EFFECT OF HIGH PRESSURE TREATMENT OF MILK ON MICROBIAL DESTRUCTION AS INFLUENCED BY PRODUCT AND PROCESS RELATED FATCTORS

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DEDICATION

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ABSTRACT

The traditional way of processing milk is the application of heat to destroy undesirable microorganisms. Though heat is an effective means of doing the job, it is associated with several limitations. High pressure (HP) processing has the potential for eliminating microorganisms without affecting the natural quality of the raw material. As a result, it has become a promising technique in recent years. Many factors are reported to influence HP destruction of microorganisms, the most important ones include food composition (i.e., lipid, carbohydrate and protein contents), water activity, process temperature, and mode of pressure treatment. Therefore, the objectives of this research were to: a) evaluate the effect of milk composition on destruction of *E. coli* by HP, b) evaluate kinetic models for spoilage and pathogenic microorganism in milk and the effect of different pressure mode (pulse and static) on the destruction rates; and c) to evaluate the effect of milk type (UHT and raw milk) and temperate on destruction of microorganism.

High pressure destruction of non-pathogenic *Escherichia coli* in milk as affected by milk composition was investigated. The application of HP resulted in significant reduction of *E. coli* K12 population in buffer solution and peptone water, while milk offered significant baro-protection to the organism. It was observed that 5 min treatment at 300 MPa and room temperature resulted in 3.7 log reductions in CFU/mL in buffer solution, 2 log reductions in peptone water, and 0.8 log reductions in pasteurized milk. The microbial destruction pattern in milk with different fat contents (0-5%) or milk with added casein (2-4%) or lactose (4-8%) were not significantly different ($p > 0.05$). However, when 1% casein was added to buffer it produced significant baro-protective effect. Addition of 1% lactose to the buffer solution also showed similar effects, albeit to a less significant degree. This finding suggests low concentrations of casein and lactose add to baro-protection while higher levels do not increase the level of protection further.

The pressure-pulse and pressure-hold inactivation kinetics of *E. coli* were evaluated in pasteurized milk. D-values were 4, 13 and 70 minutes at the following pressure levels 400, 300 and 200 MPa, respectively. The z_p value and ΔV values were

200 MPa and $-7.0*10^{-5}$ m^3 $mole.^{-1}$ The decimal destruction values (D and N_D) decreased with increasing pressure, holding time and number of pulses. Actual death time (28 min) from the experiment (400 MPa, 28 min at 20°C) was the same as predicted by kinetics study suggesting a good fit to the first order kinetic model. Comparison of pressure pulse vs pressure hold approach showed that hold approach to be more feasible at higher pressures and pulse approach could offer significant time saving incentives at lower pressures.

The pressure kinetics for the destruction of the pathogenic *E. coli* strain and *Listeria monocytogenes* were investigated in both ultra high temperature (UHT) and raw milk. Both types of milk samples used in this experiment, exhibited baroprotective effects on microbial destruction by HP, however, the effect was much stronger in raw milk compared with UHT milk. The D values for *E. coli* O157: H7 were 6.5, 11, 23.4 minutes and 12.6, 23, 35 minutes for UHT and raw milk samples, respectively. However, raising the temperature from 20°C to 35°C did not show any increase in lethality. The pathogenic *E. coli* was more pressure resistant than the non-pathogenic strain; however *Listeria monocytogenes* was more pressure resistant than both.

RÉSUMÉ

La méthode traditionnelle de conservation du lait est l'application de chaleur pour détruire les micro-organismes non-désirables. Bien que la chaleur soit un moyen efficace d'arriver à cette fin, il y a de nombreuses limitations y étant associées. Le traitement Haute Pression (HP) a le potentiel d'eliminer les micro-organismes sans affecter la qualité naturelle du produit brut ce qui a comme résultat d'en faire une des technique les plus prometteuse des dernières années. Plusieurs facteurs influencent la destruction des micro-organismes par traitement Haute Pression, les plus importants étant la composition des denrées (contenu en lipides, hydrates de carbone et protéines), l'activité au niveau de l'eau, la température ambiante et le mode de pression utilisé. Ainsi, les objectifs de cette recherche étaient de: a)évaluer les effets de la composition du lait sur la destruction de *Escherichia coli* par traitement HP, b) évaluer les modèles cinétiques pour les mico-organismes pathogènes et de déterioration dans le lait de même que l'effet des différents modes de pression (pulsatoire et statique) sur les taux de destruction, et c) évaluer les effets du type de lait utilisé (UHT et lait cru) et de la température sur la destructions des micro-organismes.

La destruction par Haute Pression de *E. coli* non-pathogène dans le lait tel qu'affecté par la composition du lait fut étudiée La résultante du traitement Haute Pression fut une réduction significative de la population de *E. coli* K12 (non-pathogène) dans une solution tampon et dans l'eau peptonée, alors que le lait offrit une baro-protection significative à l'organisme. Il fut observé que 5 minutes de traitement à 300 MPa et température ambiante eut comme résultante une réduction de 3.7 au niveau des logarythmes des CFU/ml dans la solution tampon, une réduction de 2 logarythmes dans l'eau peptonée, et une réduction de 0.8 logarythme dans le lait pasteurisé. Les modèles de destruction microbienne dans du lait avec des teneurs en gras différentes (0-5%) ou avec du lait auquel fut ajouté de la caséine (2-4%) ou de la lactose (4-8%) ne différèrent pas de façon significative ($p > 0.05$). Cependant, losque 1% de caséine était ajouté à la solution tampon, cela produisait un effet baro-protecteur significatif. L'addition de 1% de lactose à la solution tampon démontra aussi des effets similaires, quoiqu'à un moindre degré. Ces résultats tendent à suggérer que de faibles concentrations de

caséine et lactose augmentent la baro-protection mais que l'augmentation de ces concentrations ne vient pas renforcir cette protection.

Les modèles cinétiques d'inactivation de *E. coli* par pression pulsatoire et pression statique furent évalués dans le lait pasteurisé. Les valeurs-D étaient 4, 13 et 70 minutes aux niveaux de pression de 400, 300 et 200 Mpa respectivement. La valeur z_p et les valeurs ΔV étaient 200 Mpa et–$7.0*10^{-5}$ m^3 $mole.^{-1}$. Les valeurs de destruction décimales (D et N_D) diminuèrent avec l'augmentation de la pression, du temps de rétention et du nombre de pulsations. Le temps de mort (28 min) de l'expérience (400 Mpa, 28 min à 20°C) était tel que prédit par les études cinétiques suggérant une bonne concordance avec le modèle cinétique de premier ordre. La comparaison de la pression pulsatoire versus la pression statique démontra que la pression statique est plus facilement utilisable avec des pressions élevées et que la pression pulsatoire pourrait offrir des réductions de temps intéressantes avec des pressions moindres.

Les dynamiques de l'effet de la pression sur la destruction de *E. coli* de souche pathogène et de *Listeria monocytogenes* furent étudiées dans le lait ultra haute température (UHT) et le lait cru. Les deux types de lait utilisés dans l'expérience montrèrent des effets baro-protecteur sur la destrcution microbienne par HP, cependant, cet effet était nettement plus marqué avec le lait cru qu'avec le lait UHT. Les valeurs D pour *E. coli* 0157: H7 étaient de 6.5, 11, 23.4 minutes et 12.6, 23, 35 minutes pour le lait UHT et le lait cru respectivement. Cependant, l'augmentation d e la température de 20°C à 35°C n'indiqua aucune augmentation dans le taux de mortalité. La souche pathogène de *E. coli* fut plus résistante à la pression que la souche non-pathogène; cependant *L. monocytogenes* fut le micro-organisme offrant le plus de résistance.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS i
ABSTRACT iii
RSUME V
TABLE OF CONTENTS Vii
LIST OF TABLES X
LIST OF FIGURES Xii

CHAPTER 1. INTRODUCTION 1

CHAPTER 2. LITERATURE REVIEW 4

High hydrostatic pressure processing technology 4
Fundamental principles 5
Advantages of high pressure technology 6
Commercial application of high pressure technology 8
Effects of high-pressure on food and factor influencing HHP 10
Product system factors 10
Composition 10
pH 12
Microorganism 12
Processing system factors effects 12
Temperature 12
Pressure 13
Rate of compression and decompression 14
Equipment for HPP treatment 14
Critical control points during experimentation 15
Application of high pressure in food industry 16
Effect on microbial inactivation 16
Inactivation mechanism 17
Kinetics of microbial inactivation 18
Examples of applications of high-pressure in food industry 20
Food preservation technology and milk processing 21
Safety concern about milk 21
Common microorganisms in milk 22
Thermal processing milk and its limitation 24

Application of high pressure processing in milk 25
Effect of high pressure on milk constituents 25
Milk proteins 25
Milk enzymes 25
Milk fat 26
Milk lactose 27
Effect of high pressure on microorganisms in milk 27
High pressure processing affected by milk composition 28
Research on high pressure processing of milk in McGill 28
Limitations of high pressure technology 28
Future prospects of high pressure technology in dairy industry 29

CHAPTER 3. COMPOSTION EFFECTS ON THE HIGH PRESSURE *DESTRUCTION OF ESCHERICHIA COLI* IN MILK 31

Abstract 31
Introduction 32
Materials and methods 34
Preparation of culture and inoculation of test samples 34
Buffer and milk composition 35
High hydrostatic pressure treatment 35
Enumeration of survivors 36
Statistical analysis 37
Results and discussion 37
Milk, peptone water and buffer as dispensing media 37
Influence of fat on pressure destruction of *E. coli* 40
Influence of casein added to milk on the destruction of *E. coli* 42
Influence of lactose added to milk on the destruction of *E. coli* 43
Influence of casein and lactose added to buffer on the destruction of *E. coli* 45
Conclusion 47

Chapter 4. DUAL-EFFECT HIGH PRESSURE DESTRUCTION KINETICS OF *ESCHERICHIA COLI* IN MILK 55

Abstract 55
Introduction 56
Materials and methods 58
Microbial analysis and samples preparation 58
Sample preparation and high pressure treatment 58
Data analysis 59

Results and discussion 61
Kinetic study 61
Total destruction of *E. coli* 63
Pulse effect study 63
Conclusion 66

CHAPTER 5. HIGH PRESSURE DESTRUCTION OF *ESCHERICHIA COLI* O 157:H 7 AND *L. MONOCYTOGENS* IN MILK 69

Abstract 69
Introduction 70
Materials and methods 71
Bacteria strain and culture conditions 71
Preparation of samples 71
High pressure treatment 72
Kinetic data analysis 72
Microbiological assay 73
Results and discussion 74
Destruction kinetics of pathogenic *E. coli* O 157 :H 7 74
Pressure pulse affect on *E. coli* destruction 75
High pressure inactivation of *L. monocytogenes* in milk 75
High pressure destruction of *L. monocytogenes* as affected by temperature 78
Conclusion 78

CHAPTER 6. GENERAL CONCLUSIONS 82

REFFENRENCE 84

APPENDIZ APPLICATION OF USE BIOHAZZARDOUS MATERIALS 98

LIST OF TABLES

3.1	Influence of dispensing medium (milk, peptone water and buffer) on high pressure destruction on *E. coli*	37
3.2	The analysis of variance (ANOVA) OF the effect of independent variables (lactose, holding time) on high pressure (200, 300 and 400 MPa) destruction of *E. coli*	41
3.3	The analysis of variance (ANOVA) of the effect of independent variables (casein, holding time) on high pressure (200, 300 and 400 MPa) destruction of *E. coli*.	43
3.4	The analysis of variance (ANOVA) of the effect of independent variables (lactose, holding time) on high pressure 200, 300 and 400 MPa) destruction of *E. coli.*	44
3.5	Baro-resistance of *E. coli* in milk and buffer with added 1, 2, 4% casein and lactose at 300 MPa for 5 min or 200 MPa for 15 min.	46
3.6	Microbial destruction of *E. coli* K12 in milk and buffer solutions with and without added casein or lactose (1%) at 300 MPa after various treatment times.	47
4.1	Pressure levels, holding time and pulses used for HP treatment of milk inoculated with *E. coli.*	58
4.2	The high-pressure destruction kinetics of *E. coli* in milk at room temperature.	62
4.3	Pressure pulse effect related parameters for *E. coli* in milk.	64
4.4	Pressure pulse Vs pressure hold process for *E. coli* in milk.	65
4.5	Pressure pulse destruction of *E. coli*	65
5.1	E. coli survivor data and computed D value of *E. coli* O 157:H7 in UHT (2% fat) and raw milk.	76
5.2	Pressure pulse effect related parameters for *E. coli* in UHT milk.	77
5.3	Pressure pulse effect related parameters for *E. coli* in raw milk.	77

5.4 HPP log reduction values for *L. monocytogenes* in UHT and raw milk samples at 400 Mpa at various holding times. 77

LIST OF FIGURES

3.1 HP destruction of *E. coli* inoculated into milk, buffer solution and peptone water treated under (1) 300 MPa for 0 min; 300 MPa for 5 min; (3) 200 MPa for 0 min and (4) 200 MPa for 15 min. 49

3.2 HP destruction for *E. coli* in milk as affected by fat content: (a) HP 400 MPa , (b) 300 MPa and (c) 200 MPa 50

3.3 HP destruction for *E. coli* in homogenized whole milk (3.25% fat) as affected by added casein: (a) HP 300 MPa and (b) 200MPa. 51

3.4 HP destruction for *E. coli* in homogenized whole milk (3.25% fat) as affected by added lactose: (a) HP 300 MPa and (b) 200. MPa. 52

3.5 HP destruction for *E. coli* in milk, buffer and buffer with adding 1, 2 and 4% casein or lactose and subjected to two high pressure treatment: (300 MPa, 5 min and 200 MPa, 15 min). 53

3.6 HP destruction for *E. coli* in milk, buffer and buffer with adding 1, 2 and 4% casein or lactose and subjected to two high pressure treatment at 300 MPa for 0-7.5 min). 54

4.1 Survival curve of *E. coli under different pressure and holding time* at room temperature. 67

4.2 Pressure sensitivity of *E. coli* in milk at room temperature 67

4.3 Survival curve of *E. coli* 25 °C at 400 MPa. 64

4.4 Survival curve of *E. coli* under different pressure pulse treatment. 68

5.1 Effect of pressure level and treatment time on the destruction of Pathogenic *E. coli* O 157 H: 7 in raw milk. 79

5.2 Effect of pressure level and treatment time on the destruction of Pathogenic *E. coli* O 157 H: 7 in raw milk. 80

5.3 Pressure sensitivity of pathogenic *Escherichia coli* in UHT milk (2% fat) and raw milk. 81

5.4 High pressure (400 MPa) inactivation of *L. monocytogenes* in UHT and raw milk samples. 80

5.5 High pressure inactivation of *L. monocytogenes* at various pressure levels and temperature (20 and 35 °C). 81

CHAPTER 1

INTRODUCTION

Thermal pasteurization and sterilization have been predominantly used to achieve food product safety and stability for centuries. However, excessive heat treatment applied by such technologies may cause undesirable sensory changes such as non-enzymatic browning, as well as loss of vitamins and other nutrients and volatile flavor compounds. (Laso and Yousef, 2002)

There has been an increased consumer demand for natural taste and flavor of food, minimally processed, additive free and microbiologically safe foods. This stimulated the food scientists and industries to investigate new processing methods to meet this demand. Non-thermal alternative technologies such as electric or magnetic fields, ionizing radiation, light pulses, and high pressure hydrostatic pressure processing (HPP) have been investigated intensively in the past 30 years (Farkas 1998; Hoover et al., 1989; Smelt, 1998; Qin and Pothakamury, 1996; Kuob et al., 1997; Bintsis et al., 2000). Among these new non-thermal processing techniques, high hydrostatic processing has become one of the most promising methods for the food treatment and preservation at room temperature (Cheftel, 1992).

High pressure (HP) processing also described as high hydrostatic pressure (HHP) or ultra high pressure (UHP) processing, subjects liquid and solid foods, with or without packaging, to pressures between 100 and 800 MPa. Process temperature during pressure treatment can be specified from below 0°C (to minimize any effects of adiabatic heat) to above 100°C. Commercial exposure times to high pressure can range from a millisecond pulse (obtained by oscillation pumps) to treatment times of over 20-min.

Milk is one of the two original nutritional liquid foods (the other one is honey). Milk products processed even by modern thermal technologies, such as high temperature short time (HTST) pasteurization and (UHT) sterilization, still lack the fresh flavor and texture (Beatrice et al., 2002).

Application of high pressure processing to milk has been investigated by many authors. Most of them have focussed on evaluation of high pressure effects on microbial destruction kinetics, enzyme inactivation, and on the functional properties of milk. HP processing may be applied in two modes: a static mode which refers to holding pressure at the set level for a period of time, or the pulse mode involving instantaneous pressure releases once the optimum pressure level is achieved. Pulsed pressure treatments have been found to be more effective than static applications over comparable lengths of time (Aleman et al., 1994; 1996).

In the past decade, the increase in foodborne infections has become a worrisome public health concern worldwide. Some outbreaks of infections caused by *Listeria monocytogenes* were recently reported to be transmitted by milk and cheese samples. The source of *Listeria* in these outbreaks has generally been raw or inadequately pasteurized milk. *L. monocytogenes, Salmonella, E. coli O157:H7, Campylobacter jejuni* and *Yersinia enterocolitica* are the pathogens of most public health concern in recent years. *E. coli* K-12 is a common contaminant / spoilage bacterium in milk. It belongs to the Enterobacteriaceae family and is part of the flora in the intestine of humans and warm-blooded animals. Because of its typical habitat, it is considered to be a good index of direct or indirect contamination of faecal origin.

Apart from temperature, a number of factors, including the magnitude and duration of pressure treatment are known to affect the resistance of bacteria to high pressure. The composition of the suspending medium affects the sensitivity of bacteria to pressure. The presence of lipids, carbohydrates, proteins and a reduced a_w all offer resistance to high-pressure treatment (Simpson & Gilmore, 1997). Certain food ingredients have been shown to affect pressure resistance of vegetative cells. For example, increasing NaCl concentrations in the medium increased the baroresistance of *E. coli* and *Rhodotorula rubra* substantially (Oxen and Knorr, 1993), and similar effects were observed with increasing glucose concentrations or sucrose concentrations in ewe's milk by high hydrostatic pressure. Food is a complex system and different foods show considerable variation in composition. Thus, it is necessary to use a case by case approach when examining pressure effects on real food systems.

Listeria monocytogenes is more susceptible to pressure in buffer solution than in milk, as the milk proteins, carbohydrates and fats protect the bacterial cell (Styles et al. 1991; Patterson et al. 1995; Simpson 1995). UHT milk provides more protection against pressure inactivation than raw milk (Patterson et al. 1995). It is speculated that the less protective raw milk may contain heat-labile anti-microbial compounds which could act in concert with high hydrostatic pressure to increase the inactivation of *L. monocytogenes.*

Recently the effect of milk fat on high pressure pasteurization was investigated and it was concluded that all milk fat had a baroprotective effect. However, there was no progressive protective effect by increased fat content of milk (Gervilla et al., 2000). These workers studied a wide range of fat content from 0 –50 % fat in ewe` milk. These effects could be overcome by increasing pressure and operating at higher processing temperatures.

The objectives of this investigation were a) to determine the influence of fat, casein and lactose concentration of milk on the survival of *E. coli* K12 during high pressure processing; b) to compare the kinetics (estimate D value) of high-pressure inactivation of *E. coli* K12 by static and pulse pressure modes; c) to study the kinetics of HP inactivation and baroprotection on pathogenic and non pathogenic strains of *E. coli*, and d) to investigate the effect of temperatures and product type on the destruction of *Listeria monocytogenes*.

CHAPTER 2

LITERATURE REVIEW

High hydrostatic pressure processing technology

High pressure is being safely and reliably used in various industries such as chemical, ceramics, metal carbide and the plastics industry. High-pressure (HP) treatment has been known to be applicable for food processing purposes almost 100 years ago (Hite, 1899). Hite et al. (1914) showed that various foods could be preserved for an extended period of time by pressure treatment. In spite of these early studies, it was not until recently (about 15 years ago), that large-scale high-pressure engineering made sufficient progress to permit it to be adapted to the needs of the food industry (Mertens, 1993). Since then, HP processing has been at the centre of food research and development activities, especially since the emergence of commercial products on the Japanese market in 1991. Initially, emphasis was directed towards food preservation with the goal of extending the product shelf life with minimum impact on product quality. Subsequently, the potential of HP processing for physical modification of structure and function of food and food constituents for as well as the possibility for new process development (i.e. pressure-assisted freezing or thawing) was recognized (Palou et al. 1999). Commercial products from HHP technology (i.e., avocado puree, orange juice and milk) are now available in Europe and the USA. Batch and semi-continuous equipment for HHP processing are available on an industrial scale for food processing (Knorr, 1999).

High pressure processing uses significantly higher levels of pressure (100-1000MPa) to treat foods for a few minutes (Gervilla, 2000). Although the outcome of high-pressure treatment is similar to that of heat, the concept is totally different. The distinct advantages of high pressure processing are that they inactivate microorganisms without the need of severe heating, and therefore avoiding thermal degradation of food components; thereby retaining the natural flavor, color and nutrients found in natural foods (Knorr, 1993). Energy (pressure) is transmitted evenly and instantaneously throughout the product. This ensures the absence of dead spots and localized over-processing which are problematic with other treatments (Datta and Deeth 1999).

Fundamental principles underlying the effects of high-pressure on foods

Two fundamental principles, namely the Le Chatelier's principle and the isostatic principle, govern the behavior of materials under pressure, and these principles are of particular interest for food application.

The Le Chatelier's Principle: As a thermodynamic parameter, pressure has far-reaching effects on the conformation of macromolecules, the transition temperature of lipids and water and a number of chemical reactions (Cheftel, 1992; Johnston, 1992; 1995; Tauscher, 1995). Phenomena that are accompanied by a decrease in volume ($\Delta V<0$, where ΔV = volume of products –volume of reactants) are enhanced by pressure, and vice –versa (principle of Le Chatelier). Thus, under pressure, reaction equilibria are shifted toward the most compact state, and the reaction rate constant (k) is either increased or decreased, depending on whether the 'activation volumes' (ΔV) of the reaction is negative or positive . Pressure primarily affects the volume of the system. The influence of pressure on the reaction rate can be described by the transition-state theory, where the rate constant of a reaction in a liquid phase is proportional to the quasi equilibrium constant for the formation of active reactions. Based on this assumption, it was reported that at constant temperature, the pressure dependence of the reaction velocity constant (k) is due to the activation volume of the reaction ((ΔV):

$$\left[\frac{\Delta \ln(k)}{\Delta p}\right]_T = -\frac{\Delta V^*}{R \cdot T} \qquad (2.1)$$

$$\ln k = \ln ko - \Delta V / RT * P \qquad (2.2)$$

where P is the pressure, T is the gas constant (8.314 cm^3/MPa/K/mol) and T is the temperature (K).

The second principle is Pascal's law or isostatic principle, which states that pressure, is transmitted in a uniform and quasi-instantaneous manner throughout the biological sample or solution (this may not hold when a large volume of gas is present).

The time necessary for pressure processing is therefore independent of sample size, in contrast to the situation prevailing for thermal or mechanical processes. Thus, no part of the product is subjected to over-treatment.

Food is a biological material, and most biochemical reactions result in a change in volume, so food processes are influenced by pressure application. From the processing point of view, these two principles have several advantages over thermal processing as discussed below.

Advantages of high pressure technology

The most important advantage of high pressure application in food processing is its ability to destroy spoilage and pathogenic microorganisms at ambient or low temperature. Studies have shown that subzero temperature could be more effective with regard to the inactivation of microorganism or some enzymes (Hayashi, 1988; Knorr, 1995). Low temperature can help to retain nutritional quality and functionality of raw material treated with pressure and could allow maintenance of consistently low temperatures during post harvest treatment, processing, storage, transportation, and distribution periods of the life cycle of food systems.

Le Chatelier's principle, as it relates to pressure shifts freezing and pressure thawing, have been described by Bridgman (1912). Pascal's principle allows uniform and instant transmission of high pressure throughout food systems. This pressure transmission is independent of size and geometry of samples, and this feature represents a major advantage over conventional thermal processing where size and geometry can be limiting factors. For example, size reduction required in conventional thermal processing to improve heat and mass transfer is often accompanied by elevated losses of nutrient and subsequent environmental pollution (e.g., in hot water blanching processes). Such independence of size and geometry of samples could not only reduce process severity and thus lead to higher product qualities; it could also increase process flexibility and ultimately revolutionize food processing by making requirements for size reduction obsolete. In addition, since there is no pressure penetration profiles involved, the process calculation methods will be simpler than those used in thermal processing.

Pressure processing is most commonly used in a liquid pressure-transmitting medium such as water. The sample is protected from direct contact by using sealed flexible packaging. Water is a suitable pressure-transmitting medium because it is compatible with food materials and is easy to work with. More importantly the compressibility of water is so small and results in negligible compression energy even at extremely high pressures. Usually, at 22°C, the compression of water ranges from 4% at 100 MPa to 15% at 600 MPa (Farkas, 1993; Hayashi, 1989; Sawamura *et al.*, 1989).

When an aqueous solution is compressed, the compression energy E is approximately equal to E = 2/5 x P x C x Vo, where P is the pressure (Pa), C the compressibility of the solution, and Vo the initial volume (m^3). As a result, the estimated compression energy of 1 liter of water at 400 MPa = 19.2 kJ. This energy is small enough to be compared to 20.9 kJ that required heating 1 litre of water from 20-25°C. (Cheftel and Culioli, 1997).

The low energy levels involved in pressure processing allow the preservation of covalent bonds in food constituents (83 and 50 kcal/mole bond energy for C-C and S-S respectively), and only non-covalent bonds are affected (Hahashi and Hayashida , 1989). Hence small molecules such as amino acids, vitamins, pigments and flavor/fragrant components that are responsible to sensory and nutritional characteristic that are mostly stabilized by covalent bonds, are not affected appreciably by HHP treatments. Consequently processed products retain the initial color, flavor /fragrant and nutritional qualities that are mostly sacrificed when traditional treatments are used.

Since high pressure affects the non-covalent bonds (1-7 kcal /mole bond energy), larger molecules such as proteins, enzyme, polysaccharides and lipids, etc., (relatively large biopolymers) whose function depends on the quaternary, tertiary and secondary structures are denatured by high pressure. Like thermal processing, high pressure also induces a variety of modifications in food systems, such as, protein denaturation, inactivation or activation of enzymes, gel formation, tenderization, and texturization, etc. The appearance of pressure reduced protein denatured products are more attractive and maintain their original color and flavor as well as produce texture like cooked product. Texturization of

most vegetables and fruits indicates that without any additive, pressure can improve the texture of the product.

High pressure processing is an energy efficient process since pressure is generated with a pump, once the pressure is reached, the pump is stopped, valves are closed and the pressurized liquid is maintained in a steel cylinder of adequate thickness and resistance, and keeping the sample under pressure for extended period of time does not require any additional input of energy (Farr, 1990). It only requires electricity for pressure build-up. The potential for future omission of size reductions of foods prior to high-pressure processing could substantially reduce food-processing wastes and save time and labor, so it is environmentally friendly.

Moreover the method can be readily to be combined with other technologies, such as, temperature, antibacterial agent, supercritical CO_2, etc., to increase its efficiency.

A summary of advantages and limitations of high-pressure treatment as related to food processing is provided in Table 2.1.

Commercial application of high-pressure technology

The first commercial high-pressure processed food products (i.e., high acid jams from strawberry, raspberry, kiwi fruit and apple) were introduced to the Japanese market in April 1990 (Galazke and Ledward 1995). The jam retained the fresh fruit flavor and color. The first commercially pressure-processed product on the US market was guacamole product produced in Mexico (Mermelstein 1997). Thus far, there are no HP processing food products commercially available on their Canadian market.

The majority of these products are high-acid foods such as yogurt, fruit jellies and jams, fruit juices, salad dressings, and wine. However, the range of pressure-processed foods is increasing, and now extends to low acid foods such as rice and cakes, fish, ham, avocado products (guacamole). Raw oysters, shucked and pasteurized by HPP are available since 2000 (Cheftel, 1998; Smelt, 1998). But its commercial application is still small (Manvel, 1997; Rowe et al. 1997; Mermelstein 1997).

Table 2.1 Advantages of high-pressure treatment for food processing operations

Treatment	Advantages
Instant response	Immediate distribution throughout product (in the absence of gas)
Even distribution	Independence of sample size and geometry
Low/ambient temperature	Reducing thermally generated qualities reduction /loss.
Application affects (directly) non-covalent bonds	Quality retention (i.e., flavor, color, nutrients)
Increase reaction rates	Increased bioconversion rates; increased metabolite production; improved separation processes
Affects phase transition	Process and product development (i.e., gelling, melting, crystallization)
Degassing	Improved heat transfer, reduced oxidation
Membrane permeability	Aids separation processes
Waste-free technology	Environmentally friendly process
Volume compression	Compacting, forming, coating
Affects enzyme activity	Food preservation
Affects microbial activity	Food preservation
Differs from thermal effects	Selective process/product development (i.e., pressure induced gelling)
Adiabatic heating	Additional temperature effect
pH reduction	Additional pH effect

Effects of high-pressure on food and factor influencing HPP

There exist a big difference in chemical effects produced in food between pressure and heat. Pressure processing affects ionic and hydrophobic bonds, thus pressure effects only secondary and tertiary structural changes in large molecules such as proteins, polysaccharides, and complex molecules. Consequently, enzymes and carbohydrates in an aqueous environment may undergo reversible or irreversible conformation changes, e.g., protein denaturation, dissociation, aggregation, or gelation (Heremans, 1982; Balny and Masson 1993; Kunugi 1993). Whereas the heat breaking the covalent bonds in both small and large molecules causes changes to color, flavor and other sensory properties that are not observed with pressure treatment.

A number of factors, apart from the temperature magnitude and duration of pressure treatment are known to affect the resistance of bacteria to high pressure. The stage of growth of the bacteria is important in determining pressure resistance (Isaacs & Chilton, 1995). The composition of the suspending medium affects the sensitivity of bacteria to pressure. The presence of lipids, carbohydrates, proteins and reduced A_w all confer resistance (Simpson and Gilmore, 1997). This suggests that the pressure resistance of bacteria vary among foods. Therefore, it is important to validate processing parameters in foods and not extrapolate results from buffers and laboratory media (Smelt, 1998). A summary of the factors influencing HPP is provided below:

Product system factors effects

Composition

Water and water activity of the food: Water is the major constituent of most foods and is highly affected by high pressure since it is reduced in volume by 4% and 15 % under 100 and 600 MPa, respectively. According to Le Chatelier's principle, this has a major influence on the chemical changes in food. The adiabatic compression of water increases the temperature -3°C per 100 MPa. Self ionization of water is also promoted by NPP lowering the pH. Phase transition of water can be performed under pressure. At –1000 MPa water freezes at room temperature, whereas the freezing point can be lowered to -4 to -22°C under pressure from 50 to 210 MPa. This phenomenon allows sub-zero food storage without ice formation, rapid thawing, and pressure-shift crystallization. This is done by the

sudden release of pressure when cooling the product to sub-freezing point, and it results in frozen products containing very small ice crystal, hence improved quality (Kalichevsky et al. 1995).

Oxen and Knorr (1993) showed that a reduction of water activity from 0.98-1.0 to 0.94-0.96 resulted in a marked reduction in inactivation rates for microbes suspended in a food. Reducing the water activity appears to protect microbes against inactivation by HPP. It is reported in the literature that when water activity is near or below 0.9, vegetative cells enter a dormant state in which the cell membrane is modified and becomes more pressure resistant (Knorr et al., 1992). On the other hand, it is to be expected that microbial cells may be sub lethally injured by pressure, and recovery of sub lethally injured cell can be inhibited by low water activity. Consequently, the net effect of water activity may be difficult to predict.

Salt and sugar: Foodstuffs / food products offer more pressure-protection to microorganisms than buffers of microbiological media. Furthermore, food constituents also affect baroresistance of enzymes (Ogawa et al., 1990; Seyderhelm et al.; 1990; Asaka and Hayashi, 1991). Certain food ingredients have been shown to affect pressure resistance of vegetative cells (Oxen and Knorr, 1993).

Increasing NaCl concentration in the medium increased the baroresistance of *Escherichia coli* and *Rhodotorula rubra* substantially. Similar effects could be obtained with increasing glucose concentration or sucrose concentrations. Baroprotective effect of sugar was observed on the inactivation of enzymes (Ogawa et al., 1992; Horie et al., 1991). Significant baroprotective effects of NaCl or glucose were noted with suspension of *Z. rouxii* and *S. cereviceae* (Hayakawa et al. 1994).

Protein and Enzyme: The Le Chatelier Principle governs structural rearrangements, taking place in proteins under pressure. Covalent bonds are almost unaffected by high pressure, so the primary structure of protein is retained. Pressure over 300 MPa causes irreversible protein denaturation at room temperature, whereas lower pressure results in reversible changes in protein structure (Knorr, 1999).

Effects of HPP on enzymes have been studied; at low pressures of 100-200 MPa have been shown to activate monomeric enzymes, whereas higher pressures generally induce enzyme inactivation. Pressure resistance of enzymes is not related to thermal resistance.

pH

Increasing the pressure on water from 100-1000 MPa caused a decrease in pH value of about one unit. This is explained by the ionic dissociation of water and various weak acids (acetic, phosphoric etc.) and enhanced hydrogen ionic concentrations. Volume is reduced by pressure under pressure (Brandts et al., 1970; Zipp and Kauzemann, 1973). The one unit decrease in pH has a significant effect in biochemistry process such as protein denaturation, enzyme inactivation and destruction of microorganism (Hinrichs et al., 1996).

Pressure inactivation rate will be enhanced by exposed to acidic pH. Compression of foods may shift the pH of the food as a function of imposed pressure. This can be explained as below:

Ionic bonds such as those responsible for the folding of proteins can be disrupted. Ogawa et al. (1990) observed that the destruction of yeast and molds in mandarin juices are not affected by the presence of organic acids in juices such as citric, tartaric, lactic or acetic acids. It is possibly due to the fact that pressure favors ionization while most organic acids are particularly inhibitory to destruction in their undissociated form. For acidic food products, especially for fruit juices, a treatment at 500 MPa will cause pH shift of about one unit to acid side. Therefore pH can be an important factor that can influence the effects of pressure on the kinetic properties of enzyme and microorganisms especially vegetative bacteria. The reduction of pH due to this effect is expected to be less pronounced since the volume change of ionization becomes smaller at higher pressure.

Microorganism

See "applications of high pressure in food industry" page 17.

Processing system factors effects

Temperature

Pressure affects primarily the volume of a system, while temperature changes botthevolume and the energy of a system. The process of pressure treating a food always results in a temperature increase due to the work of compression. By contrast, the warming

of a food by heat transfer (at 0.15M0a) does not result in a pressure increase in the food. Temperature increases due to compression can be 3 °C or more per 100 MPa, depending on the composition of the food (Zook and et al. 1999). For example, if the food contains a significant amount of fat, such as butter or cream, the temperature rise can be larger. Foods cool down to their original temperature on decompression if no heat is lost to or gained from the walls of pressure vessel during the hold time at pressure.

For this reason care must be taken in keeping a food sample at constant temperature during pressure treatment or by determining the temperature of the food during compression and decompression. The best that can be done at this time is to define a process using the parameters of initial temperature, compression time, product temperature, process pressure, and process hold time at pressure, and reproduce these conditions for every batch of food treated.

Effect of high-pressure treatment is greatly influenced by temperature. Temperatures in the range of 45 to 50° C appear to increase the rate of inactivation of food pathogens and spoilage microbes. Process temperatures in the range of 90-110 ° C in conjunction with pressures of 500-600 MPa have been used to inactivate spore-forming bacteria such as *C.botulinum*. The use of elevated temperatures as part of a specified value.

Pressure

Aleman et al. (1994; 1996), Basak and Ramaswamy (2001) and Pandy et al. (2002) have conducted studies on comparison of static versus pulsed pressure applications. Aleman et al. (1994) studied the inactivation of *S. cerevisiae* in pineapple juice. These authors found that pulsed pressure treatments were more effective than static applications over comparable lengths of time. For example, it was shown that a total exposure time of 100 s with repetitive pulses inactivated 4 log CFU/ml of *S. cerevisae*, but on the contrary, a comparable reduction using one static holding mode at the same pressure required 5 to 15 min.

Rate of compression and decompression

The rates of compression and decompression are other important factors that require a more detail investigation. So far there is not much reports cited in the literature to examine their significance in process operation.

Equipment for HPP treatment

A schematic diagram of basic equipment design used for HP processing is illustrated in Figure 2.1 (Source: High Pressure Processing of Foods, edited by DA Ledward DE Johnston RG Earnshaw APM Hasting.1995, p181-190)

. A typical HP system consists of four main parts: 1. A high-pressure vessel and its closure. 2. A pressure –generating system. 3. A temperature-control device. 4. A material-handing system. (Mertens and Deplace, 1993).

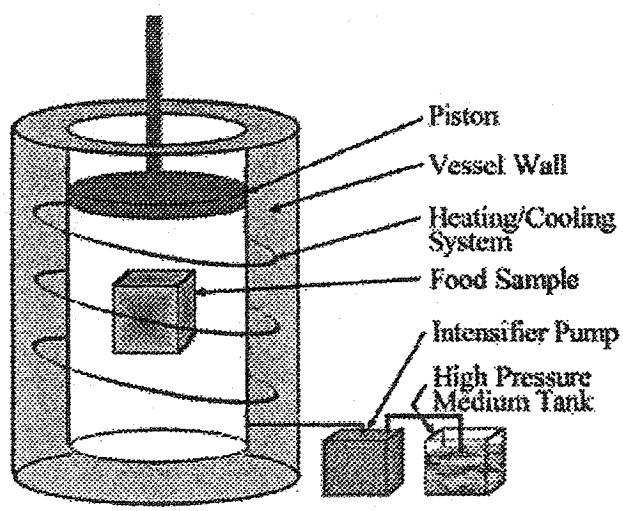

Fig. 2.1. Schematic diagram of basic equipment design for high pressure processing of foods

The most important part is the pressure vessel, which is usually a forged monolithic cylindrical vessel constructed of low-alloy steel of high tensile strength. (Mertens & Deplace, 1993). Once loaded and closed, the vessel is filled with a pressure-transmitting medium; in food processing, water added mineral oil (Myllymäki, 1996). Air must be removed from the vessel, by compressing or heating the medium, before pressure is generated (Deplace, 1995).

Three kinds of pressure treatment are used in food processing: (1) batch operation where the liquid or solid food is pressurized for a given holding time and then decompressed. Batch processes are necessary for packaged foods; (2) semi-continuous mode, in which the liquid food to be treated is introduced periodically into the high-pressure processing chamber. The combination of multiple cells, which work sequentially and which a central high-pressure compressor feeds, can be seen to produce greater continuity in the process: and (3) continuous operation mode which is suitable for liquid food such as milk.

In the food industry, vessels with a volume of several thousand liters are in use, with typical operating pressures in the range 100 - 500 MPa and holding times of about 5 - 10 min (Myllymäki, 1996). Laboratory-scale HP equipment capable of reaching pressures up to 1000 MPa is also available. Westerlund (1994) estimated the processing cost of a continuous operation at 600 MPa with a throughput of 300 to 6000 litres/h to be between 3-20 pence a litre.

Critical control points during experimentation

To ensure that an experiment can have reproducible results, the following critical control points must be considered for a treatment:

Temperature in the high-pressure vessel chamber prior to processing: This will ensure that the initial temperature is at the proper target temperature prior to the prepackaged foods.

Product temperature and uniformity of temperature throughout the product: The product must be at the initial homogenous target temperature and there must be no cold spots, otherwise the product will not achieve the target temperature during pressurization to achieve the designed process.

Ratio of pressurizing fluid to product in the vessel chamber: Since the specific heat of the pressurizing fluid differs from that of the food products, the ratio of the food products to the pressurizing fluid must be kept the same to obtain repeatable results.

Package integrity (materials and seals): Prior to loading into the high-pressure vessel, the package must be checked to ensure that there is hermetic sealing.

***Depressurization time*:** The depressurization time must be the same for reproducibility in the process. Like the pressurization time, it will affect the loss of heat through the sidewalls of the vessel and can affect the end temperature, especially on the second pulse. (Meyer et al., 2000).

Applications of high pressure in food industry

Effect on microbial inactivation

Gram-negative bacteria are inactivated to a greater extent at a lower pressure than Gram-positive bacteria. The lower resistance of Gram-negative bacteria has been attributed to their lack of teichoic acid, which is responsible for the rigidity for the cell wall of Gram-positive bacteria (Elaamadi et al., 1996). This fact explains why yeasts and molds are the most sensitive to pressure.

Bacterial spores are generally the most resistant to inimical processes. This has been attributed to protection afforded by dipicolinic acid of the spore proteins against solvation and excessive ionization, which 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 (Clouston and Wills, 1969; Gould and Sale, 1970). Germination can be markedly increased to 95-99% when spores are treated in the presence of L-alanine (Gould and Sale 1970). Repeated cycling between high and low pressures has been recommended to eliminate spores (Mozhaev et al., 1994). The increase in temperature to 70°C may have weakened the physical strength of the spore coat and increased its susceptibility to rupture when the high pressure in the cell was suddenly reduced to zero during pulse pressurization.

ZoBell (1997) found that bacteria are more resistant in the stationary phase than in the early log phase of growth. Considerable variation in pressure resistance within strains of the same species has been demonstrated in both gram-positive and gram- negative bacteria.

Mild pressure treatment (300-600 MPa) at ambient temperature was widely believed to sufficiently inactivate vegetative bacteria for the purpose of food pasteurization.

However, this view has been challenged recently by a number of findings. The efficiency of cell destruction is 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, sorbic and benzoic acids, and other additives enhance the destructive effect of pressure on micro-organisms, this permitting lower pressures, lower temperatures or shorter application times to be used to achieve safe and high quality products (Mozhaev et al., 1994).

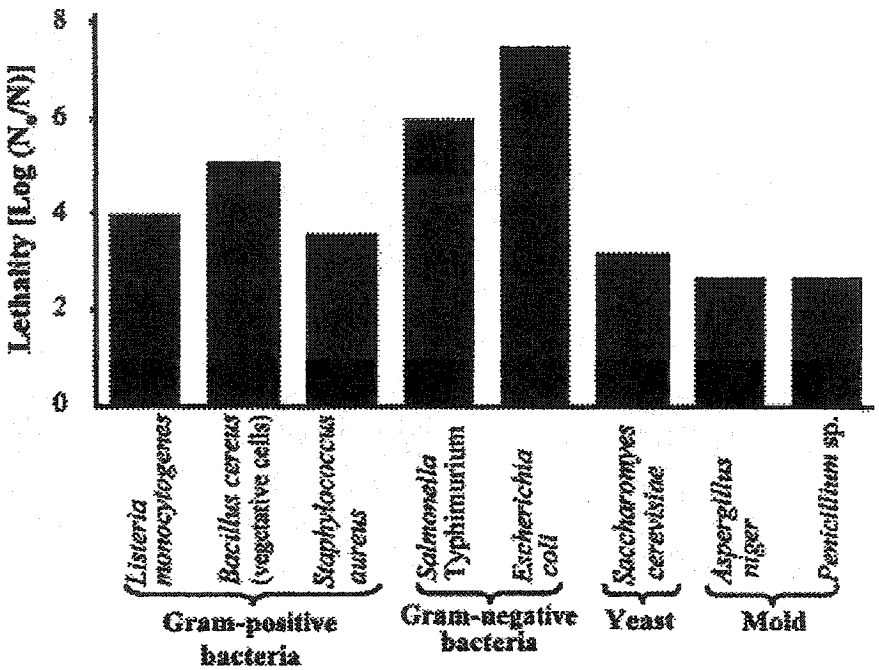

Figure 2.2. Lethality of microorganisms after high-pressure processing at 300 MPa (5°C) for 30 min (Adapted from Arroyo et al., 1999).

Inactivation Mechanisms

Pressure treatment at high temperature has been found to be effective in killing the heat-tolerant bacterial spores. The mechanism of inactivation of microorganisms by high pressure is mainly attributed to changes in the membrane structure and functionality. The changes almost always includes:

Perturbation of the cell membrane and loss of cell membrane function (Smelt, 1998). Cell membranes are destroyed via irreversible changes to the structure of the

membrane macromolecules, particularly proteins (Chong and Cossins, 1983). The nucleic acids and ribosomes involved in the synthesis of proteins are disrupted (Landar, 1967)

An increase in extracellular ATP (Smelt et al., 1994) and increased uptake of propidium iodide and ethidium bromide (Benito et al., 1999). High pressure also inhibits or inactivates essential enzyme systems in bacterial cells. In particular, membrane-bound ATPase may be denatured or displaced so that active transport can no longer take place. This would inhibit the efflux of protons and the cell would die due to acidification (Cheftel, 1995).

1. Morphological changes in microbial cells, the homogeneity of the intermediate layer between the cell wall and the cytoplasmic membrane is disrupted such as compression of gas vacuoles, cell lengthening, separation of the cell membrane from the cell wall, formation of pores in the cell wall (Cheftel, 1995) and the destruction of ribosomes, which would lead to widespread impairment of cell functions (Earnshaw et al., 1995). High-pressure inactivation is thought to be the result of a combination of these factors and not due to any single process.

2. Microbial growth is retarded at pressures in the range of 20-180 MPa; these pressures also inhibit protein synthesis (Figure 2.3) (Hoover, 1989; Hauben et al., 1997; Hauben et al., 1996). Microorganisms are more likely stressed or injured than killed in foods processed by alternative preservation technologies. Adaptation of microorganisms to stress during processing constitutes a potential hazard. Sub-lethal stress induces the expression of cell repair systems (Figrue 2.4) (Beatrice et al., 2002).

Kinetics of microbial inactivation

The destruction of microorganisms is generally described by a first order model:

$$\text{Ln } (N/No) = - kt \qquad (2.3)$$

where k = constant reaction rate; N = number of surviving microorganisms after pressure treatment for time t and No = initial number of microorganisms

The decimal reduction time (*D*-value) corresponds to the treatment time required to reduce the microbial population by 90% at constant treatment intensity. The *D*-value is calculated from the following equation:

$$D = (t_2-t_1) / (\log N_1 / N_2) \quad (2.4)$$

with N_1 and corresponding to the viable counts after treatment times t_1 and t_2, respectively. The reaction rate k is inversely related to D:

$$k = 2.303/D \quad (2.5)$$

The pressure dependence of k is related to the pressure by Arrhenius type reaction:

$$\ln k = \ln k_o - (\Delta V * P/RT) \quad (2.6)$$

$$\Delta V = -RT \text{ (slope)} \quad (2.7)$$

where ΔV is the activation volume in (m^3 $mole^{-1}$), P is the pressure in MPa, k is the rate constant (min^{-1}), T is the absolute temperature (K), R is the gas constant ($8.314*10^{-6}$ m^3 $mole^{-1}$ MPa $^oK^{-1}$) (Erying and Magee, 1942).

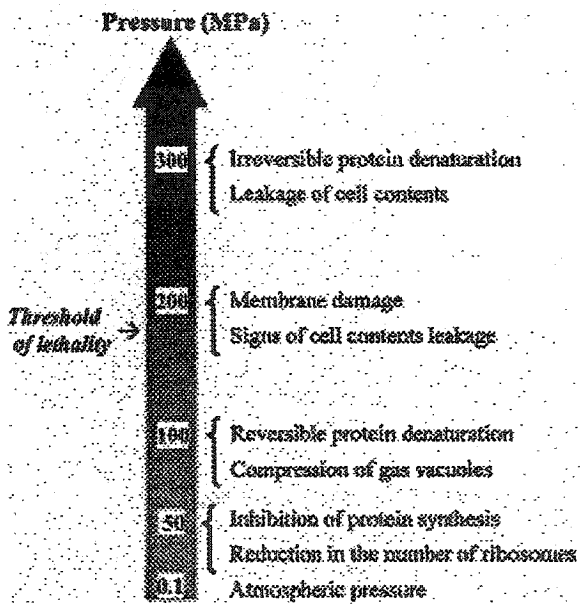

Figure 2.3. Structural and functional changes in microorganisms at different pressures.

Two parameters (i.e. N_D and PE) are derived to compare the pressure pulse with pressure hold results. N_D describes the pressure pulse effect while the D value indicates a measure of the pressure hold effect N_D can be established in terms of the number of pressure pulses required to result in one decimal reduction in microbial population, and

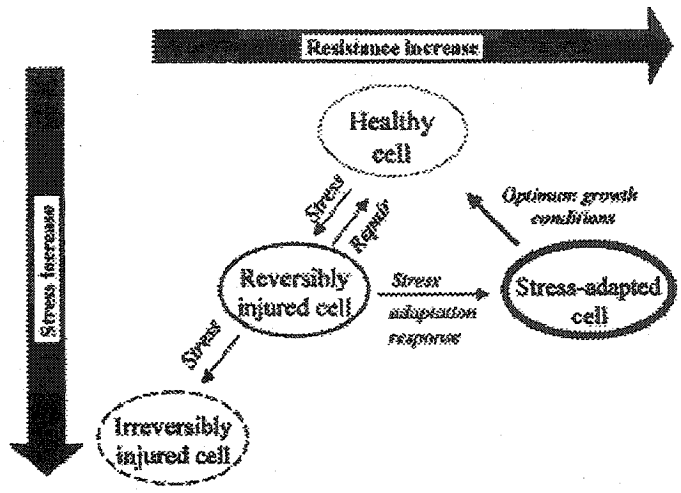

Figure 2.4. Microbial stress, injury, adaptation and resistance to processing.

it generally obtained by a negative reciprocal of slope of the log (No/N) vs. pulse number curve. Since PE represents a logarithmic reduction in microbial population due to a single pulse, it can be obtained by PE = 1/ N_D. Alternatively, the D_{IPK} value equivalence as minutes of decimal reduction achieved by one pressure pulse, which is simply the use of N_D times min which is needed to operate one pulse (Pandey et al., 2002). The time difference between pulse manner and hold manner to achieve one D destruction can be calculated by (D_P - D).

Examples of applications of High pressure in food industry

Today three major potential applications for high pressure in the food industry are recognized – in preservation: killing bacteria, reducing enzyme activities, etc. modification: changing composition, functionality, etc. Phase transition: freezing, thawing and sub-zero storage without freezing. The following are more application examples:

Pressure-assisted dehydration / rehydration processes

Pressure - assisted frying processes

Pressure-assisted extraction processes

Bio-conversion processes

Preservation processes

Gelling of protein and polysaccharides

Reduction / removal of anti-nutritional factors

Plant tissue texture retention/enhancement

Pressure shift freezing

Pressure thawing

Food preservation technology and milk processing

The trend of preservation techniques is in line with meeting consumer needs. These needs are: more natural food, less extremely preserved (e.g. less salt, less sugar, additive-free, less severely heated), more convenient and high confidence in microbiological safety (Knorr, 1999).

Preservation techniques can influence the growth and survival of microorganisms through physical, chemical, enzymic and microbial reactions. The various forms of spoilage and food poisoning caused by microorganisms are preventable by a number of traditional preservation techniques. Most of them prevent or slow microbial growth. These include: freezing, chilling, drying and it acts through lowering water activities, hence affect the microorganism growth and some of the physical, chemical and enzymic changes that lead to deterioration. Foods have also been preserved by curing, conserving, vacuum packing, and modified atmosphere packing, acidifying, fermenting, and adding preservatives. Other techniques such as aseptic processing and packaging restrict access of microorganisms to food products.

Only a few techniques such as heating (pasteurization and sterilization) (also named thermal processing) can inactivate the microorganisms. New and emerging preservation techniques can inactivate microorganism. They include the application of ionizing radiation, high hydrostatic pressure, high voltage electric discharge, high intensive light, ultrasonication in combination with heat or slightly raised pressure, addition of bacteriolytic enzymes, bacteriocins, and other naturally-occurring antimicrobials.

Safety concerns about milk

According to a report of the World Health Organization, hundreds of millions of people worldwide suffer from diseases caused by contaminated food. Recent surveillance data from the US Centers for Disease Control and Prevention (CDC) suggest that food-borne diseases cause approximately 76 million illnesses, 325,000 hospitalizations, and 5000 deaths each year in the US alone (Mead et al., 1999)

Satisfactory evaluation of a new preservation technology depends on reliable estimation of its efficacy against pathogenic and spoilage food-borne microorganisms. Raw milk or other dairy products made from it have been the source of transfer of most food-borne diseases.

Other potential food poisoning organisms include *Listeria monocytogenes*, causing listeriosis, *Yersinia enterocolitica*, *Staphylococcus aureus* and *Escherichia coli*. The fact that *L.monocytogenes*, can grow at refrigeration temperatures, is of particular concern in chilled foods, such as dairy products and in cooked meat. More recently, concern has been expressed about certain types of *E. coli* and awareness of the Vero toxin producing strains, such as *E. coli* O 157:H7 in particular, is increasing. High-pressure treatment has the potential to improve the microbiological safety and quality of certain foods, including meat, milk and their products.

Common microorganisms in milk

Milk is a suitable culture medium for many microorganisms. Microorganisms are undesirable in milk because they can be pathogenic or non-pathogenic but produce enzymes that cause undesirable transformations in the milk.

Pathogenic microorganisms that enter milk can be pathogenic for humans or animals. Human pathogens are usually classified into those causing food infection and those causing food poisoning. Food infection implies that the food, e.g., milk, acts as a carrier for the microorganism, which enters the human body through milk. So a person can become ill, often not until a day or so after drinking the milk. In food poisoning the microorganism forms a toxin in the food (or such a toxin contaminates the food by another route). The consumer rapidly falls ill. Large numbers of the pathogenic microorganism are

usually needed to cause food poisoning, unlike food infection; food poisoning does not imply that the pathogenic organism is still in the food. Some toxins are more heat-resistant than the toxin-producing microorganism itself.

Non-pathogenic microorganisms by themselves would not impair milk quality. It is that the organisms require nutrients, which are obtained by producing enzymes that hydrolyze lactose, protein, fat, or other substances in the milk, in order to yield compounds suitable for their growth.

These conversions cause the milk to develop off-flavors and to be less suitable for processing into retail milk and milk products, because of a decreased heat stability of the milk. Furthermore, most heating processes applied in dairy processing do not destroy all microorganisms or all microbial enzymes.

Coliform bacterica: Coliforms belong to the *Enterobacteriaceae* and are widespread in the digestive tract. They include *Escherichia coli* and *Aurobacter aerogenes.* They grow rapidly in milk, especially above 20° C, and attack proteins and lactose, as a result, gas is formed and flavor of the milk becomes "unclean". Some of the *E. coli* strains are pathogenic for humans. Low pasteurization kills the coliforms to virtually the same extent as *Mycobacterium tuberculosis.* This, as well as the fact that the organisms occur widely, has led to their use

as indicator organisms. If coliforms are absent, the heated product has been heated sufficiently and has most likely not been recontaminated, and so pathogenic microorganisms, apart from heat-resistant ones, will most likely be absent. The common sources of coliform bacteria are: feces, milking utensils, contaminated water, growth in raw milk, pathogenicity: mastitis, intestinal disorder, spoiled milk and cheese.

***Escherichia coli* 405 CECT:** It is considered a good index of indirect contamination of fecal origin.

***Pseudomonas fluorescens* 378 CECT:** It is an indicator of *Pseudomonas spp.*, major components of the spoilage flora of refrigerated milk.

Listeria innocua (910 CECT) vegetative types of bacteria, non-pathogenic has become a favorite surrogate for the food borne pathogen, and human-pathogen *L. monocytogenes.*

***Staphylococcus aureus* 534 CECT** is a major component of the spoilage flora of mastitis milks.

***Lactobacillus helveticus* 414 CECT** is a microorganism non-pathogen but representative of lactic flora.

Listeria monocytogenes is an important causal agent of food borne diseases. This pathogenic bacterium is a non-spore forming facultative anaerobic hard gram-positive rod and is a psychotropic microorganism that is commonly found in a range of raw foods. Both animal and plant derivatives also can grow and develop in milk and milk products. So, this results in a risk for consumer's health. It proved reasonably resistant to HPP Hence, *L monocytogenes* is a logical test organism for HPP validation.

***Escherichia coli* O 157:H7** is a growing concern to the food industry as it can cause severe symptoms and may be fatal, particularly in young and the elderly (Keyle and Cliver, 1990). Patterson and coworkers have examined a initial isolate of *E. coli* O 157: H: 7 that can endure exposures to HPP almost equivalent to that for spores of Bacillus and Clostridium. A nonpathogenic strain of bacillus may be useful, since spore suspensions are more easily stored and contained than vegetative bacteria.

Thermal processing milk and its limitations

Thermal processing technique has been used for milk preservation for a over a century. It has been used for sterilization of milk that is low in acid and high in water activities. Pasteurization of milk is required to eliminate public health concerns about bacteria such as *Salmonella, Listeria* or the relatively heat-sensitive spores of non-proteolytic strains of *C. botulinum* from chill-stored foods. Heat gives milk a prolong shelf life with assurance of microbial safety, but sacrificed the nature flavor, texture and some nutrients in the milk.

Non-thermal techniques are being employed already or being researched at the laboratory or pilot plant scale. They are less extremely preserved and allow preservation to

be achieved without need or use of high levels of heat which are necessary when using heat alone (Lopez et al., 1994).

Applications of high-pressure processing in milk

Effect of high pressure on milk constituents

Despite the inhibition and destruction of microorganisms, HP influences the physicochemical and microbiological properties of milk. HPP largely affects ionic and hydrophobic bonds, thus pressure mostly affects the higher orders of structures in large molecules such as proteins, polysaccharides and complex molecules. Consequently, enzymes and carbohydrates in an aqueous environment undergo reversible or irreversible conformational changes, resulting in denaturation, dissociations, aggregations or gelation (Heremans, 1982; Balny and Masson 1993; Kunugi 1993).

Milk proteins

The effects of high hydrostatic pressure on milk proteins have been investigated by several authors. At 230 MPa, casein micelles undergo irreversible changes and are reduced in size and this causes a decrease in the turbidity and whiteness, and an increase in the viscosity of milk (Hinrichs et al., 1996). Whey proteins can undergo partial, but fully reversible, unfolding of their native molecular structures under suitable pressures (100-300 mPa). Nakai and Li-Chan (1988) concluded the changes to the conformational structure of the proteins indicate increased exposure of hydrophobic groups, which may also alter the functional properties of the system. Hence, the forming, emulsifying, gelling and water binding capacities of the proteins may be influenced. This could lead to the development of a range of functional food ingredients prepared form milk proteins by controlled unfolding of their structure.

Milk enzymes

Several authors have investigated the effectiveness of HPP for inactivation of indigenous enzymes in milk. It is of interest due to their possible use as markers of severity of treatment, analogous to the use of alkaline phosphatase as an index of pasteurization of milk. Mussa and Ramaswamy (1997) suggested that a percentage destruction of alkaline phosphatase in UHP milk might have to be used as an indicator of bactericidal efficacy,

since complete destruction may not be feasible. As in the case in thermal processing, milk enzymes such as alkaline phosphatase can be used as an indicator of pasteurization. They found a Zp value of 368 MPa for alkaline phosphatase compared with 168 MPa for microorganisms. This is mainly due to the fact that milk enzymes are much less sensitive to pressure than most of microorganisms (Kolakowski et al., 2002). It has been shown that only alkaline phosphatase and protease completely lost their activity at 1000 MPa. HP treatment of milk at higher temperatures generally increases inactivation of alkaline phosphatase (Seyderhelm et al., 1996; Ludikhuyze et al., 2000), Indigenous milk lactoperoxidsase (Lopez-Fandino et al., 1996; Seyderhelm et al., 1996), phosphohexoseisomerase (Rademacher et al., 1998) and glutamyltransferase (Rademeacher et al., 1998) are also resistant to pressures up to 400 MPa at 20-25°C. The relatively high stability of these enzymes makes them unsuitable for use as markers for the severity of HP treatment of milk.

Milk fat

High-pressure treatment at 100-400 MPa, induces crystallization of milk fat in cream, the effect being greatest at 200 MPa (Buchheim et al, 1992; Buchheim et al, 1996b). The induction or acceleration of crystallization of milk fat by HP is probably due to the shift in the phase transition temperature under pressure. At up to 200 MPa, the crystallization and melting temperatures of milk fat are increased by 16.3 C and 15.5 C /100 MPa, respectively (Frede and Buchheim, 2000). The lower extent of milk fat crystallization at higher pressures (> 350 MPa) may be due to reduced crystal growth because of reduced molecular mobility at higher pressure (Buchheim et al., 1996a, b). Thus high-pressure treatment reduces the aging time of ice-cream mixes and enhances the physical repining of cream for making butter (Bouchheim and El-Nour 1992; Buchheim et al. 1996).

Pressures at 400 MPa did not affect the milk fat globule membrane diameter or milk fat globules size distribution (Kanno et al., 1998), this provides an advantage of the pressure treatment of milk, because fat globules would not be destroyed (Kanno et al., 1998). But higher pressures (400-800 MPa) increased the diameter of milk fat globule membranes, and broadened the milk fat globules size distribution.

Milk lactose

Recent studies have examined the effects of HP on milk fat (Huppertz et al. 2002). It is thought that HP treatment of milk may affect the Maillard reaction or the mutarotation equilibrium of lactose. Most research has focused on the effects of HP on skim milk, thus effects of HP on milk fat the and on other constituents and the properties of milk is another aspect of interest.

Effect of high pressure processing on microorganisms in milk

Research into the application of HP processing for milk preservation began with Hite (1899), interest in high-pressure applications on milk and dairy products has increased recently. In addition to microbial destruction, it has been reported that HP improves rennet or acid coagulation of milk without detrimental effects on important quality characteristics, such as taste, flavor, vitamins and nutrients (Trujillo, 2002).

Many studies on the inactivation of pathogenic and spoilage microorganisms (naturally present or introduced) by HP have been performed in milk during these years and have generally demonstrated that it is possible to obtain "raw" milk pressurized at 400-600 MPa with microbiological quality comparable to that of pasteurized (72°C, 15 s) milk depending on microbiological quality of milk (Buffa et al 2001; Kolakowski et al 1997; Mussa and Ramaswamy 1997), but not sterilized milk due to HP resistant spores. A number of researchers have investigated the combined efficacy of HP in combination with mild temperatures (30-50°C) and / or with bacteriocins ('lysozyme", nisin, pediocin, lacticin) for the inhibition of food-borne bacteria and spores. Meyer (2000) reported that using pulsed high pressure in conjunction with heat can sterilize in low-acid foods. This study demonstrated that this type of combination treatment enhances the efficiency of HP treatment (Farcia-Risco et al., 1999; Morgan et al., 2000).

While a reasonable shelf life of milk nay be obtained with pressure treatments of 400 MPa or 500 MPa, it must be noted that some strains of the pathogenic bacteria *Listeria monocytogenes, Staphylococcus aureus* and *E. coli* 0157:H7 are quite pressure resistant and may not be sufficiently inactivated. *E. coli* growth was only reduced by 2 log cycles at 600 MPa for 30 min (Rademacher et al., 1997). Some mutant strains of *E. coli* have been shown to be particularly barotolerant (Hauben et al., 1997) which has significant implications for

the use of high pressure for treatment of milk and other foods. The effect of pressure on the milk indicator organism, *Mycobacterium tuberculosis*, has not yet been reported (Rademacher et al., 1997).

High pressure processing affected by milk composition

The effect of ovine milk composition on HPP inactivation of microorganisms was studied by Gervilla (1998). They used milk with fat content ranging from 0-50% fat content, *Escherichia coli, Pseudomonas fluorescence, Listeria innocua, Staphylococcus aureus*, and *Lactobacillus helveticus*. It was conclude that ovine milk showed a baroprotective effect on all microorganism but did not show a progressive baroprotective effect. Kinetics of destruction of *Escherichia coli* and *Pseudomonas fluorescence* inoculated in ewe' milk by HPP is studied. One successful study about pathogens inactivation, which uses the combined effect of HHP and mild heat or antimicrobial peptides. It shows that for *E, coli o157:H7*, a 15min treatment of 400 MPa at 50°C resulted in approximately a 5 log reduction in milk, while a smaller (1 log) reduction was achieved with either treatment alone.

Styles et al., (1991) studied HPP inactivation of *L.monocytogenes* at 23°C in different media. Greater inactivation occurred in raw milk than UHT milk.

Lopez-Caballero et al., (2002), determined that microbial reduction due to pressure treatments was higher in ground pork patties than in sliced cooked ham.

High pressure processing research on dairy product at McGill

Several milk research activities are under way at McGill University. The food science group, the earliest to study HPP in Canada, has carried out a lot of research on HPP on vegetables, seafood, meat, fruit juices, milk and cheeses. Some work relevant to the topic of this research are: Mussa and Ramasway (1998) on HPP in milk. Pandey (2002) on the effect of HP treatment of milk on cheese process; Shao (2003) on the effect of HP inactivation of microorganism in raw milk cheese.

Limitations of high-pressure technology

Like any other process, high-pressure processing also has certain disadvantages:

- Feed enzymes and bacterial spores are very resistant to pressure and require very high pressure for their inactivation.
- The residual enzyme activity and dissolved oxygen results in enzymatic and oxidative degradation of food components.
- Most of pressure-processed foods need low temperature storage and distribution to retain their sensory qualities.
- Changes in product color and appearance may limit the usefulness of HPP treatment pressures above 200-300 MPa.

Ongoing research on the effects of high pressure on food is expected to help optimize high-pressure processing and overcome some of the disadvantages associated with its application. A summary of limitations is provided in Table 2.2.

Future prospects of high pressure technology in food and dairy industry

Nowadays, the technology and associated processing equipment have been developed to the stage where it is feasible to establish milk processing plants. There has been very limited commercial use of high-pressure treatment of milk or milk products (Datta and Deeth, 1999). The difficulty of destroying bacterial spores currently limits the bactericidal applications of high pressure to those presently covered by pasteurization. However, the combined use of pulsed-pressure treatment and temperature shows considerable promise for eliminating spores, as well as vegetative cells (Datta and Deeth, 1999). With the combination of other technologies, high pressure may be used to produce pressure-sterilized milk with a fresh milk taste, but not like UHT milk which has its distinctive heated flavor.

As with all alternative technologies, there will need to be very sound reasons for the dairy industry to change form the universally accepted heat treatments to high-pressure treatments. Since this technology is more costly than the traditional heat technologies (up to 20 times for equivalent capacity systems (Manvel, 1997), high-pressure technology will need to offer other substantial advantages.

Table 2.2 Limitation of high pressure processing

Treatment	Limitations
Membrane permeabilization	Stress reaction (plants, microorganisms), texture effects
Residual enzyme activity	Quality effects
Incomplete microbial inactivation	Safety and quality effects
Reaction enhancement	Quality effects (i.e., enzymatic browning)
Temperature effects	Adiabatic heating , heat of fusion
Volume effects	Compression of water

Finally, high pressure has potential for a wide range of food and biotechnological applications, especially, with regards to membrane related effects, sub-lethal stress induced biosynthetic effects on plants and microorganisms, and a tremendous potential for physical or physico-chemical modification as a result of temperature-pressure interactions (e.g. structure engineering) (Knorr, 1999). High pressure can be used in new type of product development or products with superior quality, unachievable by other technologies, also the development of the future technologies based on HPP, such as pressure-assisted freezing, storing or thawing of sensitive biological materials (i.e. biological tissues or organs) can afford a competitive advantage for high pressure technology (Buchheim, 1998).

CHAPTER 3

COMPOSITION EFFECTS ON THE HIGH PRESSURE DESTRUCTION OF *ESCHERICHIA COLI* IN MILK

Abstract

High pressure (HP) destruction of non-pathogenic *Escherichia coli* (K-12) in milk as affected by composition was investigated in this study. *E. coli* culture was suspended in buffer (pH 7.0) and 1% peptone water and in various commercial milk samples - skim milk, homogenized milk (3.25% fat), cream (5% fat), homogenized milk supplemented with 1-4% casein and lactose, filled in to small plastic bags, heat sealed and subjected to various high pressure treatments (100-400 MPa, 0-30 min) at room temperature. The HP treatment resulted in highest destruction of *E. coli* K12 in buffer solution, followed by peptone water and then milk. Samples treated for 5 min at HP 300 MPa at 20°C showed approximately 3.7 log reduction in CFU/mL in buffer solution, 2 log reductions in peptone water, around 0.8 log reductions in pasteurized milk. It is apparent from this study that milk has baroprotective effect on *E. coli* destruction. No significant effect ($p>0.05$) on destruction of *E. coli* K12 was observed between milk with different fat content or milk and milk samples supplemented with casein and lactose.

However, when casein and lactose were added to buffer, they resulted in a significant ($p<0.05$) baro-protective effect on the survival kinetics. Casein (1%) or lactose (2-3%) added to buffer produced the same protective effect as milk; adding beyond these threshold levels did not enhance the baro-protection. Casein appears to offer more protection than lactose. Since casein and lactose are present in milk in concentrations beyond these threshold levels, further addition of the components did not show any significant effect. The results confirm the baro-protective role of milk in HP destruction of *E. coli* in milk and the active components which contribution to baroprotective effect of milk are casein and lactose.

Introduction

There is an ever increasing growing consumer demand for minimally processed, additive-free and pathogen-free foods with high nutritional and sensory qualities. This has stimulated the food industry to explore new processing methods to address public concerns and preserve the nutrients. New process techniques such as high pressure (HP) processing and application of pulsed electric or magnetic fields, pulsed light and electron-beam irradiation are receiving much attention in recent years. High pressure processing is a physical process that applies high hydrostatic pressure (100 - 1000 MPa) to achieve microbial destruction without affecting the natural flavor and sensory characteristics of foods. Liquid milk processing is one of the largest food industries in Canada and represents one of the most nutritious products on the market. Pasteurization of milk for the destruction of pathogenic microorganisms and reduction of the natural micro-flora has been traditionally carried out by heat treatment. It has been recognized that heat has an undesirable effect on the wholesomeness of milk, for example, loss in flavor and vitamins, browning of milk and fouling of the heat transfer surface.

Hite pioneered and postulated the concept of HP preservation of liquid foods in 1899; his investigation was based on milk, fruits and vegetable products. Since then, a number of studies have been conducted on HP destruction of microorganisms in liquid milk (Mussa and Ramaswamy, 1996; Gervilla et al., 1997; Patterson and Kilpatrick, 1997; Garcia-Graells et al., 2000; Linton et al., 2001). The effectiveness of the destruction was studied in buffer solutions (Styles et al., 1991; Patterson et al., 1995). Phosphate buffer solution was used to evaluate efficacy of high hydrostatic pressure on destruction of microbial cells of several food-borne pathogen (Metrick et al., 1989; Shigehisa et al., 1991; Styles et al., 1991; Patterson et al. 1995). It has also been shown that the magnitude of cell destruction by pressure was more effective in phosphate buffers than in food systems (Carlex et al., 1993; Raffalli et al., 1994; Patterson et al., 1995; Garcia-Graells et al., 1999). Basak and Ramaswamy (2001) studied the effect of destruction kinetics of spoilage microorganisms in single strength and concentrated orange juice. They showed that the HP destruction of microorganisms depended on the concentration of sugars in the juice with

sugar contributing to baro-protection. Milk likewise has potential to exert a baro-protective effect on HP destruction of microorganisms, due to lactose, casein and fat components.

A number of factors, apart from temperature, magnitude and duration of pressure treatment (which are the most commonly studied variables), are known to affect the resistance of bacteria under high pressure during treatment. The phase of growth of the bacteria is important in determining its resistance to pressure, with the cells in the stationary phase being usually more resistant. The composition of the suspending medium affects the sensitivity of bacteria to pressure. Thus, many food constituents appear to have baro-protective effects on HP destruction of microorganisms (Linton et al., 2001). The presence of lipids, carbohydrates and proteins coupled with reduced water activity was shown to have enhanced resistance to HP (Simpson and Gilmour, 1997). This suggests that the pressure resistance of bacteria varies among foods. Therefore, it is important to validate processing parameters in real foods rather than extrapolating results from buffers and laboratory media (Smelt, 1998). It is still not clear as to why and how added substances can enhance or weaken the pressure destruction of microorganisms. This is mainly due to the fact that, limited data of physical and chemical properties e.g., solubility, conductivity, viscosity, freezing point etc., of added substances under high pressure are available, which would affect the high pressure inactivation of microorganisms (Hahsizume et al., 1995). Gervilla et al. (1999) and Gervilla (2000) in their studies on ovine milk with adjusted fat content (0, 6, 50%) inoculated with various microorganisms reported that ovine milk with all fat levels showed baro-protective effect on all microorganisms, but milk with fat content (6 and 50%) showed no progressive baro-protective effect under all pressurization conditions for all microorganisms tested.

Escherichia coli belongs to the family *Enterobacteriaceae*. This enteric bacterium, which is gram-negative, rod shaped and facultative anaerobc bacteria lives in the intestinal tracts of animals (Koodie *et al.*, 2001; Toder, 2002). The pathogenic *E. coli* (O157:H7) infections have traditionally been associated with animal products, but outbreaks associated with dairy product have been reported with increasing frequency. Outbreaks of *E. coli* have been associated with several foods including ground beef, raw milk and contaminated water (Neil, 1989; Padhye and Doyle, 1992; Rice *et al.*, 1992). Because of its typical habitat, *E. coli* is considered to be a good index of direct or indirect contamination from fecal origin.

E. coli is a key concern in the development of effective high-pressure treatment (FDA, 2000).

These studies, in general, indicate that HP processing is a reliable alternative technology that could be applied to milk preservation. Several products prepared from milk have added or altered concentration levels of the normal milk constituents. Very limited information is available on destruction kinetics of microorganisms in liquid milk as affected by its composition. The concentration of casein and lactose are important parameters that could influence the HP destruction kinetics, and yet no detailed studies have been focused on it. Relatively little is known about the pressure destruction kinetics of *E. coli* in pasteurized cow's milk as affected by milk components, which is the principal objective of this study.

Materials and Methods

Preparation of culture and inoculation of test samples

A freeze-dried culture of *Escherichia coli* K12 (ATCC-29055) was obtained from the American Type Cultural Collection (ATTC, Rockville, MD) and was supplied in vials and was stored at –80°C until use. The freeze dried culture was rehydrated in 10 mL of brain heart infusion (BHI) broth (Difco laboratories Inc., Detroit, MI) at 37°C for 24 h. Subsequently, few loops of cultured broth were inoculated into 50 mL of fresh BHI broth and incubated at 37°C for 24 h. Three such transfers were made in succession to obtain the stock culture with viable counts of 10^8- 10^9 CFU/mL. These broth cultures were used to prepare slant cultures on the BHI agar (Brain Heart Infusion; Difco 237500 Detroit, MI), after incubation for 24 h at 37°C, they were stored at 4°C in a refrigerator. The slants were maintained at 4°C and transferred monthly to provide fresh stock cultures. In each experiment, one loop from a tube of stock culture was suspended in 50 mL BHI broth and incubated at 37°C for 24 h to obtain a population of approximately 10^8 - 10^9 CFU/mL.

Pasteurized skim milk, whole homogenized milk with 3.25% fat content, and 5% cream were obtained from a local supermarket, and were aseptically transferred to stomacher bags. One mL of microbial suspension was inoculated to 99 mL of milk and

mixed. The initial concentration for *E. coli* was thus reduced approximately to 10^6 - 10^7 CFU/mL. The inoculated samples were then transferred aseptically into sterile polyethylene pouches (Dual Peel Sterilization sachet-Baxter Corp., Mississauga, ON), each pouch containing about 10 mL of test sample. The bags were heat-sealed after expelling air, and placed and sealed in another bag. Samples without HP treatment (controls) were prepared by the same method. The prepared pouch samples were kept immersed in an ice bath to prevent microbial growth during the time interval between preparation and pressure treatment (about 2 h maximum). They were then pressure treated in duplicates at selected pressure levels and holding times. Each experiment was conducted in triplicate. The study was carried out in several parts to evaluate the influence of compositional factors on the microbial resistance to HP destruction.

Buffer and milk composition

The phosphate buffer (pH 7.0) used in the study was composed of Na_2HPO_4 (0.2 mol/L) and NaH_2PO_4 (0.2 mol/L). These salts served as a base composition for non-nutritional material. Peptone water (0.1g) was dissolved in 100 mL distilled water and used to formulate the nutrient medium. They both were sterilized before use. Then appropriate amounts of these solutions were placed in a sterile stomacher bag, and casein and lactose were added at 1%, 2% or 4%, 8% levels. In some studies, pasteurized homogenized milk was also enriched with casein and lactose in the same manner. After thorough mixing, these samples placed (individually) in sterile bottles and pasteurized at 110°C for 1 min in an autoclave (J.P. Selecta S.A., Abrera, Spain). The bottles were cooled to room temperature and inoculated by the same procedure as outlined above.

High hydrostatic pressure treatment

High-pressure treatments were conducted at room temperature (~25°C) in an isostatic press (Model CIP 42260, ABB Autoclave System, Columbus, OH) in a cylindrical pressure chamber (10 cm diameter and 56 cm high). The maximum pressure level attainable was 414 MPa. The pressurization medium was distilled water mixed with 2% mineral oil (Autoclave Engineers, Part No. 5019, Columbus, OH). The pressure come-up-time was dependent on the pressure level and ranged from 45 s at 100 MPa to 180 s at 400 MPa, and the depressurization time was less than 15s. The pressure come-up-time and

depressurization were not included in the pressure hold-time because of the relatively smaller come-up-time relative to the holding times. A pressure pulse was defined as pressure treatment without any holding time; in other words, the sample was only subjected to pressure-come-up and depressurization. The processing temperature depended on each experimental condition and was maintained with circulation of water around the pressure chamber. The temperature of the pressurizing medium and sample increase during the pressure treatment due to adiabatic heating (Zimmerman and Bergman, 1993). Therefore, the temperature of pressurizing medium and sample were kept at lower initial temperature than desired, to cater for the temperature increment due to pressurization. Temperature of the pressurization medium was monitored by a thermocouple attached to a data-logger (HP-34970A, Hewlett Packard, Loveland Co.) during the experiment. The sample temperature was kept below 30°C at all times well below the point at which thermal destruction of microorganisms might occur (Basak and Ramaswamy, 2001).

Samples in sealed test pouches were equilibrated to the desired temperature and submerged in HP medium inside the HP-vessel. Cold water below the desired temperature was circulated through the jacket during the entire duration of the experimental runs. Each experiment was conducted in triplicate and the results were averaged for each sample. The pressure treated samples were immediately immersed in an ice water bath after treatment, and kept for 4 h to allow the pressurized cells to recover from pressure-stress.

Enumeration of survivors

After 4 h period of resuscitation, the samples were aseptically opened and serial dilutions (10^0-10^{-7}) were prepared in 0.1 % peptone water. In determining *E. coli* counts, 1-mL volumes of the diluted samples were pour-plated in duplicate on violet red bile agar (VRBA) (Difco Laboratories Inc., Detroit, MI) and incubated at 37°C for 24 h. The purple colonies with hollow centers of the same color were counted, and multiplied by the dilution factor to get the survivors in CFU/mL. The initial number of cells was based on the counts from the non-pressurized samples, which were used as control. Microbial destruction were expressed by log-cycle reductions (log N_o/N) in CFU/mL, where N_o is the initial number of cells and N is the number of cells after the HP treatment. The enumeration results were averaged from 4 measurements (2 samples / treatment and two plates/dilution).

Statistical Analysis

Each set of experiments was run in three replicates with duplicate samples. An analysis of variance (ANOVA) was performed and the mean comparison was conducted by using the Duncan Multiple Range Test (DMRT) and all evaluations were based at a level of significance ($P< 0.05$). The analysis was done with SAS system software (Version 8, 1999, SAS Institute Inc., Cary, NC, USA)

Results and discussion

Milk, peptone water and buffer as dispensing media

The pressure destruction data of *E. coli* K12 dispensed in milk, peptone water and buffer solution treated for 0 min and 5 min at 300 MPa and 200 MPa are presented in Figure 3.1 as log (N_0/N), i.e., logarithmic reduction in counts, vs time, and the significance of their differences by Duncan's test is shown in Table 3.1.

Table3.1 Influence of dispensing medium (milk, peptone water and buffer) on high-pressure destruction *E. coli*.

Samples	300 MPa 0 min	300 MPa. 5 min	200 MPa, 0 min	200 MPa 15 min
Milk	0.45a	1.05a	0.55a	1.18a
Peptone	1.55b	1.58b	1.08b	1.60b
Buffer	1.69b	3.38c	1.31b	1.96c
SEM	0.054	0.054	-0.102	-0.063

SEM = Standard error of the mean, numbers in a given column not sharing the same letter are significant differences ($p < 0.05$).

As can be expected the extent of *E. coli* destruction increased with pressure and treatment time in each medium. Mean logarithmic cycle reductions in *E. coli* in buffer and peptone water were not significantly different ($p>0.05$) either at both pressure when the treatment time was 0 min (a pressure pulse process), although they both were significantly higher than in milk. However, as the treatment time increased, much higher destruction was observed in buffer than in peptone water and milk. Overall, the mean log reduction of *E. coli* in milk, peptone water and buffer were significantly different ($p < 0.05$) which meant that they provided different pressure resistance to the destruction of *E. coli*. The extent of destruction of *E. coli* K12 at any given pressure level and treatment time was highest in buffer and lowest in milk. Thus, the order of baro-protection of the three media was: buffer^{a}<peptone water ^{b} <milk ^{c} ($p < 0.05$). Among the three media tested, buffer did not have any nutrients to support the growth of microorganisms. Peptone water had a small amount of nutrient that could support microbial growth while is almost a complete food providing all basic nutrients. More rapid death of *E. coli* was observed in buffer solution that contained no food ingredients. The range of destruction was 1.31-3.38 log cycle reductions in buffer, 1.08-1.60 log cycles in peptone water and 0.45-1.18 log cycle reductions in milk. The order of support for pressure resistance therefore appears to be in the increasing order of the presence of nutrients in the medium. Milk used as the dispensing medium was found to exhibit the highest protective effect against pressure inactivation of *E. coli* K12.

Patterson et al. (1995) investigated the effect of HP treatment (600 MPa; 20 °C; 5-30 min) on selected microorganisms in 10 mM phosphate-buffered saline solution at pH 7.0 and UHT milk, and found *E. coli* destruction to be more severe in buffer than in UHT milk which was confirmed in this study. Crawford (1996) and Yuste and Kalchayanand (1998) also reported similar observations. Milk is a nutrient rich carbohydrate, protein and fat containing medium and thus usually more protective than an aqueous buffer-medium (Garcia-Graells et al., 2000). The authors reported a 7.0 and 1.7 log-cycle reductions of *E. coli* population in buffer solution as compared with 2 and 0.5 log-cycles in skim milk subjected to high pressure 600 and 200 MPa, respectively, after a 15 min holding time. This compares well the results presented for milk in Table 3.1 for 200 MPa treatments. Gervilla et al. (1999) reported 5.5 and 3 log-reductions in ringer solution and skim milk,

respectively, at 300 MPa after a 15 min holding treatment at 25°C, somewhat higher than observed with the homogenized whole milk found in this study. Hauben et al. (1997) reported similar observations in their study of the variability of *E. coli* strains by HP treatments in potassium phosphate buffer (pH 7.0, 10 mM).

Takahashi (1992) used a phosphate (pH 7.0) to obtain a reduction of about 4 log units of *E. coli* population when treated at 200 MPa for 20 min at 20°C as compared with 2 log reductions in this study. At a higher temperature (40°C) and longer treatment times (10, 20 and 25 min) and in a sterile saline solution, Butz and Ludwig (1991) showed reductions of 4, 6 and $\geq$ 7.5 log units for *E. coli* population. There are likely strain to strain variations in pressure sensitivity in addition to those induced by medium (Garcia-Craells et al., 2000; Alpas et al., 1999; Simpson and Filmour, 1997; Isaacs et al., 1995; Styles et al., 1991). Garcia-Craells et al. (2000) reported 3 log units difference in inactivation between the most sensitive and the most resistant strains of both *E. coli* and *L. innocua.* Isaacs et al. (1995) studied survival curves for *E. coli* in whole milk at different pressures, temperatures and times. The reported that to obtain a 4-log unit destruction in a reasonable time (5-10 min), pressures above 400 MPa should be employed.

The different responses obtained when comparing similar pressurization of *E. coli* indicates that there could be diverse factors that influence the response of microorganisms to HP treatment. One of the factors is the substrate in which the microorganism finds itself when being pressurized. It is a well-known fact that certain constituents that exist in substrates may exert a baro-protective effect or vice versa (Knorr et al., 1992, Maggi et al., 1994). A second important factor is the level of baro-resistance between different species of the same genus and different strains of the same species (*E. coli*) as well as the conditions of growth and possible states of pre-pressurization. These factors should be considered when making recommendations to the industry and results of the same microorganism studied in different substrata or foods should not be extrapolated. (Gervilla et al., 1999)

Next to water, the major components of milk are fat, casein and lactose. Since milk offered baro-protection, the protection could be expected to come from one of these components. In the next set of experiments, the role of these components was examined.

Influence of fat on pressure destruction of *E. coli*

The pressure destruction curves for inoculated *E. coli* K12 in skim milk, milk containing 3.25% and 5% fat at selected pressure levels as a function of time are presented in Figure 3.2. The purpose of this experiment was not quantifying the destruction kinetics, but rather a comparison of the effect of fat content at different pressure time combinations and hence associated data are not presented in the traditional log-linear graphs. But the first- order rate of destruction is somewhat evident from the curves and an analysis of variance (ANOVA) revealed that pressure and holding times were statistically significant ($p< 0.001$) for the pressure destruction *E. coli* in milk.

It is apparent from the figure that relatively small differences existed in the extent of *E. coli* destruction (shown on a logarithmic scale) when milk at the three different fat content were compared as the choice of media for dispensing the microbial cells. This is true at any given pressure level and treatment time. For example, for samples treated at 400 MPa, 1.1, 1.4, 1.6 log-cycles destruction of *E.coli* was achieved in milk containing 0, 3.25 and 5% fat, respectively, with a zero min treatment time (a pressure pulse) while they increased to 4.4, 3.9 and 4.1 log-cycles with a pressure holding time of 4 min and to 4.9, 5.6 and 4.6 log-cycles after an 8 min treatment, respectively. The ANOVA results, as presented in Table 3.2, confirm the statistical insignificance ($p>0.05$) of the role of fat.

These results are partially in agreement with the results of Gervilla et al. (2000) who evaluated the baro-protective effect of fat (0, 6 and 50%) in ovine milk for different microorganisms (*E. coli, Pseudomonas fluorescens, Listeria innocua, Staphylococcus aureus*, and *Lactobacillus helveticus*). They reported that ovine milk of all fat contents demonstrated a baro-protective effect, but the fat content produced different results depending on the pressure, temperature and microorganism assayed. For example, it was reported that with *L. innocua* an increase in fat content resulted in a progressive protection against pressure inactivation. With *E. coli. S. aureus* and *L. helveticus*, baro-protection was observed in milk but there was no progressive protection between 6 and 50% fat content. On the contrary, high fat content (50%) was more lethal than intermediate 6% fat content of on the pressure destruction of *P. fluorescens*. Raso et al. (1998) also observed no protective effect of milk fat during high-pressure pasteurization of milk.

Table 3.2. The analysis of variance (ANOVA) of the effect of independent variables (fat, holding time) on high-pressure (200, 300, 400 MPa) destruction of *E. coli*.

a) 400 MPa (holding times; 0, 4 and 8 min)

Source	Degree of freedom	Sum of Square	Mean Square	F-value	Pr
Fat	2	0.1651	0.0825	0.64	0.551^{ns}
Time	2	45.2559	22.6279	174.7	0.001**
Fat x Time	4	1.6680	0.4170	3.22	0.067^{ns}
Error	9	1.1656	0.1295		
Total	17	48.2546			

** Highly significant at 1%; ns not significant at 5%.

b) 300 MPa (holding times; 0, 8 and 16 min)

Source	Degree of freedom	Sum of Square	Mean Square	F-value	Pr
Fat	2	0.3288	0.1644	3.04	0.098^{ns}
Time	2	21.1187	10.5593	195.20	0.0001**
Fat x Time	4	0.3059	0.0764	1.41	0.3050^{ns}
Error	9	0.4869	0.0541		
Total	17	22.2403			

** Highly significant at 1%; ns not significant at 5%.

c) 200 MPa (holding times; 0, 16 and 32 min)

Source	Degree of freedom	Sum of Square	Mean Square	F-value	Pr
Fat	2	0.2846	0.1423	1.79	0.2212
Time	2	9.0455	4.5227	56.97	0.0001**
Fat x Time	4	0.615	0.154	1.94	0.189^{ns}
Error	9	0.7145			
Total	17	10.6595			

* Significant at 5%; ** highly significant at 1%; ns not significant at 5%.

Since skim milk has no fat, these results also indicate that fat content in milk did not have any influence on the destruction of *E. coli* by pressure. The baro-protective effect of milk observed by researchers for pressure destruction of microorganisms therefore comes probably from other components of milk. Hence the next logical step was to evaluate the influence of casein and lactose.

Influence of casein added to milk on the pressure destruction of *E. coli*

The high-pressure destruction of *E. coli* K12 inoculated into milk with added casein (2 and 4%) as affected by selected pressure treatment (300 and 200 MPa; holding times 0-32 min) are shown in Figure 4.3. The respective analysis of variance (ANOVA) results are shown in Table 3.3.

The extent of pressure destruction of *E. coli* in milk with added casein above that that already existed was not statistically different ($p>0.05$) at any given pressure level or treatment time. Thus, while milk itself was found to be baro-protective as observed before (for example at 300 MPa pulse pressure treatment, microbial destruction in milk was about 0.4-1.0 log cycles as compared to nearly 2 log-cycle reduction in buffer), milk supplemented with casein (2 and 4%) did not result any increased protection level. For example, the reduction in log units were 1.0, 0.59, and 0.42 after a pressure pulse process, 1.9, 2.3, and 2.2 after an 8 min treatment and 4.24, 3.96, and 4.09 after 16 min treatment in milk and milk with 2 % and 4 % casein at 300 MPa, respectively. When milk is subjected to HPP, the casein micelles are irreversibly disintegrated into smaller particles (Trujillo, 2002). Kanno et al. (1998) reported that pressure intensity up to 400 MPa had no effect on size and size distribution of the milk fat globules, and did not damage the membrane envelope integrity. No studies have been carried out on the effects of protein and lactose in milk on HP destruction of *E. coli*, therefore it is difficult to make a direct comparison our results with published data. Moerman et al. (2002) reported that fat, protein and carbohydrate did not have any effect on HP destruction of *Bacillus stearothermophilus*.

Influence of lactose added to milk on the pressure destruction of *E. coli*

The high-pressure log (N_0/N) destruction of *E. coli* K12 inoculated into milk with added lactose (4 and 8%) as affected by selected pressure treatment (400, 300 and 200 MPa; holding times 0-32 min) are shown in Figure 3.4. The respective analysis of variance (ANOVA) results are shown in Table 3.4.

Table 3.3 The analysis of variance (ANOVA) of the effect of independent variables (casein, holding time) on high pressure (200, 300, 400 MPa) destruction of *E. coli*.

a) 300 MPa (holding times; 0, 8 and 16 min) added casein

Source	Degree of freedom	Sum of Square	Mean Square	F-value	Pr
Casein	2	0.0821	0.04105	0.27	0.7676^{ns}
Time	2	35.404	17.702	117.49	0.0001**
Casein x Time	4	0.5011	0.1253	0.83	0.5379^{ns}
Error	9	1.3561	0.1507		
Total	17	37.344			

** Highly significant at 1%; ns not significant at 5%.

b) 200 MPa (holding times; 0, 16 and 32 min) added casein

Source	Degree of freedom	Sum of Square	Mean Square	F-value	Pr
Casein	2	0.6360	0.3180	3.79	0.0641^{ns}
Time	2	32.5835	16.2917	193.96	0.0001**
Fat x Time	4	0.2593	0.0648	0.77	0.5701^{ns}
Error	9	0.7559	0.0839		
Total	17	34.2348			

* Significant at 5%; ** Highly significant at 1%; ns not significant at 5%.

Table 3.4 The analysis of variance (ANOVA) of the effect of independent variables (lactose, holding time) on high pressure (200, 300, 400 MPa) destruction of *E. coli*.

(a) 400 MPa (holding time 0, 4, 8 min) added lactose

Source	Degree of freedom	Sum of Square	Mean Square	F-value	Pr
Lactose	2	0.0148	0.0074	0.06	0.9456^{ns}
Time	2	35.7504	17.875	135.89	0.0001**
Lactose x Time	4	0.3191	0.0798	0.61	0.6681^{ns}
Error	9	1.1839	0.1315		
Total	17	37.2683			

** Highly significant at 1%; ns not significant at 5%.

(b) 300 MPa (holding time 0, 8, 16 min) added lactose

Source	Degree of freedom	Sum of Square	Mean Square	F-value	Pr
Lactose	2	0.04403	0.0220	0.56	0.5909^{ns}
Time	2	19.0844	9.542	241.92	0.0001**
Lactose x Time	4	0.05773	0.01443	0.37	0.8271^{ns}
Error	9	0.355	0.0394		
Total	17	19.5412			

** Highly significant at 1%; ns not significant at 5%.

(c) 200 MPa (holding time 0, 16, 32 min) added lactose

Source	Degree of freedom	Sum of Square	Mean Square	F-value	Pr
Lactose	2	0.5068	0.2534	1.37	0.3033^{ns}
Time	2	10.269	5.1345	27.68	0.0001**
Lactose x Time	4	0.4376	0.1094	0.59	0.6786^{ns}
Error	9	1.6696	0.855		
Total	17	12.883			

** Highly significant at 1%; ns not significant at 5%.

As with casein, the extent of pressure destruction of *E. coli* in milk with added lactose above that already existed was not statistically different ($p>0.05$) at any given pressure level or treatment time. In addition, the effect of lactose was found to be lower than that of casein, even though the concentration of lactose added was twice as large. For example, reduction in log units for milk and milk supplemented with 4 % and 8 % lactose at 300 MPa were 0.64, 0.42, and 0.44 for 0 min for the pressure pulse process, 2.05, 2.03, and 2.08 after 8 min treatment and 3.06, 3.04, and 2.88 after 16 min treatment at 300 MPa, respectively.

These results thus indicated that there was no significant progressive effect in the pressure destruction of *E. coli* in milk when supplemented with various amounts of fat, casein and lactose. However, there was clear evidence of baro-protection in milk as compared with buffer. Hence the next logical step was to enrich the buffer with casein and lactose within the range of their normal levels in milk (1-4%) and compare their baro-resistance with milk and buffer controls.

Influence of casein and lactose added to buffer on the destruction of *E. coli*

The results of the high-pressure microbial destruction [log (N_0/N)] of *E. coli* K12 in milk and buffer solution with addition of 1, 2, 4 % casein and lactose treated by 300 MPa for 5 min or 200 MPa for 15 min are presented in Figure 4.5. The mean comparison of the results is shown the Table 3.5.

It is quite obvious that the largest destruction at a given pressure was associated with buffer solution followed by buffer with buffer supplemented with different lactose and casein solutions as shown in Figure 4.5. The lowest destruction was generally observed in milk. The results by comparison of the mean values show no significant differences between buffer with added 1%, 2% and 4% casein or no significant differences between buffer with added 1%, 2% and 4% lactose. However, the addition of casein or lactose (1-4%) to buffer solution significantly decreased ($p < 0.05$) the destruction of *E. coli* population at all conditions tested as compared to destruction the buffer alone. The *E. coli* destruction in buffers supplemented with 1-4% casein or lactose were not significantly different from either each other or in milk. Thus any supplemental addition of casein and lactose to buffer appear to be baro-protective against HP destruction of *E. coli*.

Table 3.5 Baro-resistance of *E. coli* in milk and buffer with added 1, 2, 4% casein and lactose at 300 MPa for 5 min or 200 MPa for 15 min.

Sample	Count reduction (log-cycles) 200MPa - 15 min	Count reduction (log-cycles) 300MPa - 5 min
Buffer+4% casein	1.00a	1.24a
Buffer+2% casein	1.33a	1.44a
Buffer+1% casein	1.28a	1.51a
Milk	1.23a	1.54a
Buffer+4% lactose	2.00b	2.40b
Buffer+2% lactose	2.43b	2.44b
Buffer+1% lactose	2.42b	2.53b
Buffer	2.77c	3.63c
SEM	0.12	0.18

SEM, Standard error of the mean; Numbers in the same column sharing the same letter is not significantly different ($p > 0.05$).

To re-confirm baro-protective action of casein and lactose at the observed threshold level of 1%, additional experiments were carried out at this level, and tested for pressure inactivation at 300 MPa by varying the treatment times (0, 2.5, 5, 7.5 min). The results of this study are shown in Figure 3.6 and mean comparisons are shown in Table 3.6.

The results show that that the destruction in buffer was significantly ($p<0.05$) different from the destruction in all other samples. Buffer supplemented with 1% casein was no different from milk in terms of the destruction achieved. However, the destruction achieved in buffer plus lactose was considerably higher than in milk alone or in buffer plus casein supplement. The longer the treatment times the higher the destruction of *E. coli* in all samples. In this reconfirmation study, the buffer solution supplemented with 1% casein showed a baro-protective effect undistinguishable from that in milk. However, this effect was not apparent at 1% lactose supplement as found in the previous test. Perhaps the

threshold level for lactose could be slightly higher (~2%). In any case, the results confirm the role of casein and lactose in contributing to baro-protective activity in milk.

Table 3.6. Microbial destruction of *E. coli* K12 in milk and buffer solutions with and without added casein or lactose (1%) at 300 MPa after various treatment times.

Sample	Count reduction (log-cycles) 200MPa - 0 min	Count reduction (log-cycles) 200MPa - 0 min	Count reduction (log-cycles) 200MPa - 0 min	Count reduction (log-cycles) 200MPa - 0 min
milk	0.36 a b	0.27 a	1.23 a	1.82 a
Buffer+casein	0.42 b	0.36 a	1.25 a	1.54 a
Buffer lactose	0.54 c	1.32 b	2.62 b	2.99 b
Buffer	2.29 d	2.56 c	3.72 c	4.2 c
SEM	0.7756	0.999	0.1243	0.1473

SEM, Standard error of the mean; Numbers in the same column sharing the same letter are not significantly different ($p > 0.05$).

Conclusions

The applications of high pressure treatment resulted in significant reduction of *E. coli* K12 population in buffer, peptone water and milk and in that specific order with reference to their effectiveness. No significant differences on the effect of *E. coli* K12 destruction was observed between milk with different fat contents or milk with added 4% casein or lactose.

However, when casein and lactose were added to buffer, they provided significant baro-protection to *E. coli*. Casein (1%) or lactose (2-3%) added to buffer offered the same protective behavior as milk; however additional amounts did not add further to the protection.

This protective effect due to casein and lactose added to milk was noticeable probably because concentrations level of casein and lactose exist in milk beyond the threshold levels. The study demonstrates that milk has baroprotective effect on *E. coli* destruction and components contributing to the protection are likely casein and lactose.

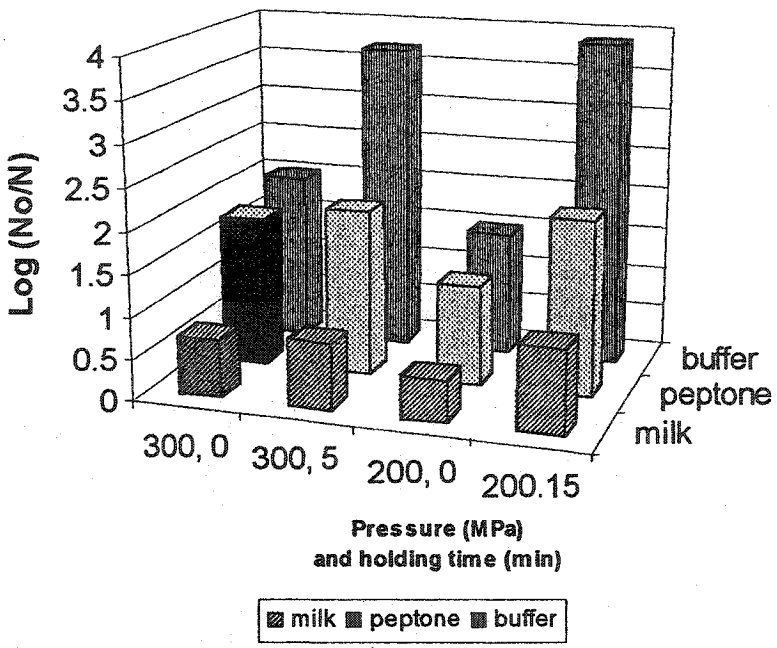

Figure 3.1 HP destruction of *E. coli* inoculated into milk, buffer solution and peptone water treated under (1) 300 MPa for 0 min; (2) 300 MPa for 5 min; (3) 200 Ma for 0 min and (4) 200 for 15 min.

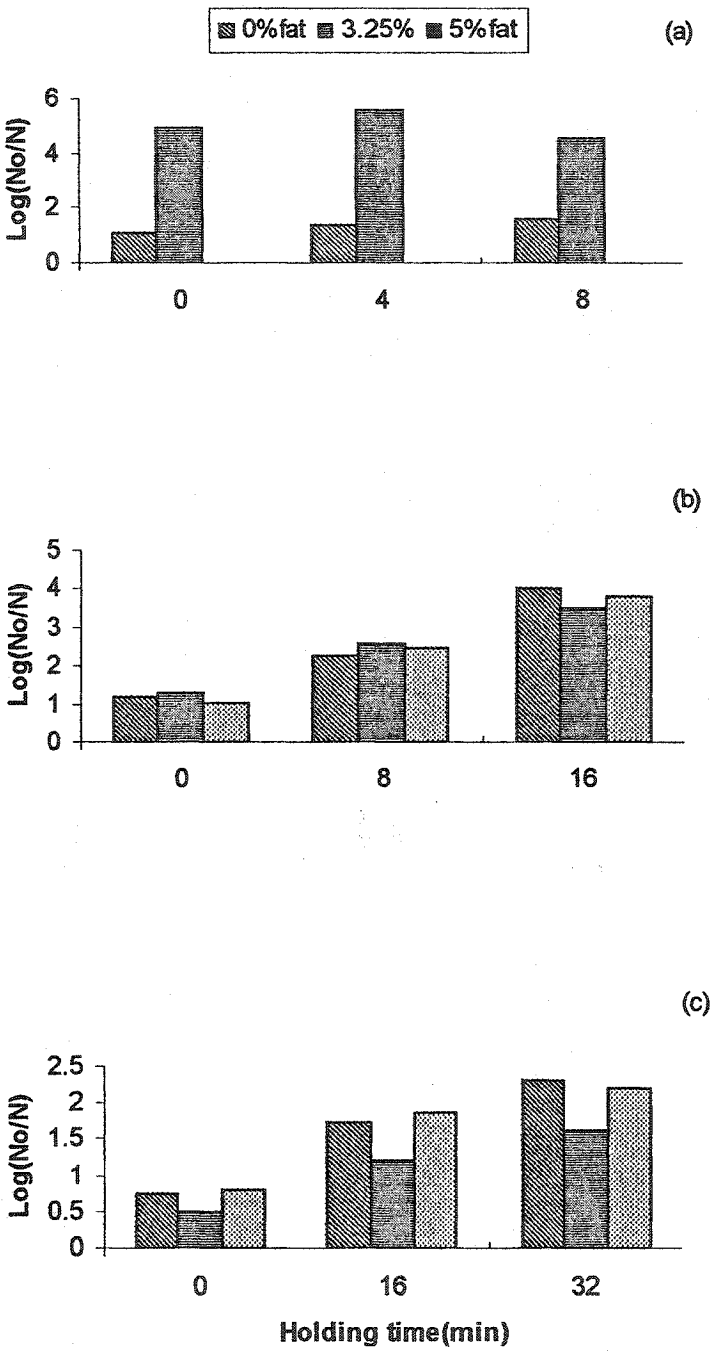

Figure 3.2 High pressure destruction for E. coli in milk as affected by fat content: (a) HP 400 MPa (b), 300 MPa (c) and 200 MPa

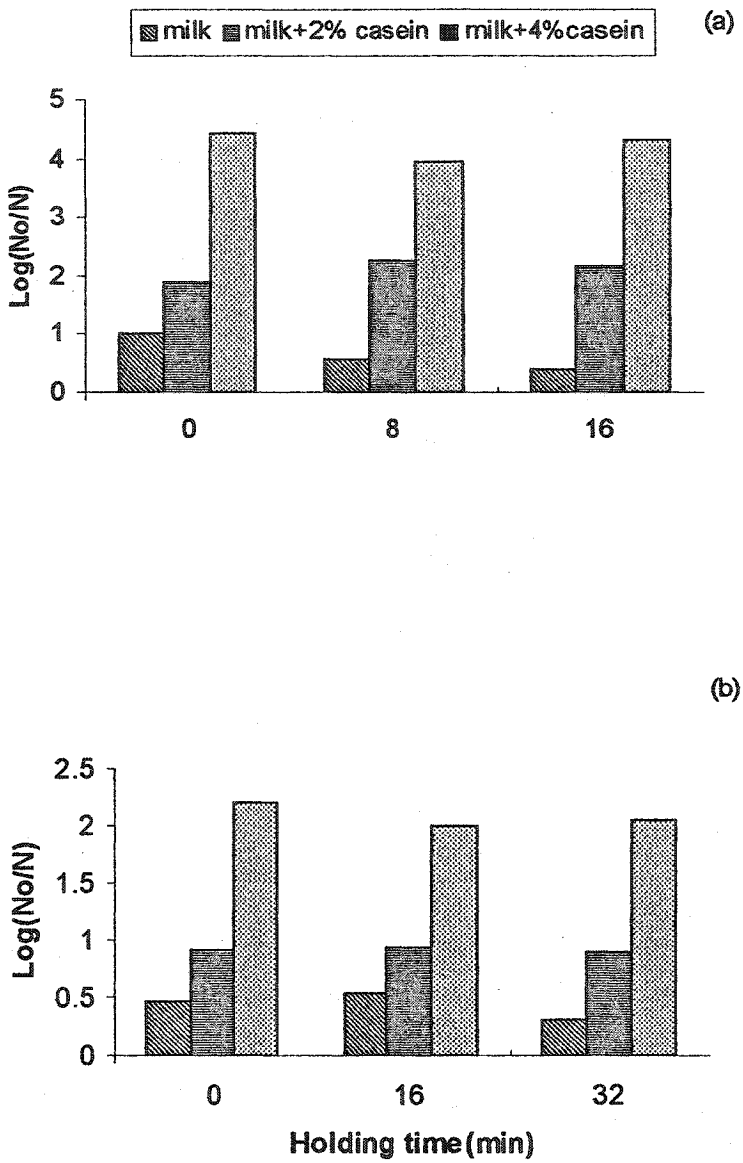

Figure 3.3 High pressure destruction for E. coli in homogenized whole milk (3.25% fat) as affected by added casein: (a) HP 300 MPa (b), 200 MPa.

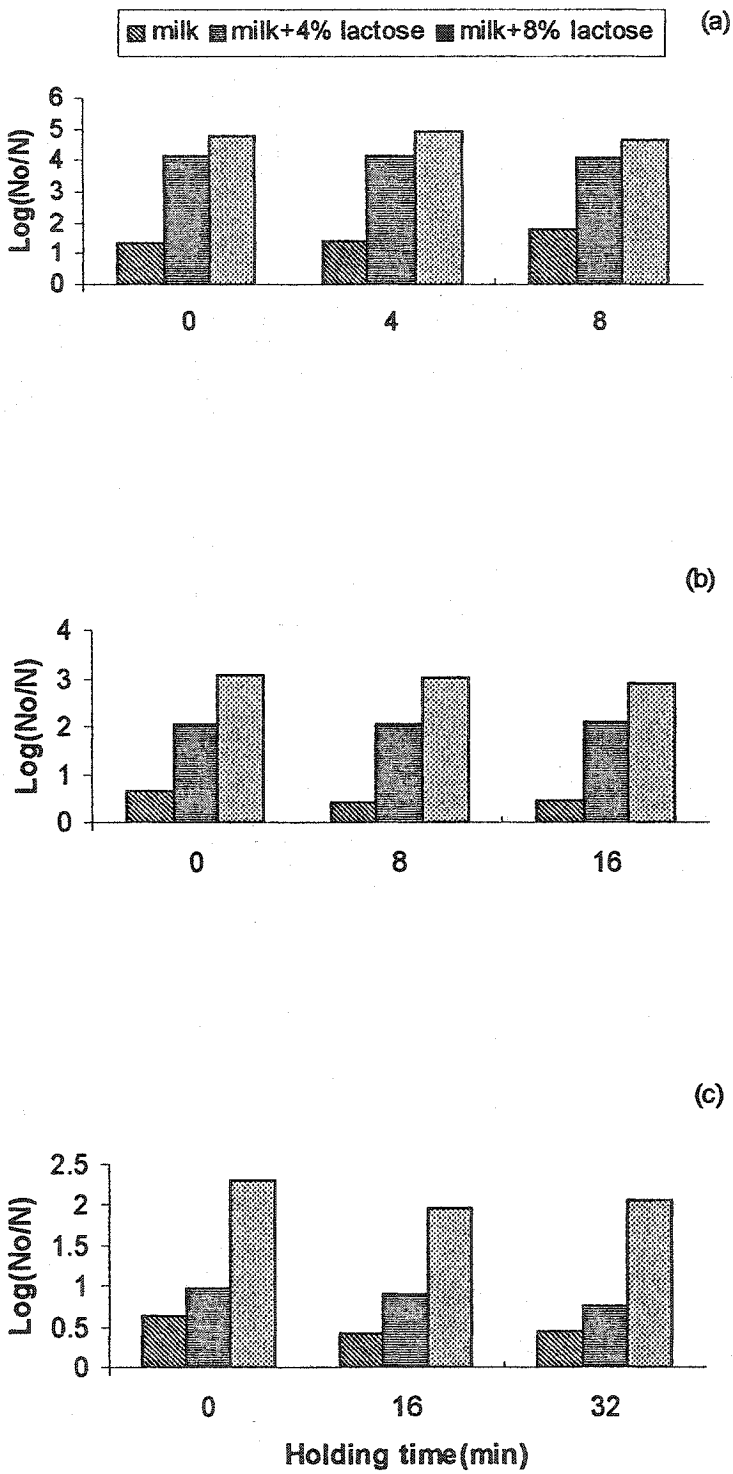

Figure 3.4 High pressure destruction for E. coli in homogenized whole milk (3.25% fat) as affected by added lactose: (a) HP 300 MPa (b), 200 MPa.

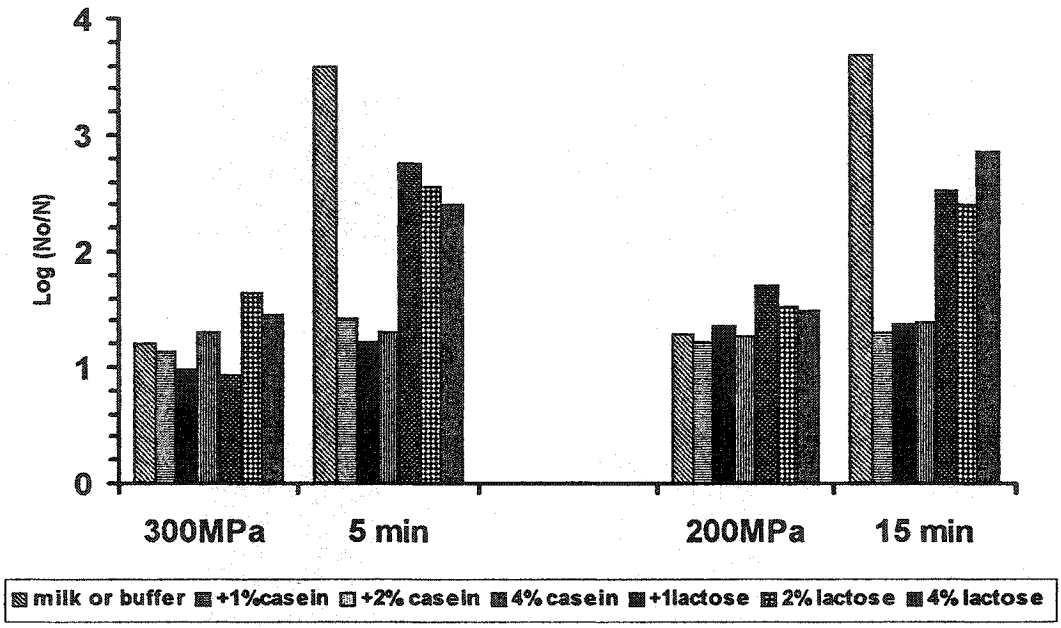

Figure 3.5 High pressure destruction of *E. coli* in milk, buffer, and buffer with added 1, 2 and 4% casein or lactose and subjected to two high pressure treatments: (300 MPa, 5 min and 200 MPa, 15 min)

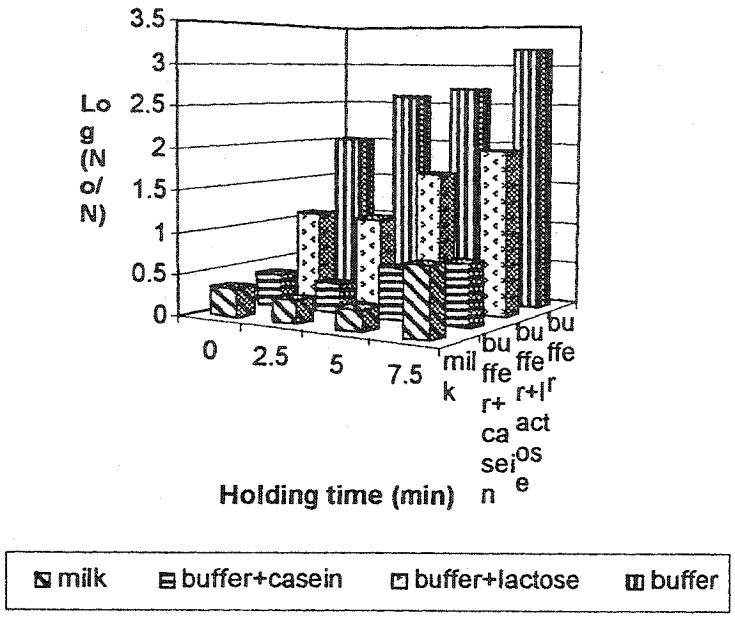

Figure 3.6. High pressure destruction of *E. coli* in milk, buffer, and buffer with added 1% casein or lactose and subjected to high pressure treatments at 300 MPa for 0-7.5 min.

CHAPTER 4
DUAL-EFFECT HIGH-PRESSURE DESTRUCTION KINETICS OF *ESCHERICHIA COLI* IN MILK

Abstract

The dual effect high pressure destruction kinetics of *Escherichia coli* in milk was investigated in this study. *E. coli* culture was inoculated into commercial pasteurized homogenized milk (3.25% fat), filled in to small plastic bags, heat sealed and subjected to various high pressure treatments (100-400 MPa, 0-30 min; 1-3 pulse cycles) at room temperature (25°C). *E. coli* destruction due to pressure was modeled based on a dual destruction behavior comprising of (i) a pressure pulse effect (PE) due to pressurization-depressurization with zero hold-time and (ii) a subsequent semi-logarithmic (first order) destruction during the pressure hold-time. The pressure dependency of destruction rate was evaluated by the pressure-z value approach and Arrhenius models. High pressure treatment at 400 MPa for 30 min completely destroyed *E. coli* population of 10^7 CFU/mL. The pressure destruction increased with pressure, holding time and number of pulses. The time advantage of pulse over hold approach was more noticeable at lower pressures. The single pulse effect was 0.71, 0.57 and 0.26 log cycle reductions at 400, 300 and 200 MPa, and the respective D values were 4, 13 and 70 min. The pressure-z value and volume of activation were 200 MPa and -7.0×10^{-5} m^3 $mole^{-1}$.

Introduction

Among the modern technologies in the food industry, the most important are those involving non-thermal treatments of the product. High pressure (HP) processing (100-1000 MPa) is one of the most promising methods for the food treatment and preservation at room temperature (Cheftel 1992). Research into the application of HP processing for milk preservation began when Hite (1899) demonstrated that the shelf life of milk and other food products could be extended by pressure treatment. The advances achieved in ceramics and metallurgical industries in the use of HP techniques during the seventies and eighties of the last centuries opened the possibility of treating food by this method at industrial level.

Unlike thermal treatments, where covalent as well as non-covalent bonds are affected, HP treatment at room and mild temperatures only disrupts relatively weak chemical bonds (hydrogen bonds, hydrophobic bonds, and ionic bonds). Thus, small molecules such as vitamins, amino acids, small sugars and flavor compounds remain unaffected by the HP treatment (Sierra et al., 2000). Garcia-Risco et al., (2000) found that HP treatments at 400MPa for 15 min at 25 - 60°C maintained the organoleptic properties of milk, suggesting that these combined treatments could be used to produce milk of good sensory properties with an increased shelf life.

Liquid milk is heat-treated using a range of conditions to provide acceptable safety and shelf life. But heat treatments adversely affect the nutritive value and flavor of fresh milk. Several studies on the inactivation of pathogenic and spoilage microorganisms (naturally present or introduced), have shown that HP treatments can be used to obtain "raw" milk pressurized at 400-600 MPa with a microbiological quality comparable to that of pasteurized (72°C, 15s) milk depending on the microbiological quality of milk (Mussa and Ramaswamy, 1997; Buffa et al., 2001a; Kolakowski et al., 1998). Later reports suggest that complete sterilization for milk by HP that requires higher temperatures combined with higher pressures.

Escherichia coli (non-pathogenic) in milk and milk products is not necessarily life threatening, but is indicative of inadequate and unhygienic handling practices in processing operations. Although the presence or absence of *E. coli* in milk may only show the effectiveness of plant hygiene; however, if it can often be used as a surrogate against its pathogenic variant, E. coli O157:H7, the evaluation process could be lot easier to handle

(Pandey and Ramaswamy 2002). It is well known that the pathogenic *E. coli* is able to cause different intestinal diseases. *E. coli* infections have been associated with the consumption of a range of products, including ground beef, raw milk and contaminated water, and the outbreaks have been increasing (Neil, 1989; Padhye and Doyle, 1992; Rice *et al.*, 1992; Koodie *et al.*, 2001; Toder, 2002).

Some previous studies have determined kinetic data useful for HP pasteurization of milk. The majority of these were carried out with the objective of establishing pressure-processing conditions, including process time and temperature. Only few studies have explored the application and evaluation of pulse effect on destruction of microorganisms. Pressure process can be applied in two modes – pulse or hold mode. In the pulse mode, once the desired level of pressure is reached, it is immediately released without any holding time. In the hold mode, when the pressure level is achieved at the preset value, it is held at that level for a period (hold time) and then released. It is should be noted that a complete pressure cycle comprises of a pressure pulse with or without any holding time, a pressure cycle without hold thus constitutes a pressure pulse. The pressure destruction of microorganism has been shown to follow a dual behavior involving pressure pulse and pressure hold effects. This concept was initially observed by Hayakawa et al. (1994), but mostly quantified in later studies (Basak and Ramaswamy, 1996; Mussa et al., 1998; Pandey et al., 2002; Riahi et al., 2003). Pandey et al. (2002) showed that pulse mode has advantages over the hold approach for destruction of microorganisms. Riahi et al. (2003) compared the pulse vs. hold approach for pressure destruction kinetics of microorganisms in apple juice and elucidated conditions under which one can be better than the other. Although, some comparison between the pressure pulse and hold approaches have been made in these earlier studies, more appears to be desirable.

In the previous section (Chapter 3), the role of milk components on the destruction kinetics of *E. coli* were detailed. The objective of this study was to evaluate the HP destruction kinetics of *E. coli* in milk and to compare the pulse vs hold effects for the destruction rate of *E. coli*.

Materials and Methods

Microbial analysis and samples preparation

All procedures used for microbial analysis and sample preparations were as described previously in the composition study (Chapter 3).

Sample preparation and high pressure treatment

Pasteurized milk obtained from a local market was inoculated with *E. coli* K12 and subjected to various pressure treatments (200- 400 MPa) with various holding times and pulses treatments as detailed in Table 4.1. The destruction kinetics of *E. coli* was studied at room temperature. Since compression heat will increase the temperature of the pressure medium by about 3°C per 100 MPa, the pressure chamber and media were temperature conditioned with a continuous circulation of temperature-controlled water around the pressure chamber. To ensure that the sample temperature was below 25°C (i.e., the point at which thermal destruction of microorganisms might occur, Basak and Ramaswamy 2001), the pressure medium initial temperature was set at 13, 15 and 18°C for treatment at 400, 300, 200 MPa, respectively, before the start of the pressure treatment. With water circulation around the chamber, thermocouple was used to sense temperature of the pressurizing fluid. The prepared samples were then subjected to various combinations of high-pressure treatment; at 200 - 400 MPa for 1 to 3 cycles with or without holding times (Table 4.1). Each experiment was replicated three times with duplicate analysis in each replication The enumeration results were averaged from 4 measurements (2 samples / treatment and two plates/dilution).

Table 4.1 Pressure levels, holding time and pulses used for HP treatment of milk inoculated with *E. coli*

Pressure (MPa)	Holding time (min)	Number of pulses
200	0, 20, 40, 60	1, 2, 3
300	0, 10, 20, 30, 40	1, 2, 3
400	0, 5, 10, 15	1, 2, 3

Data analysis

Pressure destruction of *E. coli* was analyzed as a twofold effect: a change in survivor counts due to pressurization and depressurization (constituting a pressure pulse), and a first order rate of destruction during the pressure hold time. The change in activity of PME due to the pressure pulse was defined by Basak and Ramaswamy (1996) as an instantaneous pressure kill (IPK) value and was obtained by subtracting activity (logarithmic scale) from the initial activity after subjecting the sample to one pressure pulse. This is redefined as a pressure pulse effect (PE) to be more explicit and meaningful (Riahi et al., 2003). The pressure pulse inactivation behavior has been confirmed to be also true with microorganisms (Mussa, 1999; Basak, 2001, Riahi, 2003).

Pressure destruction during the hold time was modeled based on the first order rate kinetics:

$$\text{Log}_e(N/N_o) = -kt \qquad \textbf{(4.1)}$$

where N = survivor count after a pressure treatment for time t (min), N_o = initial count before pressure treatment or at zero time, and k = reaction rate constant (min^{-1}). The treatment time at any given pressure that will result in 90% destruction of cells, i.e. resulting in one decimal reduction in the survivors, is referred to as the decimal reduction time or D value. This was obtained as the negative reciprocal slope of the $\log_{10}(N/N_o)$ *vs.* time (or time taken to traverse one logarithmic cycle) and is therefore reciprocally related to k:

$$D = 2.303/k \qquad \textbf{(4.2)}$$

when D values at different pressures are plotted on a semi-logarithmic scale, the pressure range for one log-cycle change in D values represents the z value. Thus, the pressure z value (z_p) of the process is defined as the pressure range between which the D values change by factor of ten. The z (z_p) value can be expressed by the following equation:

$$z_p = [P_2 - P_1] / [\log D_{P1} - \log D_{P2}] \qquad \textbf{(4.3)}$$

This is analogous to treatment of thermal death time (TDT) data of microorganisms widely used in thermal process calculations. The z_p value was calculated from the regression of $\log_{10}$ (D) *vs.* pressure data as the negative reciprocal of the slope.

The relative effect of pressure pulse (PE) and pressure hold-time was assessed using N_D values (Mussa, 1999). N_D, which represents the number of pressure cycles required to achieve one decimal reduction in enzyme activity (thus equivalent to a D value), can be calculated as follows:

$$N_D = 1 / (PE) \quad (4.4)$$

D_P or decimal reduction time equivalent of PE, represents the holding time in minutes at a given pressure level which results in an equivalent inactivation achieved by one pressure cycle. These values were obtained as follows:

$$D_P = \text{D value} * [(PE)] \quad (4.5)$$

The pressure sensitivity of the kinetic parameters wan also be analyzed by the Arrhenius approach. In the Arrhenius type, the activation volume (ΔV) which is measure of net pressure effect at constant temperature was obtained by plotting ln k value against pressure:

$$\left[\frac{\Delta \ln(k)}{\Delta p}\right]_T = -\frac{\Delta V^*}{R \cdot T} \quad (4.6)$$

or $$\ln (k) = \ln (ko) - (\Delta V \ P/RT) \quad (4.7)$$

or $$\Delta V = -RT \text{ (slope)} \quad (4.8)$$

where ΔV is the activation volume in (m^3 mole $^{-1}$), P is the pressure in MPa, k is the rate constant (min^{-1}), T is the absolute temperature (K), R is the gas constant ($8.314*10^{-6}$ m^3 $mole^{-1}$ MPa $^{o}k^{-1}$) (Erying and Magee, 1942).

The parameters (i.e. N_D and D_P) were derived to compare the pressure pulse with pressure hold results. N_D describes the pressure pulse effect while the D value indicates a measure of the pressure hold effect. N_D can be established in terms of the number of pressure pulses required to result in one decimal reduction in microbial population, and it can be obtained as a negative reciprocal of the slope of log (N_o/N) vs. pulse number curve. Since PE represents a logarithmic reduction in microbial population due to a single pulse, it can be obtained by PE = 1/ N_D. Alternately, D_P value as equivalent minutes of decimal reduction (PE x D) achieved by one pressure pulse can be used. The difference D-D_P gives the relative difference between the two approaches (Pandey et al., 2002). However, in terms of operation, the pulse mode processing time (PT_{pulse}) would be relatively higher than D_P because of the need to accomplish multiple pressurization and depressurization. It is best obtained by multiplying N_D by the time needed to operate one pulse. Likewise, this single pulse time also needs to be added to the pressure hold process as well (PT_{hold}). The time difference between the pulse and hold modes of pressurization to achieve one D destruction can then be: (PT_{pulse} - PT_{hold}).

Results and discussion

Kinetic study

The survival curves for *E. coli* K12 inoculated into whole milk following pressure treatment at various levels as a function of holding time is presented in Figure 4.1. The linearity of the curves and the associated high R^2 values indicate that the first order rate model is suitable to be used for the analysis pressure destruction. The kinetics parameters (D and k values) computed from the survivor curves at different pressure is shown in Table 4.2. The pressure sensitivity of D values is shown as a z-value plot in Figure 4.2. A similar plot of ln k vs P for activation volume is not shown, however, the pressure sensitivity z value (z_p) and volume of activation computed from the kinetic data are shown in Table 4.2

Table 4.2. The high-pressure destruction kinetics of *E. coli* in milk at room temperature

Pressure	D value (min)	R^2	k-value (min^{-1})	ΔV (x 10^{-5} m^3 $mole^{-1}$)	z_p (MPa)	R^2
200	70	0.95	-0.03	-7.0	161	0.99
300	13	0.94	-0.17			
400	4.0	0.98	-0.57			

From Figure 4.1 and Table 4.2, D values of *E. coli* were found to be 4.0, 13 and 70 min at 400 300 and 200 MPa, respectively. Statistically, the D values at different pressure levels were significantly deferent ($p< 0.05$), and decreased (and k values increased) with an increase in pressure level. Isaacs et al. (1995) studied survival curves for *E. coli* in whole milk at different pressures, temperatures and times. Their results showed that *E. coli* in the stationary phase of growth was more baro-resistant, and to obtain a reduction of > 4 log units in 5-10 min, it was necessary to apply pressures ≥ 400 MPa. Our results show a 3-log reduction in 12 min at 400 MPa. The D value (13 min at 300 MPa) was also higher than that reported by Gervilla et al. (1999) for ewe's milk (5.19 min at 300 MPa) under similar conditions. It is well recognized that certain constituents exist in substrates may exercise a baro-protective effect or vice versa (Knorr et al., 1992; Maggi et al., 1994). Further, the level of baro-resistance between different species of the same genus and different strains of the same species (*E. coli*), as well as the conditions of growth and possible states of pre-pressurization stress can affect the destruction kinetics. These factors should be considered when making recommendations to the industry and results of the same microorganism studied in different substrata or foods should not be extrapolated (Gervilla *et al.*, 1999). The destruction kinetics of *E. coli* in milk by high pressure has also been studied previously in our lab (Mussa, 1999; Mussa et al., 1998; Pandey et al., 2002). These studies found the D value for *E. coli* in raw milk to be 4 - 15 min in the pressure range, 400-300 MPa. This range of D value is similar to our results (4 to 13 min for pressure range 400-300 MPa). The pressure sensitivity of *E. coli* is also in the same range (z_p value: 205 vs 160 MPa found in this study).

Total destruction of *E. coli*

From Table 4.2, it is clear, that in order to achieve 1 log cycle destruction, the pressure processing time required is 4.0, 13 and 70 min at 400 MPa, 300 MPa, and 200 MPa, respectively. The large difference in the D-values (70 - 4 min) between 200 and 400 MPa is easily noticeable and arises form the log-linear relationship between D value and pressure. In such situations, obviously treatment at higher pressures would be more effective than a prolonged treatment at lower pressure. In order to test the usefulness of kinetic data for pressure processing application, test samples were inoculated with an initial count of 10^7 CFU/ml of *E. coli* and HP treatment at 400 MPa was applied up to 32 min to achieve 7 - 8 log reduction. The survival curves for *E. coli* treated under 400 MPa for prolonged time periods are presented in Figure 4.3. There was no survival found after 28 min under 400 MPa, confirming the validity of previously established kinetics. D value calculated from the survival curve of the confirmation run was also 4.0 min at 400 MPa as was observed earlier. Some researchers (Helge et al., 2000; Raso et al., 1998) have found a tailing of pressure destruction curves with microbial spores and ascribed it to the presence of different strains that reacted in different ways under HP processing conditions No tailing was observed in this study.

Pulse effect study

A careful analysis of survivor curves (Figure 4.1) show that the destruction of microorganisms by high-pressure treatment followed the first order rate kinetic model. However, this was only after an initial drop (at time zero, due to a pulse effect of pressure). Thus, this study demonstrated the dual effect behavior of pressures destruction characterized by a step-change in the number of survivors with application of a pressure pulse (pressurization and depressurization without any hold-time) and a first order rate of destruction during the pressure hold. The destruction due to pressure pulse (PE) increased with the pressure level.

Separate experiments were conducted in order to determine the pressure pulse effect on *E. coli*. Three pulses were used at each of the three pressure levels: 200, 300 and 400 MPa. The progressive destruction of *E. coli* following the pulse pressure application is demonstrated in Figure 4.4 which demonstrates a traditional log-linear trend with pulse number with a high R^2 value. The number of pulses required to achieve a decimal reduction

in microbial population can be obtained from the negative slope and PE can be obtained as the reciprocal N_D value. The computed N_D, the associated R^2 value, PE and D_P and ($D-D_P$) values and are shown in Table 4.3.

Table 4.3 Pressure pulse effect related parameters for *E. coli* in milk.

Pressure	N_D (number of pulses)	R^2	PE (log-cycle reductions)	D_P (D x PE) (min)	($D-D_P$) (time saving) (min)
400	1.41	0.97	0.71	2.8	1.2
300	1.75	0.94	0.57	7.4	5.6
200	3.85	0.95	0.26	18	52

Several useful informations can be obtained from Figure 4.4 and Table 4.3. First it shows that higher the number of pulses, greater is the extent of destruction. Therefore, higher the pressure, lower is the number of pressure pulses required to achieve a given amount of destruction, like N_D which represents the number of pulses per decimal reduction in microbial population. Some comparison between the pulse and hold approaches can also be made. At 400 MPa, the calculated number of pulses required to achieve one decimal reduction in microbial population is 1.41 or approximately 7 pulses are required for a 5-log reduction. Seven pulses are quire excessive from a practical standpoint. Even so, considering the fact that a pressure pulse process at 400 MPa takes about 5 min, the total time involved would be 35 min. The decimal reduction time at 400 MPa is 4 min and for 5 log reduction, it would take 4 x 5 = 20 min. Hence a pressure cycle time of 3.5 min together with a holding time of 20 min for a total time of 23.5 min would yield similar results. This would offer a time savings of 11.5 min (33%). On the other hand, in situations in which mild low pressure applications are desired, for example equivalent of one decimal reduction, a different scenario can be explored. At 200 MPa, the N_D is 3.95, i.e., four pressure pulses are required for achieving the desired task. The pressure cycle time at 200 MPa is 2.5 min and hence the process would require 2.5 x 4 = 10 min. On the other hand, the hold approach would require a holding time of 70 min giving a treatment time of 73 min. In this case the pressure pulse approach gives a saving of 63 min (84%), certainly a big advantage. These time advantages can be realized by ($D-D_P$) column in Table 4.3. $D-D_P$

values only show the difference in magnitude in pressure contact time between two approaches. When these are converted to operational parameters by taking in to account the pressure come up and come down periods, the differences in real time can be realized as shown in Table 4.4.

Table 4.4. Pressure pulse vs pressure hold process for *E. coli* in milk

Pressure (MPa)	N_D (number of pulses)	Pressure pulse time (min)	Total time* PT_{pulse} (min)	D Value (min)	Total time PT_{hold} (min)	PT_{pulse} - PT_{hold} (min)
400	1.41	3.5	7.0	4.0	7.5	-0.5
300	1.75	3.0	6.0	13	16	-10
200	3.85	2.5	10.0	70	72.5	-62.5

*Based on rounded pulse numbers

The individual pulse effects (log reduction in microbial population) at each cycle are listed in Table 4.5. The results demonstrate fair consistency of pressure pulse application. A given pulse resulted in similar magnitude of destruction around the average value. Thus the pulse effect can be considered cumulative. This gives justification for the data presented earlier for the pressure pulse vs pressure hold approach. In order for the pressure pulse effect to be useful, the multiple pulse effects must be cumulative and quantifiable. In order to be fully reproducible, the process must be well controlled. Multiple quick pulse can result in warming up of the equipment, resulting in higher kill in later pulses. Conceptual use of multiple pulse techniques have been proposed in earlier research (Mussa, 1998; Basak, 2000; Pandey, 2001 and Riahi, 2003); however, adequate validation data were not provided to demonstrate its usefulness.

Table 4.5 Pressure pulse destruction of *E. coli*

Pressure	Pulse Effect (PE) (log cycle reductions)				N_D
	Pulse 1	Pulse 2	Pulse 3	Avg.	
200	0.20	0.48	0.13	0.27	3.70
300	0.52	0.23	0.56	0.44	2.27
400	0.39	0.72	1.01	0.71	1.41

Conclusions

Pressure destruction kinetics and pulse effect of *E. coli* in pasteurized milk were studied for 200- 400 MPa pressure treatments with holding times of 0-60 min and 1-3 pulses. Results indicate and prove the dual effect pressure destruction of microorganism with a step change in destruction due to a pressure pulse followed by a first order holding time effect. Both modes of pressure application were well described by a first order rate model (pulse application with multiple pulses). Higher pressures, longer holding times and more pulses produced more destruction of microorganisms. Higher pressure gave little higher pulse effect on destruction of microorganism, but the time advantage of pulse pressure was more apparent at lower pressures, while the kill effect was more obvious at higher pressures.

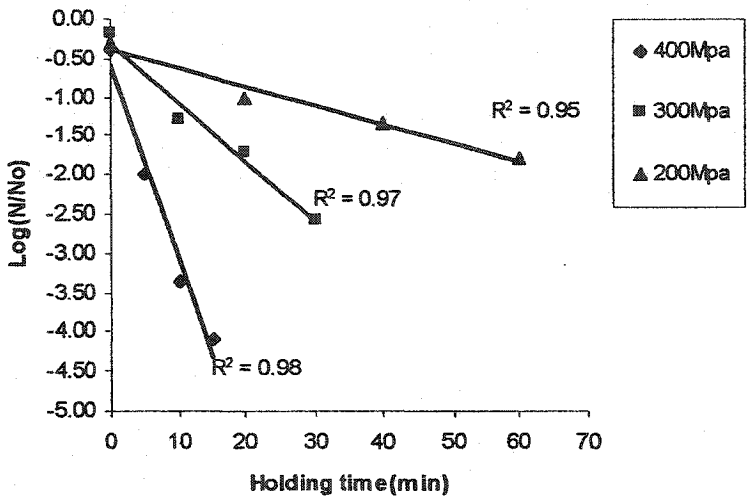

Figure 4.1 Survival curve of *E. coli* under different pressure and holding time at room temperature

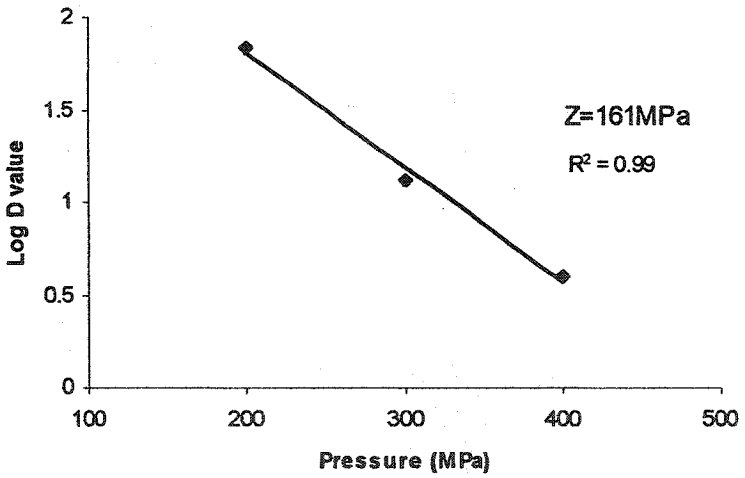

Figure 4.2 Pressure sensitivity of *E. coli* in milk at room temperature

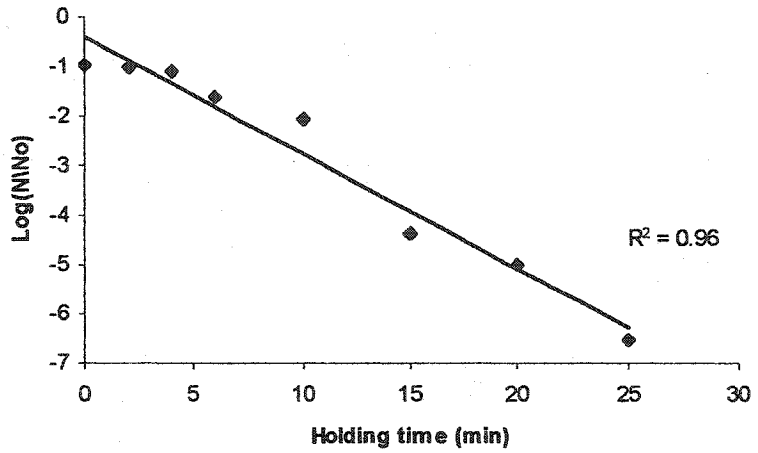

Figure 4.3 Survival curve of *E. coli* at 25 °C at 400 MPa.

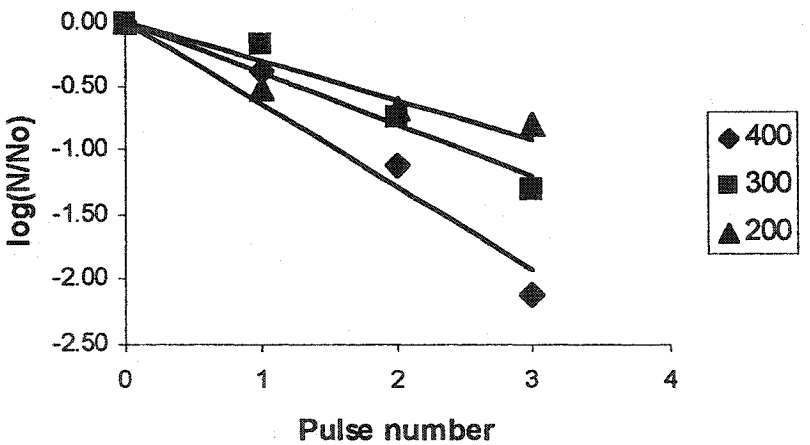

Figure 4.4 Survival curve of *E. coli* under different pressure pulse treatment

CHAPTER 5

HIGH PRESSURE DESTRUCTION OF *ESCHERICHIA COLI* O157:H7 AND *LISTERIA MONOCYTOGENS* IN MILK

Abstract

Two pathogenic strains of *Escherichia coli O157:H7* and *Listeria monocytogenes* were inoculated in raw milk and commercially processed ultra high temperature (UHT) milk at a concentration of 10^7 CFU/mL, and exposed to high pressure (HP) (300, 350, and 400 MPa) treatment for up to 90 min. Both strains showed first–order kinetics of destruction and their survival in UHT milk was lower than in raw milk. D values of *E. coli* O157 H:7 at 300-400 MPa were 6.5-23 min in UHT milk and 13-35 min in raw milk. Generally the associated z values of 179-196 MPa for UHT and raw milk, were within the broad range of values reported in the literature. The pathogenic strain of *E. coli* was found to be more resistant than the non-pathogenic strain for HP destruction.

The influence of temperature on the HP destruction of *L. monocytogenes* in UHT milk was investigated in the pressure range 300-400 MPa treated for 0 - 60 min at 20 and 35°C. As with *E. coli*, the D value for *L. monocytogenes* in UHT milk (12.9 min at 400 MPa) was lower than in raw milk (16.5 min at 400 MPa). No significant differences in inactivation rate were observed between the two temperatures. *L. monocytogenes* was more resistant than *E. coli O157:H7* for high pressure destruction.

Introduction

High pressure processing is an emerging technology which is stimulating a lot attention among researchers in the area of non thermal processing and inactivation of potent microorganism (Mertens and Knorr, 1992). Non-thermal processing technique is becoming a popular method of microbial inactivation of thermolabile food products, as a result of consumer preference for product of high nutritional and sensory quality, that are minimally processed, and additive free (Farr, 1990, Hoover et al., 1989). This method is gaining popularity in the food industry because of its positive influence on the functional properties of food products (Gervilla et al., 1999). High pressure has been successfully used to extend shelf life of high-acid foods such as refrigerated fruit juices, jellies, and jam (Kimura et al., 1998). High pressure processing is a method which exposes food material to extremely high pressure (greater than 100 MPa), and this method has been known for more than 100 years (Hite, 1899). One of the primary considerations is its ability to eradicate pathogenic micro-organisms and thus ensure food safety.

The risk of *Escherichia coli O 157* infection is a particular problem for the food industry. It causes haemorrhagic colitis, has a low infective dose and may give rise to life threatening conditions such as haemolytic-uremic syndrome. Food-poisoning outbreaks have often been associated with the consumption of foods of animal origin, including hamburger and raw milk (Armstrong *et al.* 1996). Outbreaks of listeriosis have been particularly associated with soft cheeses and pate, but also with milk (Farber and Peterkin 1991).

A lot of research has been conducted on high pressure inactivation of pathogens such as *E. coli* and *Listeria monocytogenes* in milk products (Patterson and Kilpatrick, 1998; Gercia-Graells et al., 2000). Several researchers have shown that many factors can influence the sensitivity of micro-orgainism to high pressure treatment (Patterson and Kilpatrick, 1998). In general, microbial cells in the exponential phase are more susceptible to pressure inactivation than those within the stationery phase. Gram-negative bacteria are more pressure sensitive than the gram-positive bacteria. It was reported that the gram negative bacteria cell membrane structure are complex, and sensitive to environmental changes caused by pressure treatment (Shigehisa et al., 1999). The nature of substrates can also influence the effect of pressure on microbial inactivation. For example, pressure

treatment at 20°C in UHT milk offers more protection to *E. coli* and *L. monocytogenes* than in poultry meat as reported by Patterson et al. (1999). Listerial strain was found to be more pressure resistant in liquid UHT dairy cream than in mince beef (Carlez et al., 1993; Raffalli et al., 1994). Mussa et al. (1998) reported the destruction of *L. monocytogenes* Scott A and indigenous microorganism present in milk. They found the pathogens to be more pressure resistant than the indigenous micro-organisms and recommended using the destruction of *L. monocytogenes* as the criterion for pasteurization of milk. In high pressure treatments, process variables such as temperature and pulse time have been found to be critical factors to achieve sterility (Meyer et al., 2000).

The objective of this study were to investigate the effect of high pressure inactivation of *E. coli* and *L. monocytogenes* in milk, and to compare the influence of process variables such as temperature and type of milk on their destruction kinetics.

Materials and Methods

Bacterial strain and culture conditions

Freeze dried strain of *E. coli* O157:H7 and *Listeria monocytogenes* strain Scott A were obtained by Dr. Smith's laboratory (McGill University, Department of Food Science). Cultures were prepared by inoculating a loop full of frozen cultures in glycerol into MOX agar (OXOID CM 856, Listeria selective agar base (Oxford formulation) with added SR 140 E (Listeria supplement), Oxford, Toronto, Canada), and incubating at 37°C for 48 h. This culture was used to prepare a subculture by inoculating 1 colony of this first culture into 10 ml of fresh BHI broth (Brain Heart Infusion; Difco 237500; USA.) and incubating for 24 h at 37°C. After the incubation, the cell population reached about 10^9 CFU/mL (stock culture).

Preparation of samples

Raw milk was obtained from the Macdonald campus (McGill University) dairy farm, and commercial UHT milk (2% fat) was obtained from a local supermarket and placed in a sterile glass bottle and stored at 4°C. Test samples were prepared by inoculating 1 mL *L. monocytogenes* or pathogen *E. coli* O157:H7 stock culture in 99 mL of milk to obtain approximately 10^7 CFU/mL cell concentration. Aliquotes of inoculated milk

samples were transferred to dual peel sterilization pouches (Nasco Plastic, New Hamburg, ON) and sealed. Each pouch contained about 10 mL inoculated test sample in raw or UHT milk before pressure treatment. An untreated control was kept in each case to evaluate the initial count. Inoculated milk samples were stored in ice bath for 3 h before high pressure treatment to allow the cells to acclimatise to the new environment.

High Pressure treatment

Pasteurized milk obtained from a local market was inoculated with *E. coli* K12 and subjected to various pressure treatments (200- 400 MPa) with various holding times (0-80 min). The destruction kinetics of *E. coli* was studied at room temperature. Since compression heat will increase the temperature of the pressure medium by about 3°C per 100 MPa, the pressure chamber and media were temperature conditioned with a continuous circulation of temperature-controlled water around the pressure chamber. To ensure that the sample temperature was below 25°C (i.e., the point at which thermal destruction of microorganisms might occur, Basak and Ramaswamy 2001), the pressure medium initial temperature was set at 13, 15 and 18°C for treatment at 400, 300, 200 MPa, respectively, before the start of the pressure treatment. With water circulation around the chamber, thermocouple was used to sense temperature of the pressurizing fluid. The prepared samples were then subjected to various combinations of high-pressure treatment, at 200 - 400 MPa for different holding times. Each experiment was replicated three times with duplicate analysis in each replication. The enumeration results were averaged from 4 measurements (2 samples / treatment and two plates/dilution).

Kinetic Data Analysis

Pressure destruction during the hold time was modeled based on the first order rate kinetics:

$$\mathrm{Ln}\,(N/N_o) = -kt \qquad (5.1)$$

where N = survivor count after a pressure treatment for time t (min), N_o = initial count before pressure treatment or at zero time, and k = reaction rate constant (min^{-1}). The treatment time at any given pressure that will result in 90% destruction of cells, i.e. resulting in one decimal reduction in the survivors, is referred to as the decimal reduction time or D value. This was obtained as the negative reciprocal slope of the $\log_{10}(N/N_o)$ *vs.*

time (or time taken to traverse one logarithmic cycle) and is therefore reciprocally related to k:

$$D = 2.303/k \qquad (5.2)$$

when D values at different pressures are plotted on a semi-logarithmic scale, the pressure range for one log-cycle change in D values represents the z value. Thus, the pressure z value (z_p) of the process is defined as the pressure range between which the D values change by factor of ten. The z (z_p) value can be expressed by the following equation:

$$z_p = [P_2 - P_1] / [\log D_{P1} - \log D_{P2}] \qquad (5.3)$$

This is analogous to treatment of thermal death time (TDT) data of microorganisms widely used in thermal process calculations. The z_p value was calculated from the regression of $\log_{10}$ (D) *vs.* pressure data as the negative reciprocal of the slope.

The relative effect of pressure pulse (PE) and pressure hold-time was assessed using N_D values (Mussa, 1999). N_D, which represents the number of pressure cycles required to achieve one decimal reduction in enzyme activity (thus equivalent to a D value), can be calculated as follows:

$$N_D = 1 / (PE) \qquad (5.4)$$

D_P or decimal reduction time equivalent of PE, represents the holding time in minutes at a given pressure level which results in an equivalent inactivation achieved by one pressure cycle. These values were obtained as follows:

$$D_P = D \text{ value} * [(PE)] \qquad (5.5)$$

Microbiological assay

After pre-pressure treatments, appropriate decimal dilutions in peptone water diluents were prepared from each sample, and the number of survivors in the pressure-treated samples and the untreated control were enumerated by the spread plate method

(Collins & Lyne, 1976), by plating 0.1 ml on duplicate plates. For *L. monocytogenes* the MOX agar {OXOID CM 856; Listeria selective agar base (Oxford formulation); with added SR 140 E Listeria supplement} was used and the plates were incubated for 48 h at 37°C; for *E. coli* O157:H7, the media was same as for non-pathogenic *E. coli* K-12 described previously (in Chapter 3). The above procedure was carried out in duplicate and each dilution plated on two plates for each pressure-time combinations for both strains.

Results and Discussion

Destruction kinetics of pathogenic *E.. coli* O157:H7

From the studies detailed in Chapters 3 and 4, the D value of *E. coli* was expected be in the 5 - 15 min range between 300 and 400 MPa. Since the pathogenic strain was to be completely eliminated by pressure processing, the pressure treatments were kept relatively long (40-80 min). In the initial studies the commercial UHT (2%) milk was used as the base, but then since the application would be intended for raw milk the experiments were repeated in raw milk. The survivor counts of *E. coli* O157:H7 in raw and UHT milk as affected by pressure and treatment time are shown in Figures 5.1 and Kinetic details and D values are summarized in Table 5.1. The results show that high pressure had a considerable effect on the inactivation of pathogenic *E. coli* in both media. The destruction increased with pressure and treatment time.

For example, the microbial destruction in UHT milk samples was 7 log-cycles with at 400 MPa after a 40 min treatment, 5 log-cycles after 60 min at 350 MPa and only 3 log-cycles after 80 min at 200 MPa. Thus even 50-100% longer holding times were not adequate to achieve a similar destruction when the pressure was dropped from 400 to 300 to 200 MPa. Combination effect of different treatment times and pressures could bring about equivalent lethal effects: higher pressure shorter holding time, and lower pressure longer holding time.

The D-values showed a decreasing trend with a pressure increase, as expected (Table 5.1). The relatively high R^2 indicate the pressure destruction to be well fit by the first-order kinetics rate, as has been reported by several researchers (Styles et al., 1991; Szezawinski et al., 1996; Mussa and Ramaswamy, 1997; Mussa et al., 1999). The authors

used different media, foods and various types of microorganisms. The D-values found in this study were 6.5 - 23.4 min at 400 - 300 MPa in UHT milk and 12.6 - 35.2 min, respectively, in raw milk. Thus the *E. coli* is sensitive to pressure applications in UHT milk than in raw milk. Perhaps some components formed during the UHT process is responsible for sensitizing the pressure destruction, or perhaps the presence of other microorganisms in raw milk might have intervened in the pressure destruction of E coli in raw milk although a species specific medium was used for enumeration.

The associated D values found for the pathogenic strains were considerably higher than those found for the non-pathogenic *E. coli* as detailed in previous two sections. Such behaviour was previously reported by Mussa (1999) for *L. monocytogenes* in raw milk. The z-value plot is illustrated in Figure 5.3 and the computed z-values were: 179 and 196 MPa in UHT and raw milk, respectively.

Pressure pulse effect on *E. coli* destruction

As observed in the previous studies, the dual effect pressure destruction was evident in this study as well. As can be observed from Figures 5.1 and 5.2, although the destruction followed the first order model, it is only after an initial drop in counts as result of the pressure pulse. The pressure pulse effect can be seen in the reduced counts at zero time which are values following a pressure pulse (zero hold time). The PE values and other pressure pulse related values are listed in Table 5.2 and 5.3. These data are somewhat similar to those described in Chapter 4.

High pressure inactivation of L. monocytogenes in milk

High pressure inactivation of *L. monocytogenes* in UHT and raw milk samples were investigated. The treatments were performed at 400 MPa for 0 to 90 min. The residual survivors for the pressure destruction of *L. monocytogenes* for in both milks are shown in Figure 5.4 and the kinetic data are summarized in Table 5.4. The results confirm the first order nature of destruction again; however the pulse effect was not evident since the lines almost originated from the origin. At an intermediate holding time of 60 min at 400 MPa, the destruction of *L. monocytogenes* was about 50% higher (4.7 log cycle reductions) in UHT milk as compared with that in raw milk (3.2 log cycles). Therefore, as observed with *E. coli* the UHT milk again appeared to be a more sensitive medium for *L. monocytogenes.* These are again evident from the associated D values: 12.8 min in UHT milk and 16.9 min

in raw milk. The associated D value for *L. monocytogenes* also is much larger than that for the pathogenic *E. coli* and hence *L. monocytogenes* should be a better target for establishing the process.

Table 5.1 *E. coli* survivor data and computed D value of *E. coli* O157:H7 in UHT (2%fat) and raw milk

Pressure (MPa)	Product	Time (min)	Log(N/N0)	D value (min)	R^2
400 MPa	UHT	0	-0.6	6.5	0.94
		12	-3.35		
		20	-4.95		
		30	-5.95		
		40	-6.95		
	Raw	0	-0.5	12.6	0.87
		10	-2.6		
		20	-2.55		
		30	-3.19		
		40	-4.17		
350 MPa	UHT	0	0	11	0.98
		15	-3.32		
		30	-3.98		
		45	-5.2		
		60	-5.85		
	Raw	0	0	22.5	0.85
		15	-1.94		
		30	-2.4		
		45	-2.64		
		60	-2.98		
300 MPa	UHT	0	0	23.4	0.90
		20	-0.94		
		40	-1.89		
		70	-2.73		
		80	-3.67		
	Raw	0	0	35.2	0.92
		20	-0.27		
		40	-1.55		
		70	-2.02		
		80	-2.09		

Table 5.2 Pressure pulse effect related parameters for *E. coli* in UHT milk

Pressure	PE (log-cycle reductions)	N_D (1/PE) (number of pulses)	D (min)	D_P (D x PE) (min)	$(D-D_P)$ (time saving) (min)
400	1.1	0.9	6.5	7.15	-0.65
350	0.9	1.11	11	9.9	1.1
300	0.26	3.84	23.4	6.08	16.9

Table 5.3 Pressure pulse effect related parameters for *E. coli* in raw milk

Pressure	PE (log-cycle reductions)	N_D (1/PE) (number of pulses)	D (min)	D_P (D x PE) (min)	$(D-D_P)$ (time saving) (min)
400	0.9	1.1	12.6	11.3	1.3
350	0.7	1.4	22.5	15.8	6.7
200	0.16	6.3	35.2	5.6	29.6

Table 5.4 HPP log reduction values for *L. monocytogenes* in UHT and raw milk samples at 400 MPa at various holding times.

Time (min)	UHT milk log(N/No)	Raw milk log(N/No)	UHT milk	Raw milk
0	0	0		
15	-0.9	-0.4	D value (min)	D value (min)
30	-2.04	-1.63	12.8	16.9
45	-3.15	-2.23	$R^2 = 0.99$	$R^2 = 0.98$
60	-4.69	-3.16		
75	-6.39	-4.35		
90	-6.69	-4.89		

These D values for *L. monocytogenes* found in this study are higher than those reported by Mussa et al. (1999) and Patterson et al. (1999) reported a D-value of 9.3 min for inactivating *L. monocytogenes* in raw milk However, *L. monocytogenes* was reported to be more pressure-resistant in liquid UHT diary cream (Carlez et al., 1993; Raffalli et al., 1994), than to *Escherichia coli* at the applied pressures.

High pressure destruction of L. monocytogenes as affected by temperature

The effect of temperature on high-pressure inactivation of *L. monocytogenes* was investigated and the results are shown in Figure 5.5. The effect of temperature (20-35 °C) on the pressure inactivation of *L. monocytogenes* was not significant ($p>0.05$) as could be observed from the curves in Figure 5.4. These were also evident from the computed D values at 20 and 35°C: 14.7 and 15 min at 400 MPa; 53 and 55 min at 300 MPa and 53 and 56 min at 300 MPa, respectively. Generally, the microbial inactivation by high-pressure treatment was markedly affected by temperature, but temperature sensitivity depends on the type of microorganism and the pressure levels. Patterson and Kilpatrick (1998) reported the lethality of the combined effect of temperature and pressure on the inactivation of *E. coli* to be significant above 200 MPa and below this pressure the sub lethal temperature had to be greater than or equal to 55°C. However, *Listeria* exhibited a much greater resistance to HP inactivation as reported by (Mussa, et al, 1999, Simpson and Gilmour, 1997).

Conclusions

Pressure treatment caused greater microbial inactivation in UHT milk than raw milk for both pathogenic *E. coli* O157:H7 and *L. monocytogenes*. The destruction pattern was again described as dual behavior demonstrating pulse and hold effects. The pathogenic strain *E. Coli* were more resistant than the non-pathogenic one. *E. coli* O157:H7 was more pressure sensitive than *L. monocytogenes* for high pressure destruction. The temperature (20 to 35°C) effect on pressure destruction of *Listeria monocytogenes* in milk was not significant.

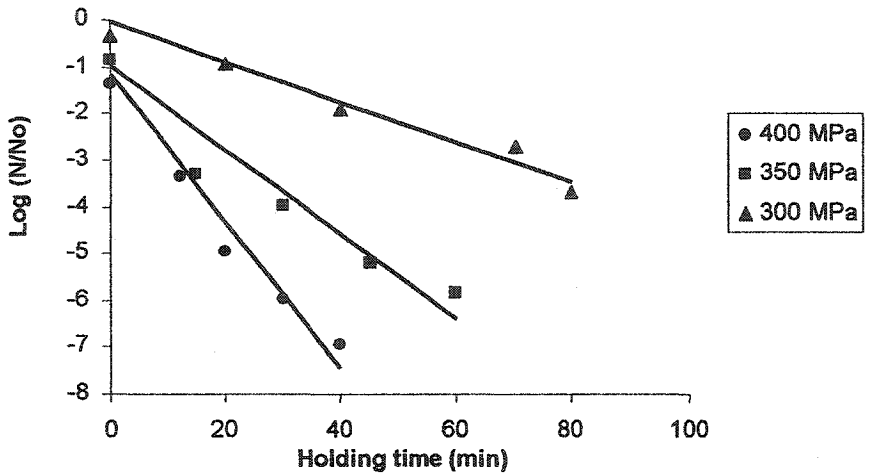

Figure 5.1 Effect of pressure level and treatment time on the destruction of Pathogenic *E. coli* O157:H7 in UHT milk

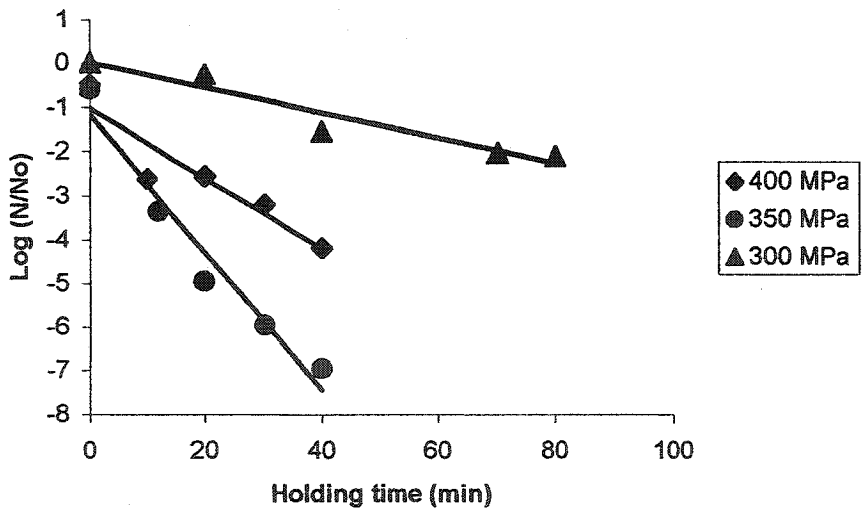

Figure 5.2 Effect of pressure level and treatment time on the destruction of Pathogenic *E. coli* O 157 H: 7 in raw milk

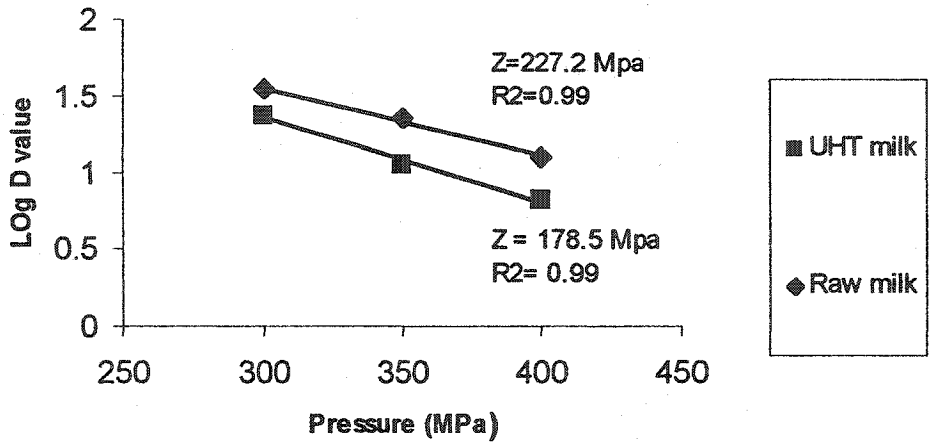

Figure 5.3 Pressure sensitivity of pathogenic *Escherichia coli* in UHT milk (2% fat) and raw milk

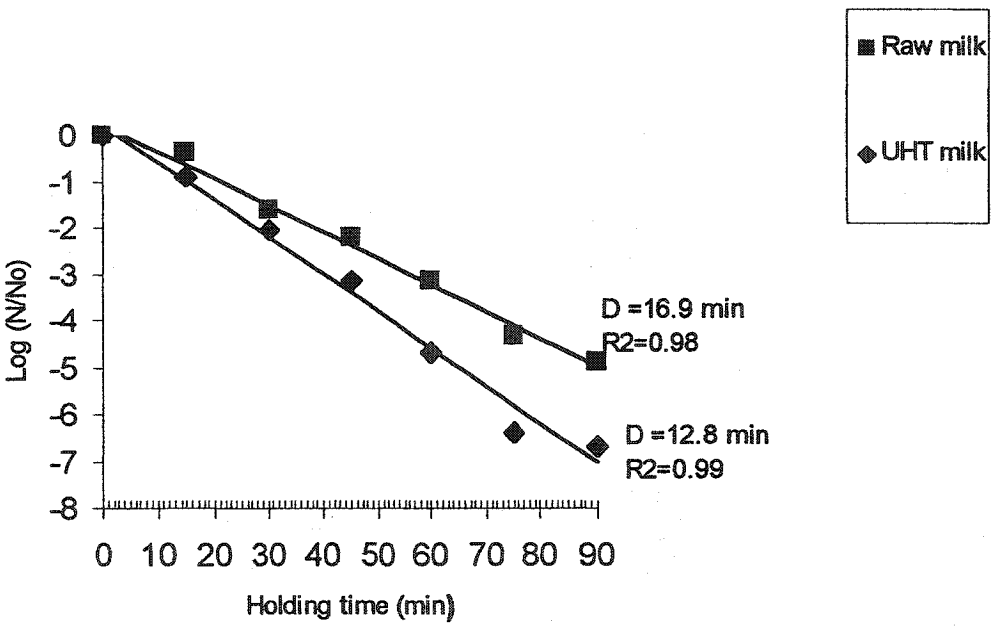

Figure 5.4 High pressure (400 Mpa) inactivation *Listeria monocytogenes* in UHT and raw milk samples.

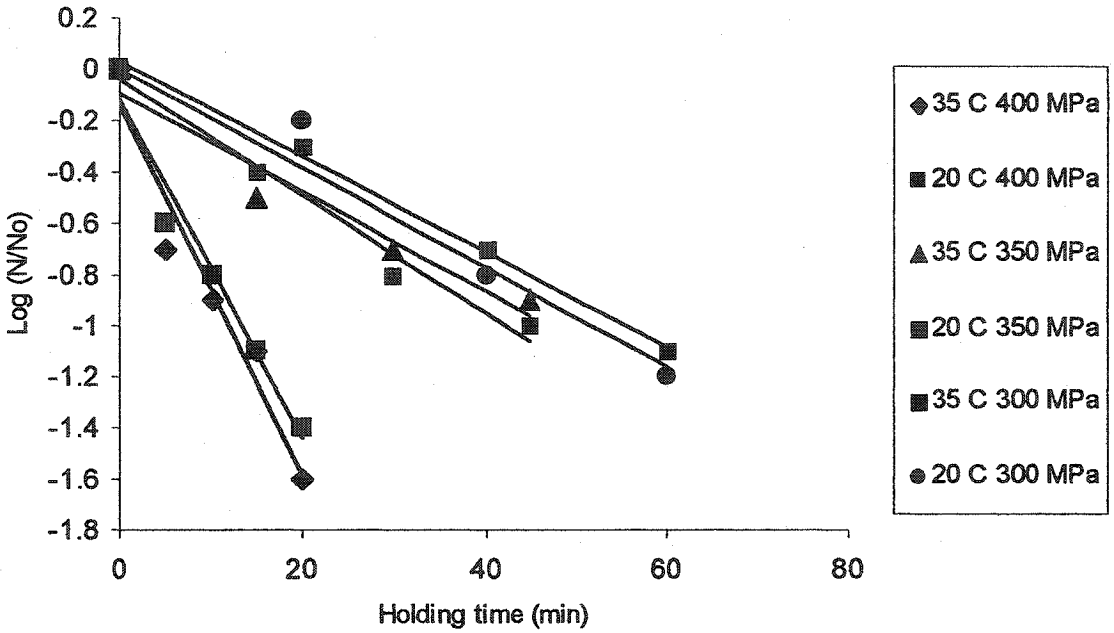

Figure 5.5 High pressure inactivation of *Listeria monocytogenes* at various pressure levels and temperature (20 and 35°C).

CHAPTER 6
GENERAL CONCLUSIONS

Kinetics of HP destruction of microorganisms in milk was the focus of this study. Only limited information is available in the literature on the effect of milk composition on microbial destruction. Some reports demonstrate that milk show a baro-protective effect for high pressure inactivation of microorganisms, but effect is not due to fat content.

The HP destruction of microorganisms (*Escherichia coli K-12)* in milk as affected by milk compositions (fat, casein and lactose) was the first challenge of this study. In this study the effect of microbial inactivation in buffer solutions, peptone water and milk were subsequently tested. Milk had baro-protective effect on HP inactivation of *E. coli,* although no significant difference in protective effect in milk with different fat contents (0-5%) or between milk and milk supplemented with casein and lactose (2-8%). Natural milk has casein and lactose in the range 2-4% which may offer protective effect on the destruction of *E. coli*. But increase of casein and lactose beyond what was already present in milk did not show an increase in protective effect. Hence, further studies were conducted to investigate the protective threshold limits of casein and lactose, by adding lower concentrations of these to buffer. This revealed that when 1% casein was added to buffer it produced significant baro-protective effect. Addition of 1% lactose to the buffer solution also showed similar effects, albeit to a less significant degree. This suggests that low concentrations of casein and lactose add to baro-protection while higher levels do not increase the level of protection further.

The kinetics of pressure destruction and pulse effects were studied on *E. coli* during HP treatment. The result of the study shows a first order kinetics model. The D-value for the different pressure levels decreased as pressure increased. A D-value of 4, 13, 70 min was observed at 400, 300 and 200 MPa at 20°C. This value is higher than most values reported in the literature. The study also demonstrated a biphasic behavior of pressure destruction characterized by a step-change in the number of survivors with application of pressure pulse (pressurization and de-pressurization). The pulse mode had a noticeably high time advantage with respect to microbial inactivation at lower pressures.

High pressure had lethal effect on pathogens *(E. coli* and *Listeria momocytogenes)*. The lethality increased with pressure and holding time. The inactivation of pathogen data had a good fit to the log-linear model, thus first order reaction kinetic model was observed. Higher lethality was observed in UHT processed milk than in raw milk. The inactivation of *Listeria monocytogenes* as affected by temperature was also investigated. Temperature range of 20- 35 °C did not contribute immensely to the pressure inactivation of pathogens. The pathogenic *E. coli* 0157:H7 was more pressure resistant than the non-pathogenic strain and *L. monocytogenes* was more pressure resistant than *E. coli.*

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McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: H.S. Ramaswamy TELEPHONE: 398-7919

ADDRESS: Macdonald Campus FAX NUMBER: 398-7977

E-MAIL: ramaswamy@macdonald.mcgill.ca

DEPARTMENT: Food Science & Agricultural Chemistry

PROJECT TITLE: Novel concepts in high pressure processing of foods

2. FUNDING SOURCE: MRC ☐ NSERC ☒ NIH ☐ FCAR ☐ FRSQ ☐

INTERNAL ☐ OTHER ☐ (specify) _____

Grant No.: G201010 Beginning date Nov. 2000 End date October 2003
NSERC STPGP 235021-00

3. Indicate if this is

☒ Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date October 2003

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency _____ Approval End Date _____

☐ New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

Containment Level (circle 1): 1 (2) 3 4

Principal Investigator or course director: _____ (SIGNATURE) date: Nov 5/2002 (day month year)

Chairperson, Biohazards Committee: _____ (SIGNATURE) date: _____ (day month year)

Approved period: _____ beginning _____ (day month year) ending _____ (day month year)

* as defined in the "McGill Laboratory Biosafety manual"

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
Dr. H. Ramaswamy	Food Science & Ag. Chem	✓				
Dr. Jim Smith	Food Science & Ag. Chem	✓				
Hong Jin	Food Science & Ag. Chem				✓	
Mr. Bernard Cayouette	Food Science & Ag. Chem		✓			

5. EMERGENCY: Person(s) designated to handle emergencies

Name: Dr. Jim Smith Phone No: work: 398-7923/8623 home: 457-2262

Name: Mr. Bernard Cayouette Phone No: work: 398-8623 home: 527-0364

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues) & designated biosafety risk group

Bacteria: *Listeria monocytogenes* Scott A (Risk Group 2)

Escherichia coli 0157 (Risk Group 2)

ii) the procedures involving biohazards

All stock cultures are prepared from frozen cultures in glycerol and grown overnight at 30 to 37°C by Mr. Bernard Cayouette. Working cultures (~ 10 mL) are prepared the following day and food (milk, cheese, ~50g) are inoculated, in duplicate, in double lined plastic bags with various levels of these pathogens to give final inoculum levels of 10^2-10^4CFU/g. The inoculated samples are transferred to the Pilot Plant in a Styrofoam© container with ice where they are subjected to various high pressure treatments. After each high pressure cycle, the bags are returned to the lab, again in a Styrofoam© container, for microbiological analysis. Plates are incubated under selected conditions and enumerated within 24-48 hours. All contaminated containers, plates, dilution bottles etc., are sterilized by autoclaving prior to discarding and are clearly identified as "Autoclaved and Sterilized before pick-up by janitorial staff. Preparation of all cultures, dilutions and plates, as well as inoculation of all food samples, are done under aseptic conditions in a biological safety cabinet. All staff/students wear face masks, lab coats and rubber gloves during all microbiological procedures. No pipetting is done by mouth. Benches are routinely washed with a 1% hypochlorite solution at the end of each day while the safety cabinet is swabbed with 70% ethanol and the UV light left on overnight.

iii)

iv) the protocol for decontaminating spills

Spills seldom occur in our laboratory since good housekeeping is encouraged and monitored on a regular basis to prevent such events. However, in the event of a spill, the protocol for decontaminating spills of type 2 microorganisms is as outlined in both the McGill and Department laboratory safety manuals. If the spill occurs on the bench, it is absorbed by the white paper bench cloth routinely placed on each bench. The cloth is removed and the "contaminated" area soaked with 1% hypochlorite for ~ 30 mins and then wiped dry. Then the area is then wiped with paper towels soaked with 70% ethanol. If the spill occurs in the safety cabinet, the spill is wiped up and then the area is soaked with 70% ethanol for ~ 30 minutes, dryed with clean paper towels and the U.V. light left on for ~ 1 hour prior to resumption of work.. Students/staff do not enter into the lab for at least 1 hour after a spill. All towels, contaminated paper cloths etc, lab coats, masks are placed in autoclavable bags and decontaminated by sterilization. All bags are clearly labelled "Autoclaved and Sterilized.

If a spill occurs on the body, the clothing is removed and sterilized. Splashes to the face are washed with germicidal soap and hot water. All spills are reported to the laboratory supervisor and a follow up session is done with the students to go over the cause(s) of the spill and to re-enforce preventive measures.

Copies of the McGill and departmental safety protocols are in the lab at all times for perusal by the students. Furthermore, most of the work done in our laboratory is under the constant supervision of Mr. Bernard Cayouette.

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)?

No. Cultures are prepared in 10 mL amounts, in triplicate, for use in the inoculation studies.

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

N/A

9. What precautions are being taken to reduce production of infectious droplets and aerosols?

All inoculation/enumeration procedures are done under the strictest of aseptic conditions in a biological safety cabinet. All students/staff were appropriate protective clothing when handling cultures or inoculated foods.

10. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
Macdonald Stewart	1-055	Labconco	36205-04	247196	11/09/01

Equipment Certification

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- ✓ NSF STD. 49
- ✓ CAN/CSA-Z 316.3-95
- ◯ MANUFACTURER'S SPECS.
- ◯ OTHER

Performed by: [signature]

Date: NOV. 8 2001

NSF Next Inspection Recommended

Date: NOV. 2002

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McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: H.S. Ramaswamy TELEPHONE:

   ADDRESS: Macdonald Campus FAX NUMBER:

   E-MAIL:

   DEPARTMENT: Food Science & Agricultural Chemistry

   PROJECT TITLE: Microbiological and process modeling for promoting safety and quality of high pressure treated foods

2. FUNDING SOURCE: MRC ☐ NSERC ☐ NIH ☐ FCAR ☐ FRSQ ☐

   INTERNAL ☐ OTHER ☐ (specify) COPPAO

   Grant No.: C201658 / CORPAO-101014 Beginning date April 2001 End date March 2004

3. Indicate if this is

   ☒ Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
   Approval End Date March 2004

   ☐ New funding source: project previously reviewed and approved under an application to another agency.

   Agency _____ Approval End Date _____

   ☐ New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

Containment Level (circle 1): 1 (2) 3 P4

Principal Investigator or course director: _____ date: NOV/2001 (day month year)

Chairperson, Biohazards Committee: _____ SIGNATURE date: 25 11 02 (day month year)

Approved period: _____ beginning 25 11 02 (day month year) ending 31 03 04 (day month year)

* as defined in the "McGill Laboratory Biosafety manual"

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

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1. PRINCIPAL INVESTIGATOR: M.S. Ramaswamy TELEPHONE:

   ADDRESS: Macdonald Campus FAX NUMBER:

   E-MAIL:

   DEPARTMENT: Food Science & Agricultural Chemistry

   PROJECT TITLE: Microbiological and process modeling for promoting safety and quality of high pressure treated foods

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Containment Level (circle 1): 1 (2) 3

Principal Investigator or course director: _____ date: Nov 5/2001
(day month year)

Chairperson, Biohazards Committee: _____ date: _____
SIGNATURE (day month year)

Approved period: _____ beginning _____ ending _____
(day month year) (day month year)

* as defined in the "McGill Laboratory Biosafety manual"

2nd REVISION, JANUARY 1996

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
Dr. H. Ramaswamy	Food Science & Ag. Chem	✓				
Dr. Jim Smith	Food Science & Ag. Chem	✓				
Hong Jin	Food Science & Ag. Chem				✓	
Mr. Bernard Cayouette	Food Science & Ag. Chem		✓			

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Bacteria: *Listeria monocytogenes* Scott A (Risk Group 2)

Escherichia coli 0157 (Risk Group 2)

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Macdonald Stewart	1-055	Labconco	36205-04	247196	11/09/01

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- ◯ MANUFACTURER'S SPECS.

Performed by:

Date: NOV. 8 2001

NSF Next Inspection Recommended

Date: NOV. 2002

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