can be an effective method for inactivating pressure-resistant and pressure-sensitive strains of *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Salmonella* in organic acid solutions. This combination treatment indicates possible pressure pasteurization applications for effective control of pathogens in low pH liquid foods, that still needs further studies.

It can also be concluded that, high hydrostatic pressure (345 MPa, 50°C, 5 min) applied to high acid foods and for low acid foods (when combined with bacteriocins) can be successfully applied to obtain high levels of microbial destruction with respect to the inoculated foodborne pathogens. The recommended shelf life of these products can also be extended even at elevated storage temperatures.

CHAPTER VII

RECOMMENDATIONS

Pressure pulse technique for microbial destruction can be highly effective and reproducible. Although the process conditions should be further optimized to maximize bacterial inactivation, pressure cycling strongly enhances the pressure sensitivity of vegetative cells and is a promising approach to increase efficacy and safety of HHP pasteurization of food products, particularly in combination with bacteriocins of lactic acid bacteria such as nisin and pediocin and particularly with regard to the problem of pressure-resistant bacterial strains.

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Table A.1. Composition of various TGE media used (% in 100 ml).

Ingredients	Broth for Culture Maintenance	Broth for Pediocin and Nisin Production	Soft Agar	Plating Agar
Trypticase	0.500	1	0.500	0.500
Glucose	0.500	1	1	0.500
Yeast Extract	0.500	1	0.500	0.500
Tween 80	0.100	0.200	0.200	0.100
Mn^{2+}	0.005	0.005	0.005	0.005
Mg^{2+}	0.005	0.005	0.005	0.005
Agar	-	-	0.750	1.500

Table A.2. Average results of pooled data of OD 600 nm readings for foodborne bacteria.

Time (h)	***L. monocytogenes***	***S. aureus***	***E. coli* O157:H7**	***Salmonella***
0	0.027	0.070	0.068	0.054
2	0.115	0.228	0.522	0.362
4	0.351	0.757	0.837	0.679
6	0.511	0.946	0.888	0.748
8	0.506	0.925	0.911	0.767
10	0.506	0.910	0.913	0.774
12	0.506	0.904	0.913	0.783

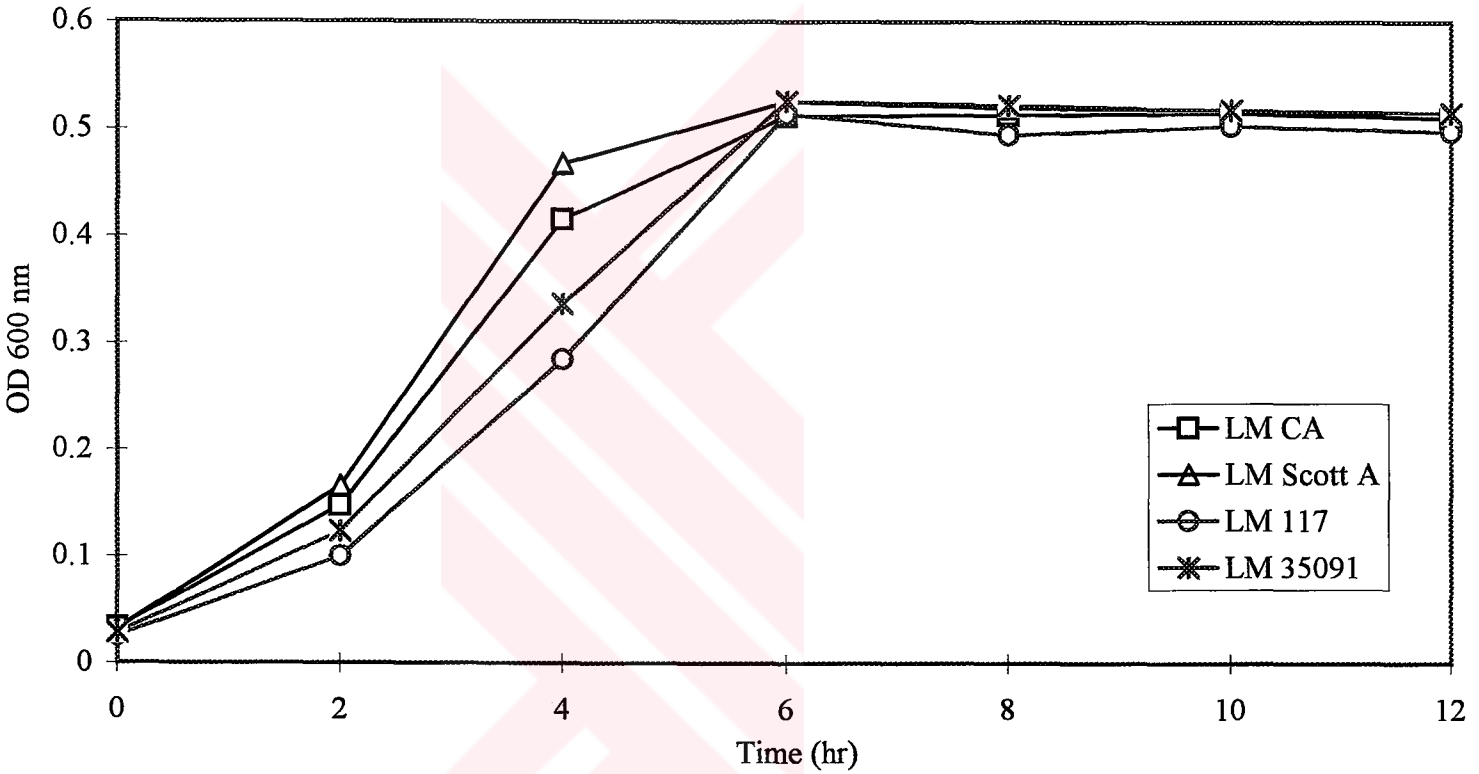

Figure A.3.A. Growth curve of *Listeria monocytogenes* strains Ca, Scott A, 117 and 35091.

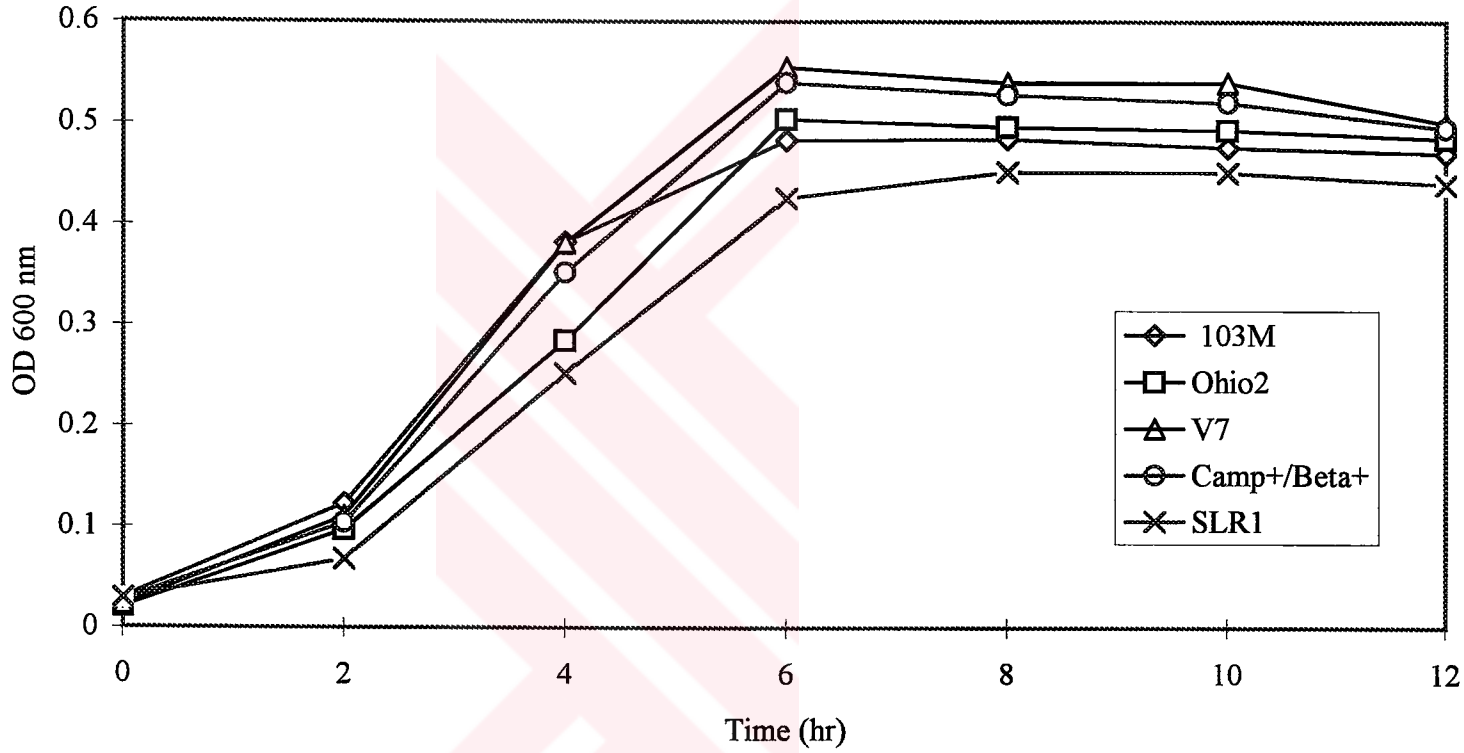

Figure A.3.B. Growth curve of *Listeria monocytogenes* strains 103M, $Ohio_2$, V7, $Camp^+/Beta^+$ and SLR1.

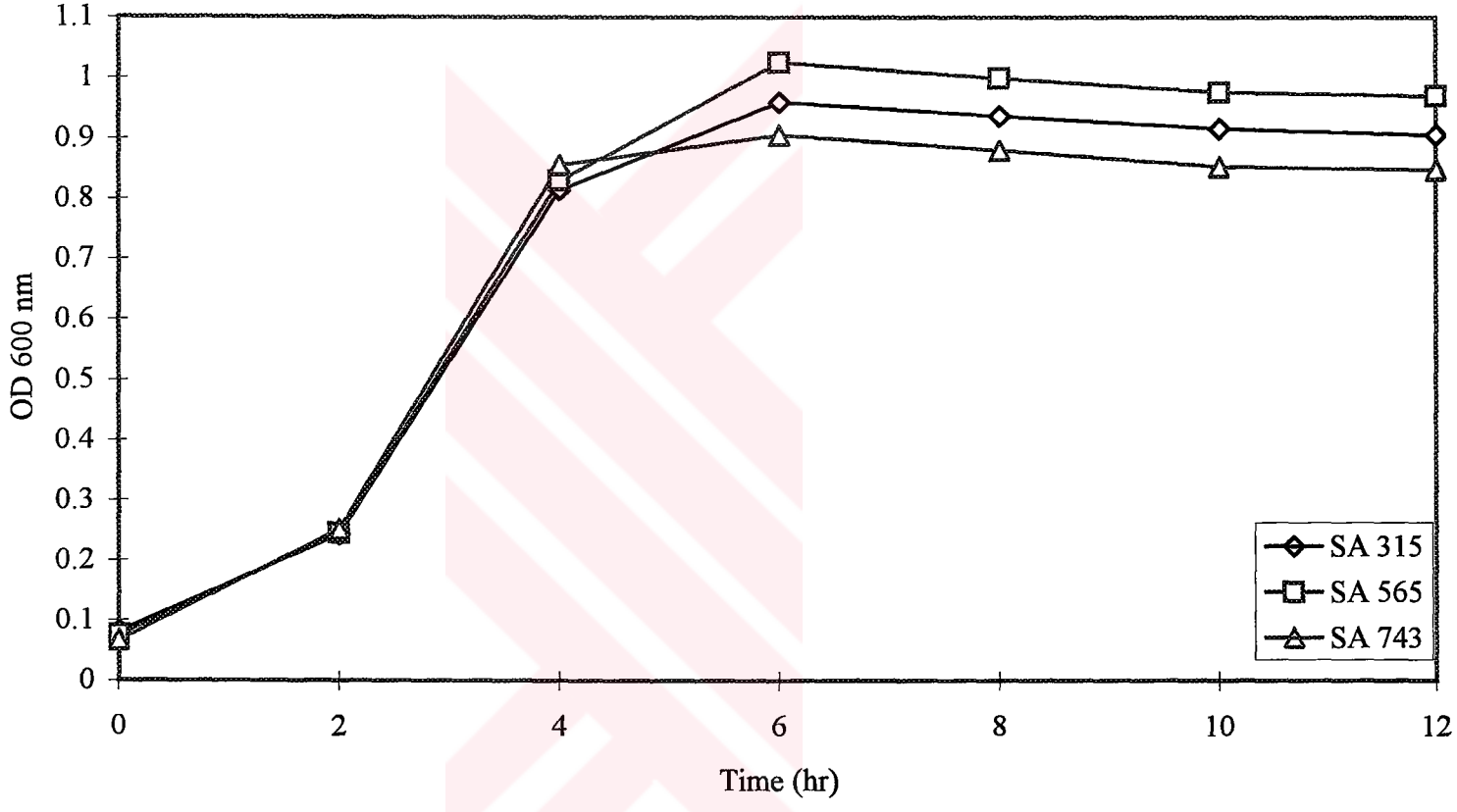

Figure A.3.C. Growth curve of *Staphylococcus aureus* strains 315, 565 and 743.

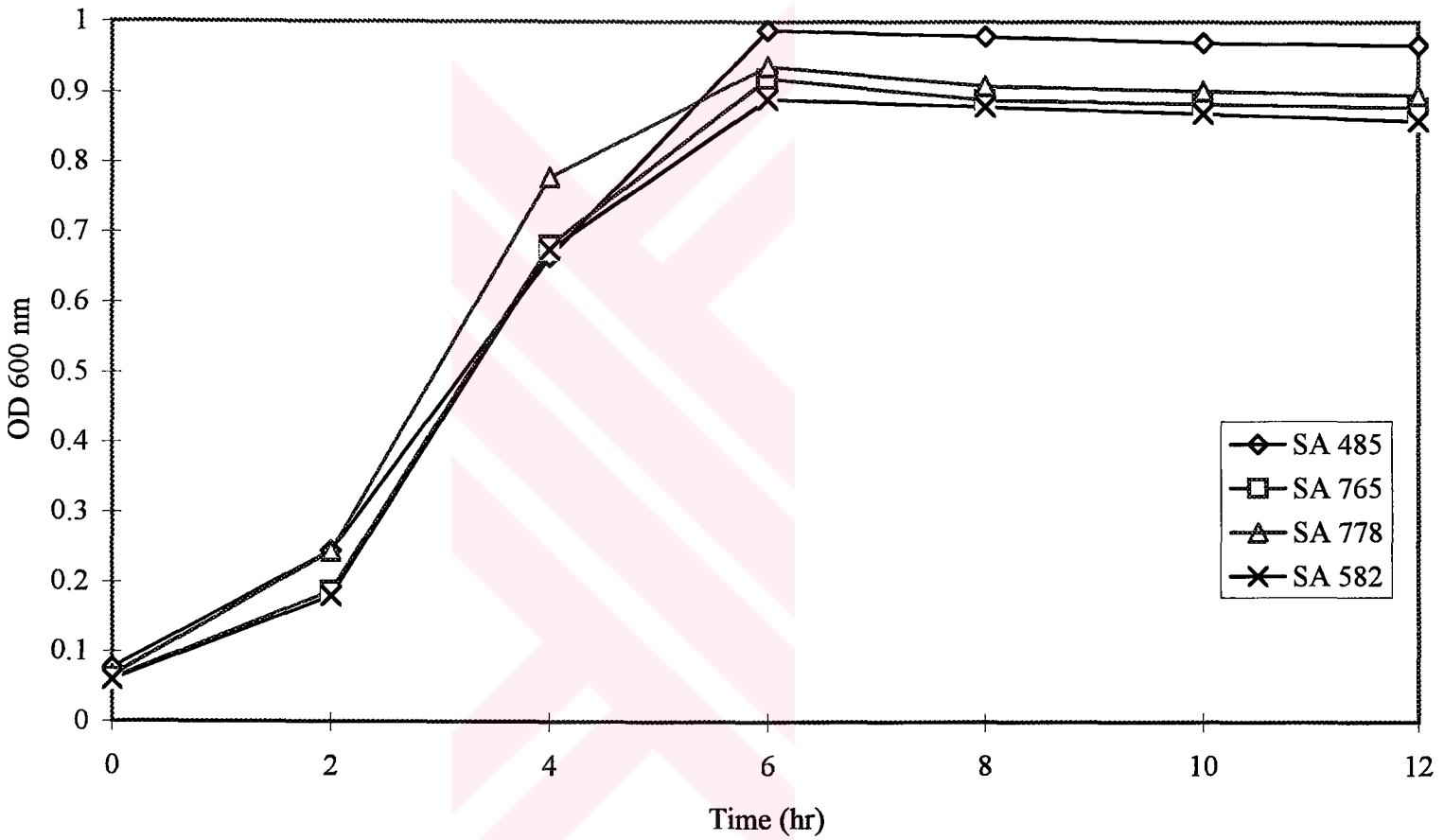

Figure A.3.D. Growth curve of *Staphylococcus aureus* strains 485, 765, 778 and 582.

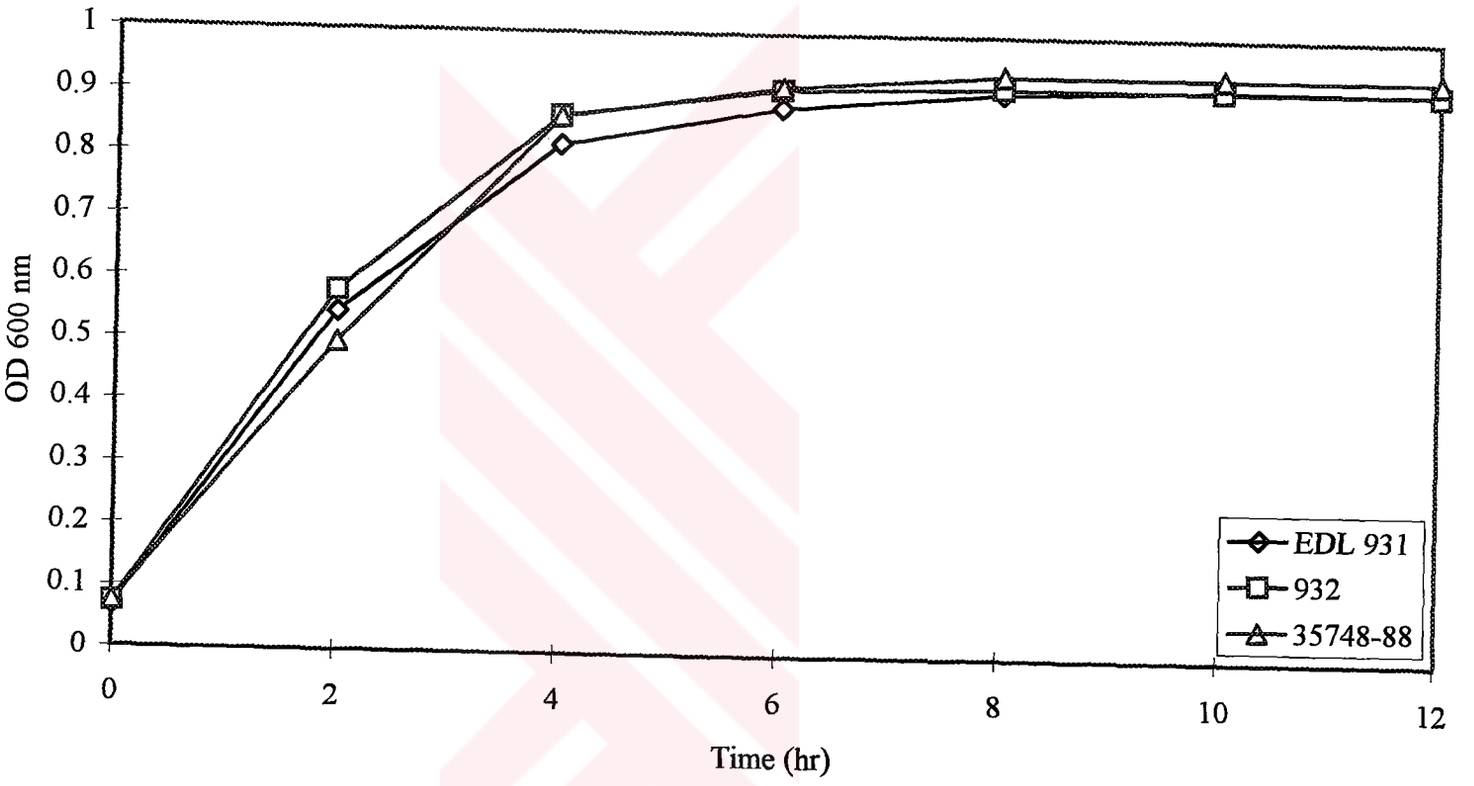

Figure A.3.E. Growth curve of *Escherichia coli* O157:H7 strains EDL 931, 932 and 35748-88.

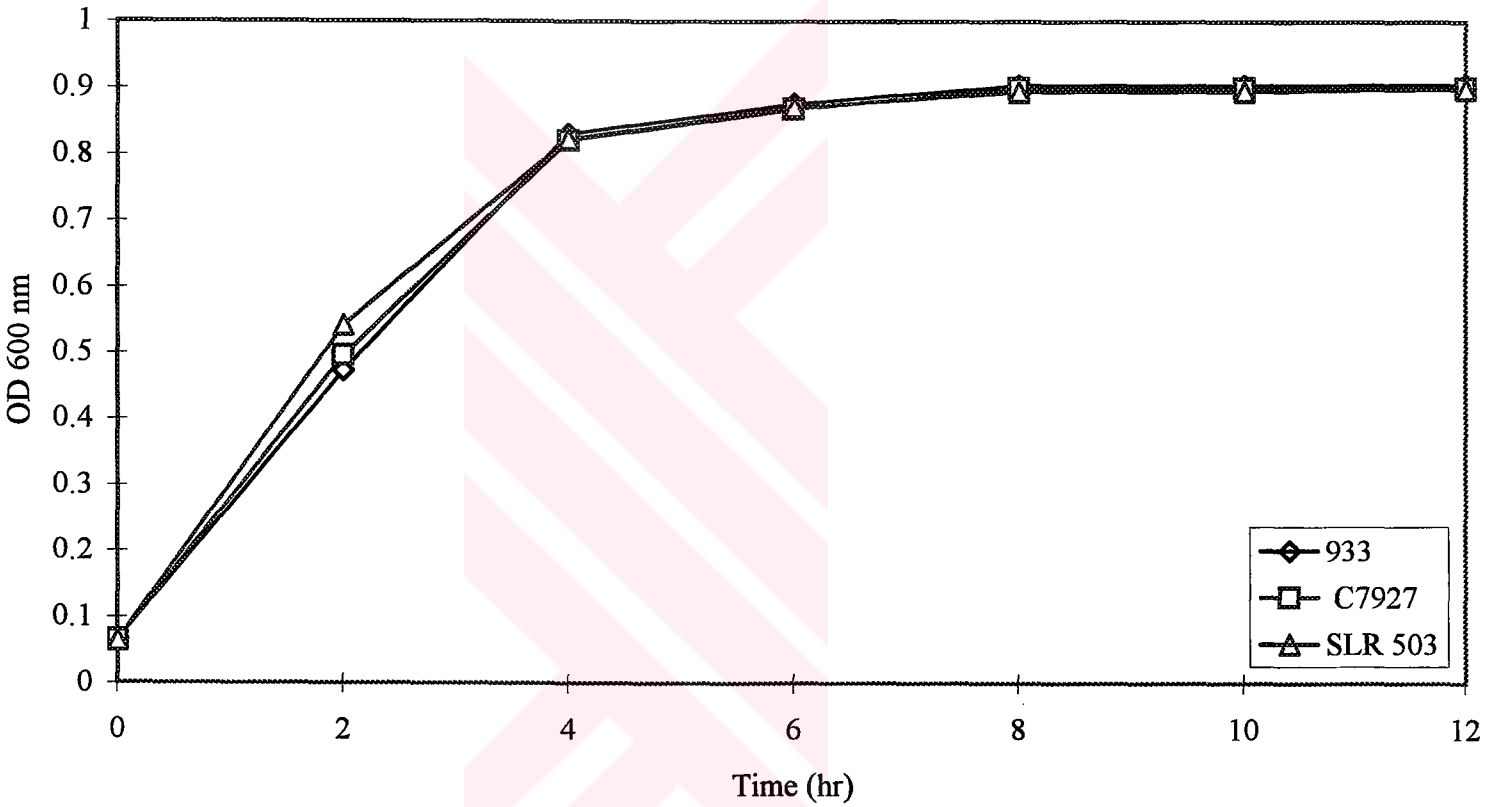

Figure A.3.F. Growth curve of *Escherichia coli* O157:H7 strains 933, C7927 and SLR 503.

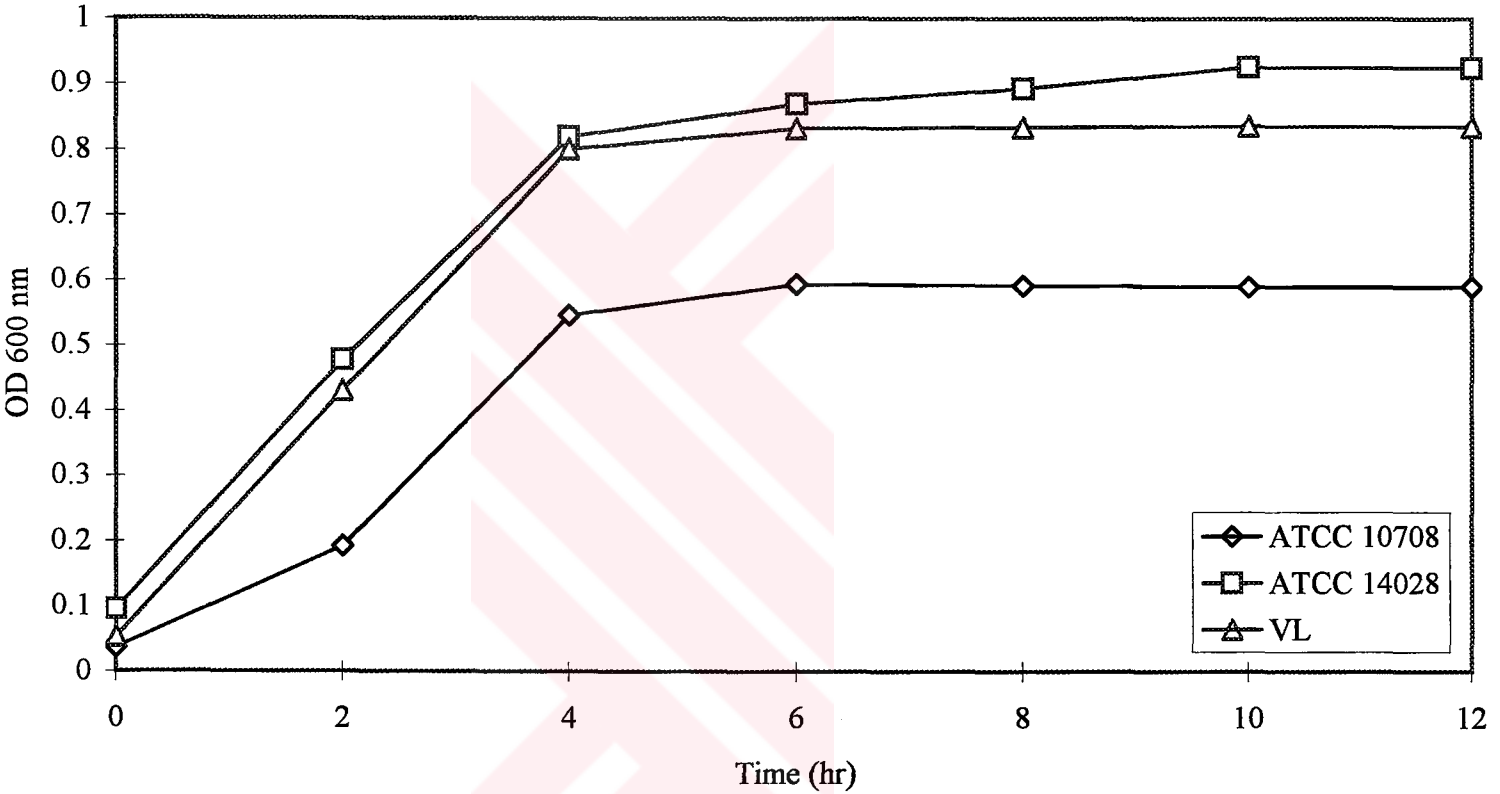

Figure A.3.G. Growth curve of *Salmonella* strains ATCC 10708, ATCC 14028 and VL.

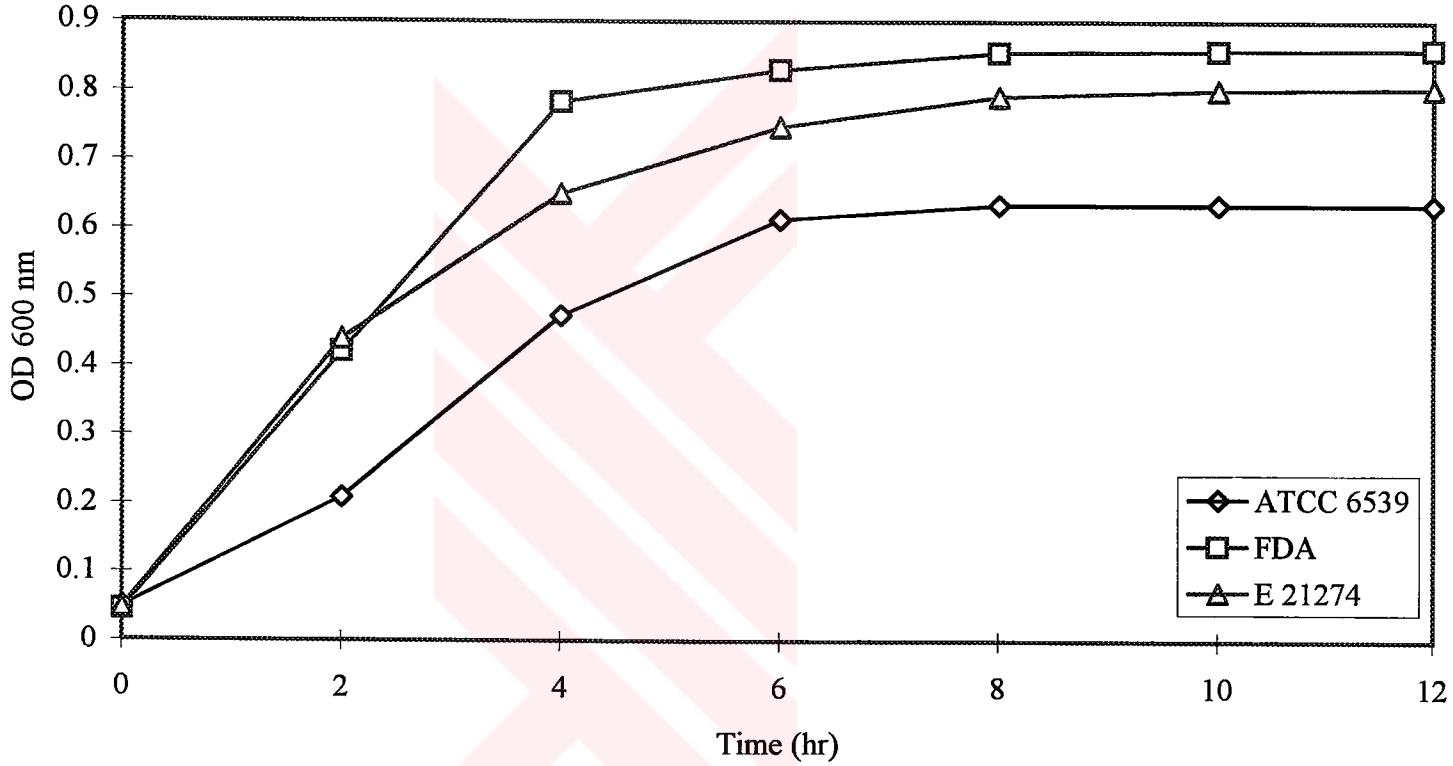

Figure A.3.H. Growth curve of *Salmonella* strains ATCC 6539, FDA and E 21274.

Table A.4.A. Individual plate counts of *Listeria monocytogenes* strains during pressurization at 345 MPa at 25°C for 5 min.

Control				Individual Plate Counts			
cfu/ml	log cfu/ml	Standard Deviation	Average	cfu/ml	log cfu/ml	Standard Deviation	Average
***L. monocytogenes* CA**							
5.00E+07	7.70	0.168	8.00	8.00E+06	6.90	0.134	7.08
6.00E+07	7.78			8.00E+06	6.90		
6.00E+07	7.78			8.00E+06	6.90		
1.00E+08	8.00			8.00E+06	6.90		
1.00E+08	8.00			1.10E+07	7.04		
1.00E+08	8.00			1.50E+07	7.18		
1.00E+08	8.00			1.50E+07	7.18		
1.20E+08	8.08			1.50E+07	7.18		
1.40E+08	8.15			1.50E+07	7.18		
1.20E+08	8.08			1.50E+07	7.18		
1.40E+08	8.15			1.50E+07	7.18		
1.80E+08	8.26			1.60E+07	7.20		
***L. monocytogenes* Scott A**							
5.00E+07	7.70	0.125	8.00	7.00E+06	6.85	0.109	7.04
8.00E+07	7.90			7.00E+06	6.85		
8.00E+07	7.90			1.00E+07	7.00		
1.00E+08	8.00			1.00E+07	7.00		
1.00E+08	8.00			1.10E+07	7.04		
1.00E+08	8.00			1.10E+07	7.04		
1.00E+08	8.00			1.10E+07	7.04		
1.10E+08	8.04			1.10E+07	7.04		
1.20E+08	8.08			1.40E+07	7.15		
1.30E+08	8.11			1.40E+07	7.15		
1.40E+08	8.15			1.40E+07	7.15		
1.40E+08	8.15			1.50E+07	7.18		
***L. monocytogenes* 117**							
4.00E+07	7.60	0.118	7.78	1.30E+05	5.11	0.098	5.30
3.00E+07	7.48			1.40E+05	5.15		
7.00E+07	7.85			1.80E+05	5.26		
6.00E+07	7.78			1.80E+05	5.26		
7.00E+07	7.85			1.90E+05	5.28		
7.00E+07	7.85			1.90E+05	5.28		
6.00E+07	7.78			2.20E+05	5.34		
7.00E+07	7.85			2.20E+05	5.34		
6.00E+07	7.78			2.20E+05	5.34		
7.00E+07	7.85			2.20E+05	5.34		
7.00E+07	7.85			2.70E+05	5.43		
7.00E+07	7.85			2.70E+05	5.43		

Control				Individual Plate Counts			
cfu/ml	log cfu/ml	Standard Deviation	Average	cfu/ml	log cfu/ml	Standard Deviation	Average
***L. monocytogenes* 35091**							
5.00E+07	7.70	0.140	8.00	3.00E+05	5.48	0.243	5.90
6.00E+07	7.78			4.00E+05	5.60		
9.00E+07	7.95			5.00E+05	5.70		
9.00E+07	7.95			5.00E+05	5.70		
1.00E+08	8.00			7.00E+05	5.85		
1.00E+08	8.00			7.00E+05	5.85		
1.10E+08	8.04			9.00E+05	5.95		
1.20E+08	8.08			1.20E+06	6.08		
1.20E+08	8.08			1.20E+06	6.08		
1.30E+08	8.11			1.50E+06	6.18		
1.30E+08	8.11			1.50E+06	6.18		
1.50E+08	8.18			1.50E+06	6.18		
***L. monocytogenes* 103**							
6.00E+07	7.78	0.113	7.90	2.20E+05	5.34	0.036	5.41
6.00E+07	7.78			2.20E+05	5.34		
6.00E+07	7.78			2.50E+05	5.40		
7.00E+07	7.85			2.50E+05	5.40		
7.00E+07	7.85			2.60E+05	5.41		
7.00E+07	7.85			2.60E+05	5.41		
7.00E+07	7.85			2.70E+05	5.43		
8.00E+07	7.90			2.70E+05	5.43		
1.00E+08	8.00			2.70E+05	5.43		
1.00E+08	8.00			2.70E+05	5.43		
1.20E+08	8.08			2.80E+05	5.45		
1.20E+08	8.08			2.80E+05	5.45		
***L. monocytogenes* Ohio$_2$**							
6.00E+07	7.78	0.079	7.95	8.00E+04	4.90	0.180	5.15
7.00E+07	7.85			8.00E+04	4.90		
8.00E+07	7.90			8.00E+04	4.90		
8.00E+07	7.90			1.10E+05	5.04		
9.00E+07	7.95			1.30E+05	5.11		
9.00E+07	7.95			1.40E+05	5.15		
9.00E+07	7.95			1.60E+05	5.20		
1.00E+08	8.00			1.70E+05	5.23		
1.00E+08	8.00			1.80E+05	5.26		
1.00E+08	8.00			1.90E+05	5.28		
1.10E+08	8.04			2.50E+05	5.40		
1.10E+08	8.04			2.50E+05	5.40		

Control				Individual Plate Counts			
cfu/ml	log cfu/ml	Standard Deviation	Average	cfu/ml	log cfu/ml	Standard Deviation	Average
***L. monocytogenes* V7**							
6.00E+07	7.78	0.108	8.04	1.00E+06	6.00	0.112	6.11
8.00E+07	7.90			1.00E+06	6.00		
1.00E+08	8.00			1.00E+06	6.00		
1.00E+08	8.00			1.00E+06	6.00		
1.10E+08	8.04			1.10E+06	6.04		
1.20E+08	8.08			1.20E+06	6.08		
1.20E+08	8.08			1.20E+06	6.08		
1.20E+08	8.08			1.30E+06	6.11		
1.30E+08	8.11			1.50E+06	6.18		
1.30E+08	8.11			1.80E+06	6.26		
1.40E+08	8.15			1.80E+06	6.26		
1.40E+08	8.15			2.00E+06	6.30		
***L. monocyotogenes* Camp+/Beta+**							
7.00E+07	7.85	0.122	8.00	1.20E+06	6.08	0.083	6.26
7.00E+07	7.85			1.50E+06	6.18		
8.00E+07	7.90			1.60E+06	6.20		
8.00E+07	7.90			1.60E+06	6.20		
8.00E+07	7.90			1.70E+06	6.23		
1.00E+08	8.00			1.80E+06	6.26		
1.10E+08	8.04			1.90E+06	6.28		
1.10E+08	8.04			2.00E+06	6.30		
1.20E+08	8.08			2.00E+06	6.30		
1.20E+08	8.08			2.10E+06	6.32		
1.20E+08	8.08			2.20E+06	6.34		
1.80E+08	8.26			2.40E+06	6.38		
***L. monocytogenes* SLR1**							
8.00E+07	7.90	0.051	8.00	2.00E+04	4.30	0.135	4.47
9.00E+07	7.95			2.00E+04	4.30		
9.00E+07	7.95			2.00E+04	4.30		
1.00E+08	8.00			2.00E+04	4.30		
1.00E+08	8.00			3.00E+04	4.48		
1.00E+08	8.00			3.00E+04	4.48		
1.00E+08	8.00			3.00E+04	4.48		
1.00E+08	8.00			4.00E+04	4.60		
1.00E+08	8.00			4.00E+04	4.60		
1.00E+08	8.00			4.00E+04	4.60		
1.10E+08	8.04			4.00E+04	4.60		
1.30E+08	8.11			4.00E+04	4.60		

Table A.4.B. Individual plate counts of *Staphylococcus aureus* strains during pressurization at 345 MPa at 25°C for 5 min.

Control				Individual Plate Counts			
cfu/ml	log cfu/ml	Standard Deviation	Average	cfu/ml	log cfu/ml	Standard Deviation	Average
***S. aureus* 315**							
1.30E+08	8.11	0.050	8.18	1.40E+07	7.15	0.075	7.28
1.30E+08	8.11			1.50E+07	7.18		
1.40E+08	8.15			1.70E+07	7.23		
1.40E+08	8.15			1.70E+07	7.23		
1.40E+08	8.15			1.80E+07	7.26		
1.40E+08	8.15			1.80E+07	7.26		
1.50E+08	8.18			2.00E+07	7.30		
1.50E+08	8.18			2.10E+07	7.32		
1.60E+08	8.20			2.10E+07	7.32		
1.70E+08	8.23			2.10E+07	7.32		
1.80E+08	8.26			2.30E+07	7.36		
1.80E+08	8.26			2.50E+07	7.40		
***S. aureus* 565**							
1.30E+08	8.11	0.067	8.26	1.00E+07	7.00	0.104	7.14
1.50E+08	8.18			1.00E+07	7.00		
1.50E+08	8.18			1.00E+07	7.00		
1.80E+08	8.26			1.10E+07	7.04		
1.90E+08	8.28			1.20E+07	7.08		
1.90E+08	8.28			1.50E+07	7.18		
1.90E+08	8.28			1.50E+07	7.18		
1.90E+08	8.28			1.60E+07	7.20		
1.90E+08	8.28			1.60E+07	7.20		
2.00E+08	8.30			1.70E+07	7.23		
2.10E+08	8.32			1.70E+07	7.23		
2.20E+08	8.34			1.90E+07	7.28		
***S. aureus* 743**							
1.00E+08	8.00	0.095	8.14	1.50E+07	7.18	0.048	7.28
1.10E+08	8.04			1.70E+07	7.23		
1.10E+08	8.04			1.80E+07	7.26		
1.10E+08	8.04			1.80E+07	7.26		
1.20E+08	8.08			1.90E+07	7.28		
1.40E+08	8.15			2.00E+07	7.30		
1.40E+08	8.15			2.00E+07	7.30		
1.50E+08	8.18			2.00E+07	7.30		
1.60E+08	8.20			2.00E+07	7.30		
1.70E+08	8.23			2.10E+07	7.32		
1.70E+08	8.23			2.20E+07	7.34		
2.00E+08	8.30			2.20E+07	7.34		

Control				Individual Plate Counts			
cfu/ml	log cfu/ml	Standard Deviation	Average	cfu/ml	log cfu/ml	Standard Deviation	Average
***S. aureus* 485**							
1.10E+08	8.04	0.068	8.11	2.20E+07	7.34	0.042	7.41
1.10E+08	8.04			2.20E+07	7.34		
1.10E+08	8.04			2.30E+07	7.36		
1.20E+08	8.08			2.50E+07	7.40		
1.20E+08	8.08			2.50E+07	7.40		
1.20E+08	8.08			2.60E+07	7.41		
1.30E+08	8.11			2.60E+07	7.41		
1.40E+08	8.15			2.60E+07	7.41		
1.40E+08	8.15			2.70E+07	7.43		
1.40E+08	8.15			2.80E+07	7.45		
1.60E+08	8.20			2.90E+07	7.46		
1.80E+08	8.26			2.90E+07	7.46		
***S. aureus* 765**							
1.00E+08	8.00	0.070	8.11	3.00E+06	6.48	0.088	6.60
1.10E+08	8.04			3.00E+06	6.48		
1.10E+08	8.04			3.00E+06	6.48		
1.20E+08	8.08			4.00E+06	6.60		
1.20E+08	8.08			4.00E+06	6.60		
1.20E+08	8.08			4.00E+06	6.60		
1.20E+08	8.08			4.00E+06	6.60		
1.30E+08	8.11			4.00E+06	6.60		
1.40E+08	8.15			5.00E+06	6.70		
1.50E+08	8.18			5.00E+06	6.70		
1.60E+08	8.20			5.00E+06	6.70		
1.70E+08	8.23			5.00E+06	6.70		
***S. aureus* 778**							
1.30E+08	8.11	0.054	8.18	2.00E+07	7.30	0.099	7.48
1.30E+08	8.11			2.00E+07	7.30		
1.30E+08	8.11			3.00E+07	7.48		
1.40E+08	8.15			3.00E+07	7.48		
1.40E+08	8.15			3.00E+07	7.48		
1.40E+08	8.15			3.00E+07	7.48		
1.50E+08	8.18			3.00E+07	7.48		
1.60E+08	8.20			3.00E+07	7.48		
1.60E+08	8.20			3.00E+07	7.48		
1.70E+08	8.23			4.00E+07	7.60		
1.70E+08	8.23			4.00E+07	7.60		
1.90E+08	8.28			4.00E+07	7.60		

Control				Individual Plate Counts			
cfu/ml	log cfu/ml	Standard Deviation	Average	cfu/ml	log cfu/ml	Standard Deviation	Average
***S. aureus* 582**							
4.00E+08	8.60	0.130	8.80	1.00E+01	1.00	0.000	1.00
4.00E+08	8.60			1.00E+01	1.00		
5.00E+08	8.70			1.00E+01	1.00		
5.00E+08	8.70			1.00E+01	1.00		
6.00E+08	8.78			1.00E+01	1.00		
6.00E+08	8.78			1.00E+01	1.00		
7.00E+08	8.85			1.00E+01	1.00		
7.00E+08	8.85			1.00E+01	1.00		
8.00E+08	8.90			1.00E+01	1.00		
9.00E+08	8.95			1.00E+01	1.00		
9.00E+08	8.95			1.00E+01	1.00		
9.00E+08	8.95			1.00E+01	1.00		

Table A.4.C. Individual plate counts of *Escherichia coli* O157:H7 strains during pressurization at 345 MPa at 25°C for 5 min.

Control				Individual Plate Counts			
cfu/ml	log cfu/ml	Standard Deviation	Average	cfu/ml	log cfu/ml	Standard Deviation	Average
***E. coli* 931**							
1.80E+08	8.26	0.043	8.34	1.00E+05	5.00	0.060	5.08
1.90E+08	8.28			1.00E+05	5.00		
2.00E+08	8.30			1.10E+05	5.04		
2.10E+08	8.32			1.10E+05	5.04		
2.10E+08	8.32			1.20E+05	5.08		
2.10E+08	8.32			1.20E+05	5.08		
2.20E+08	8.34			1.20E+05	5.08		
2.30E+08	8.36			1.20E+05	5.08		
2.30E+08	8.36			1.30E+05	5.11		
2.40E+08	8.38			1.40E+05	5.15		
2.40E+08	8.38			1.50E+05	5.18		
2.50E+08	8.40			1.50E+05	5.18		
***E. coli* 932**							
1.30E+08	8.11	0.085	8.26	360	2.56	0.054	2.62
1.40E+08	8.15			360	2.56		
1.50E+08	8.18			360	2.56		
1.60E+08	8.20			360	2.56		
1.80E+08	8.26			430	2.63		
1.80E+08	8.26			430	2.63		
1.90E+08	8.28			430	2.63		
1.90E+08	8.28			430	2.63		
2.00E+08	8.30			450	2.65		
2.00E+08	8.30			450	2.65		
2.20E+08	8.34			500	2.70		
2.60E+08	8.41			500	2.70		
***E. coli* 35748-88**							
1.70E+08	8.23	0.039	8.30	6.00E+03	3.78	0.098	3.95
1.70E+08	8.23			6.00E+03	3.78		
1.90E+08	8.28			7.00E+03	3.85		
1.90E+08	8.28			9.00E+03	3.95		
2.00E+08	8.30			9.00E+03	3.95		
2.00E+08	8.30			1.00E+04	4.00		
2.00E+08	8.30			1.00E+04	4.00		
2.00E+08	8.30			1.00E+04	4.00		
2.10E+08	8.32			1.00E+04	4.00		
2.10E+08	8.32			1.10E+04	4.04		
2.20E+08	8.34			1.10E+04	4.04		
2.30E+08	8.36			1.10E+04	4.04		

Control				Individual Plate Counts			
cfu/ml	log cfu/ml	Standard Deviation	Average	cfu/ml	log cfu/ml	Standard Deviation	Average
***E. coli* 933**							
1.60E+08	8.20	0.041	8.28	2.00E+05	5.30	0.131	5.48
1.80E+08	8.26			2.00E+05	5.30		
1.80E+08	8.26			2.00E+05	5.30		
1.90E+08	8.28			3.00E+05	5.48		
1.90E+08	8.28			3.00E+05	5.48		
1.90E+08	8.28			3.00E+05	5.48		
1.90E+08	8.28			3.00E+05	5.48		
2.00E+08	8.30			3.00E+05	5.48		
2.00E+08	8.30			4.00E+05	5.60		
2.00E+08	8.30			4.00E+05	5.60		
2.00E+08	8.30			4.00E+05	5.60		
2.40E+08	8.38			5.00E+05	5.70		
***E. coli* C7927**							
1.50E+08	8.18	0.037	8.25	2.10E+05	5.32	0.052	5.39
1.60E+08	8.20			2.10E+05	5.32		
1.70E+08	8.23			2.10E+05	5.32		
1.70E+08	8.23			2.20E+05	5.34		
1.80E+08	8.26			2.50E+05	5.40		
1.80E+08	8.26			2.50E+05	5.40		
1.90E+08	8.28			2.60E+05	5.41		
1.90E+08	8.28			2.60E+05	5.41		
1.90E+08	8.28			2.70E+05	5.43		
1.90E+08	8.28			2.70E+05	5.43		
1.90E+08	8.28			2.80E+05	5.45		
2.00E+08	8.30			2.90E+05	5.46		
***E. coli* SLR 503**							
1.90E+08	8.28	0.044	8.32	7.00E+04	4.85	0.115	5.04
1.90E+08	8.28			8.00E+04	4.90		
1.90E+08	8.28			8.00E+04	4.90		
1.90E+08	8.28			9.00E+04	4.95		
2.00E+08	8.30			1.10E+05	5.04		
2.00E+08	8.30			1.10E+05	5.04		
2.00E+08	8.30			1.10E+05	5.04		
2.20E+08	8.34			1.20E+05	5.08		
2.30E+08	8.36			1.30E+05	5.11		
2.30E+08	8.36			1.40E+05	5.15		
2.40E+08	8.38			1.50E+05	5.18		
2.50E+08	8.40			1.60E+05	5.20		

Table A.4.D. Individual plate counts of *Salmonella* strains during pressurization at 345 MPa at 25°C for 5 min.

Control				Individual Plate Counts			
cfu/ml	log cfu/ml	Standard Deviation	Average	cfu/ml	log cfu/ml	Standard Deviation	Average
***S. choleraesuis typhi* ATCC 6539**							
3.00E+07	7.48	0.112	7.70	0			
4.00E+07	7.60			0			
4.00E+07	7.60			0			
4.00E+07	7.60			0			
5.00E+07	7.70			0			
5.00E+07	7.70			0			
5.00E+07	7.70			0			
6.00E+07	7.78			0			
6.00E+07	7.78			0			
6.00E+07	7.78			0			
7.00E+07	7.85			0			
7.00E+07	7.85			0			
***S. choleraesuis* ATCC 10708**							
1.80E+08	8.26	0.063	8.34	0			
1.90E+08	8.28			0			
1.90E+08	8.28			0			
1.90E+08	8.28			0			
2.00E+08	8.30			0			
2.30E+08	8.36			0			
2.30E+08	8.36			0			
2.30E+08	8.36			0			
2.50E+08	8.40			0			
2.60E+08	8.41			0			
2.60E+08	8.41			0			
2.70E+08	8.43			0			
***S. typhimurium* ATCC 14028**							
1.60E+08	8.20	0.061	8.30	10	1.00	0.000	1.00
1.70E+08	8.23			10	1.00		
1.80E+08	8.26			10	1.00		
1.90E+08	8.28			10	1.00		
1.90E+08	8.28			10	1.00		
1.90E+08	8.28			10	1.00		
2.00E+08	8.30			10	1.00		
2.00E+08	8.30			10	1.00		
2.00E+08	8.30			10	1.00		
2.10E+08	8.32			10	1.00		
2.50E+08	8.40			10	1.00		
2.60E+08	8.41			10	1.00		

Control				Individual Plate Counts			
cfu/ml	log cfu/ml	Standard Deviation	Average	cfu/ml	log cfu/ml	Standard Deviation	Average
***S. enteritidis* VL**							
3.00E+08	8.48	0.160	8.78	15	1.18	0.094	1.30
4.00E+08	8.60			15	1.18		
4.00E+08	8.60			15	1.18		
5.00E+08	8.70			20	1.30		
5.00E+08	8.70			20	1.30		
6.00E+08	8.78			20	1.30		
7.00E+08	8.85			20	1.30		
7.00E+08	8.85			20	1.30		
8.00E+08	8.90			20	1.30		
9.00E+08	8.95			25	1.40		
9.00E+08	8.95			25	1.40		
9.00E+08	8.95			30	1.48		
***S. enteritidis* FDA**							
1.00E+08	8.00	0.072	8.14	430	2.63	0.032	2.69
1.10E+08	8.04			480	2.68		
1.20E+08	8.08			480	2.68		
1.20E+08	8.08			490	2.69		
1.30E+08	8.11			490	2.69		
1.40E+08	8.15			490	2.69		
1.50E+08	8.18			490	2.69		
1.50E+08	8.18			490	2.69		
1.60E+08	8.20			490	2.69		
1.60E+08	8.20			500	2.70		
1.60E+08	8.20			510	2.71		
1.60E+08	8.20			600	2.78		
***S. typhimurium* E 21274**							
2.00E+08	8.30	0.144	8.60	720	2.86	0.028	2.89
3.00E+08	8.48			750	2.88		
3.00E+08	8.48			750	2.88		
3.00E+08	8.48			770	2.89		
4.00E+08	8.60			770	2.89		
4.00E+08	8.60			770	2.89		
4.00E+08	8.60			770	2.89		
5.00E+08	8.70			770	2.89		
5.00E+08	8.70			790	2.90		
5.00E+08	8.70			790	2.90		
6.00E+08	8.78			880	2.94		
6.00E+08	8.78			900	2.95		

Table A.5.A. Individual plate counts of *Listeria monocytogenes* strains during pressurization at 345 MPa at 50°C for 5, 10 and 15 min.

Control		Individual Plate Counts After					
	log		log		log		log
cfu/ml	cfu/ml	5 min	cfu/ml	10 min	cfu/ml	15 min	cfu/ml
***L. monocytogenes* CA**							
1.10E+08	8.04	0		0		0	
1.20E+08	8.08	0		0		0	
1.30E+08	8.11	0		0		0	
1.30E+08	8.11	0		0		0	
1.30E+08	8.11	0		0		0	
1.40E+08	8.15	0		0		0	
1.40E+08	8.15	0		0		0	
1.40E+08	8.15	0		0		0	
Average	8.11						
Standart Deviation	0.03						
***L. monocytogenes* Ohio$_2$**							
8.00E+07	7.90	0		0		0	
8.00E+07	7.90	0		0		0	
9.00E+07	7.95	0		0		0	
9.00E+07	7.95	0		0		0	
1.10E+08	8.04	0		0		0	
1.10E+08	8.04	0		0		0	
1.20E+08	8.08	0		0		0	
1.30E+08	8.11	0		0		0	
Average	8.00						
Standart Deviation	0.08						

Table A.5.B. Individual plate counts of *Staphylococcus aureus* strains during pressurization at 345 MPa at 50°C for 5, 10 and 15 min.

Control		Individual Plate Counts After					
	log		log		log		log
cfu/ml	cfu/ml	5 min	cfu/ml	10 min	cfu/ml	15 min	cfu/ml
***S. aureus* 485**							
9.00E+07	7.95	4.50E+02	2.65	8.00E+01	1.90	5.00E+01	1.70
9.00E+07	7.95	4.50E+02	2.65	8.00E+01	1.90	5.00E+01	1.70
1.10E+08	8.04	5.00E+02	2.70	9.00E+01	1.95	6.00E+01	1.78
1.10E+08	8.04	5.00E+02	2.70	9.00E+01	1.95	6.00E+01	1.78
1.30E+08	8.11	5.00E+02	2.70	1.20E+02	2.08	6.00E+01	1.78
1.40E+08	8.15	5.00E+02	2.70	1.10E+02	2.04	7.00E+01	1.85
1.40E+08	8.15	5.00E+02	2.70	1.20E+02	2.08	7.00E+01	1.85
1.60E+08	8.20	6.50E+02	2.81	1.20E+02	2.08	7.00E+01	1.85
Average	8.08		2.70		2.00		1.78
Standart Deviation	0.09		0.05		0.07		0.06
***S. aureus* 765**							
8.00E+07	7.90	0		0		0	
9.00E+07	7.95	0		0		0	
1.20E+08	8.08	0		0		0	
1.50E+08	8.18	0		0		0	
1.50E+08	8.18	0		0		0	
1.50E+08	8.18	0		0		0	
1.60E+08	8.20	0		0		0	
1.70E+08	8.23	0		0		0	
Average	8.11						
Standart Deviation	0.11						

Table A.5.C. Individual plate counts of *Escherichia coli* O157:H7 strains during pressurization at 345 MPa at 50°C for 5, 10 and 15 min.

Control		Individual Plate Counts After					
	log		log		log		log
cfu/ml	cfu/ml	5 min	cfu/ml	10 min	cfu/ml	15 min	cfu/ml
***E. coli* 933**							
1.80E+08	8.26	0		0		0	
1.80E+08	8.26	0		0		0	
1.80E+08	8.26	0		0		0	
2.00E+08	8.30	0		0		0	
2.00E+08	8.30	0		0		0	
2.10E+08	8.32	0		0		0	
2.20E+08	8.34	0		0		0	
2.20E+08	8.34	0		0		0	
Average	8.30						
Standart Deviation	0.04						
***E. coli* 931**							
1.20E+08	8.08	0		0		0	
1.30E+08	8.11	0		0		0	
1.40E+08	8.15	0		0		0	
1.40E+08	8.15	0		0		0	
1.60E+08	8.20	0		0		0	
1.80E+08	8.26	0		0		0	
1.80E+08	8.26	0		0		0	
1.80E+08	8.26	0		0		0	
Average	8.18						
Standart Deviation	0.07						

Table A.5.D. Individual plate counts of *Salmonella* strains during pressurization at 345 MPa at 50°C for 5, 10 and 15 min.

Control		Individual Plate Counts After					
cfu/ml	log cfu/ml	5 min	log cfu/ml	10 min	log cfu/ml	15 min	log cfu/ml
***S. enteritidis* FDA**							
1.20E+08	8.08	0		0		0	
1.20E+08	8.08	0		0		0	
1.40E+08	8.15	0		0		0	
1.50E+08	8.18	0		0		0	
1.50E+08	8.18	0		0		0	
1.60E+08	8.20	0		0		0	
1.80E+08	8.26	0		0		0	
2.10E+08	8.32	0		0		0	
Average	8.18						
Standart Deviation	0.08						
***S. typhimurium* E 21274**							
1.90E+08	8.28	0		0		0	
2.00E+08	8.30	0		0		0	
2.40E+08	8.38	0		0		0	
2.40E+08	8.38	0		0		0	
2.70E+08	8.43	0		0		0	
2.70E+08	8.43	0		0		0	
2.80E+08	8.45	0		0		0	
2.90E+08	8.46	0		0		0	
Average	8.39						
Standart Deviation	0.06						

Table A.6.A. Individual plate counts of *Listeria monocytogenes* strains during thermal inactivation at 50°C for 5, 10 and 15 min.

Control		Individual Plate Counts After					
	log		log		log		log
cfu/ml	cfu/ml	5 min	cfu/ml	10 min	cfu/ml	15 min	cfu/ml
***L. monocytogenes* CA**							
1.40E+08	8.15	2.80E+07	7.45	1.00E+07	7.00	6.00E+06	6.78
1.40E+08	8.15	3.00E+07	7.48	1.40E+07	7.15	9.00E+06	6.95
2.00E+08	8.30	3.00E+07	7.48	1.60E+07	7.20	1.00E+07	7.00
2.20E+08	8.34	4.30E+07	7.63	1.60E+07	7.20	1.00E+07	7.00
2.20E+08	8.34	5.00E+07	7.70	1.80E+07	7.26	1.00E+07	7.00
2.30E+08	8.36	5.00E+07	7.70	2.00E+07	7.30	1.10E+07	7.04
2.40E+08	8.38	5.00E+07	7.70	2.20E+07	7.34	1.20E+07	7.08
2.40E+08	8.38	5.00E+07	7.70	2.30E+07	7.36	1.40E+07	7.15
Average	8.30		7.60		7.23		7.00
Standart Deviation	0.09		0.11		0.11		0.10
***L. monocytogenes* Ohio$_2$**							
9.00E+07	7.95	7.00E+06	6.85	4.00E+06	6.60	2.00E+06	6.30
1.10E+08	8.04	8.00E+06	6.90	8.00E+06	6.90	4.00E+06	6.60
1.20E+08	8.08	1.10E+07	7.04	8.00E+06	6.90	4.00E+06	6.60
1.40E+08	8.15	1.10E+07	7.04	8.00E+06	6.90	5.00E+06	6.70
1.60E+08	8.20	1.20E+07	7.08	8.00E+06	6.90	6.00E+06	6.78
1.60E+08	8.20	1.30E+07	7.11	9.00E+06	6.95	7.00E+06	6.85
1.60E+08	8.20	1.30E+07	7.11	1.00E+07	7.00	8.00E+06	6.90
1.80E+08	8.26	1.40E+07	7.15	1.00E+07	7.00	8.00E+06	6.90
Average	8.14		7.04		6.90		6.70
Standart Deviation	0.10		0.10		0.12		0.19

Table A.6.B. Individual plate counts of *Staphylococcus aureus* strains during thermal inactivation at 50°C for 5, 10 and 15 min.

Control		Individual Plate Counts After					
	log		log		log		log
cfu/ml	cfu/ml	5 min	cfu/ml	10 min	cfu/ml	15 min	cfu/ml
***S. aureus* 485**							
8.00E+07	7.90	7.00E+07	7.85	3.00E+07	7.48	2.20E+07	7.34
8.00E+07	7.90	1.10E+08	8.04	3.00E+07	7.48	2.80E+07	7.45
1.30E+08	8.11	1.20E+08	8.08	4.00E+07	7.60	2.80E+07	7.45
1.30E+08	8.11	1.20E+08	8.08	4.00E+07	7.60	2.90E+07	7.46
1.40E+08	8.15	1.20E+08	8.08	4.00E+07	7.60	3.00E+07	7.48
1.40E+08	8.15	1.50E+08	8.18	4.00E+07	7.60	3.00E+07	7.48
1.40E+08	8.15	1.50E+08	8.18	5.00E+07	7.70	4.00E+07	7.60
2.50E+08	8.40	1.50E+08	8.18	5.00E+07	7.70	4.00E+07	7.60
Average	8.11		8.08		7.60		7.48
Standart Deviation	0.15		0.10		0.08		0.08
***S. aureus* 765**							
8.00E+07	7.90	2.70E+07	7.43	9.00E+06	6.95	8.00E+06	6.90
9.00E+07	7.95	4.00E+07	7.60	9.00E+06	6.95	8.00E+06	6.90
1.00E+08	8.00	2.90E+07	7.46	1.30E+07	7.11	1.00E+07	7.00
1.20E+08	8.08	2.90E+07	7.46	1.30E+07	7.11	1.10E+07	7.04
1.30E+08	8.11	2.90E+07	7.46	1.50E+07	7.18	1.10E+07	7.04
1.40E+08	8.15	2.90E+07	7.46	2.00E+07	7.30	1.10E+07	7.04
1.60E+08	8.20	2.90E+07	7.46	2.00E+07	7.30	1.50E+07	7.18
1.60E+08	8.20	2.90E+07	7.46	2.00E+07	7.30	1.50E+07	7.18
Average	8.08		7.48		7.15		7.04
Standart Deviation	0.11		0.05		0.14		0.10

Table A.6.C. Individual plate counts of *Escherichia coli* O157:H7 strains during thermal inactivation at 50°C for 5, 10 and 15 min.

Control		Individual Plate Counts After					
	log		log		log		log
cfu/ml	cfu/ml	5 min	cfu/ml	10 min	cfu/ml	15 min	cfu/ml
***E. coli* 933**							
9.00E+07	7.95	4.00E+06	6.60	2.00E+06	6.30	9.00E+05	5.95
1.10E+08	8.04	6.00E+06	6.78	3.00E+06	6.48	2.00E+06	6.30
1.30E+08	8.11	6.00E+06	6.78	4.00E+06	6.60	2.00E+06	6.30
1.30E+08	8.11	7.00E+06	6.85	4.00E+06	6.60	2.00E+06	6.30
1.30E+08	8.11	8.00E+06	6.90	4.00E+06	6.60	2.00E+06	6.30
1.30E+08	8.11	8.00E+06	6.90	4.00E+06	6.60	2.00E+06	6.30
1.30E+08	8.11	8.00E+06	6.90	6.00E+06	6.78	3.00E+06	6.48
1.90E+08	8.28	1.00E+07	7.00	7.00E+06	6.85	3.00E+06	6.48
Average	8.04		7.30		6.95		6.48
Standart Deviation	0.08		0.11		0.16		0.15
***E. coli* 931**							
6.00E+07	7.78	1.20E+07	7.08	5.00E+06	6.70	2.00E+06	6.30
1.00E+08	8.00	1.30E+07	7.11	7.00E+06	6.85	2.00E+06	6.30
1.10E+08	8.04	1.40E+07	7.15	8.00E+06	6.90	3.00E+06	6.48
1.10E+08	8.04	1.60E+07	7.20	9.00E+06	6.95	3.00E+06	6.48
1.10E+08	8.04	1.70E+07	7.23	1.00E+07	7.00	3.00E+06	6.48
1.10E+08	8.04	3.00E+07	7.48	1.00E+07	7.00	4.00E+06	6.60
1.40E+08	8.15	3.00E+07	7.48	1.00E+07	7.00	4.00E+06	6.60
1.60E+08	8.20	5.00E+07	7.70	1.60E+07	7.20	4.00E+06	6.60
Average	8.11		6.84		6.60		6.30
Standart Deviation	0.12		0.21		0.14		0.12

Table A.6.D. Individual plate counts of *Salmonella* strains during thermal inactivation at 50°C for 5, 10 and 15 min.

Control		Individual Plate Counts After					
	log		log		log		log
cfu/ml	cfu/ml	5 min	cfu/ml	10 min	cfu/ml	15 min	cfu/ml
***S. enteritidis* FDA**							
1.20E+08	8.08	1.30E+07	7.11	8.00E+06	6.90	3.00E+06	6.48
1.80E+08	8.26	1.40E+07	7.15	1.00E+07	7.00	4.00E+06	6.60
2.00E+08	8.30	1.40E+07	7.15	1.00E+07	7.00	4.00E+06	6.60
2.00E+08	8.30	1.40E+07	7.15	1.10E+07	7.04	4.00E+06	6.60
2.20E+08	8.34	2.00E+07	7.30	1.30E+07	7.11	5.00E+06	6.70
2.40E+08	8.38	2.00E+07	7.30	1.50E+07	7.18	6.00E+06	6.78
2.70E+08	8.43	3.00E+07	7.48	1.50E+07	7.18	8.00E+06	6.90
2.80E+08	8.45	3.00E+07	7.48	1.60E+07	7.20	9.00E+06	6.95
Average	8.32		7.26		7.08		6.70
Standart Deviation	0.11		0.14		0.10		0.15
***S. typhimurium* E 21274**							
7.00E+07	7.85	2.00E+06	6.30	1.10E+06	6.04	8.00E+05	5.90
1.00E+08	8.00	4.00E+06	6.60	3.00E+06	6.48	8.00E+05	5.90
1.00E+08	8.00	4.00E+06	6.60	3.00E+06	6.48	8.00E+05	5.90
1.20E+08	8.08	4.00E+06	6.60	3.00E+06	6.48	1.00E+06	6.00
1.30E+08	8.11	4.00E+06	6.60	3.00E+06	6.48	1.00E+06	6.00
1.30E+08	8.11	4.00E+06	6.60	3.00E+06	6.48	1.10E+06	6.04
1.30E+08	8.11	5.00E+06	6.70	3.00E+06	6.48	1.20E+06	6.08
2.20E+08	8.34	6.00E+06	6.78	8.00E+06	6.90	1.40E+06	6.15
Average	8.08		6.60		6.48		6.00
Standart Deviation	0.13		0.13		0.22		0.08

VITA

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