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EFFECT OF COMPRESSION AND DECOMPRESSION RATES OF HIGH HYDROSTATIC PRESSURE PROCESSING ON INACTIVATION OF MICROORGANISMS IN DIFFERENT MATRICES

THESIS SUBMITTED TO FULFILL THE REQUIREMENT FOR DEGREE OF DOCTOR OF PHILOSPHY (PhD) IN FOOD SCIENCE & TECHNLOGY.

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HACEN CONSTAR: que el Licenciado en Ciència i Tecnologia dels Aliments SYED QAMAR ABBAS ha realizado, bajo nuestra dirección, en el Centre Especial de Recerca Planta de Tecnologia dels Aliments (CERPTA), Departamento de Ciencia Animal y de los Alimentos de la Universitat Autònoma de Barcelona, el trabajo titulado ¨EFFECT OF COMPRESSION AND DECOMPRESSION RATES OF HIGH HYDROSTATIC PRESSURE PROCESSING ON INACTIVATION OF MICROORGANISMS IN DIFFERENT MATRICES¨ que presenta para optar al grado de doctor, considerándolo acabado, autorizamos su presentación para que sea juzgada por la comisión correspondiente.

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SUMMARY

The effect of compression and decompression rates of high hydrostatic pressure (HHP) for inactivation of bacteria has been scarcely studied. The available literature presented contradictory results. This study was designed to analyze the effect of different rates of compression and decompression during HHP treatments of selected matrices (Tris buffer, skimmed milk and orange juice) inoculated with selected strains of vegetative (*Escherichia coli* & *Staphylococcus aureus*) and spore forming (*Bacillus subtilis*) bacteria. The HHP experiments were conducted using different HHP machines in different research centers and samples were analyzed by microbiological and biotechnological procedures. Results revealed that higher inactivation of vegetative bacteria can be achieved by using fast compression rates during HHP processing. While slow compression is supposed to induce a stress response in microbial cells that leads to lower inactivation effect of the process. On the other hand, bacterial spores are more sensitive to slow compression rates during HHP processing. Under our experimental conditions slow decompression resulted in higher inactivation of vegetative and spore forming bacteria as compared to fast decompression. Slow decompression is attributed with extended processing time. When cells are sublethally injured by compression treatments and pressure holding time, they become more sensitive to upcoming processing stages. Further studies to investigate the sublethal injuries, revealed that fast compression and slow decompression resulted in highest number of sublethally injured cells in all microorganisms. A part of this sublethally injured population is able recover their injuries in low acid environment (like milk and Tris buffer) and may challenge the food safety.

While in high acid environment (like orange juice), these sublethally injured bacterial are unable to recover and become dead during 15 days storage.

RESUMEN

El efecto de las tasas de compresión y descompresión a altas presiones hidrostáticas (HHP) en la inactivación de bacterias ha sido poco estudiado. La literatura disponible presenta resultados contradictorios. Este estudio se diseñó para analizar el efecto de diferentes tasas de compresión y descompresión durante los tratamientos de HHP sobre matrices seleccionadas (tampón Tris, leche desnatada y zumo de naranja) inoculados con cepas bacterias seleccionadas en estado vegetativo (*Escherichia coli* y *Staphylococcus aureus*) y formadoras de esporas (*Bacillus subtilis*). Los experimentos HHP se llevaron a cabo utilizando diferentes máquinas en diferentes centros de investigación y las muestras se analizaron por procedimientos microbiológicos y biotecnológicos. Los resultados revelaron que el aumento de la inactivación de las bacterias vegetativas se puede lograr mediante el uso de la compresión rápida durante el procesamiento HHP. La compresión lenta se supone que induce una respuesta de estrés en las células microbianas que conduce a un menor efecto de inactivación del proceso. Por otro lado, las esporas bacterianas son más sensibles a la ralentización de las tasas de compresión durante el procesamiento HHP. Bajo nuestras condiciones experimentales, la descompresión lenta dio como resultado una mayor inactivación de las bacterias formadoras de esporas y de las células vegetativas en comparación con la descompresión rápida. Una descompresión lenta se relaciona con un tiempo de procesamiento prolongado, causa las células ya han recibido un daño subletal durante la fase de compresión y de mantenimiento de presión resultan más sensibles al tratamiento prolongado. Estudios adicionales realizados para investigar los daños subletales, revelaron que la compresión rápida y

descompresión lenta causaron un mayor número de células con daño subletal en todos los microorganismos estudiados. Una parte de esta población con daño subletal es capaz de recuperar sus lesiones en el entorno de baja acidez (como la leche y el tampón Tris) y puede comprometer la seguridad alimentaria. Si bien en el ambiente de elevada acidez (como el jugo de naranja), estas bacterias subletal no pueden recuperarse y terminan muriendo durante los15 días de almacenamiento.

CHAPTER 1

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INTRODUCTION AND LITERATURE REVIEW

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FACTORS AFFECTING BACTERIAL INACTIVATION DURING HIGH HYDROSTATIC PRESSURE PROCESSING OF FOODS – A REVIEW

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ABSTRACT

Although, the High Hydrostatic Pressure (HHP) technology has been gaining gradual popularity in food industry since last two decades, intensive research is needed to explore the missing information. Bacterial inactivation in food by using High Hydrostatic Pressure (HHP) applications can be enhanced by getting deeper insights of the process. Some of these aspects have been already studied in detail (like pressure, time and temperature, etc.), while some others still need to be investigated in more details (like pH, rates of compression and decompression etc.). Selection of process parameters is mainly dependent on type of matrix and target bacteria. This intensive review provides comprehensive information about the variety of aspects that can determine the bacterial inactivation potential of HHP process indicating the fields of future research on this subject including pH shifts of the pressure treated samples and critical limits of compression and decompression rates to accelerate the process efficacy.

Keywords: High Hydrostatic Pressure (HHP), bacterial inactivation, effect of matrix, compression rate, decompression rate, effect of microbial strains.

INTRODUCTION

The application of High Hydrostatic Pressure (HHP) as emerging technology in food processing has steadily increased during past 20 years and has received particular attention as a viable alternative (economically and technologically) to thermal processes (Patterson, 2005). Several products, such as high-pressure-pasteurized orange juice (France), cooked ham (Spain), oysters (UK, USA), and fruits (Germany) have been evaluated with common agreement that the HHP treatment has not caused significant changes in the composition or the structure of the products affecting their nutritional value, metabolism or the amounts of undesirable substances (Eisenbrand, 2005).

Processing of foods by HHP offers unique advantages over traditional thermal treatments, as it exerts anti-microbial affects without changing the sensory and nutritional quality of food (Juan et al., 2007). One of the principal advantages of the HHP process is the expanded shelf-life and improvement of food safety due to large inactivation of microbial population. The other major advantages of HHP for processing and preservation of foods are elimination or significant reduction of heating, thus avoiding thermal degradation of food components; high retention of flavor, color and nutritional values; uniform and instant treatment of the product under pressure; reduced requirement for chemical additives; and potential for the design of new products due to the creation of new textures, taste and functional properties (Datta & Deeth, 1999).

In spite of remarkable advantages, HHP processing is still not widely accepted by food manufacturers. That is because of high initial cost, limited research information and lack of technical guidance to the manufacturers. On the other hand, important unsolved scientific and technological problems restrain wide scale application of HHP in food industry. With respect to microbiological

safety, quantifying inactivation of important food related pathogens by HHP is most urgent and critical in the establishment of high pressure processing. Although the number of kinetic studies on HHP inactivation of microorganisms is steadily increasing, a number of issues remain unresolved.

High pressure technology should be considered as an additional option for preserving and/or processing raw materials, intermediate constituents or complete foods, as there are already in the market a significant number of HPP-treated products thanks to the existence of industrial equipments from different manufacturers. Moreover, much research is being conducted by groups located in numerous different research institutes and universities in many countries. These works cover a very wide range of aspects of applying high pressures: from the improvement and maintenance of equipments to microbiological, physicochemical, sensory and nutritional aspects of components and foods, as well as its economic aspects and environmental impact.

HHP is based on the application of two physical principles: Le Châtelier's principle and the principle of pressure transmission in a uniform and instantaneous manner. According to Le Châtelier's principle if a chemical system at equilibrium experiences a change in concentration, temperature, volume, or partial pressure, then the equilibrium shifts to counteract the imposed change and a new equilibrium is established. In addition to these principles, there are some factors that may affect the bacterial inactivation by HHP. Processing of food products by high pressure obviously implies a much higher pressure than the atmospheric one, usually in the range of 500–1000 times higher. It will, therefore, be essential to note that changes in the components will follow the thermodynamic constraints that involve working at a given pressure, which implies that one of the basics to keep in mind is that

components' phase-changes will not follow the conditions (thermal, volume) which are usually considered when working at 0.1 MPa. Therefore, an extrapolation from what happens in many processes at atmospheric pressure to what is happening in high pressure should not be expected. Changes that imply a positive variation in volume will be hindered when increasing pressure, and in the same way elevated pressure will favor processes that involve changes associated with a reduction in volume, which is known as Le Châtelier's principle. As water-phase changes (dissociation constant) are clearly different under pressure, chemical balances shift to different equilibrium values depending on pressure value (Mor-Mur and Saldo, 2012).

In present work, research information has been reviewed about effect of different factors that should be considered during application of HHP in food processing. Some of these aspects are still under investigation to discover the unknown facts and also to enhance the microbial inactivation efficiency of HHP treatments.

1- PRESSURE, TEMPERATURE & TIME MODELS

1.1-Pressure Level and Holding Time

Research has revealed that bacterial inactivation is directly proportional to the pressure level and pressure holding time. Earnshaw (1995) studied the inactivation of *Staphylococcus carnosus* by HHP in nutrient broth. He found the reductions of 0, 0.5, 1.5 and 2.5 log units at 300, 400, 500 and 600 MPa respectively, at 20 ℃ for 30 minutes. Alpas et al. (2000) reported that increasing pressurization time from 5 to 10 min at 207 MPa, had significant effect on inactivation of *S. aureus* and *L. monocytogenes* suspended in Tryptone Soy Broth with yeast extract.

Yuste et al. (2004) while working with *S. aureus* found that among different combinations of pressure and time during HHP treatments, in some cases, time influences the level of inactivation more than pressure.

Chen et al. (2012) studied the microbial survival parameters under HHP. They developed optimization algorithm to estimate survival parameters through finding the best fitting-curves to non-isobaric survival data. They used published data on *E. coli* ATCC 25922 to test the algorithm, and concluded that the optimization algorithm developed provides an accurate estimation of microbial survival parameters from non-isobaric survival data, presented in terms of microbial log reduction versus time and corresponding pressure profiles used to achieve that inactivation. Although the alogrithm was tested by using Weibull model, it should be applicable to other inactivation kinetic models since a specific inactivation kinetics model does not need to be assumed.

A number of authors proposed first-order inactivation kinetics for various bacteria (Mussa et al., 1999; Ponce et al., 1998). Gervilla et al., (1999) treated *Staphylococcus aureus* in ovine milk at different pressure-time combinations. They reported a linear decline in *S. aureus* log-count with increase in pressure level and holding time. Pressure studies (Erkmen & Dogan, 2004; Erkmen & Karatas, 1997; Van Opstal et al., 2005) indicated that at a given pressure, counts of microorganisms, including *S. aureus* (Figure 1.1) and *E. coli* O157:H7, decrease exponentially with holding time, and a decimal reduction time (*D* value) can be calculated for each organism. For example, pressure inactivation of *E. coli* MG1655 was investigated at 256 different combinations of pressure ranging from 150 to 600 MPa and temperatures in the range of 5 – 49.5 ºC. In fresh carrot juice, linear relationship was suitable to fit the

inactivation, in $\log_{10}$ values, and treatment times under all pressure-temperature combinations (R^2 –values > 0.91) (Van Opstal et al., 2005).

On the other hand, also significant deviations from linearity have been reported, sometimes for the same bacteria and food matrix (Buzrul, et al., 2008; Chen & Hoover, 2003; Tassou et al., 2008). The inactivation curves induced by milder pressure treatment rarely obey first-order model, and tailing is frequently observed. (Patterson et al., 1995).

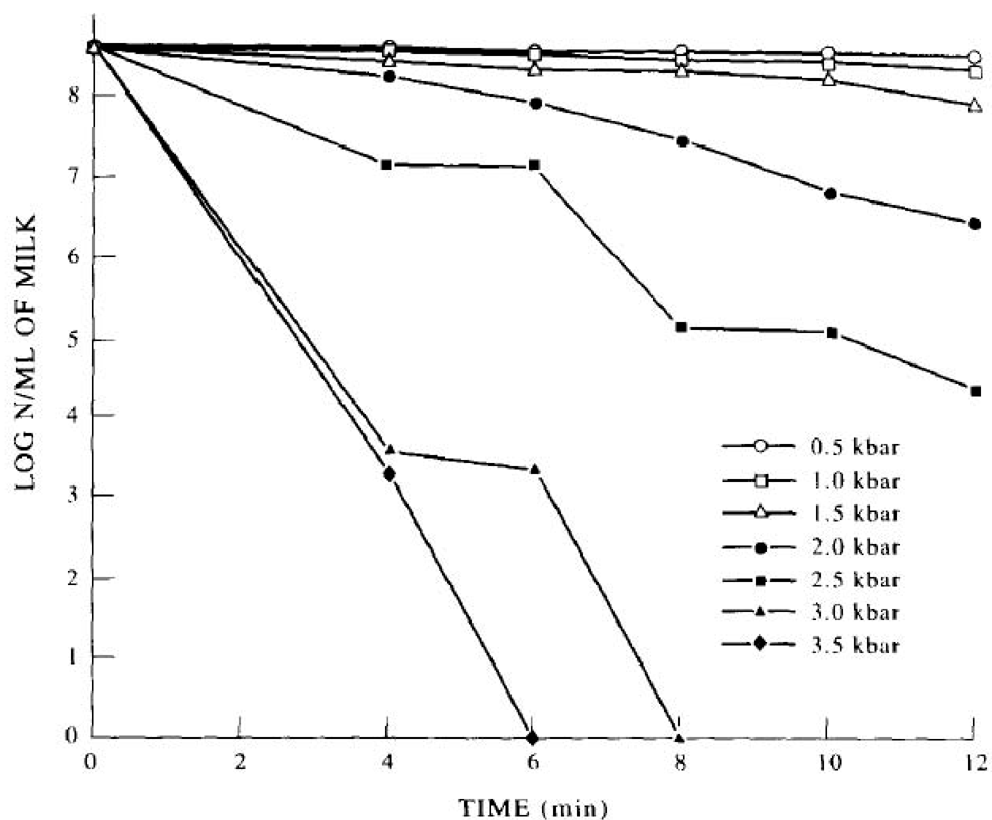


Figure 1.1. Inactivation of *S. aureus* in cow's milk at different pressure vs time combinations at 20 ºC
. Source: Erkman and Karatas, 1997 (reproduced with permission)

Typically, isobaric survival curves exhibit a tail (Ahn et al., 2007; Margosch et al, 2006; Panagou et al., 2007; Rajan et al., 2006 ; Tossou et al., 2008 ; Van Boejen et al., 2008). It can be described by either Weibull model or other nonlinear models (Ahn et al., 2007; Celik et al., 2009; Chen, 2007; Tassou et al., 2008; Van Boejen et al., 2008).

Successful use of *D*-value for defining conditions of food pasteurization relies on the inactivation rate being log linear. Neverthless, *D*-value is often calculated from a linear portion in a non-linear survival curves (Pfeifer and Kessel, 1994). Noma et al., (2006) suggested that the presence of genetically pressure-resistant subpopulation was responsible for tailing. Accorging to the authors, cytoplasmic membrane of the tail-culture cells had higher stability to pressure treatment at 100 MPa for 10 min than that of the original cells. It has been further discussed under physiological state of microorganisms.

An effective pressure inactivation of microorganisms under mild conditions requires the elucidation of the mechanism of tailing and the potential means to avoid tailing.

1.2-Treatment Temperature

Temperature is another important factor that affects the inactivation level of vegetative bacteria. It is well know that the temperature of the product and pressure fluid can affect microbial resistance, with larger inactivation rates obtained above or below the ambient temperature. In fact, elevated temperature (30-50 ºC) promotes pressure inactivation of microorganisms but the effect of low temperature (<20 ºC) on inactivation is still not clear (Alpas el at., 2000). Therefore the process efficacy is influenced by the processing temperature (Alpas et al., 2000; Bayindirli et al., 2006). Bacterial cells are relatively less

sensitive to hydrostatic pressure at 20 - 35 ºC, but become more sensitive to pressurization above 35 ºC, due to phase transition of membrane lipids (Kalchayanand et al., 1998). Cheftel (1995) correlates the decrease in resistance to pressure at low temperatures with changes in membrane structure and fluidity through weakening of hydrophobic interactions and crystallization of phospholipids. Moderate heating (40 – 60 ºC) can enhance microbial inactivation by pressure (Carlez et al., 1993), which in some cases makes it possible to get desired results at lower pressures. Patterson and Kilpatrick (1998) applied HHP to *E. coli* O157:H7 and *S. aureus* in milk and poultry. Their findings showed a practical necessity for combined use of pressure (400-500 MPa and elevated temperatures (50 ºC) for 5-6 $\log_{10}$ (cfu/g) reduction.

Pressure-temperature combinations that lead to a 5 log reduction of several pathogenic and spoilage organisms within 5 min of treatment are exemplarily shown in Figure 2. It is generally accepted that pressure and temperature act synergistically on the destruction of vegetative bacteria in the high temperature domain, which is indicated by the left end of isorate curves in Fig. 1.2 (Buckow and Heinz, 2008).

High process temperatures are mainly required when spore inactivation is concerned. The term Pressure Assisted Thermal Sterilization (PATS) is generally used while dealing with inactivation of bacterial spores (Wimalarante and Farid, 2008). Unlike vegetative cells, spores can not be killed at low temperatures during HHP processing. Bacterial endospores, as compared to vegetative cells, display a considerably higher resistance to temperature and pressure. Spores of *Clostridium botulinum* and *Bacillus* species are the key bacteria for the safety or the spoilage of low acid (heat treated) preserved goods. These spores have shown remarkable tolerance to pressures above 1000

MPa at room temperature (Margosch et al., 2006; Margosch et al., 2004). On the other hand, many other bacterial endospores, which are relevant to food, are inactivated at pressures of 600 MPa or higher in combination with an initial temperature above 60 °C (Heinz & Knorr, 2002). Often the required inactivation temperature and/or time is lowered by combination with pressure as indicated in the pressure-temperature plain of Fig.1.3 for a number of bacterial spores (Buckow and Heinz, 2008).

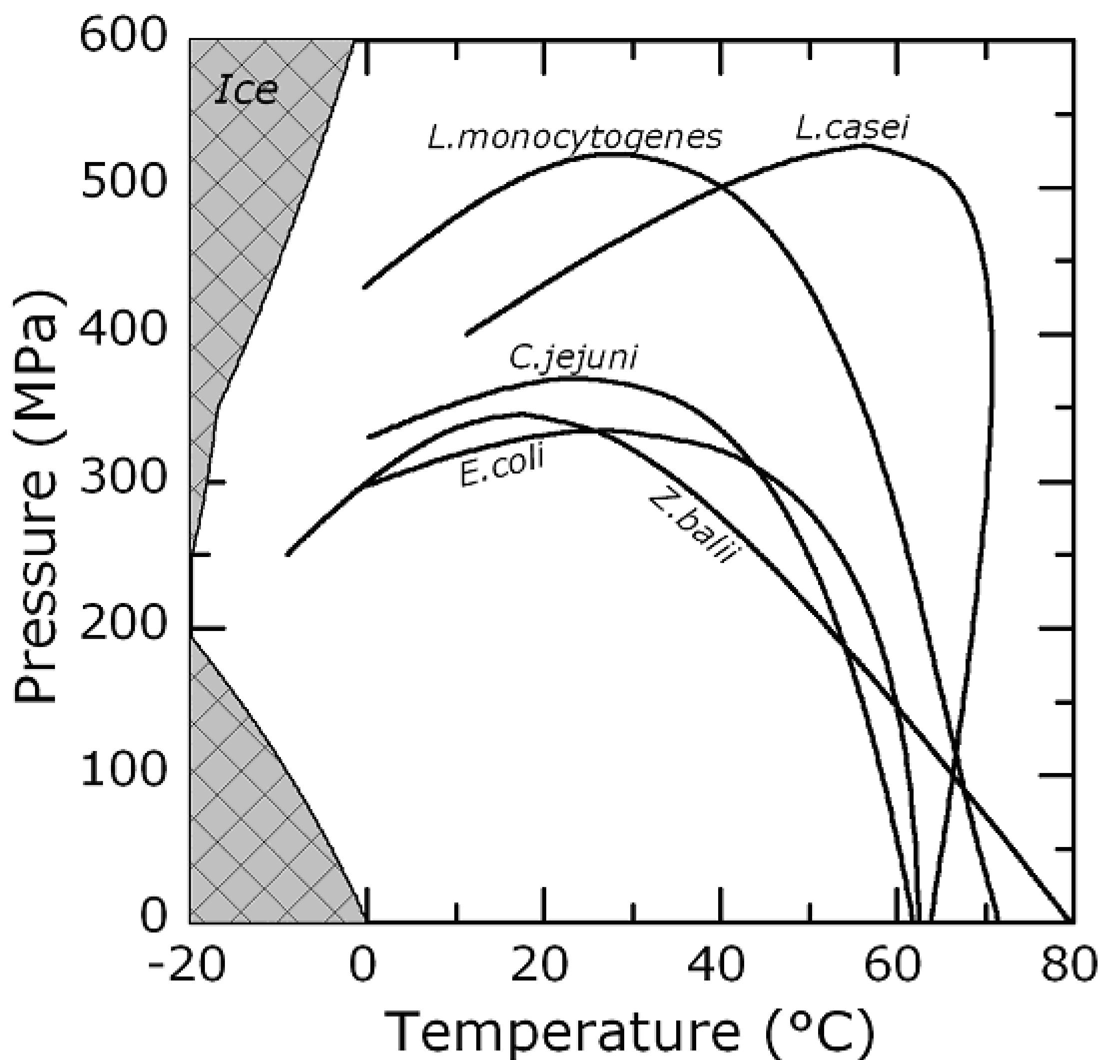


Figure 1.2. Pressure-temperature isorate diagram for 5 log inactivation of different vegetative bacteria after 5 min isothermal/isobaric treatment. Source: Buckow and Heinz (2008). Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

In dependence of the applied temperature and pressure level, bacterial endospores pass through different physiological pathways, depending on the temperature and pressure level, and this can induce spore germination or a subsequent inactivation during the treatment. In literature two- (Margosch, et al., 2006) or three-step models (Mathys, et al., 2007) for spore inactivation are discussed, but it is assumed that the spore germination is the first step of spore inactivation under pressure (Gould & Sale, 1970).

Application of HHP results in pressure-induced germination of bacterial spores (Rendueles et al., 2011) and these germinated spores could be destroyed by simultaneous use of mild heating (Clery et al., 2004; Margosch et al., 2006). *Bacillus subtilis* spores were quite resistant to 600 MPa at 20 ºC but at 60 ºC and 70 ºC the inactivation of 0.5 and 3 log cfu/ml, respectively, was observed (Syed et al. 2012).

In addition, adiabatic heating is also an important aspect while discussing microbial inactivation by pressure. Different compression media exhibit varying characteristics of heating up during compression. Pressure transmitting fluids are used for uniform transfer of pressure to the food. Many of early laboratory machines were not made from stainless steel and necessitated the use of oils as the pressure medium. Solutions of castor oil, silicone oil, water and glycol are commonly used as pressure transmitting fluids. In general it is accepted that when water is used as compression medium, physical compression results in a temperature increase of 2-3 ºC per 100 MPa (Cheftel, 1995). When organic solvents or oils are employed as pressure transmitting fluid, the temperature increase is greater than that of water due to their higher compressibility, lower thermal conductivity and lower heat capacity (Makita, 1992).

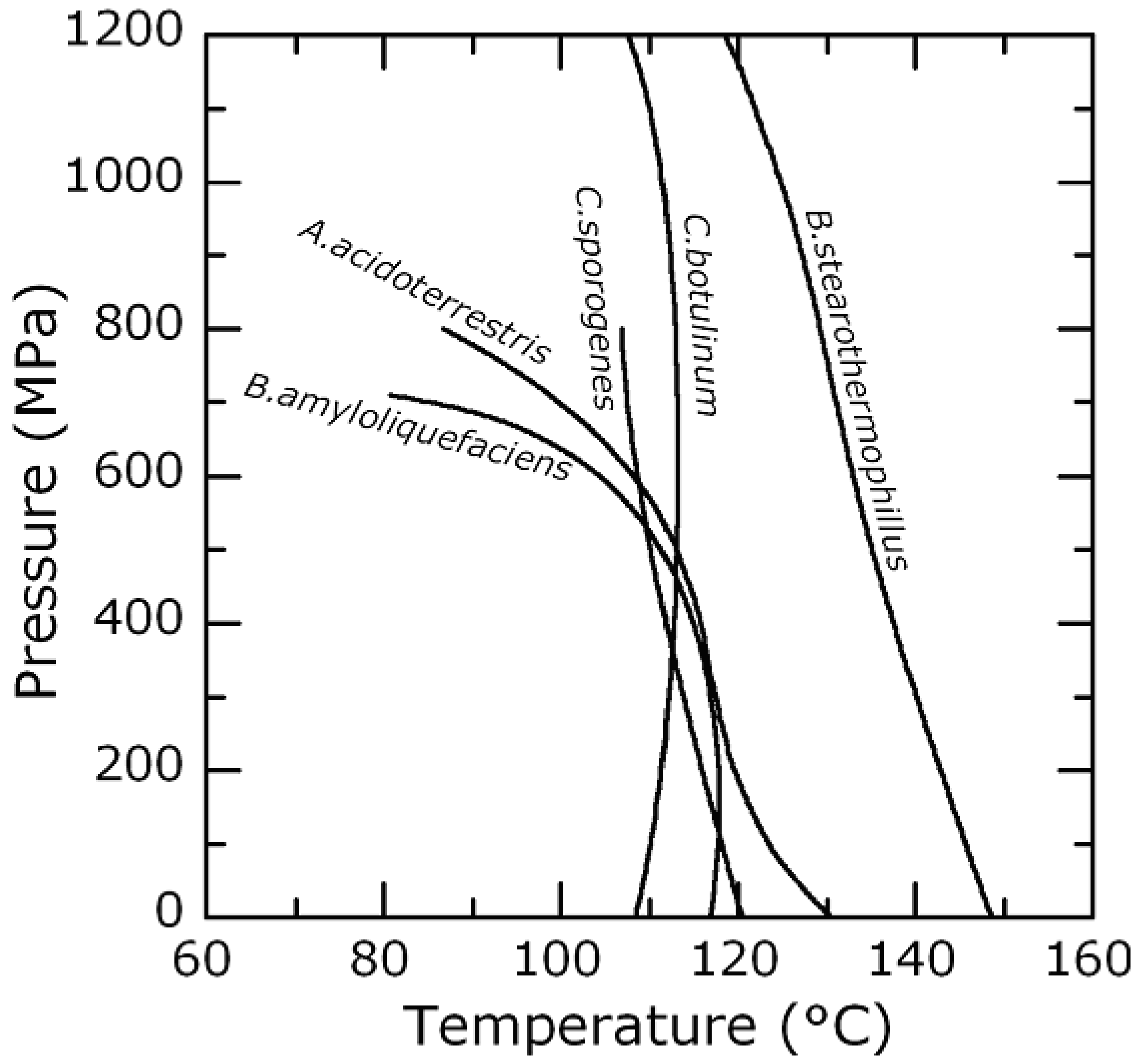


Figure 1.3. Pressure-temperature isorate diagram for 5 log inactivation of different endospores after 5 min isothermal/isobaric treatment.
Source: Buckow and Heinz (2008). Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

Difference between compressibility of compression fluid and the food sample may result in heat transfer between compression fluid and food sample and also from compression fluid to vessel walls. The potential influence of adiabatic heating characteristics of compression media on microbial inactivation is not investigated in detail. Balasubramanian & Balasubramaniam (2003) studied the adiabatic heating characteristics of different concentration of water-glycol mix. They reported that pressure transmitting fluid containing highest percentage of glycol (25:75 water-glycol) showed highest temperature increase.

The apparent temperature increase in compression medium during HHP is influenced by the target pressure, holding time, product compressibility, initial temperature and rate of heat loss to surroundings. Change in compression fluid temperature as a result of adiabatic heating and subsequent heat transfer may point out the risk of deviation from isothermic conditions and consequently the risk of non-uniform treatment, therefore should be considered carefully during HHP microbial inactivation studies. Adiabatic heat transfer to food samples and equipment vessels depends upon the sample size, packaging material, thickness and type of packaging material, thickness of equipment vessel and its heat conduction characteristics.

2-RATES OF COMPRESSION AND DECOMPRESSION

There has been very limited work done on this aspect of HHP and only a few references are available regarding the effect of rate of pressurization and depressurization on inactivation of bacterial cells. The available literature on this topic is quite contradictory and there is room for more research on this aspect.

The issue was highlighted by Smelt (1998) who assumed that a slow ramp during compression might induce a stress response of microbial cells and hence leads to a lower inactivation effect of the process. In the same way, Herdegen (1998) reported that a process of rapid pressurization (6.7 MPa/s) and slow decompression (0.83 MPa/s) was more effective than a process of same maximum pressure and holding time but reverse pressurization and depressurization rates, as cited by Rademacher et al. (2002).

Noma et al. (2002), investigated the inactivation and injury effects of HHP treatments at different pressure levels (from 70 to 400 MPa) combined with

slow and fast decompression (30 s and 1 ms, respectively). The authors concluded that a very rapid decompression procedure could enhance the injury, which causes the higher bactericidal effect of HHP treatments. On the other hand, Rademacher et al. (2002) investigated effect of pressurization ramp on inactivation kinetics of *Listeria innocua* suspended in Tris buffer by using fast pressurization ramp (8.3 MPa/s) and slow depressurization ramp (1.7 MPa/s) in comparison with slow pressurization (1.7 MPa/s) and fast depressurization (8.3 MPa/s). They concluded that rate of pressurization and depressurization in the range of 1.7-8.3 MPa/s, does not affect inactivation kinetics of *L. innocua*, if the temperature changes are negligible during pressure treatment.

While working with inactivation of *Bacillus* spores Syed et al. (2012) found that processing of 600 MPa at 60 & 70 ºC was more lethal when slow compression and slow decompression rates were used. The increased lethality of slow rates was more likely linked to increased treatment times. However, combination of fast compression with slow decompression resulted in highest number of injured/germinated population.

Some controversial results on the effect of pressure increase rate can be linked to the dissipation of the adiabatic heat generated. In non-accurately designed experiments the effect of fast pressurization may be hindered by the effect of accumulation of heat generated adiabatically, while in slow compression the heat generated may be released and does not cause a high temperature increase as in the case of fast compression. Therefore, equipment performance and operation procedures may lead to differences in the log reductions of the same strain in a similar food (Mor-Mur and Saldo, 2012).

Rupture of bacterial cells due to pressurization and depressurization is considered to be the main cause of destruction of microorganisms under

different pressure regimes (Yano et al., 1998). Ramaswamy et al. (2008) discussed the effect of pressurization and depressurization as pulsing effect. This pulse effect was explained to be due to pressurization (adiabatic compression) and depressurization (rapid expansion) that leads to rupture of cells and microbial death.

Although these results gives an idea that there is difference in bacterial inactivation by changing rates of compression and decompression, but the issues still unclear are the critical limits of compression and decompression rates. It has been reported that changing rates of compression do not have linear effect on bacterial inactivation (Syed et al. 2013; Rademacher et al. 2002); rather there would be some threshold point beyond which the inactivation efficiency of process is enhanced significantly. Those threshold limits need to be investigated for each microorganism. Furthermore, development of mathematical models to estimate the resultant inactivation of bacteria by changing rates of compression and decompression also needs to be worked out.

3-MICROBIOTA

It is generally accepted that efficiency of any treatment for bacterial inactivation is directly related to initial microbial population in the subject matrix. The high initial microbial load always results in high survival counts after treatment. Hence, initial microbial count in any matrix is a primary factor that can reduce the effectiveness of any treatment. Mostly, to overcome this issue some pretreatments like initial staining and hygienic measures are practiced.

HHP usually has a higher destructive effect in organisms with a greater degree of organization and structural complexity. Prokaryotes are usually more

resistant, compared to eukaryotes (Yuste, et al., 2001). In addition, microorganisms, including pathogens, can vary significantly in their responses to high pressure and this variation exists not only between different species but also between strains of the same species. Certain strains of *E. coli* O157:H7 were found to be particularly resistant to pressure, in a variety of substrates (Benito et al., 1999). It is demonstrated that the pressure resistance of certain natural isolates of *E. coli* O157:H7 varied greatly (Robey et al., 2001). Among the pathogenic non-spore forming gram-positive bacteria, *L. monocytogenes* and *S. aureus* are the two well studied pathogens regarding the use of HHP processing. *S. aureus* appears to have high resistance to pressure (U.S. FDA, 2000).

3.1- Gram-positive Vs Gram-negative Bacteria

The different chemical composition and structural properties of the cell membrane in Gram-positive and Gram-negative microorganisms result in differences in resistance to HHP (Russell, 2002). Gram-positive bacteria are generally more resistant compared to Gram-negative (Shigehisa et al., 1991) but there is considerable overlap. For instance, *L. dextranicum* is more sensitive to HHP than several gram-negative bacteria (*S. flexneri, E. coli* and *S. typhimurium*), and the pressure resistant mutant of *E. coli* (LMM1010), is more resistant than several gram-positive bacteria (*L. dextranicum, L. innocua*, and *L. plantarum*), particularly at pressures >300 MPa (Wuytack et al., 2002).

3.2-Physiological State of Microorganisms

Experiments in model systems show that the physiological status of microbial populations subjected to HHP processing influence pressure resistance.

Vegetative cells in the growth phase are normally more sensitive to HHP than are cells in stationary phase (Manas & Mackey, 2004; Hayman et al., 2007). Chen (2007) worked with the HHP treatments of six food-borne pathogens suspended in UHT whole milk. The results indicated that small subpopulations of bacteria were much more resistant than the rest of the populations. The author explained that there may be heterogeneous sensitivities to pressure within the bacterial populations caused by different physiological states within the population. Similarly, cocci are more resistant than rods (Yuste et al. 2004). The presence of heat and cold-shock membrane proteins can increase resistance to pressure (Wemekamp-Kamphuis et al., 2002). The activation of certain genes (producers of the *RpoS* protein in *E. coli* and *SigB* in *L. monocytogenes*) directly affects the degree of resistance to pressure (Malone et al., 2006; Wemekamp-Kamphuis et al., 2004).

In food industries, the foods do not always contain a previously known population of pathogenic bacteria and their physiological state. So any standard treatment of HHP may leave a risk of viable pathogens and lead to serious consumer hazards. This may be one of the reasons for limited adaptation of HHP in food industries especially in liquid foods. The development of HHP treatment to produce commercially sterile product for all food categories might be a challenge for upcoming research groups.

4-TYPE OF MATRIX

Studies on inactivation of bacteria by HHP treatment usually showed that the inactivation of bacteria in food system was more difficult than in water or buffer system (Patterson et al. 1995).

4.1- Food Composition

The chemical composition of food is important, since the presence of fats, proteins, minerals and sugars serves as a protector and increases microbial resistance to HHP (Black, Huppertz, et al., 2007; Molina et al., 2004). Several authors reported that bacteria are more resilient in complex matrix as milk or meat, compared to a buffer at the same pH. Chen and Hoover (2003) observed a strong baroprotective effect on *Yersinia enterocolitica* in whole milk than in phosphate buffer when treated at 350-450 MPa at 22 ºC for 10 min. Microorganisms generally show higher resistance in food systems and some organic matters contribute to that resistance.

In addition, as to the effect of fat on inactivation of microorganisms, contradictory information is found in the literature. Some authors found a baroprotective effect of fat on inactivation of vegetative bacteria that increased with fat content (Styles et al., 1991; Garcia-Graells et al., 1999), while others did not observe this difference (Garcia-Risco et al., 1998; Gervilla et al., 2000). Gervilla et al. (2000) inoculated different microorganisms (*E. coli, Pseudomonas fluorescens, L. innocua, S. aureus* and *Lactobacillus helveticus*) in Ringer solution and milk samples with 0, 6 and 50% fat contents and treated with 100 to 500 MPa pressure treatments at 4, 25 and 50 ºC. They reported that ovine milk with 0, 6 and 50% fat had baroprotective effect as compared to Ringer solution, but the samples with 6 and 50% fat did not show a progressive baroprotective in all pressurization conditions or for all microorganisms. Escriu and Mor-Mur (2009) did not found any correlation between the quantity and even the quality of fat (fat saturation level) and the destruction of *Listeria* and

Salmonella in meat model systems. Both of the microorganisms exhibited mixed tendencies with different fat quantities and qualities.

Similar studies for varying levels of sugars, proteins and other food constituents need to be carried out for deeper insights of the HHP treatments. Existing studies only provides information about the protective effect of single levels of food constituents against HHP, but the comparative studies with varying levels of different food constituents are missing. Such studies will help to categorize foods for high and low pressure requirements.

4.2- pH / Acidity

The pH is another major stress factor that can bring variability in the resistance for different microorganisms (Stewart et al., 1997). Although pH alone may not be enough to inactivate microorganisms, its combination with high pressure greatly increases treatment lethality. After pressure processing, the injured cell cannot heal, and even those not showing any effect of the treatment may become more sensitive to the high acidity of medium. The dependence of the effectiveness of the pressure treatment on pH has been studied by many researchers, showing that more acidic conditions tend to enhance the effect of pressure. This linkage between high pressure and low pH is one of the examples of hurdle theory. This dependence has been evidenced in bacteria (Ritz et al., 2006) and even in viruses (Kingsley and Chen, 2009).

As pH changes under pressure are difficult to be measured, very little is known on the real effect of pH during HPP. As different buffering systems show different pH-shifts under pressure, Mathys et al. (2008) conducted experiment using different buffering systems, showing a higher inactivation of *Geobacillus*

stearothermophylus spores by pressure when suspended in phosphate buffer than when suspended in ACES buffer (pH 4.5 – 8), due to the fact that phosphate has especially high dependence on pressure, thus lowering pH during treatment.

Susceptibility to pressure increases visibly as pH deviates from neutral values (Alpas et al., 2000). HHP may inactivate membrane proteins responsible for regulating the trans-membranous flow of protons, leading to inability to maintain the homeostasis (Hoover et al., 1989). A previous adaptation to environmental conditions can modify susceptibility, and cells of *L. plantarum* grown at pH 5.0 were more resistant to pressures of 250 MPa than were cells grown at pH 7.0 (Wouters et al., 1998).

The pH variations under pressure have been scarcely studied, although the interaction of pH- pressure is well known. There is a shift in the dissociation equilibrium with a different intensity depending on the buffering system. On dairy systems the pressure produces a permanent change on pH caused by a shift in calcium equilibrium. For transient changes the information of the process is difficult to be measured, as the instrumentation suitable to withstand high pressure is limited. Several studies had assayed dyes to measure pH changes through optical methods (Hayert et al., 1999, Salerno et al., 2007); however, the problem to measure pH under pressure in actual foods still remains unsolved (Mor-Mur and Saldo, 2012).

Keeping view the quite evident role of pH in bacterial inactivation and its susceptibility to change under pressure, there is need to develop HHP equipments with pH probe that can be immersed in to the sample and measure continuous pH variations of the food sample under pressure. Such type of

development may provide process insights for better control on HHP treatments.

4.3-Water Activity (a_w)

Water content plays a crucial role in determining cellular damage under pressure with a strong relationship with water activity: the lower the a_w of the food matrix, the higher the protection of spoiling and pathogenic agents against HHP effects, so lower values of water activity (a_w) increase microbial resistance to HHP (Black, Huppertz et al., 2007; Black, Setlow, et al., 2007; Hayman et al., 2008; Patterson, 2005). For *L. monocytogenes*, Hayman et al. (2008) postulated that low a_w results in protein stabilization, which prevents protein denaturation and cell death during HHP. Moussa et al. (2009) studied the effect of a_w between 0.11 and 0.99 in *S. cerevisiae* and found that $a_w \leq 0.71$ completely prevented the cell inactivation, as when water is available in a sufficient amount high pressure could induce membrane permeability causing uncontrolled mass transfers that could lead to death. This phenomenon is observed both in synthetic models and food. The efficacy of HHP processing decreases with reduced a_w, and it is visibly observed in foods with values below 0.9 (Raso et al., 1998). This fact also applies to spores such as *B. cereus* and can be attributed to incomplete germination in conditions of low water availability (Black, Huppertz et al., 2007; Black, Setlow, et al., 2007). Ionic solutes such as NaCl or $CaCl_2$ offer more protection to *Bacillus coagulans*, compared with non ionic solutes such as sucrose and glycerol (Patterson 1999) due to effect on osmotic pressure.

5- DESTRUCTION VS SUB-LETHAL INJURIES

After HHP treatments one population of microorganisms may be killed, another population may survive, and a third population may be sublethally injured (Wu et al. 2001). In addition, a small population of bacteria has been reported to be stressed, that are not able to exhibit growth immediately after HHP treatments but during subsequent storage they can grow normally. Stress is a change in the environment that imposes either reduced growth or survival potential. For any stress, the bacterial cell has a defined range within which the rate of colony forming units (CFUs) is positive (growth), zero (survival) or negative (death). In microbial populations, healthy cells are usually countable on both nonselective and selective media, whereas stressed cells are able to form colonies on nonselective media but not countable on selective media (Comas and Rius, 2009). Injured microorganisms can be distinguished from stressed ones by their inability to grow in specific media after a certain period of stress release. A series of morphological and structural changes in the cell, such as the separation of membrane from cell wall, the lengthening of the cell, the compression of gas vacuoles (Patterson, 2005) and the condensation of nuclear material (Manas and Mackey, 2004; Wouters et al., 1998) are described.

Injured and stressed organisms are potentially as important as their normal counterparts because they can resuscitate and become functionally normal in a favorable environment with consequent danger to public health. During repair, restoration of growth capabilities will occur before normal growth occurs. Many cellular modifications are reversed and losses of cell constituents are restored to the normal state during incubation.

Syed et al. (2013) worked with *E. coli* in Tris buffer, skimmed milk and orange juice at 600 MPa/ 3 min). They found higher recovery of stressed *E. coli* cells after 24 h in Tris buffer than in milk (1.19 and 0.79 log cfu/ml, respectively) during storage at 4 ºC. However, samples of orange juice (non favorable environment because of low pH) did not allow stressed cells to recover.

The availability of some substrates or the presence of factors in the matrix such as vitamins and amino acids in the food (or growth medium) allows better recovery of sub-lethally damaged cells after HHP processing (Tassou et al., 2007). It has also been described that the kind of solute (salt or sugar) can have significant influence on cell survival after processing and especially on the resistance of the spores (Patterson 2005). Cells with sub-lethal damage, under appropriate conditions (nutrient rich substrates, appropriate temperature and storage time), can resuscitated (Bozoglu et al., 2004) and psychrotrophs such as *L. monocytogenes* can constitute a risk (Ritz et al., 2006). The microbiological analyses performed to HHP processed foods must consider the possible presence of sub-lethally injured microorganisms, whose resuscitation requires the use of methodologies and non-selective culture media, rich in nutrients and incubated at temperature and for sufficient time to permit the repair of damage (Kalchayanand et al., 1994; Patterson et al., 1995; Ritz et al., 2006; Ulmer et al., 2000).

Mussa and Ramaswamy (1997) treated milk (inoculated with *L. monocytogenes*) at 350 MPa and observed an increase of 4 log cfu/ml after 12, 18 and 25 days of storage at 10, 5 and 0 ºC, respectively. They recommended refrigeration storage after HHP treatment to inhibit the rapid recovery of microorganisms.

Generally, most stressed cells repair within 2-4 h at a suitable incubation temperature in nutritionally rich non-selective medium. Moreover, the re-synthesis of RNA lost during injury is critical in the first stage of repair (Ray, 1986).

Research has indicated that regardless of the nature of the stress imposed on a microbe, for injured vegetative cells: (a) the injuries are repaired when incubated in an appropriate environment, (b) the optimum temperature and time differ with the nature of the stressor, (c) the completely repaired cells regain normal resistance to the selective agents in the media, and (d) the repair process precedes cell multiplication (Wu, 2008). Therefore, it is desirable to allow injured cells to repair any damage before enumeration by customary procedures (Ray and Adams, 1984).

CHAPTER 2

Background, Objectives & Working plan

Background

High Pressure processing is under research by dedicated research teams around the world. One of the most fundamental goals in high pressure research is to mimic processes and phenomena similar to those occurring in the interior of the Earth and other planetary objects (phase transitions, chemical reactions, microbiological activity, to name a few) and to understand their physical, chemical, geological, and biological foundations and implications. However, the development of such scientific discipline extends the elucidation of natural processes, since it has allowed the expansion and growth of novel applications in diverse technological areas, from materials and mineral sciences to food technology.

Currently, the two main practical applications of high pressure processing are the synthesis of new materials (as the production of synthetic diamonds) and food processing. MALTA CONSOLIDER (CSD2007-00045. Grupo Español de Altas Presiones- Materia a Altas Presiones) is a research project especially developed to carry out high pressure research activities in multiple disciplines in Spain. The MALTA project seeks to set up the necessary scientific infrastructure and conditions to carry out interdisciplinary studies of the above mentioned phenomena and to investigate their fundamental aspects in a coordinated manner. The *Centre Especial de Recerca Planta de Tecnologia dels Aliments* (CERPTA) has been assigned high pressure research project related to its application in food processing. This research work is a part of the principle project under taken by MALTA CONSOLIDER.

Interest of study

It is widely accepted that the major concern in food industries is always food safety. However, the question of food quality and healthy nutrition are becoming increasingly important and are socially reflected as an increase in demand of fresh-like and natural products. High pressure treatments can retain the fresh-like characteristics of foods better than heat treatments, it has not yet been successfully introduced into the food industry, possibly due to the less known insights of the process.

Different approaches to improve the efficiency of High Hydrostatic Pressure (HHP) are being studied, like effect of HHP under different processing temperatures, pH, and at different pressure levels and holding times. Another aspect, not completely neglected but scarcely reported is changing rates of compression and decompression during HHP treatments. Earlier studies contradictorily indicate that the efficiency of HHP process can be affected by controlling rates of compression and decompression. The previous findings are quite confusing, do not giving a clear idea about what happens by changing rates of compression and decompression. Preceding research on this topic provides only limited information using any single rate of compression and decompression that makes it difficult to extract any solid conclusion.

Based on all this knowledge coming from the available literature appears the need of a research project to cast light on this topic. In this way, the present research project was designed to determine the HHP processing parameters of compression and decompression rates. Initial microbial load was selected to be 6-7 log cfu/ml. Preliminary studies were carried out to determine optimum pressure levels and holding times to obtain 2-3 log cfu/ml reduction in total microbial count. It is worthy to mention that the objective of our selected HHP

treatments was not to produce sterilized products, rather we intended to obtain mixed population of stressed, sublethally injured, dead and healthy survivals.

Selection of Matrices:

We selected a variety of liquid matrices (food & non food) with low acidic (skim milk & Tris buffer) and high acidic properties (orange juice). Milk is generally considered as most sensitive product among liquid foods to get spoil by microbial activity. It is due to the nutritional properties and neutral pH of milk. We selected skimmed milk for our experiments to avoid any possible protection to microorganisms due to presence of fat globules (Gervilla et al., 2000). Tris buffer solution has been selected with neutral pH, same like milk but having no nutritional value. Orange juice is a representative of high acidic liquid foods having all nutritional properties necessary for bacterial growth. Selection of these three matrices would help us to understand the effect of low and high pH (juice Vs milk) and effect of nutritional and non-nutritional media (Tris buffer Vs skim milk). These selected matrices were inoculated with selected bacterial strains (6-7 log cfu/ml) before HHP.

Selection of Bacteria:

A variety of bacteria, mainly concerned with human health and food safety, have been selected including vegetative (*Escherichia coli* & *Staphylococcus aureus*) and spore forming bacteria (*Bacillus subtilis*). *E. coli* is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Some serotypes of *E. coli* can cause serious food poisoning in humans, and are occasionally responsible for product recalls due to food contamination. Some strains develop traits that can be

harmful to a host animal. These virulent strains typically cause a bout of diarrhea that is unpleasant in healthy adults and is often lethal to children. More virulent strains, such as O157:H7 cause serious illness or death in the elderly, the very young or immune depressed individuals (Vogt and Dippold, 2005).

S. aureus is the most common species of staphylococcus to cause *Staph* infections and is a successful pathogen due to a combination of nasal carriage and bacterial immuno-evasive strategies. *S. aureus* can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. Its incidence ranges from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections. It is still one of the five most common causes of nosocomial infections and is often the cause of postsurgical wound infections (Kluytmans et al., 1997). In addition, *S. aureus* is found to be one of the most resistant vegetative bacteria to high pressure treatments.

B. subtilis is a rod-shaped Gram-positive, catalase-positive bacterium,. It is also heavily flagellated, which gives *B. subtilis* the ability to move quickly in liquids.It has proven highly amenable to genetic manipulation, and has become widely adopted as a model organism for laboratory studies, especially of sporulation, which is a simplified example of cellular differentiation. It has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. *B. subtilis* spores can survive the extreme heat during cooking. In terms of popularity as a laboratory model organism, *B.*

subtilis is often used as the Gram-positive equivalent of *E. coli*, an extensively studied Gram-negative bacterium.

Selection of Treatment Parameters:

Based on varying properties of selected bacterial strains, specific pressure levels and processing temperatures were selected for each strain. We conducted preliminary experiments with *E. coli* at 350 and 400 MPa for different holding times but did not found distinguishing inactivation level between different HHP treatments. So we decided to increase the pressure level up to 600 MPa. *E. coli* having only vegetative forms and being sensitive to HHP were treated at 600 MPa at 4 ℃ startup temperature, where as *S. aureus,* being pressure resistant strain were treated at 700 MPa at 4 ℃ startup temperature. As bacterial spores have been reported to be quite resistant to HHP at low processing temperatures, *B. subtilis* spores were treated with 600 MPa pressure levels but using higher processing temperatures of 20, 60 and 70 ℃.

Objectives of Study:

As described previously, the effect of changing rates of compression and decompression on bacterial inactivation has been reported contradictorily in the literature, our study mainly addresses this issue.

General Objectives

The general purpose of this study was to test the hypothesis of the influence of HHP increase and decrease rates on the viability of the microorganisms.

Specific Objectives:

- To select the high pressure conditions to guarantee the survival of microorganisms representing the stressed, injured and healthy populations for each species.
- Observe the effect of pressure changing rates on counts of *E coli* (Gram negative), *S. aureus* (Gram positive) and resistant forms of *B. subtilis*.
- Study the effect of the environmental conditions (food matrix characteristics) on the effect of pressure treatment and on the subsequent refrigerated storage.
- Study the effect of pressure treatments on the induction of the stressed and sublethaly injured populations on non sporulated microorganisms.
- Determine the effects of different pressure conditions on the behavior of spores on their thermal resistance.

Working Plan

Determination of survival counts:

Plate counting would be used for determination of healthy and dead bacteria. Tryptone Soy Agar (TSA) was used for *E. coli* and *S. aureus*, as a non-specific agar media for determination of total survival count (cfu/ml) for treated and untreated samples. For *B. subtilis* Nutrient agar was used to total survival counts.

Determination of Stressed Cells:

Stressed cells are generally considered as temporarily inactivated cells due to unfavorable environmental conditions. These cells are supposed to grow normally after a certain time from stress release. In our study, microbial analyses would be carried out once immediately after HHP treatments and after overnight storage at refrigeration temperature. It is supposed that sublethally injured cells are unable to heal their wounds during over-night under refrigeration conditions. However, stressed cells get sufficient time to manage themselves after stress release and retrieve their normal growth. In this way, the differential count between day 0 and day1 is considered as stressed cells.

Determination of Sublethally Injured cells:

Different microbiological and biotechnological techniques are used for determination of sublethally injured population. For *E. coli* the differential count between non-selective (TSA) and selective media VRBGA (Violet Red Bile Green Agar) was used as a tool for estimation of sublethally injured cells. Selective media contains some stressing salts that do not allow sublethally injured cells to retain their normal growth. While working with *S. aureus* it was observed that the selective media (Baird Parker) did not have enough stressing

effect to restrain sublethally injured cells to grow and the colony count for both non-specific and specific media were almost similar. Consequently, we decided to use lag time analysis (by BioScreen) to differentiate between healthy and sublethally injured cells. It has been reported that injured cells exhibit extended lag time as compared to normal healthy cells. So the comparison of HHP treated samples against untreated control provides an estimated population of sublethally injured cells.

For *B. subtilis* we used mild heat treatment after HHP processing. Previously, it was tested that healthy *B. subtilis* spores are not affected by thermal treatment at 80 ºC. A portion of HHP treated samples was then further subjected to thermal treatment of 80 ºC. and the difference between HHP treated and HHP+Thermal treated samples was considered as sublethally injured population. Further confirmation of sublethal impact of different HHP treatments was done by Flow Cytometric analyses of HHP treated and untreated control samples.

The further detail of each experimental procedure has been explained in upcoming chapters.

Figure 2.1: Working plan with *E. coli*

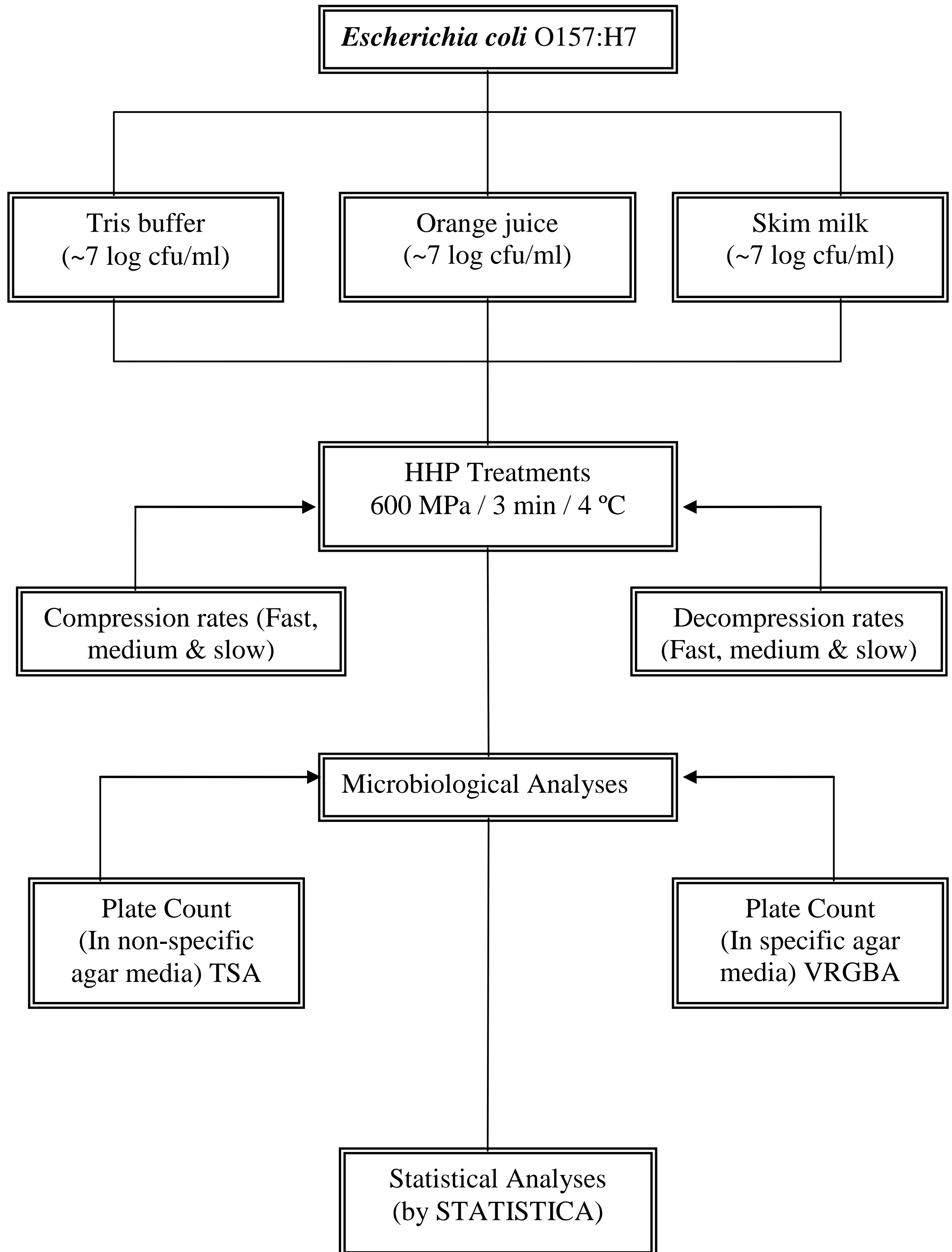


Figure 2.2: Working plan with *S. aureus*.

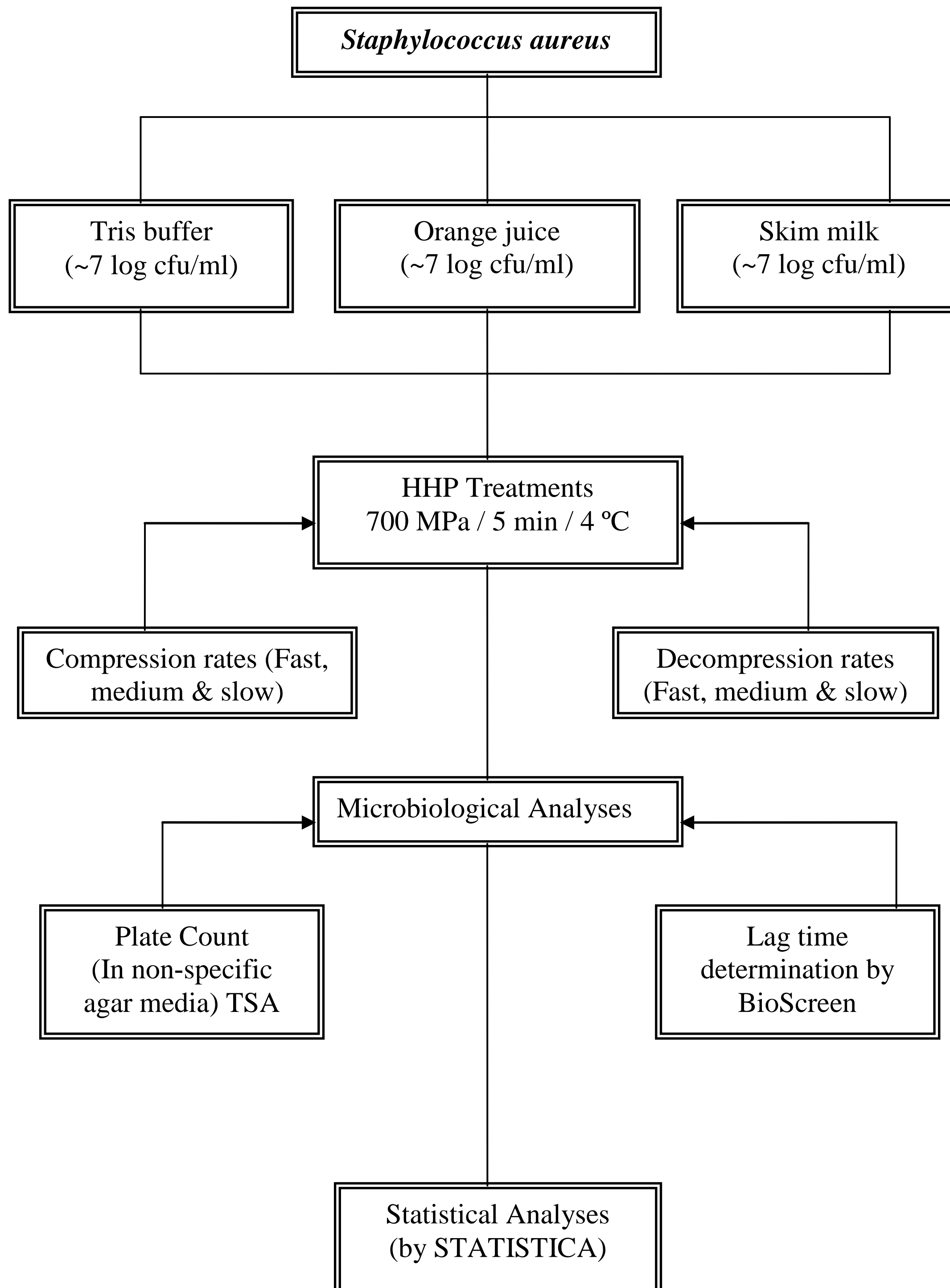
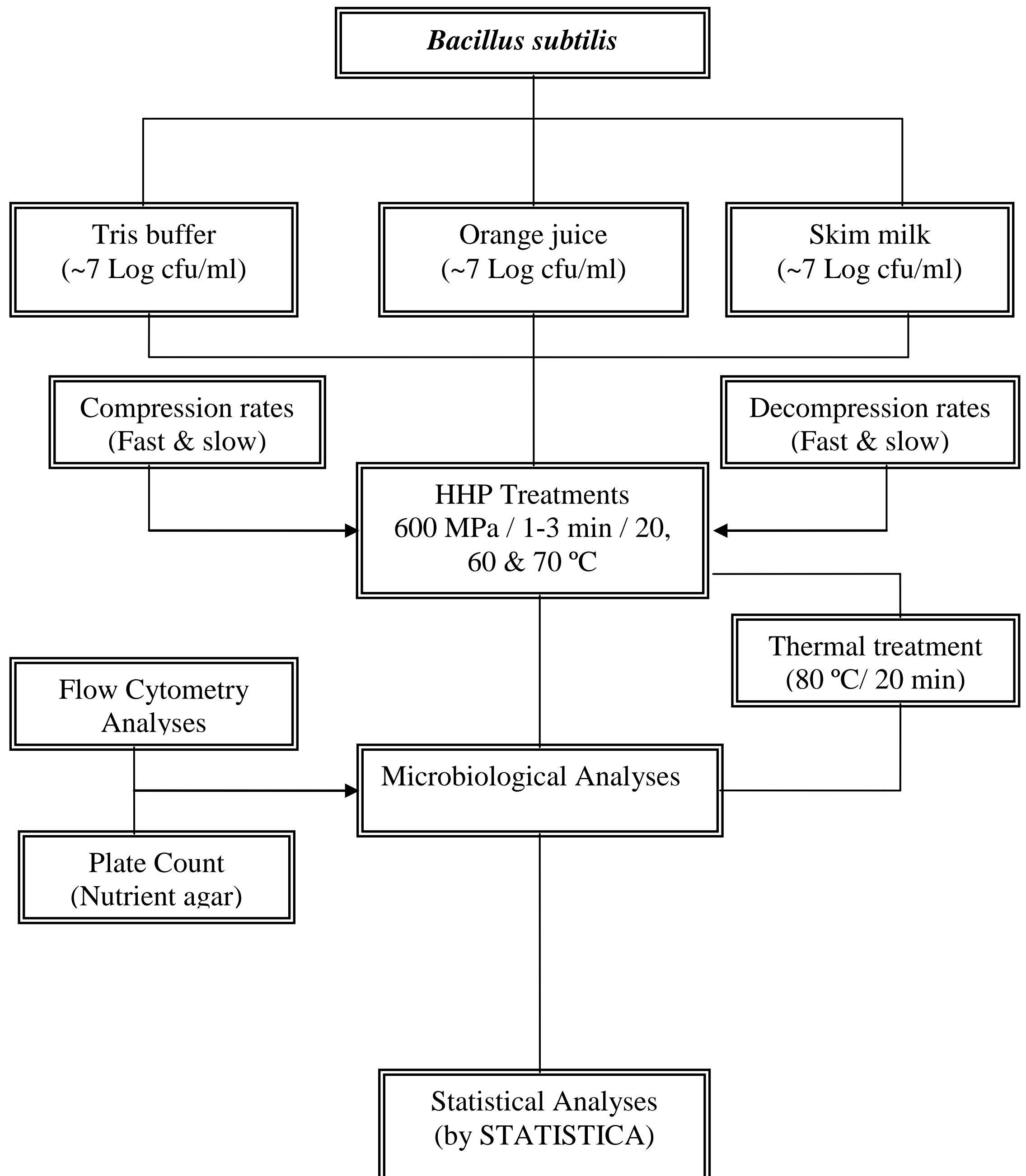


Figure 2.3: Working plan with *B. subtilis*



CHAPTER 3

ESCHERICHIA COLI O157:H7

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LETHALITY AND INJURING EFFECT OF COMPRESSION AND DECOMPRESSION RATES OF HIGH HYDROSTATIC PRESSURE ON *ESCHERICHIA COLI* O157:H7 IN DIFFERENT MATRICES

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ABSTRACT

Effect of compression and decompression rates of high-hydrostatic-pressure (HHP) on *Escherichia coli* O157:H7 was investigated. Samples of orange-juice, skimmed-milk and Tris-buffer were inoculated with *E. coli* O157:H7 and subjected to 600 MPa for 3 minutes at 4 ºC with fast, medium and slow compression and decompression. Analyses immediately after HHP treatment revealed that *E. coli* in milk and juice treated with fast compression suffered more than slow compression rates. Slow decompression resulted in higher inactivation of *E. coli* in all matrices. After overnight storage, highest stress-recovery (1.19 log cfu/ml) was observed in Tris buffer. Healthy cells were <1 log cfu/ml in milk and buffer samples but no growth was detected in orange juice for any of the treatments immediately after HHP. After 15 days at 4 ºC *E. coli* cells in skimmed milk and Tris buffer recovered significantly, whereas the recovery of sublethally injured cells was inhibited in orange juice.

Keywords: High Hydrostatic Pressure (HHP), Compression rate,

Decompression rate, *E. coli*,

INTRODUCTION

High pressure processing has been known for more than a century as a food preservation method, however commercial applications have emerged only recently (Torres and Velazquez, 2005; Rastogi, Raghavarao, Balasubramaniam, Niranjan, Knorr, 2007). Its potential to inactivate vegetative microorganisms and inhibit undesired activity of various food enzymes, with minimal changes in sensorial and nutritional properties has been widely recognized (Trujillo, Capellas, Saldo, Gervilla, Guamis, 2002).

The resistance of microorganisms to pressure in food is variable depending on HHP processing conditions (pressure, time, temperature, cycles etc.), food constituents, the properties and the physiological state of the microorganism (Smelt, 1998).

Regarding microbiological safety, quantifying inactivation of important food related pathogens by high pressure is most urgent and critical in the establishment of HHP especially in non-acid foods (including dairy products) in which microorganisms are much more resistant to pressure than in acid foods (Garcia-Graells, Hauben, Michiels, 1998). Although a lot of research has been carried out on HHP microbial inactivation, a number of issues remain unresolved, and this could be one of the reasons for limited application of HHP technology in food industry.

Mussa and Ramaswamy (1997) evaluated the practicality of application of the kinetic data by subjecting the milk to a HHP process (at 350 MPa for 32 minutes) sufficient to reduce the number of microorganisms by 4 logarithmic cycles. HHP kinetics of microorganism inactivation in milk showed a first order rate of destruction up to 350 MPa. At higher pressures the destruction

was too rapid for enumeration and often complete destruction was observed after 10 minutes exposure.

Smelt (1998) assumed that a slow ramp during compression might induce a stress response of microbial cells and hence leads to a lower inactivation effect of the process. Herdegen (1998) worked with *Listeria innocua* and reported that a process of rapid pressurization (6.7 MPa/s) and slow decompression (0.83 MPa/s) was more effective than a process of same maximum pressure and holding time but reverse pressurization and depressurization rates, as cited by Rademacher, Werner, and Pehl (2002). Noma, Shimoda, and Hayakawa (2002), investigated the inactivation and injury effects of HHP treatments at different pressure levels (from 70 to 400 MPa) combined with slow and fast decompression (30 s and 1 ms, respectively). The authors concluded that a rapid decompression procedure could enhance the injury, which causes the higher bactericidal effect of HHP treatments. On the other hand, Rademacher et al (2002) investigated effect of pressurization ramp on inactivation kinetics of *Listeria innocua* suspended in Tris buffer by using fast pressurization ramp (8.3 MPa/s) and slow depressurization ramp (1.7 MPa/s) in comparison with slow pressurization (1.7 MPa/s) and fast depressurization (8.3 MPa/s). They concluded that rate of pressurization and depressurization in the range of 1.7-8.3 MPa/s, does not affect inactivation kinetics of *Listeria innocua*, if the temperature changes are negligible during pressure treatment.

Keeping in view the controversial results of different research groups about compression and decompression rates, there is a need to investigate the behaviour of each microorganism under different treatment conditions.

Among the spoilage microbiota encountered in food, *Escherichia coli* is a common microorganism. Some pathogenic *E. coli* strains are a major public

health concern because the organism has a low infectious dose, is highly resistant to adverse environmental conditions, and causes severe disease symptoms ranging from mild diarrhoea to hemorrhagic colitis and the life threatening hemolytic-uremic syndrome. Outbreaks of *E. coli* were linked to the consumption of meat, juice and insufficiently pasteurized milk (Cody et al., 1999). Present study was conducted with *E. coli* O157:H7 inoculated in sterilized orange juice, skimmed milk and Tris buffer solution and subjected to varying rates for compression and decompression. The stressing, injuring and lethality, effects of different compression and decompression rates were evaluated by microbial count in selective and non selective media during storage at 4 ºC for 15 days.

MATERIALS AND METHODS

E. coli Culture Preparation:

E. coli O157:H7 (CECT 5947) were inoculated into 10 ml solution of Brain Heart Infusion (BHI) (OXOID Ltd. Hampshire, UK) broth at room temperature in a glass test tube and incubated at 37 ºC for 24 hours. After 24 hours the contents of test tube were added into another 50 ml of fresh sterilized BHI solution and again incubated at 37 ºC for next 24 hours to have a second culture. After 24 hours the culture was ready to inoculate into food matrices, being the culture at stationary phase.

Inoculation into Food Matrices:

10 ml of *E. coli* O157:H7 culture was added to the 1 litre sterilized samples of each matrix: Skimmed milk (Commercial brand: PASCUAL LECHE

DESNATADA), pulp-free Orange Juice (Commercial brand: PASCUAL ZUMOSOL NARANJA) (pH 3.5, Brix 12.0) and Tris Buffer (Panreac Quimica S.A, Barcelona Spain) 10 mM solution prepared in lab and pH adjusted to 6.85. The initial inoculum level in final matrices was about 7 log cfu/ml.

Sample Preparation for HHP Treatments:

Inoculated samples of all the matrices were filled into small plastic bottles (~ 30 ml capacity) leaving no head space. The filled bottles were enwrapped with Parafilm (Pechiney Plastic Packaging, Chicago, US) to prevent any leakage due to HP treatment. Finally, these plastic bottles were double packed in plastic bags under vacuum and cold stored until pressure treatment (~ 2h).

HHP Treatments:

Samples were subjected to HHP treatments (Stansted SPF-600, Stansted, UK). The pressure chamber diameter was 3.5 cm and length 12 cm, using water as compression medium. HHP treatments were carried out using slow, medium and fast compression rates (1.3, 3.6 and 11.4 MPa / s respectively) along with slow, medium and fast decompression rates (2.6, 6.9 and 12.9 MPa /s respectively) at 600 MPa pressure for 3 minutes holding time at 4 ºC starting temperature. The starting temperature 4 ºC was selected to avoid high temperatures during adiabatic heating preventing thermal shocks to microorganisms. The maximum temperatures observed during fast, medium and slow compression treatments were 30, 24 and 22 ºC, respectively. Following treatment combinations and codes were used for sample identification.

a) Fast Compression followed by medium decompression = FM

b) Slow Compression followed by medium decompression = SM

c) Medium Compression followed by medium decompression = MM

d) Medium compression followed by fast decompression = MF

e) Medium compression followed by slow decompression = MS

Figure 3.1 illustrates the compression and decompression combinations for HPP treatments. Immediately after pressure treatment the samples were kept back in refrigeration at 4 ℃ to avoid cell reproduction due to favorable temperature until microbiological analyses at specified intervals.

Figure 3.1a: Slow (Black), medium (dark grey) and fast (light grey) compression rates vs. medium decompression rate.

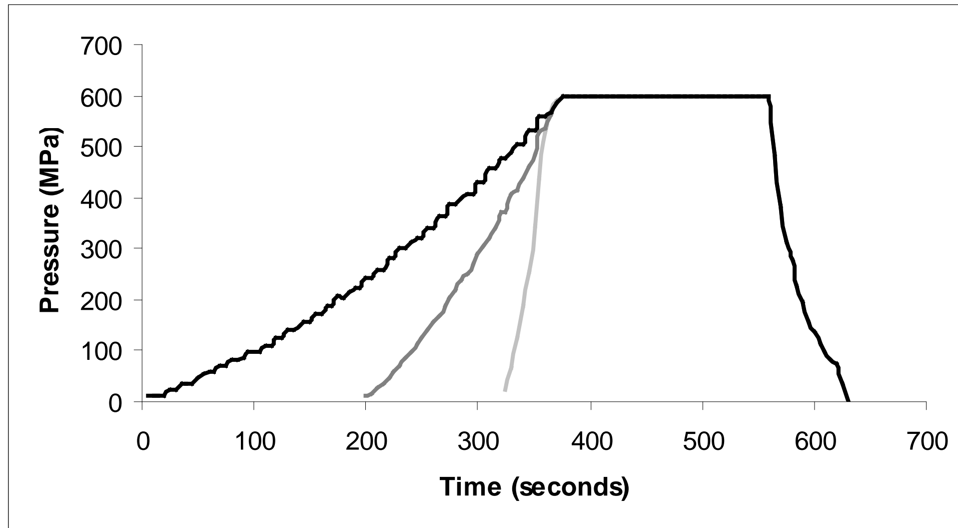
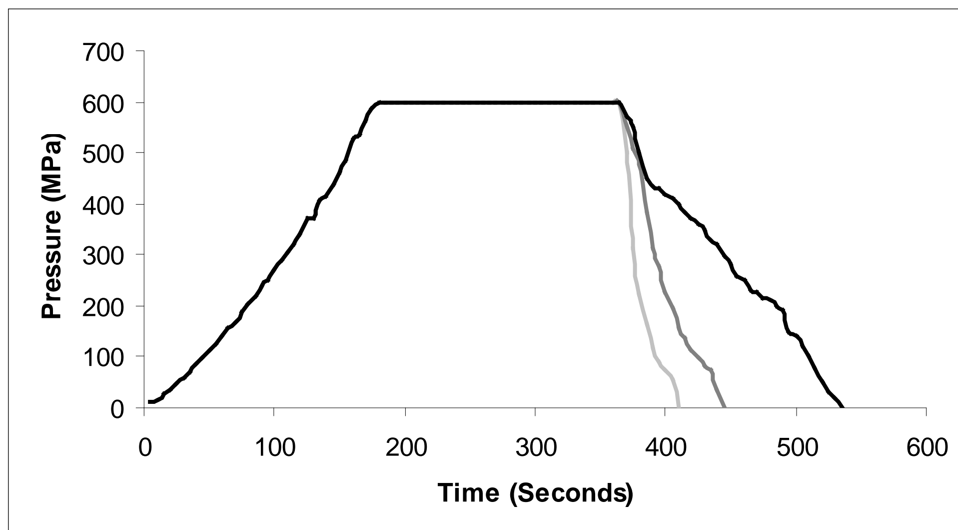


Figure 3.1b: Slow (black), medium (dark grey) and fast (light grey) decompression rates vs. medium compression rate.



Microbiological Analyses

Plate counting was used to determine the number of surviving *E. coli* cells. Decimal dilutions were prepared, when necessary, in Buffered Peptone Water (OXOID LTD. Hampshire, UK) and 1 ml of the appropriate dilution was plated in duplicate in selective media Violet Red Bile Glucose Agar (VRBGA) (OXOID LTD. Hampshire, UK) and non selective media Tryptone Soy Agar (TSA) (OXOID LTD). VRBGA contains bile salts and inhibits the growth of sublethally injured cells. Plates were incubated at 37 ºC for 24 h before enumerating colony forming units (cfu/ml). Injured microorganisms were manifested by their inability to grow on selective media. Therefore, the difference of microbial count between non-selective and selective media should be considered as sub-lethally injured population (Busta, 1978; Ray, 1996).

Stressed cells were considered to be recovered after HHP during overnight storage at 4-5 ºC. This recovery was analyzed by the difference in microbial plate count in TSA between day 0 and day 1 was supposed to be the stressed population.

Statistical Analyses

Three independent experiments were performed in separate weeks. The resulted data for log cfu/ml of all the treated and untreated samples was analyzed using ANOVA test (Newman-Keuls test) by STATISTICA 7.0 software (StatSoft Inc., Oklahoma, USA) at significance level of $p = 0.05$.

RESULTS AND DISCUSSION

Effect of Compression Rates

The effect of different HHP treatments on inactivation of *E. coli* O157:H7 is shown in Table 3.1. In general, immediately after HHP treatments (0 day), counts of *E. coli* showed a decrease of about 1.5-3 logs in all food matrices studied. These were expected results, as the study was designed for not to achieve full inactivation of bacterial cells, rather we aimed to study the different populations resulting after HHP processing, such as stressed and injured cells in viable count.

At day 0, the highest lethality was observed when fast compression was used (Table 3.1). The highest lethality was observed in skim milk (2.85 log cfu/ml) as compared to Tris buffer and orange juice 2.64 and 2.17 log cfu/ml, respectively (Table 3.1). Although Tris buffer solution showed no difference between fast and slow compression rates at day 0, but after 1 day of refrigerated storage the difference was noticeable (Table 3.1).

The effect of pressurization ramps has been previously investigated with controversial results. Faster compression rates are also attributed to greater extent of mechanical injuries in bacterial cells (Yano, Nakayama, Ishihada, Saito, 1998). These results agree with the behaviour of *E. coli* observed in our work, where the fast compression was more effective in all matrices (Fig. 3.2).

The inclusion of medium levels of compression treatment is a unique aspect of this study that enables us to have insights of the extent the compression effect during HHP treatments. As shown in Figure 3.2, we are able to develop a hypothesis that fast compression (11.4 MPa/s) is more lethal, when it is significantly faster as compared to slower (1.3 MPa/s) rates, as stated by Smelt

(1998). Otherwise, if the difference in the compression rates is not quite high (as like 1.3 MPa/ s for slow and 3.6 MPa/ s in medium), the treatment having longer treatment time (slow rate) would become more lethal, as observed in this study for Tris buffer and orange juice.

Temperature is generally assumed to be a critical processing factor in the pressure inactivation of microbial pathogens, but variations in temperature of the food sample during HHP can potentially alter the process out-comes. The situation is more obvious when pressure equipments have long come-up and decompression times e.g. > 2 minutes (Guan, Chen, Ting, and Hoover, 2006). In this study the initial temperature for processing was 4 ºC. Adiabatic heating in fast ramps raised the temperature up to 30 ºC to reach the pressure level of 600 MPa, while in case of slower ramps temperature increased up to 22 ºC. Gervilla, Mor-Mur, Ferragut, and Guamis (1999) worked with HHP processing of ewe's milk for inactivation of *E. coli* at different temperatures (10, 25 and 50 ºC). They reported that treatments temperature of 10 and 25 ºC were not significantly different for inactivation of *E. coli* at 300 MPa, whereas higher inactivation was achieved at 50 ºC. Since the differences in temperature between slow and fast treatments in our work is moderate (8 ºC), the higher lethality of fast compression can be mainly attributed to the greater impact of mechanical injuries of microorganism due to HHP.

Effect of Decompression Rates

At day 0, the more lethal effect in microbial count was observed when slow decompression speed was used (Table 3.1). The highest lethality of slow decompression treatment (MS) was observed in skimmed milk (3.12 $\log_{10}$ cfu/ml) as compared to Tris buffer and orange juice (2.86 and 1.94 cfu/ml,

respectively).The effect of decompression after overnight storage at 4-5 ºC is shown in Figure 3. HHP treatments with slower decompression rates (2.6 MPa/s) resulted in high inactivation of *E. coli* in all orange juice and Tris buffer, as compared to faster ones (12.9 MPa/s)

Table 3.1: Measuring inactivation of *E. coli* (Log_{10} cfu/ml) caused by different HHP treatments at 600 MPa for 3 minutes at 4 ºC on Tryptone Soy Agar (TSA).

Matrices	Storage Days	HHP Treatments				
		FM	SM	MM	MF	MS
Tris Buffer	0	2.64 + 0.35	2.67 + 0.14	2.21 + 0.43	2.53 + 0.64	2.86 + 0.54
	1	1.61 + 0.58	1.48 + 0.18	1.47 + 0.10	1.59 + 0.63	1.72 + 0.32
	7	1.72 + 0.42	1.81 + 0.09	1.49 + 0.20	1.84 + 0.58	2.06 + 0.27
	15	3.27 + 0.68	3.29 + 0.17	3.07 + 0.43	3.16 + 0.62	3.45 + 0.09
Skim Milk	0	2.85 + 0.78	2.55 + 0.33	2.78 + 0.27	2.77 + 0.63	3.12 + 0.74
	1	2.04 + 0.68	1.91 + 0.23	2.04 + 0.44	2.34 + 0.85	2.43 + 0.75
	7	2.31 + 0.73	2.12 + 0.42	2.26 + 0.41	2.68 + 0.81	2.80 + 0.73
	15	2.62 + 0.59	2.46 + 0.33	2.59 + 0.50	2.98 + 0.83	2.99 + 0.68
Orange Juice	0	2.17 + 0.84	1.68 + 0.41	1.49 + 0.47	1.74 + 0.65	1.94 + 0.59
	1	4.15 + 0.21	3.44 + 0.39	3.13 + 0.42	3.46 + 0.13	4.17 + 0.87
	7	N.D	N.D	N.D	N.D	N.D
	15	N.D	N.D	N.D	N.D	N.D

Table 3.2: Log_{10} cfu/ml of *E .coli* O157: H7 after different HHP treatments at 600 MPa for 3 minutes at 4 °C on Violet Red Bile Glucose Agar (VRBGA)

Matrices	Storage Days	Control (untreated)	HHP Treatments				
			FM	**SM**	**MM**	**MF**	**MS**
Tris Buffer	**0**	6.90 + 0.08	N.D	N.D	N.D	N.D	N.D
	1	6.89 + 0.08	0.05 + 1.15	0.16 + 0.91	0.54 + 0.29	0.57 + 1.32	0.17 + 0.67
	7	6.90 + 0.03	1.86 + 0.83	1.85 + 0.31	2.01 + 0.30	1.56 + 1.02	1.75 + 0.57
	15	6.82 +0.06	2.30 + 0.68	2.37 + 0.81	2.41 + 0.54	2.16 + 1.03	2.24 + 0.73
Skim Milk	**0**	6.67 +0.38	N.D	N.D	N.D	N.D	N.D
	1	6.75 +0.01	0.62 + 1.03	1.03 + 0.68	0.78 + 1.07	0.33 +1.5	N.D.
	7	6.74 +0.01	1.97 + 0.04	2.07 + 0.26	2.07 + 0.57	1.38 + 1.41	1.45 + 0.79
	15	6.73 +0.02	1.93 + 1.11	1.71 + 1.23	1.88 + 1.06	1.44 + 1.20	1.17 + 1.50
Orange Juice	**0**	6.86 +0.06	N.D	N.D	N.D	N.D	N.D
	1	6.80 +0.00	N.D	N.D	N.D	N.D	N.D
	7	6.93 +0.07	N.D	N.D	N.D	N.D	N.D
	15	6.76 +0.02	N.D	N.D	N.D	N.D	N.D

The lethal impact of decompression rates have been previously investigated by different authors with contradictory findings. Noma et al. (2002), observed higher inactivation of *E. coli* cells with fast decompression (1 ms) as compared to slow decompression (30 s), they suggested that rapid decompression could enhance the degree of pressure mediated injury, which caused the higher bactericidal effect. Whereas, Herdegen, (1998) observed high effectiveness of slow decompression (0.83 MPa/s) as compared to fast decompression (6.7 MPa/s) rates for inactivation of *L. innocua*.

In our work slow decompression was more lethal than fast, which in turn was more lethal than medium rate of decompression (Fig. 3.3). Differences in the results could be attributed to the difference in pressure release rate. It could be concluded that when decompression is fast enough, (like 1ms tested by Noma et al (2002)) it causes mechanical damages to the cells, resulting in higher lethality. Since such a fast decompression is not possible with most of the equipments being used, the comparative treatment with extended treatment time results in higher lethality.

There are only a few references that discuss the effect of compression and decompression ramps of HHP treatments, moreover they only compared slow and fast rates. This is the first work, in which three levels of compression and decompression rates have been studied. HHP treatments with medium rates of compression and decompression (MM), showed quite outfit results while comparing with slower and faster compression and decompression (Fig. 3.2 and 3.3). It is evident that slow and fast compression rates have higher lethal impact on *E. coli* as compared to medium rates (Figure 3.2). The lethal impact of fast compression is more visible in orange juice as compared to other matrices. In Tris buffer and orange juice, medium treatments (MM) resulted in

least inactivation of *E. coli* cells as compared to other HHP treatments (Table 3.1). The lesser effectiveness of MM treatment may be due to lack of both virtues of higher and slower rates as discussed earlier.

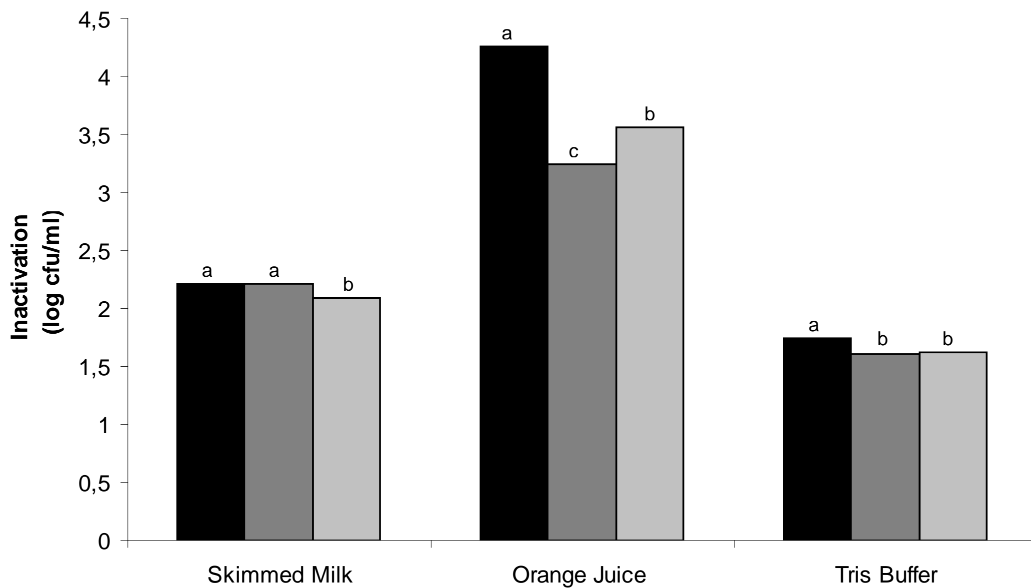
Study of stressed and injured cells during storage

Table 3.1 show that after overnight storage there are important changes in microbial count for all treatments, suggesting that some of the cells could recover from the stressing effect of HHP treatments. Because the storage conditions (4 ºC) do not allow the microorganisms to reproduce and this increase is noticeable even in Tris buffer samples where there are no nutrients. These changes in microbial counts could be considered as stress recovery. In this way, the population of stressed cells can be estimated by differences in total bacterial counts between day 0 and day 1 (Table 3.1). Furthermore, our results show that the analyses of samples after 1 day of HHP treatment may avoid the over-estimation of process effectiveness and measuring error due to differences in plating time, and pointing to the significance of time when microbial analyses are carried out.

In skimmed milk and Tris buffer samples, analyses immediately after HHP treatment (day 0) revealed higher lethality of HHP treatments as compared to day 1 analyses (Table 3.1). In Tris buffer the highest recovery of stressed cells was found in medium compression and the lowest in slow compression treatments. Similarly in skimmed milk, slow decompression resulted in least recovery of stressed cells as compared to medium and fast treatments. The stressing effect of faster decompression and faster compression was even greater than the medium rates. While in orange juice no recovery of injured cells could be observed, rather the number of viable bacterial count decreased

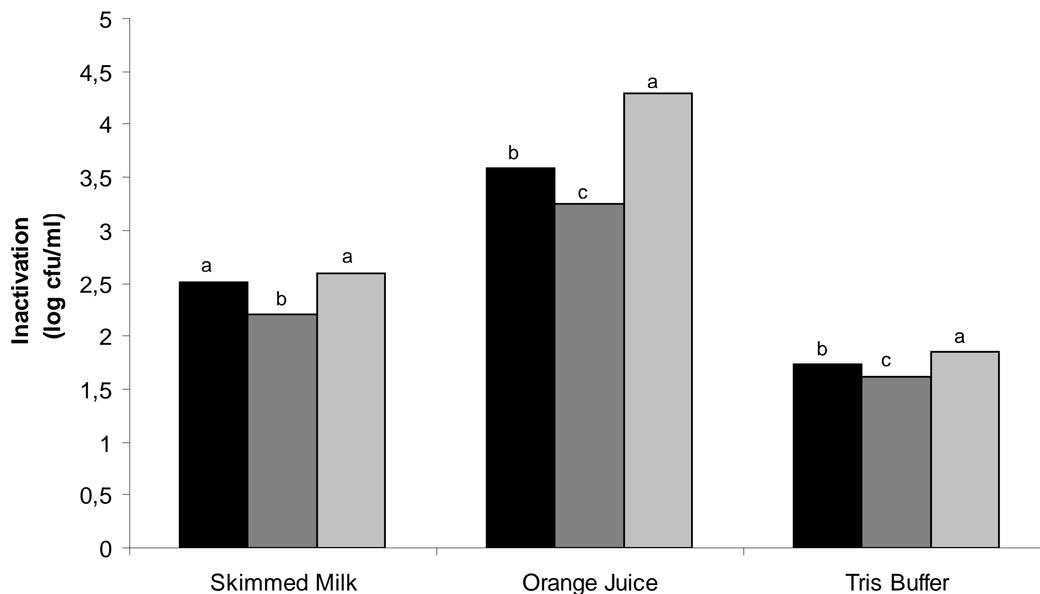
during storage. The cell count in untreated orange juice remained unchanged during storage.

Figure 3.2: Comparing effect of compression rates of HHP on *E. coli* O157:H7 after 1 day of refrigerated storage ■ Fast ▩ Medium □ Slow.



*Different alphabets in the same matrices represent significant difference at *p*=0.05.

Figure 3.3: Comparing effect of decompression rates of HHP on *E. coli* O157:H7 after 1 day of refrigerated storage. ■ Fast , ▩ Medium, □ Slow.



*Different alphabets in the same matrices represent significant difference at *p*=0-05

Bacterial cells, able to grow in general media (TSA) but unable to grow in selective media, are considered as sub-lethally injured population (Busta, 1978; Ray, 1996), since selective media contains ingredients inhibiting the growth of depressed or injured cells. In this work violet red bile glucose agar (VRBGA) was used as selective media to cultivate only healthy bacterial cells.

In skimmed milk, healthy cells were able to grow after 7 days (Table 3.2), with highest population in medium and slow compression treated samples. Whereas, the number of injured cells resulted by slow and fast compression was greater (2.62 and 2.54 log cfu/ml respectively) than medium compression treated samples (2.48 log cfu/ml).

After 15 days, further increase in healthy cells was observed, whereas, the untreated samples of all matrices showed quite consistent results throughout 15 days storage. In Tris buffer, the slow and fast compression rates resulted in same level of lethality (2.40 and 2.38 log cfu/ml respectively) but it was higher than medium rates (2.18 log cfu/ml). The slow decompression resulted in higher lethality (2.56 log cfu/ml) as compared to fast and medium rates (2.27 and 2.18 log cfu/ml respectively). In skimmed milk, after 15 days the lethality effect of fast and medium compression (2.83 and 2.81 log cfu/ml) was found to be greater than slow compression rates (2.68 log cfu/ml). While slow and fast decompression were more lethal (3.2 log cfu/ml and 3.19 log cfu/ml) than medium decompression rates (2.81 log cfu/ml). In orange juice no growth could be detected after 7 and 15 days of storage.

It is evident that the effect of HHP on *E. coli* O157:H7 cells suspended in different matrices is different. When suspended in skimmed milk, these microorganisms were found to be more sensitive to HHP treatment at 600 MPa pressure level with 2.43 log cycle reductions (by MS treatment at day 1) in

non-selective media (TSA), compared to Tris buffer solution, 1.72 log cycle reduction (by MS at day 1) in TSA count. The trend of *E. coli* O157:H7 in orange juice was quite opposite to milk and Tris buffer. There was significant reduction in total survival count and no growth was detectable in specific media after overnight storage. During subsequent storage of 7 and 15 days, same trend persisted in orange juice and microbial count decreased to lower than detectable limit (1 cfu/ml) in TSA media. Whereas in skimmed milk and Tris buffer a tendency of slight decrease in TSA count was observed after 7 days of storage, *E. coli* count in VRBGA go on rise continuously even after 15 days.

The microbial recovery trend in Tris buffer and skimmed milk can be justified by favorable pH conditions for *E. coli* cells where they were able to recover the minor cellular injuries and pressure shock effects. Comparing with Tris buffer, the greater sensitivity of microorganisms in HHP treated milk can be attributed to the changes in pH during pressure treatments. A low pH value makes microbial cells more sensitive to HHP treatments (Ananta, Heinz, Schluter, and Knorr, 2001). Milk is more vulnerable to HHP induced pH shifts (Buchheim, Schrader, Morr, Frede, and Schutt, 1996), while Tris buffer has more stable pH during pressure treatments. In case of orange juice, strong acidic environment prevents the injured microorganism to recover and grow normally, as well as expressed additional detrimental effect on the injured cells (Linton, McClements, Patterson, 1999) leading to their death after 7 days of storage. Untreated orange juice had the same counts on TSA and VRBGA during all the 15 days storage (Table 3.2). The continuous decrease in TSA count in HHP treated orange juice samples points out an effect of sublethal injuries that could not be recovered, and the synergistic effect of HHP and harsh environment.

The increasing population of *E. coli* in skim milk and Tris buffer may be alarming for the researchers, indicating that inactivation by HHP treatments may not be the destruction of bacteria, rather it may be cellular injuries that can be healed up with time in non-stressing media (like milk and Tris buffer).

CONCLUSION

It is concluded that during HHP processing of orange juice and skimmed milk fast compression and slow decompression rates are more effective, as compared to slow and medium compression and fast and medium decompression rates. Either of the extremes in both compression and decompression rates are even more effective than medium levels. The initial effect of HHP on *E. coli* O157:H7 is more lethal in skimmed milk and Tris buffer solution (pH 6.85) as compared to orange juice (pH 3.5). But during subsequent storage at 4 ºC, skimmed milk and Tris buffer are more susceptible to get spoiled by microbial recovery and activation. Orange juice inhibits the recovery of sub-lethally injured and depressed cells during storage.

CHAPTER 4

STAPHYLOCOCCUS AUREUS

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Effect of compression and decompression rates of high hydrostatic pressure on inactivation of *Staphylococcus aureus* in different matrices

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ABSTRACT

Staphylococcus aureus ATCC6538 was inoculated in skimmed milk, orange juice and Tris buffer samples. Inoculated samples were subjected to HHP treatments at 700 MPa for 5 minutes at 4 ºC starting temperature with fast, medium and slow rates of compression and decompression. Immediate effect of different HHP treatments was not significantly different. However, during subsequent storage in refrigeration, highest microbial inactivation was the result of treatments with fast compression and slow decompression rates in all matrices. Sublethally injured population was estimated by lag time analyses based on optical density. Orange juice does not let the sublethally injured cells of *S. aureus* to recover and resume normal growth. In skimmed milk and Tris buffer, slow decompression rates resulted in highest population of sublethally injured cells.

Keywords: High Hydrostatic Pressure (HHP), Compression rate, Decompression rate, *S. aureus*.

INTRODUCTION

High Hydrostatic Pressure (HHP) processing is a method which has shown great potentials in the food industry. Similar to heat treatment, high pressure inactivates microorganisms, denatures proteins and extends the shelf life of food products (Rosina, 2006; San Martín et al., 2004). The research on the effect of high pressure on food was first carried out in the nineteenth century (Hite, 1899) describing an increase in shelf-life for products such as milk, fruit and other foods, but the scientific development, its application in the food industry, and the foodstuff marketing are much more recent and have taken place in the past two decades (Considine et al., 2008). At present, due to technological improvements in equipment, industrial application is widespread for a range of pressures between 100 and 800 MPa, depending on the desired objective (Xi, 2006). The process is isostatic, i.e. the pressure is transmitted uniformly and instantly, and adiabatic, which means that no matter the food shape or size, there is a little variation in temperature with increasing pressure (the temperature increases approximately 3 °C per 100 MPa, depending on the composition of the food) (Otero et al., 2010). This prevents the food from being deformed or heated which would modify its organoleptic properties.

Previous studies on HHP has shown many factors that can influence the efficiency of HHP to inactivate microorganisms, such as magnitude of pressure, pressurization time and temperature, microbial types, cell growth phase, suspending media and the presence of antimicrobial compounds such as bacteriocins and lysozyme (Shigehisa et al. 1991; Kalchayanand et al. 1994; Patterson et al. 1995; Benito et al. 1999).

Few researchers have reported the effect of compression and decompression rates on microbial inactivation during HHP processing but a very little work

has been done on this aspect and the available information is contradictory to each other. The issue was highlighted by Smelt (1998) who assumed that a slow ramp during compression might induce a stress response of microbial cells and hence leads to a lower inactivation effect of the process. Herdegen (1998) reported that a process of rapid pressurization (6.7 MPa/s) and slow decompression (0.83 MPa/s) was more effective than a process of same maximum pressure and holding time but reverse pressurization and depressurization rates, as cited by Rademacher et al (2002).

Noma et al. (2002), investigated the inactivation and injury effects of HHP treatments on several vegetative bacterial strains (*E. coli, Salmonella typhimurium, Pseudomonas aeruginosa* and *Vibrio parahaemolyticus*) at different pressure levels (from 70 to 400 MPa) combined with slow and fast decompression (30 s and 1 ms, respectively). The authors concluded that a rapid decompression procedure could enhance the injury, which causes the higher bactericidal effect of HHP treatments. On the other hand, Rademacher et al (2002) investigated the effect of pressurization ramp on inactivation kinetics of *Listeria innocua* suspended in Tris buffer by using fast pressurization ramp (8.3 MPa/s) and slow depressurization ramp (1.7 MPa/s) in comparison with slow pressurization (1.7 MPa/s) and fast depressurization (8.3 MPa/s). They concluded that rate of pressurization and depressurization in the range of 1.7-8.3 MPa/s, does not affect inactivation kinetics of *L. innocua*, if the temperature changes are negligible during pressure treatment.

We conducted previous studies with *E. coli* O157:H7 and *Bacillus subtilis* suspended in Tris buffer, skimmed milk and orange juice at 600 MPa for 3 min. It has been reported that fast compression and slow decompression treatments were more lethal for *E. coli* (Syed et al 2013) while in case of *B.*

subtilis slow compression and slow decompression resulted in highest bacterial inactivation (Syed et al 2012).

Present study was conducted with *Staphylococcus aureus,* a major cause of foodborne illness (Eifert et al., 1996) and highly resistant to HHP (Yuste et al., 2004). *S. aureus* was suspended in Tris buffer, skimmed milk and orange juice and treated with three different rates of compression and decompression at pressure of 700 MPa held for 5 min. The inactivation and injuring effects were measured by using plate count and optical density, respectively.

MATERIALS AND METHODS

S. aureus Culture Preparation:

Staphylococcus aureus (ATCC 6538) were inoculated into 10 mL solution of Tryptone Soya Broth (TSB) (OXOID Ltd. Hampshire, England) at room temperature in a glass flask and incubated at 37 ºC for 22 hours in water-bath with shaking. After 22 hours the contents of test tube were added into another 50 ml of fresh sterilized TSB solution and again incubated at 37 ºC for next 22 hours. After 22 hours the culture was ready to inoculate into food matrices, being the culture at early stationary growth phase.

Sample Preparation for HHP Treatments

10 mL of *S. aureus* ATCC6538 culture was added to the 1 L of each matrix, sterilized skimmed milk, orange juice and Tris buffer solution. The initial inoculum level was about 1 x 10^7 cfu/ml. Inoculated samples of all matrices were filled in to heat sealed plastic packs of approximately 5 ml volume leaving no headspace.

HHP Treatments:

Samples inoculated with *S. aureus* ATCC6538 were subjected to 700MPa for 5 minutes using Stansted High Pressure Micro FoodLab, Stansted, UK, model SFL 085-9-W. HHP treatments were carried out using fast, medium and slow rates of compression (33, 10 and 5 MPa/s, respectively) followed by fast, medium and slow rates of decompression (70, 28 and 5 MPa/s, respectively). The starting temperature 4 ºC was selected to avoid high temperatures during adiabatic heating preventing thermal shocks to microorganisms. Following treatment combinations and codes were used for sample identification.

f) Fast Compression followed by medium decompression = FM
g) Slow Compression followed by medium decompression = SM
h) Medium Compression followed by medium decompression = MM
i) Medium compression followed by fast decompression = MF
j) Medium compression followed by slow decompression = MS

Immediately after HHP treatment the samples were kept back in refrigeration at 4 ºC to prevent cell reproduction under favorable temperature until microbiological analyses at specified intervals.

Microbiological Analyses:

Plate counting was used to determine the number of viable *S. aureus* cells. Decimal dilutions were prepared, when necessary, in Tryptone Soya Broth (OXOID LTD. Hampshire England) and 0.1 ml of the appropriate dilution was plated in duplicate on non selective media Tryptone Soy Agar (TSA) (OXOID LTD.). Plates were incubated at 37 ºC for 24 h before enumerating colony forming units (cfu/ml).

Stressed cells were considered to be recovered after HHP during overnight storage at 4 ºC. In this way, the difference in log microbial count between day 0 (D0) and day 1 (D1) was supposed to be the stressed population (Syed et al., 2013).

Measurement of Optical density:

300 µL of each sample diluted in TSB (from decimal dilutions of microbiological analyses) was poured in duplicate wells of BioScreen plate (Bioscreen C). The optical density (OD) of samples was measured at 450 nm wavelength after every 15 minutes for 24 h at 37 ºC.

Statistical Analyses:

Two independent experiments were performed in separate days. The resulted data for log cfu/ml of all the treated and untreated samples was analyzed using ANOVA test (Newman-Keuls test) by STATISTICA 7.0 software (StatSoft Inc., Oklahoma, USA) at significance level of $p = 0.05$.

RESULTS & DISCUSSION

The results of *Staphylococcus aureus* (cfu/ml) for HHP treatments at 700 MPa for 5 minutes holding time with different compression and decompression rates are shown in Table 4.1. The intensities of selected HHP treatments were for studying different sets of populations like stressed, injured, healthy and dead cells instead of total inactivation.

At D0, all treatments differ non-significantly from each other. But after overnight storage (D1), the microbial count was significantly lower for fast

compression than slow decompression treatments in all matrices. Furthermore, D1 results also show increase in colony count as compared to D0. That might be due to revival of stressed cells in favorable storage media as assumed by Syed et al (2013). Statistical analyses of D0 and D1 microbial count revealed significantly higher ($p=0.05$) number of stressed cells caused by FM treatments in skimmed milk and Tris buffer (2.28 and 1.57 log cfu/ml, respectively). Whereas, the populations of stressed cells differ non-significantly among other treatments. In orange juice no recovery of stressed cells was observed in FM treatment and total count decreased after overnight storage. The highest population of stressed cells was resulted by SM in orange juice. Analyses of difference in D1 and D0 results reveal that compression treatments are more stressing to *S. aureus* as compared to decompression treatments.

After one week (D7), among HHP treated samples, the lowest microbial counts were observed for fast compression and slow decompression treatments in skimmed milk (2.22 and 3.51 cfu/ml) and in Tris buffer (1.25 and 3.85 cfu/ml), respectively. Similar tendency of microbial counts pertains after two weeks (D15) of storage and fast compression and slow decompression treatments resulted in highest lethality for *S. aureus.*

On the other hand, orange juice was found to inhibit the survival of healthy cells of *S. aureus*, and microbial count were below detection limit (<1 cfu/ml) after D15 of refrigerated storage (4-6 ºC) for HHP treated as well as untreated samples. Whereas, the microbial count for untreated samples of skimmed milk and Tris buffer remained unchanged during refrigerated storage for two weeks.

Table 4.1: *Staphylococcus aureus* (cfu/ml+ S.D.) treated with 700MPa pressure for 5 minutes by different compression and decompression rates

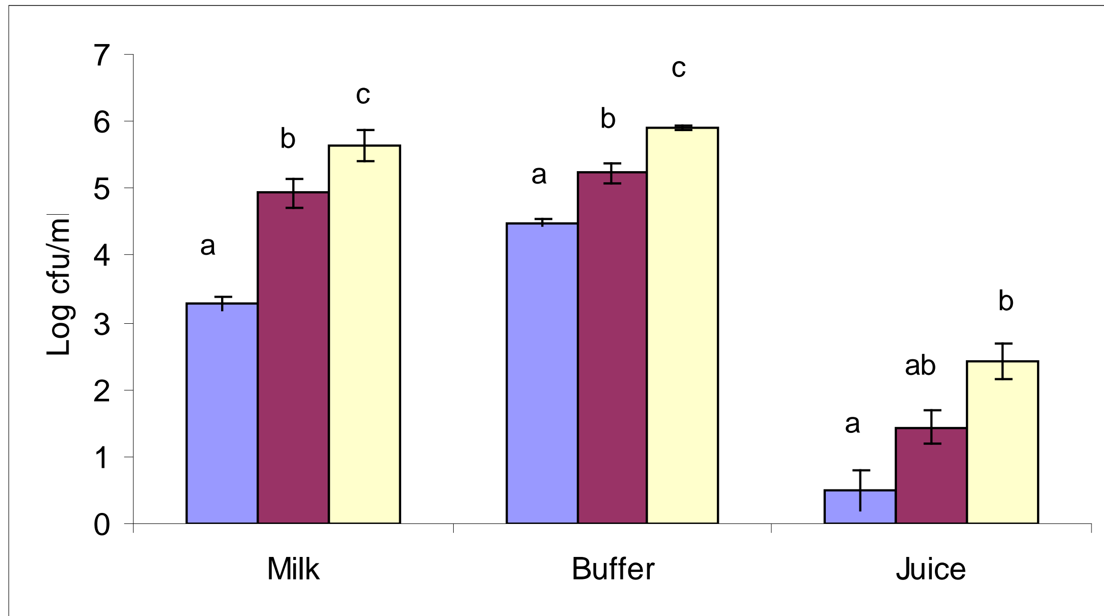
Matrices	Storage Days	Control	FM	SM	MM	MF	MS
Skimmed Milk	**D0**	7.41+0.00 a	1.00+0.00 B	3.27+0.48 ab	3.56+1.88 ab	3.55+1.03 ab	3.25+1.31 ab
	D1	7.83+0.13 a	3.28+0.2 B	5.64+0.45 c	5.23+0.80 c	5.01+0.68 c	4.54+0.87 c
	D7	7.33+0.03 a	2.22+0.15 d	4.81+0.63 bc	4.29+0.46 bc	5.61+1.46 c	3.51+0.36 b
	D15	7.26+0.06 a	2.33+0.30 c	5.89+0.06 b	4.81+1.02 b	4.81+0.71 b	3.65+0.12 d
Tris Buffer	**D0**	7.48+0.00 a	2.92+0.12 b	5.48+0.00 ab	4.68+0.57 ab	4.40+1.52 ab	4.65+1.03 ab
	D1	7.77+0.11 a	4.49+0.11 c	5.90+0.07 b	5.48+0.37 b	5.09+0.84 bc	5.12+0.20 bc
	D7	7.31+0.04 a	1.25+0.5 d	5.33+0.78 c	4.08+0.82 b	5.42+0.3 c	3.85+0.29 b
	D15	7.82+0.01 a	2.70+0.44 b	5.65+0.11 e	3.90+0.18 c	4.47+0.55 d	3.40+0.25 c
Orange Juice	**D0**	7.60+0.00 a	1.35+0.49 b	1.00+0.00 b	1.30+0.43 b	1.00+0.00 b	1.00+0.00 b
	D1	7.50+0.03 a	0.50+0.058 b	2.43+0.53 c	1.91+0.70 bc	1.28+0.91 bc	1.15+0.86 bc
	D7	5.28+0.15	N.D.	N.D	N.D	N.D	N.D
	D15	N.D.	N.D	N.D	N.D	N.D	N.D

***Different alphabets in same rows shows significant difference among values at p=0.05**
N.D. = Not detectable (<10cfu/ml).

The individual effect of compression and decompression treatments was determined by statistical analyses. Figure 4.1 shows that in all matrices, fast pressurization resulted in lower bacterial count (p =0.05) as compared to medium and slow rates. This trend prevails during further analyses of D7 and D15 (Figures 4.3 & 4.5). Although changes occurred in total count during storage for different treatments in milk and Tris buffer samples but the tendency of least viability after fast compression remained unchanged.

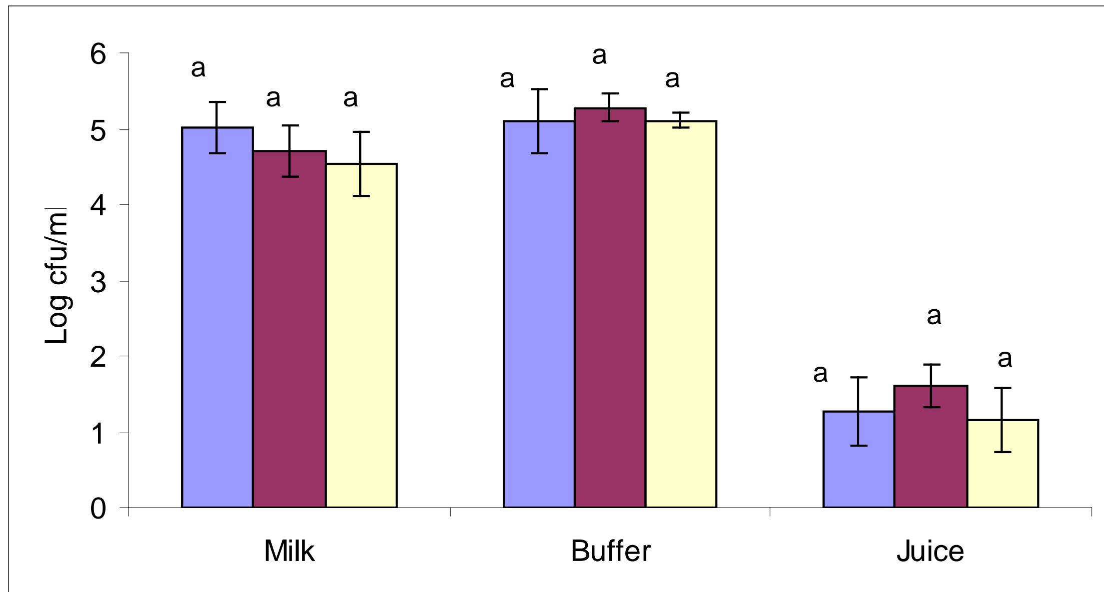
The higher lethality of fast compression might be linked with adiabatic heating during compression. In present study the initial temperature of the sample and compression fluid was set at 4 ºC. Increase in temperature due to adiabatic heating was observed to be 29, 26 and 21 ºC for fast, medium and slow rates of compression, respectively. Cheftel (1995) reported that physical compression results in temperature increase of 2-3 ºC/100 MPa. *S. aureus* is reported to be the highly resistant to pressure and temperature conditions. Gervilla et al. (1999) studied the inactivation of *S. aureus* in milk using HHP. They did not find any difference in activation at 10 and 25 ºC using 500 MPa pressure level. Sencer et al., (2008) reported if water is used as compression medium, then different compression rates do not affect adiabatic heating values for HHP processing of liquid foods. From these findings we can conclude that the higher lethality impact of fast compression is mainly due to mechanical injuries occurred during pressure changing part of HHP treatments, as described by Yano et al (1998). Whereas slow rates of compression let the microorganisms to develop the stress response, hence reduce the inactivation efficiency of the HHP process (Smelt, 1998).

Figure 4.1: Effect of different compression rates (Fast, Medium, Slow) on survival count of *S. aureus* on D1



Different letters in same matrix shows significant difference among HHP treatments at *p=0.05*

Figure 4.2: Effect of different decompression rates (Fast, Medium, Slow) on survival count of *S. aureus* on D1

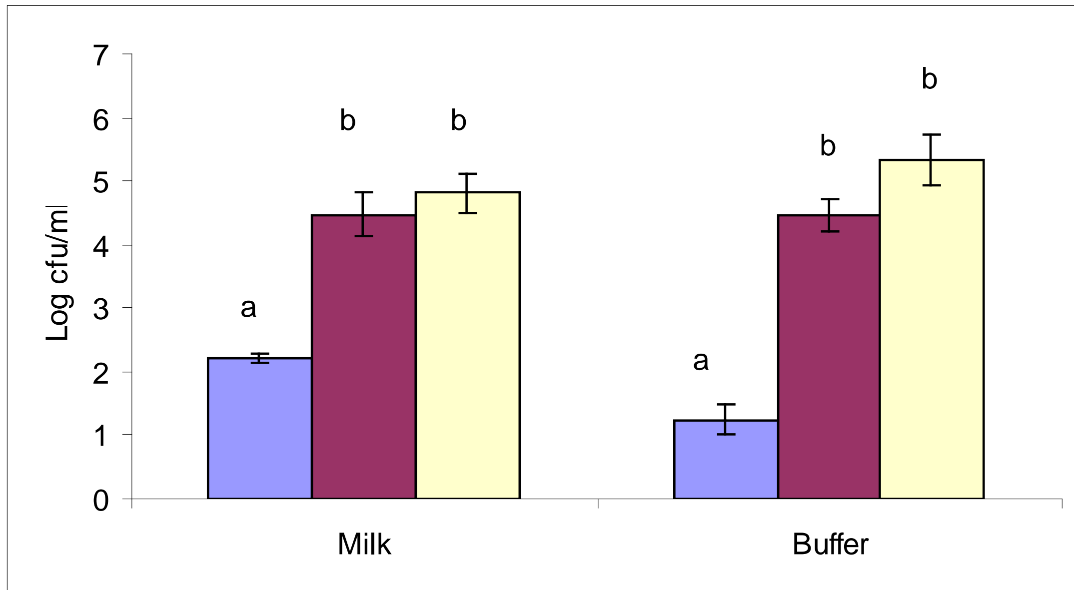


Different letters in same matrix shows significant difference among HHP treatments at *p=0.05*

Regarding decompression, initially the inactivation effect of different treatments did not varied significantly (Figure 4.2), but at D7 slow and medium rates of decompression resulted in lower bacterial counts as compared to fast decompression (Figure 4.4). Similar trend prevailed during D15 analyses and least survival count was observed by slow decompression as compared to faster rates of decompression (Figure 4.6).

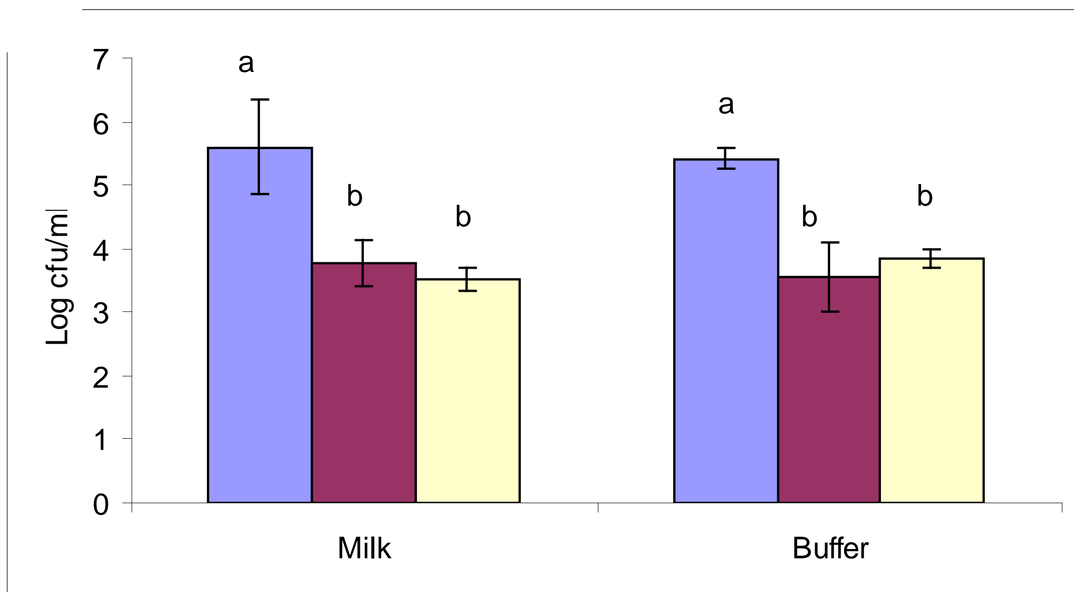
The lethal impact of decompression rates have been previously investigated by different authors with contradictory findings. Noma et al. (2002), observed higher inactivation of *E. coli* cells with fast decompression (1 ms) as compared to slow decompression (30 s), they suggested that rapid decompression could enhance the degree of pressure mediated injury, which caused the higher bactericidal effect. Whereas, Herdegen, (1998) observed high effectiveness of slow decompression (0.83 MPa/s) as compared to fast decompression (6.7 MPa/s) rates for inactivation of *L. innocua*. Rademacher et al. (2002) did not find any differences between slow and fast decompression treatments. In present study the slow rates of decompression (5 MPa/s) resulted in higher lethality than fast and medium decompression (70 & 28 MPa/s, respectively) (Fig. 4.4 and 4.6). Form this discussion, it can be concluded that since slow decompression elongates the process time after making microorganisms more sensitive during pressure holding time, it causes more inactivation of bacterial cells as reported by Syed et al., (2013). Otherwise, if the decompression is so fast (1 ms, as practiced by Noma et al., 2002), the resultant mechanical injuries can make the process more lethal. Since mostly the HHP machine being used in industries and most of research centers do not possess such a fast decompression time, it can be generally concluded that longer the decompression time is, the higher will be inactivation of bacterial cells.

Figure 4.3: Effect of different compression rates (Fast, Medium, Slow) on survival count of *S. aureus* on D7.



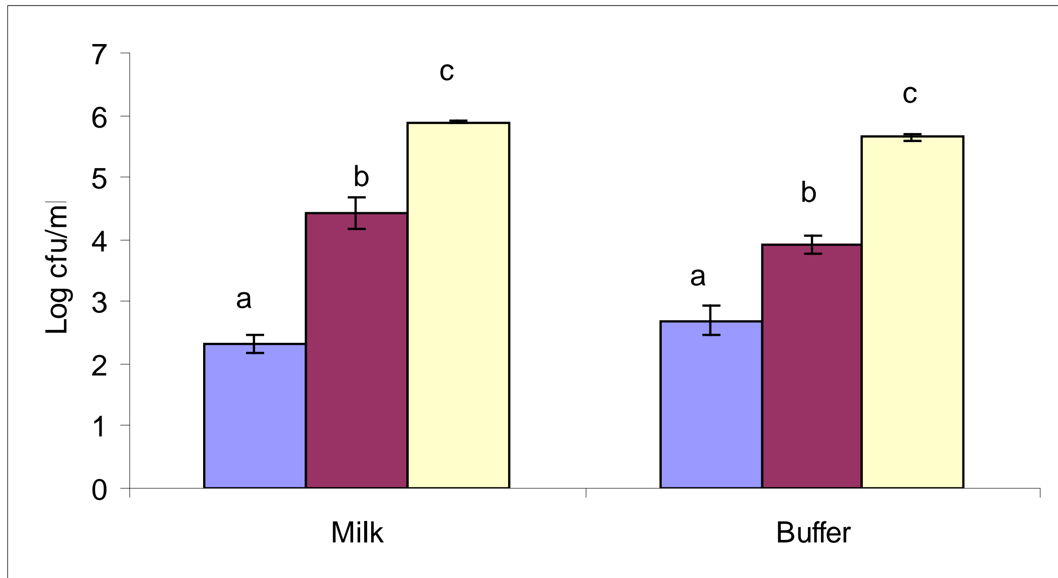
Different letters in same matrix shows significant difference among HHP treatments at *p=0.05*

Figure 4.4: Effect of different decompression rates (Fast, Medium, Slow) on survival count of *S. aureus* on D7



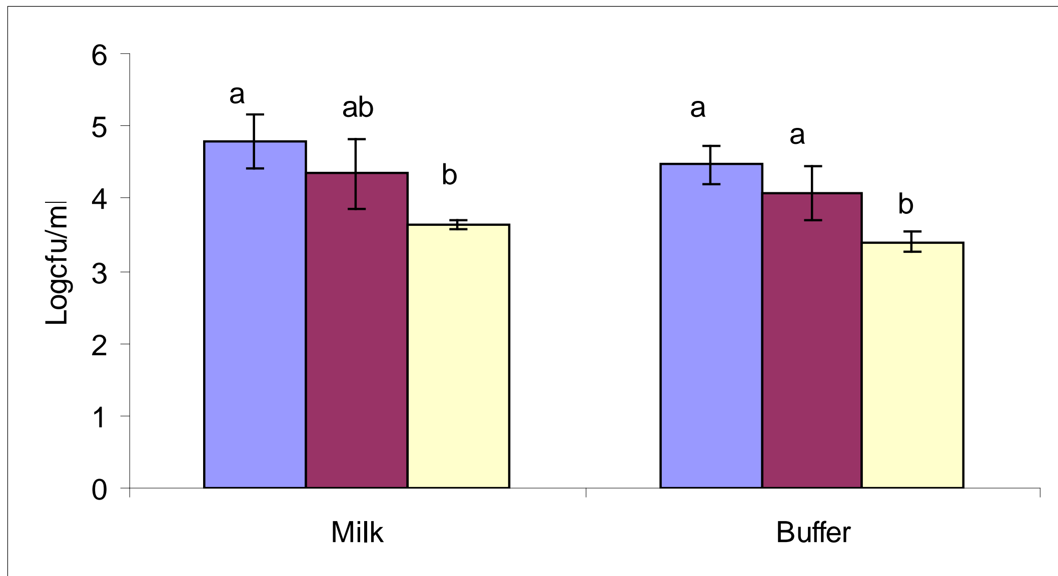
Different letters in same matrix shows significant difference among HHP treatments at *p=0.05*

Figure 4.5: Effect of different compression rates (Fast, Medium, Slow) on survival count of *S. aureus* on D15.



Different letters in same matrix shows significant difference among HHP treatments at *p=0.05*

Figure 4.6: Effect of different decompression rates (Fast, Medium, Slow) on survival count of *S. aureus* on D15



Different letters in same matrix shows significant difference among HHP treatments at *p=0.05*

Sublethally injured bacterial population in HHP treated samples was estimated by measuring OD during incubation at 37 ºC. Measuring OD threshold time (lag time) gives a good idea of injured population in samples (Baranyi and Pinn, 2004). It has been reported that samples with higher number of injured cells exhibit longer lag times as compared to healthy cells (Guillier and Augustin, 2006). Li et al. (2006) measured OD by using Bioscreen and reported longer lag times of *E. coli* cells sublethally injured by heat and acid stresses as compared to untreated control. Figure 4.7 shows typical growth curves of *S. aureus* ATCC6538 (HHP treated vs control) with changes in OD at 450 nm during incubation at 37 ºC.

Table 4.2 shows lag times of different HHP treated samples for milk and Tris buffer after overnight storage at 4 ºC. The OD for orange juice remained unchanged for treated samples possibly due to stressing effect (low pH) of media that inhibit revival of injured cells. It is evident from Table 4.2 that certain HHP treatments resulted in higher number of injured cells that delayed the lag phase. In Tris buffer, MM and MS treatments did not changed OD during 24 h incubation at 37 ºC showing the highest number of injured cells as compared to SM and FM treatments. While in milk highest injured cells were observed in MS treated samples that do not changed OD during 24 h incubation at 37 ºC. Secondly, FM treated samples resulted in longest lag time. But after certain time these injuries were healed up and cells were able to show normal growth in suitable growth media. The corresponding plate count results shows that injured cells that delayed lag time are still able to exhibit normal growth on suitable agar media.

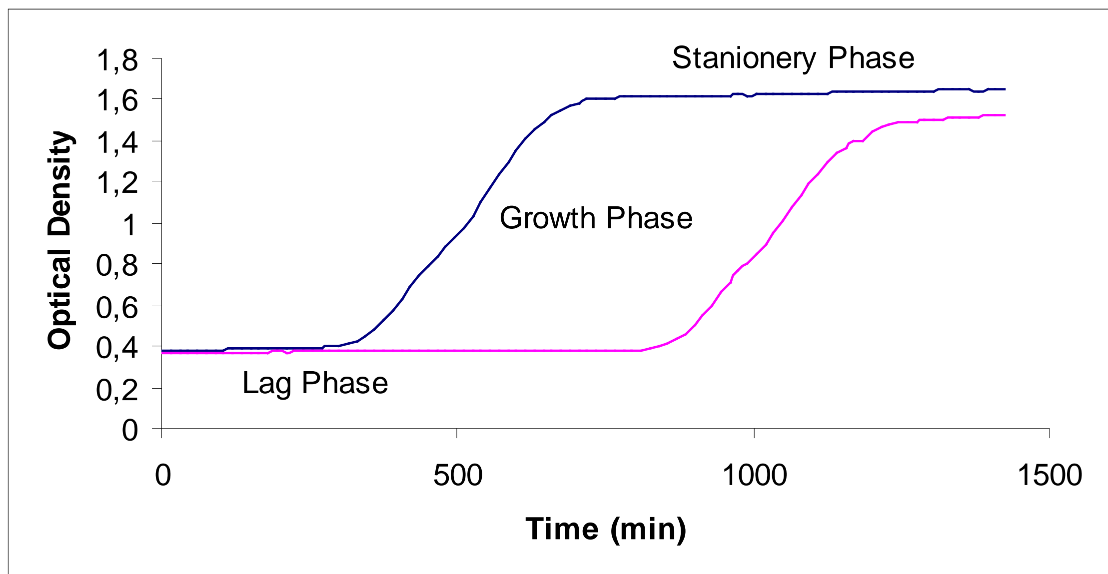
Comparison of previously discussed data about stressed cells (Table 4.1) and injured cells (Table 4.2), it can be concluded that high population of stressed

cells is resulted by extreme compression treatments, whereas higher number of injured cells are resulted by slow decompression treatments. Consequently, the lethal effect of fast compression and slow decompression rates overrides other treatments.

Table 4.2: Measurement of lag times for different HHP treatments against certain threshold limit of optical density

Matrices	Treatment	Threshold Optical density	Lag time (min)	Log cfu/ml
Tris buffer	**C**	0.96	510	7.62
	FM	0.96	885	3.98
	SM	0.97	975	5.15
	MM	0.39	>24 h.	4.97
	MF	0.97	960	3.81
	MS	0.39	>24 h.	N.D.
Skimmed milk	**C**	0.96	510	7.52
	FM	0.99	975	5.26
	SM	0.97	690	6.19
	MM	0.95	810	6.06
	MF	0.96	818	5.92
	MS	0.39	>24 h	4.57

Figure 4.7: Typical representation of optical density measurement at 450 nm for HHP treated (700 MPa/5 min) (pink line) and untreated samples (control) (blue line)



CONCLUSIONS

Fast and slow rates of compression results higher number of stressed cells than medium rates. Stressed cells can revive during storage in Tris buffer and skimmed milk but orange juice inhibits their revival. Faster rates of compression during high hydrostatic pressure processing are more lethal to inactivate to *Staphylococcus aureus* in all matrices causing more incurable sublethal injuries. Slow decompression rates during HHP processing results in high lethality, possibly due to elongated processing times. HHP mediated injured cells of *S. aureus* are quite sensitive to low pH and can easily be killed during storage even at 4 ℃. Highest lethal impact of HHP treatments was observed in orange juice leading to complete destruction after 15 days of storage. Skimmed milk and Tris buffer provides suitable environment for sublethally injured cells to maintain their survival at 4 ℃ storage. Finally, the lethal effect of fast compression and slow decompression rates overrides other treatments.

CHAPTER 5

BACILLUS SUBTILIS

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Effect of compression and decompression rates during High Hydrostatic Pressure processing on inactivation kinetics of bacterial spores at different temperatures

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ABSTRACT

We investigated the effect of changing compression and decompression rates of High Hydrostatic Pressure (HHP) treatments on inactivation of spores. *Bacillus subtilis* (PS832) spores were inoculated in Tris buffer, skimmed milk and orange juice. The samples were subjected to HHP treatments of 600 MPa for 3 minutes at 60 ºC and 70 ºC. Microbiological analyses were carried out at 0, 1, 7 and 15 days of refrigeration storage (4 -5 ºC). Flow cytometry technique was used for the estimation of sublethally injured population. After 15 days, all pressure treated matrices at 70 ºC showed higher spore inactivation caused by slower compression rates as compared to faster ones. However, at 60 ºC, the inactivation caused by slower compression was not significantly different from faster rates. Slow decompression was found to be more lethal in 60 ºC and 70 ºC HHP treated samples. It is concluded that slow compression combined with slow decompression has a greater impact on inactivation of *Bacillus subtilis* spores than any combination of fast compression and fast decompression at 60 ºC and 70 ºC processing temperatures. However the population of sub-lethally injured cells was found to be higher with fast compression and slow decompression rates.

Keywords: High Hydrostatic Pressure (HHP), *Bacillus subtilis*, compression rate, decompression rate, bacterial spores, Flow cytometery.

Introduction

High hydrostatic pressure (HHP) processing is an emerging technology to produce shelf-stable foods. The pressure treated food is subjected to pressures up to 700 MPa at chilled or ambient temperature for a certain holding time to achieve pasteurization. The unique advantage of this technology is its immediate and uniform pressure transmission throughout different media as well as a uniform and rapid heating or cooling due to the adiabatic heat of compression, if the treated food is homogeneous in its composition (Crawford, et al., 1996; Knorr 1993; O'Brien & Marshal, 1995; Patterson, et al.,, 1995; Yuste, et al.,. 1998; Heinz, et al., 2009). However, an ideal adiabatic process does not occur in practical application and consequently the temperature distribution during pressure holding time could be quite inhomogeneous. A further advantage of HPP processing is that it preserves product attributes such as color, flavor, texture and nutritional values when compared with conventional thermal processed food (Ahn and Balasubramaniam, 2007). Consequently, combinations of HHP and ambient temperatures are useful alternatives for the production of high quality and pasteurized foods.

However, to achieve a sterilized product, higher treatment temperatures are necessary. The combination of HHP and initial product temperatures between 60 °C and 90 °C could lead, due to the adiabatic heat of compression, to process temperatures between 90 °C and 130 °C. This combination of pressure and high temperatures does not only accelerate the inactivation of bacterial spores, but also the treatment temperature required for full spore inactivation can be reduced (Ardia, et al., 2004; Mathys, et al., 2009; Ahn, et al., 2007, Black et al 2006, Margosch, et al. 2006, and Margosch, et al., 2004).

Nevertheless, the mechanism of inactivation of bacterial spores by heat and pressure is still a matter of discussion.

In dependence of the applied temperature and pressure level, bacterial endospores pass through different physiological pathways, depending on the temperature and pressure level, and this can induce spore germination or a subsequent inactivation during the treatment. In literature two- (Margosch, et al., 2006) or three-step models (Heinz & Knorr, 1996; Mathys, et al., 2007b) for spore inactivation are discussed, but, it is assumed that the spore germination is the first step of spore inactivation under pressure (Gould & Sale, 1970).

At low pressures in the range of 100-200 MPa and ambient temperatures a spore germination caused by the activation of the germinant receptors is possible, but a part of the whole spore population is still in the dormant state, which could cause food borne diseases in the processed product (Heinz & Knorr, 2002), and consequently, higher pressures and temperature are needed for the sterilization of foods. The application of very high pressure (higher than 500 MPa) could induce a non-nutrient germination by a direct opening of the spore's dipicolinic acid (DPA) channels (Ca^{2+}–DPA channels), which triggers the cortex lytic enzymes (CLE) (Setlow, 2003), and may result in an incomplete germination process (Paidhungat, et al., 2002; Wuytack, et al., 1998). Moreover, during very high pressure treatments under elevated temperatures additional effects such as the dissociation equilibrium shift in buffer solution (Mathys, et al., 2008), a possible spore agglomeration (Mathys, et al., 2007b) and the adiabatic heat of compression, have to be taken into account. In the course of finding mechanisms behind this inactivation, it was shown that flow cytometry is a potent tool to gain insights in the states and

mechanisms of cell damage of pressure treated microorganisms (Black, et al., 2006; Mathys, et al., 2007a).

However, while spore inactivation by HHP was extensively investigated, there is very limited information available on the role of compression and decompression rates on bacterial spore inactivation.

In general, the compression rate of HHP equipment is fixed and normally governed by equipment design parameters. Typical come-up time in commercial scale HHP equipment falls in the range of 210 to 360 s to reach 600 to 700 MPa pressure level. Excessively longer come up times (>240 s) can increase the total process time and reduce the process throughput (Tonello Samson, C. 2010, NC Hyperbaric, personal communication).

However, variation in come up time may affect the inactivation kinetics of microorganisms (Rathphitagsanti, et al., 2008). Therefore, consistency and awareness of pressurization and depressurization rates are important in process development.

The role of compression and decompression rates on inactivation of *Salmonella typhimurium* and *Listeria monocytogenes* (Chapleau, et al. 2006), *Bacillus stearothermophilus* spores (Hayakawa et al. 1998), *Escherichia coli*, *Salmonella typhimurium* , *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus* (Noma, et al. 2002) during HHP has been studied previously. However, the results are often contradictory which may be due to variation in process parameters and experimental design.

Smelt (1998) hypothesized that fast compression might contribute to higher inactivation of vegetative cells, while a slower compression might induce a stress response that would render pressurization less effective. Further, he assumed that organisms such as yeasts are sensitive to fast depressurization due

to the vacuole in the cell that collapses, whereas vegetative bacteria are probably quite insensitive under similar conditions.

Hayakawa et al 1998 reported that rapid decompression (1.30 to 1.65 ms) enhanced microbial lethality of *Bacillus stearothermophilus* spores. Noma et al 2002, reported similar findings for the inactivation of vegetative bacteria where rapid decompression (1 ms) was more effective than slow decompression (>30s), and additionally, lower treatment pressures were required. Rademacher, et al. 2002 found no significant differences in inactivation of *Listeria innocua* between treatments of fast compression (500 MPa/min) followed by slow decompression (100 MPa/min) or slow compression (100 MPa/min) followed by fast decompression (500 MPa/min). Chapleau et al (2006) correlated the efficiency of HHP treatment with microbial reduction by using barometric power, which was estimated from the area under the pressure time curve. The authors observed the highest microbial inactivation of *Salmonella typhimurium* and *L. monocytogenes* when the slowest compression (1 MPa/s) and decompression (5 MPa/s) were applied. These correspond to highest barometric power.

Bearing in mind the contradictory results of previous researchers, systematic studies are needed to determine the influence of compression and decompression rates on inactivation of bacterial spores. The present study was conducted to evaluate the effect of compression and decompression rates on spore inactivation of *Bacillus subtilis* spores suspended in different matrices. Additionally, the experiments were conducted at different processing temperatures to estimate the impact of temperature on the inactivation by HHP.

Materials and Methods

Spores Preparation

For all HP treatment *Bacillus subtilis* PS832 spores (obtained from P. Setlow; University of Connecticut, Health Center), a prototrophic derivative of strain 168, were used. Spores were prepared at 37 °C on 2xSG medium plates without antibiotics and harvested and cleaned as described, according to the method described by (Nicholson & Setlow, 1990; Paidhungat & Setlow, 2000). The spores were cleaned by repeated centrifugation and washing with cold (6 °C) distilled water with occasional (1 min) sonication treatments, and stored in the dark at 6 °C. The final spore suspension contained <1% germinated spores or growing cells as seen by phase contrast microscopy or flow cytometery (Mathys, et al., 2007a).

Sample Preparation

Commercially available orange juice (pH 3.4) and skimmed milk (pH 6.80) were selected from a local supermarket. Tris buffer solution (Carl Roth GmbH, Karlsruhe, Germany) (10 mM) with pH 6.85 was freshly prepared and stored at 4 °C after sterilization. 10 ml of each of the three matrices were inoculated with 2 ml spore suspension to get the initial total count of ~ 10^8 cfu /ml in each matrix. Inoculated sample of each matrix was filled in sterilized small screw capped plastic tubes (Cryotube, Thermo Fisher Scientific (Nunc GmbH & Co. KG), Langenselbold, Germany) of approximately 1.6 ml volume.

HHP Treatments

HHP treatments were carried out in a multi-vessel high pressure unit (Model U111, Unipress, Warsaw, Poland) by using a constant final pressure of 600 MPa for 3 minutes pressure holding time. The compression medium was 1:3 glycol-water solution. The rate of compression was controlled manually by

pulsing compression pump and rate of decompression was also controlled manually by controlling pressure discharge valves. However the pressurization and depressurization data was recorded by data acquisition software (TestPoint 4.0, Capital Equipment Corporation) and are displayed in Fig. 5.1.

During trial run of equipment with dummy samples it was observed that pressure increase up to 600 MPa, resulted in the adiabatic heating of approximately 28 ºC in the sample (measured by infusion of thermocouple inside the dummy sample tube during pressure treatments). To overcome the effect of adiabatic heating, the following equation was a good guideline.

$T_i = T_f - 28$ ºC

Where T_i is the initial temperature of the sample before starting HHP treatment, T_f is the final required temperature during HHP treatment.

However a slight variation in adiabatic heating trend was observed between slow and fast compression rates. The temperature and time histories of HHP experiments are given in Table 5.1 (a & b). Following treatment codes were used identify the HHP treated samples.

FF = Fast compression followed by Fast decompression

FS = Fast compression followed by Slow decompression

SF = Slow compression followed by Fast decompression

SS = Slow compression followed by Slow decompression

The treatments were carried out at 20 ºC, 60 ºC and 70 ºC with repetition on different days. The samples were cooled down in an ice box immediately after the HHP treatments until the first microbiological analyses and afterward stored in refrigeration (4 – 5 ºC) during the remaining 15 days of the study.

Post-pressure thermal treatment

To estimate the amount of germinated *Bacillus subtilis* spores after the different pressure treatments at 60 °C, a part of HHP treated samples was further subjected to post-pressure thermal treatment. Therefore, 100 µL of HHP treated samples were transferred into eppendorf tubes (Eppendorf AG, Hamburg, Germany) and heat treated for 20 minutes in block heater previously heated at 80 °C (Stuart SBH130D, Bibby Scientific Limited, Stone, UK).

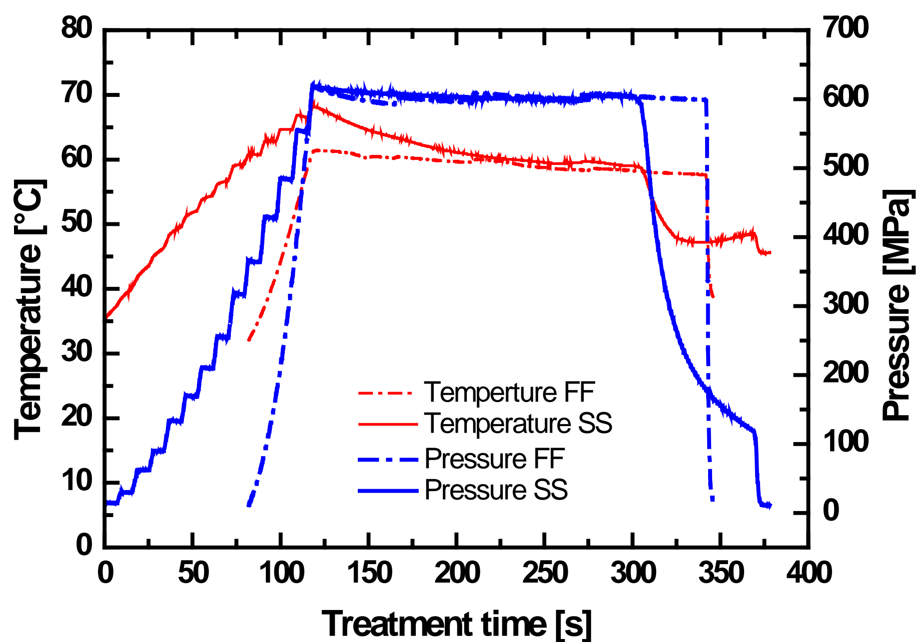


Figure 5.1: Pressure (blue) and temperature (red) profiles for fast (solid dotted line) and slow (solid line) compressions and decompressions ramps, for 600 MPa and 70 °C.

Table 5.1a: **Temperature histories during HHP treatments of 600 MPa for 3 minutes holding time.**

Treatment Code	Treatment Description	60 ºC			70 ºC		
		Starting Temperature	Maximum Temperature	Temperature during holding time	Starting Temperature	Maximum Temperature	Temperature during holding time
FF	Fast Compression with Fast Decompression	32 ºC	61 ºC	60 ºC	42 ºC	76 ºC	70 ºC
FS	Fast Compression with Slow Decompression	32 ºC	65 ºC	60 ºC	42 ºC	78 ºC	70 ºC
SF	Slow Compression with Fast Decompression	32 ºC	69 ºC	60 ºC	42 ºC	77 ºC	70 ºC
SS	Slow Compression with Slow Decompression	32 ºC	68 ºC	60 ºC	42 ºC	77 ºC	70 ºC

Table 5.1b: **Pressure come-up and pressure release timings for 600 MPa final pressure level.**

HHP Treatment	Time (Seconds)
Fast compression	40
Slow compression	165
Fast decompression	4
Slow decompression	105

Thermal Inactivation

To evaluate the thermal resistance of the used spore suspension without pressure, Tris buffer solution; orange juice and skimmed milk were inoculated with *Bacillus subtilis* spores (PS832) to get the initial total count of ~ 10^8 cfu /ml in each matrix. The spore suspension (60 µL) was filled into thin glass capillaries and hermetically sealed to prevent evaporation of the suspension due to temperatures above 100 °C. The spores were inactivated in a thermostatic bath (Huber GmbH, Offenburg, Germany), filled with silicon oil (M40.165.10, Huber GmbH, Offenburg, Germany) at various temperatures (80, and 100 ºC). After thermal treatment the glass capillaries were immediately stored at 4 °C.

Microbiological Analyses

The surface of each tube was cleaned with 70% ethanol before being opened in the sterilized microbial clean bench. After mixing the tube contents thoroughly, 30 micro liter of the sample content was serially diluted in 1/4 Ringer solution (TP887925 712, Merck KGaA, Darmstadt, Germany) on micro plates (Carl Roth GmbH, Karlsruhe, Germany) and two 50 µl samples of every dilution were drop-plated in Petri-dishes on nutrient agar (CM 003, Oxoid Ltd., Hampshire, England). The dishes were incubated at 37 °C for 48 h and the colonies were manually counted.

Flow Cytometry Analyses

Flow cytometry (FCM) of the pressure treated samples at 60 °C was performed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 15 mW air-cooled 488 nm argon-ion laser. As an operating software BD CellQuest Pro (BD Biosciences) and as a sheath fluid Isoton II (Coulter Corporation, Miami, FL, USA) were used. Spore suspension was

double stained with the fluorescent dyes SYTO 16 (Invitrogen, Oregon, USA) and propidium iodide (PI, Invitrogen, Oregon, USA), after a method which is described elsewhere (Mathys, et al., 2007a). The detection of a green fluorescent signals (SYTO 16), indicates a germination of the pressure treated spores due to a cortex hydrolysis. The detection of red fluorescent signals (PI), indicates a damage of the inner spore membrane and consequently an inactivation of the spore (Mathys, et al., 2007a; Black, et al., 2005).

Statistical Analyses

Two independent trials were carried out and two sample replicates were used in each trial. So the final data was based on average values of four replicates. The data was analyzed by using STATISTICA 7.0 (StatSoft Inc., Oklahoma, USA) software. The mean values and significant differences were determined by using Newman-Keuls test at 95% confidence interval (p =0.05).

Results and Discussions

In the preliminary stage, the Tris buffer solution; skimmed milk and orange juice were inoculated with spores and exposed to thermal treatments of 80, and 100 ºC to estimate the thermal tolerance of *Bacillus subtilis*. The results of the thermal treatments showed no inactivation at low temperature (80 °C, 20 min), therefore 80 ºC for 20 minutes was suitable treatment for the determination of post pressure thermal resistance of *Bacillus* spores.

Table 5.2 : Effect of compression and decompression rates and treatment temperatures on survival of pressure treated *Bacillus subtilis* spores.(600 MPa/3 min) during storage at 4 ºC

Process Temperatures		60 ºC (Log N/No)*				70 ºC (Log N/No)*			
HHP Treatments**		FF	FS	SF	SS	FF	FS	SF	SS
Tris Buffer	0 Day	-0.688 ± 0.240	-0.728 ± 0.130	-0.670 ± 0.099	-0.801 ± 0.066	-4.244 ± 0.805	-4.662 ± 0.276	-4.498 ± 0.708	-4.816 ± 0.357
	1 Day	-0.623 ± 0.227	-0.726 ± 0.083	-0.607 ± 0.010	-0.787 ± 0.046	-4.761 ± 0.090	-4.646 ± 0.409	-4.771 ± 0.343	-5.149 ± 0.180
	7 Days	0.132 ± 0.108	-0.011 ± 0.251	0.079 ± 0.159	-0.108 ± 0.223	-4.430 ± 0.516	-5.207 ± 0.154	-4.572 ± 0.682	-5.019 ± 0.141
	15 Days	0.007 ± 0.039	-0.255 ± 0.178	-0.115 ± 0.193	-0.418 ± 0.468	-4.493 ± 0.553	-5.135 ± 0.152	-4.565 ± 0.725	-5.057 ± 0.225
Skimmed Milk	0 Day	-0.758 ± 0.103	-0.821 ± 0.047	-0.787 ± 0.021	-0.940 ± 0.121	-4.106 ± 0.134	-4.806 ± 0.107	-4.293 ± 1.851	-4.983 ± 0.309
	1 Day	-0.651 ± 0.093	-0.704 ± 0.028	-0.719 ± 0.030	-0.859 ± 0.028	-4.224 ± 0.146	-3.983 ± 0.125	-4.412 ± 1.781	-4.846 ± 0.409
	7 Days	-1.093 ± 0.439	1.131 ± 0.359	-0.797 ± 0.065	-0.942 ± 0.084	-4.442 ± 0.094	-4.477 ± 0.446	-4.373 ± 1.696	-5.232 ± 0.243
	15 Days	-0.887 ± 0.158	-0.904 ± 0.031	-0.864 ± 0.096	-1.083 ± 0.057	-4.303 ± 0.257	-4.128 ± 0.136	-4.263 ± 1.341	-4.766 ± 0.688
Orange Juice	0 Day	-1.265 ± 0.089	-0.593 ± 0.372	-1.017 ± 0.053	-1.367 ± 0.057	-4.242 ± 0.139	-3.957 ± 0.073	-2.971 ± 0.056	-3.927 ± 1.240
	1 Day	-1.036 ± 0.056	-1.242 ± 0.130	-0.923 ± 0.052	-1.224 ± 0.080	-5.112 ± 1.184	-4.500 ± 0.532	-2.913 ± 0.023	-3.664 ± 0.899
	7 Days	-2.241 ± 0.531	-2.074 ± 0.090	-1.456 ± 0.243	-1.722 ± 0.114	-5.125 ± 1.135	-4.637 ± 0.488	-3.024 ± 0.068	-3.517 ± 0.972
	15 Days	-2.482 ± 0.315	-2.624 ± 0.040	-1.750 ± 0.024	-1.858 ± 0.119	-4.974 ± 0.735	-4.642 ± 0.532	-3.032 ± 0.095	-3.541 ± 0.735

*LogN/No) (N = cfu/ml of treated samples, No = cfu/ml of untreated samples). ± Standard. Deviation].

** FF = Fast compression - fast decompression, FS = Fast compression –slow decompression, SF= Slow compression – fast decompression, SS = Slow compression – slow decpmression

Table 5.3: Effect of compression and decompression rates (600MPa/3 min) and treatment temperature on inactivation of pressure treated *Bacillus subtilis* spores (Log_{10} cfu/ml).

Matrix	Storage days	60 ºC				70 ºC			
		Compression		Decompression		Compression		Decompression	
		Fast	Slow	Fast	Slow	Fast	Slow	Fast	Slow
Tris Buffer	0	0.71a + 0.19	0.73a + 0.11	0.67a + 0.17	0.76a + 0.11	4.91a + 1.48	4.66a + 0.55	4.37a + 0.71	5.19a + 1.28
	1	0.67a + 0.18	0.69a + 0.12	0.61a + 0.18	0.75a + 0.08	4.71a + 0.29	4.96a + 0.32	4.77a + 0.23	4.90a + 0.40
	7	0.05a + 0.19	0.02a + 0.20	0.09a + 0.13	0.06a + 0.22	4.82a + 0.54	4.80a + 0.51	4.50a + 0.56	5.11b + 0.17
	15	0.12a + 0.18	0.26a + 0.37	0.05a + 0.15	0.33a + 0.34	4.82a + 0.51	4.81a + 0.56	4.53a + 0.60	5.10b + 0.18
Skim Milk	0	0.79a + 0.09	0.86a + 0.11	0.77a + 0.07	0.88b + 0.10	4.46a + 0.39	4.64a + 1.28	4.20a + 1.22	4.90a + 0.23
	1	0.67a + 0.07	0.79b + 0.08	0.68a + 0.07	0.78b + 0.09	4.32a + 0.30	4.57a + 1.25	4.28a + 1.21	4.61a + 0.40
	7	1.11a + 0.37	0.87a + 0.10	0.94a + 0.33	1.03a + 0.26	4.46a + 0.29	4.55a + 1.15	4.41a + 1.12	4.61a + 0.39
	15	0.89a + 0.11	0.97a + 0.14	0.87a + 0.12	0.99b + 0.10	4.22a + 0.21	4.52a + 1.02	4.29a + 0.89	4.45a + 0.57
Orange Juice	0	1.43a + 0.30	1.19b + 0.19	1.14a + 0.15	1.48b + 0.27	6.21a + 2.26	3.45b + 0.96	4.63a + 2.35	5.03a +2.19
	1	1.19a + 0.11	1.07b + 0.17	1.03a + 0.12	1.23b + 0.10	4.80a + 0.91	3.29b + 0.71	4.01a + 1.40	4.08b + 0.82
	7	2.16a + 0.36	1.59b + 0.22	1.85a + 0.57	1.90a + 0.21	4.88a + 0.85	3.27b + 0.58	4.07a + 1.34	4.07a + 0.85
	15	2.52a + 0.21	1.68b + 0.07	2.07a + 0.45	2.12a + 0.49	4.81a + 0.62	3.28b + 0.56	4.00a +1.14	4.09a + 0.84

* Different letters in Fast and Slow columns shows significant difference at p =0.05. (+ Standard Deviation).

The HHP experiments were started at 20 ºC with 600 MPa pressure for 3 minutes with changing compression and decompression ramps. But the microbiological results showed no effect of HHP treatments on spore inactivation at low temperature (20 ºC) (results not shown), consequently, the process temperature was increased to 60 °C and 70 °C. The inactivation data of *Bacillus subtilis* spores are shown in Table 5.2. Different treatments of HHP at 70 ºC resulted in higher inactivation rates of *Bacillus subtilis* spores compared to 60 °C.

Microbiological analyses immediately after HHP treatments showed that in Tris buffer solution SS treatment had the strongest impact (-4.8 log_{10}) of HHP followed by FS treatment (-4.7 log_{10}) , SF (-4.5 log_{10}) and FF (-4.2 log_{10}), respectively. The same tendency was observed in skimmed milk samples. In orange juice HHP treatments at 70 ºC resulted in highest impact of FF treatment with -4.2 log_{10} followed by FS (-4.0 log_{10}), SS (-4.0 log_{10}) and SF (-3.0 log_{10}), respectively. Statistical analyses were carried out to determine the significance of the individual impact of fast and slow treatments. Table 5.3 shows that at 70 ºC in Tris buffer, the inactivation of *Bacillus subtilis* spores during initial days of storage was not significantly different by slow and fast treatments of compression and decompression. However, after 7 days inactivation by slow decompression (5.11 log cfu/ml) was significantly higher than fast decompression treatment (4.5 log cfu/ml). In skim milk 70 ºC treatments of HHP did not revealed any significant difference between fast and slow rates. In orange juice HHP treatments at 70 ºC revealed higher inactivation of *Bacillus* spores by fast compression and slow decompression. The impact of slow decompression was more visible during initial days of storage but during 15 days the difference between slow and fast decompression

become non-significant. Whereas, fast compression showed higher inactivation impact than slow compression throughout the storage period of 15 days.

However, due to relatively high inactivation for all tested pressure ramps in all matrices as well as the contradictorily result between the inactivation in Tris buffer and milk versus orange juice, the treatment intensity was lowered (60 °C) to perform analysis of the physiological state of the spores after the HPP treatments via FCM.

Table 5.2 shows the impact of different HHP treatments at 60 ºC. In Tris buffer solution the spore inactivation trend was similar to 70 ºC treatments but with lower inactivation rate. SS treatments resulted in a slightly higher inactivation of *Bacillus subtilis* spores with -0.80 log_{10}, whereas the inactivation for the other pressure ramps was equal with -0.73 log_{10} for the FS treatment followed by FF and SF (-0.69 and -0.67 log_{10} respectively). In skimmed milk and orange juice, SS treatment had higher lethality (-0.94 and -1.37 log_{10} respectively) as compared to other HHP treatments.

Furthermore, Table 5.2 presents the detailed microbiological counts during 15 days storage at 4 °C after different HHP treatments. For all tested matrices no significant increase in cell counts during storage was observed. Moreover, a continuous inactivation in orange juice was detected, which could be due to spores that are partly germinated, which are sensitive to the lower pH in the juice compared to Tris buffer or milk. These spores, which possibly only finished stage I of germination under pressure (Paidhungat, et al., 2002; Wuytack, et al., 1998) have lost their resistance against e.g., chemical substances (Setlow, 2003) and are sensitive to the low pH (Moussa-Boudjemaa, et al. 2006; Leguerinel & Mafart, 2001; Reineke, et al., 2011b) and in a sublethal state. Hence, these spores are not inactivated at neutral pH, like

in milk or Tris buffer but could recover on nutrient rich agar plates.

At 60 ºC, in Tris buffer, no significant differences in fast and slow rates of compression and decompression could be observed (Table 5.3). In skim milk, HHP treatments at 60 ºC resulted in significantly higher inactivation by slow decompression than fast decompression during 15 days storage. While the effect of fast and slow compression rates differed significantly only during day 1 analyses. In orange juice at 60 ºC, fast compression showed significantly greater inactivation of *Bacillus* spores than slow compression rates. Whereas, slow decompression revealed greater inactivation of *Bacillus* spores during initial days of storage, but after 7 days these differences became non-significant (Table 5.3).

Estimation of germinated and sub-lethally injured spore population

To estimate the impact of possible sub-lethally injured cells on the cell count during storage and to get deeper insight into the underlying inactivation mechanisms during different compression and decompression times, post-HHP thermal treatment of samples was conducted. Samples were subjected to heat treatment after HHP treatments, to estimate the amount of germinated spores after pressure treatment (Wuytack, et al., 1998) as well as FCM analysis.

Table 5.4 shows data of thermal sensitive spore population after HHP treatment of 600 MPa for 3 minutes at 60 ºC. It is quite evident that the higher number of sensitive cells was the result of FS treatment in Tris buffer (-4.3 $\log_{10}$) and skimmed milk samples (-5.6 $\log_{10}$), while in orange juice no survival could be detected in HHP plus thermal treated samples. From these results FS treatment could be declared as the most effective process condition to shift the *Bacillus subtilis* spores from the dormant state, into the germinated or even inactivated

state (-0.62 log_{10} in Tris buffer and -0. 82 log_{10} in skimmed milk). Wuytack and Michiels (2001) explained the role of low pH during HHP and heat treatments of *Bacillus subtilis* spores. They concluded that there is no germination at pH < 5. In addition, they suggested that so-called H-spores are formed due to exchange of Ca^{2+} ions with H^+. These H-spores are known to partially loose their heat resistance, so it is no surprise that they do not survive the heat treatment at 80°C that is applied in this work to kill germinated spores. However, H-spores can revert to normal spores in the presence of Ca 2+, so they should not be considered as germinated. H-spores formed by HHP treatment in acid conditions can be distinguished from vegetative cells and truly germinated spores by heat treatment at 60°C. Low pH of orange juice causes an additional inactivation effect on HHP and thermally treated spores.

Table 5.4 : Estimation of sub-lethally injured /germinated population of *Bacilus subtilis* spores at 60ºC HHP treatment (600MPa/3 min) and a thermal post-HHP treatment at 80 °C for 20 min.

Matrices	Treatments	Log N/No* after HHP treatment (A)	Log N/No* after HHP + thermal treatments (B)	Sub-lethally injured/germinated population (A-B)
Tris Buffer	FF	-0.485 ± 0.021	-3.732 ± 0.033	3.732
	FS	-0.616 ± 0.013	-4.255 ± 0.069	4.254
	SF	-0.588 ± 0.062	-3.683 ± 0.051	3.682
	SS	-0.752 ± 0.051	-3.969 ± 0.035	3.968
Skimmed Milk	FF	-0.682 ± 0.049	-5.168 ± 0.056	5.167
	FS	-0.824 ± 0.079	-5.607 ± 0.00	5.606
	SF	-0.770 ± 0.011	-5.005 ± 0.157	5.004
	SS	-0.840 ± 0.037	-4.778 ± 0.322	4.777
Orange Juice	FF	-1.329 ± 0.025	N.D**	
	FS	-1.912 ± 0.050	N.D**	
	SF	-1.043 ± 0.030	N.D**	
	SS	-1.398 ± 0.058	N.D**	

*LogN/No) (N = cfu/ml of treated samples, No = cfu/ml of untreated samples). ± Standard. Deviation]
**N.D. = Not detectable (< 1 cfu/ml)

To further evaluate the physiological state of these thermal sensitive spores, FCM analysis were performed. To exclude additional possible matrix effects, such as a pressure dependent pH shift (Mathys, et al., 2008) in e.g. skimmed milk or orange juice, *Bacillus* spores were suspended in Tris buffer solution, due to the calculated pH stability under relevant pressure temperature combinations (Mathys, Heinz, & Knorr, 2005).

The results are presented in Figures 5.2a and 5.2b for 1 s and 3 min dwell time, respectively. According to Mathys et al. (2007a), bacterial endospores pass under pressure through the physiological states dormant (R1), germinated (R2), unknown and not cultivable state (R3) and inactive (R4). For the untreated sample, spores were detected by FCM as being in the dormant state. After different pressure treatments at 60 °C with 3 min dwell time, regardless of the pressure ramps, all spores were detected in the unknown state, in which it has to mentioned that FCM provides only quantitative information about the different subpopulation if the amount is between 1 % and 100 % related to the total amount of detected particles. Hence, this correlates quite well with a high amount of thermal sensitive spores (Table 5.4). However, no information about the impact of the different pressure rates was possible, but a slight indication for a lower effectiveness of FF and SF treatment where detected by the FCM, due to a small amount of spores which still in the germinated state (R2) and not shifted to R3.

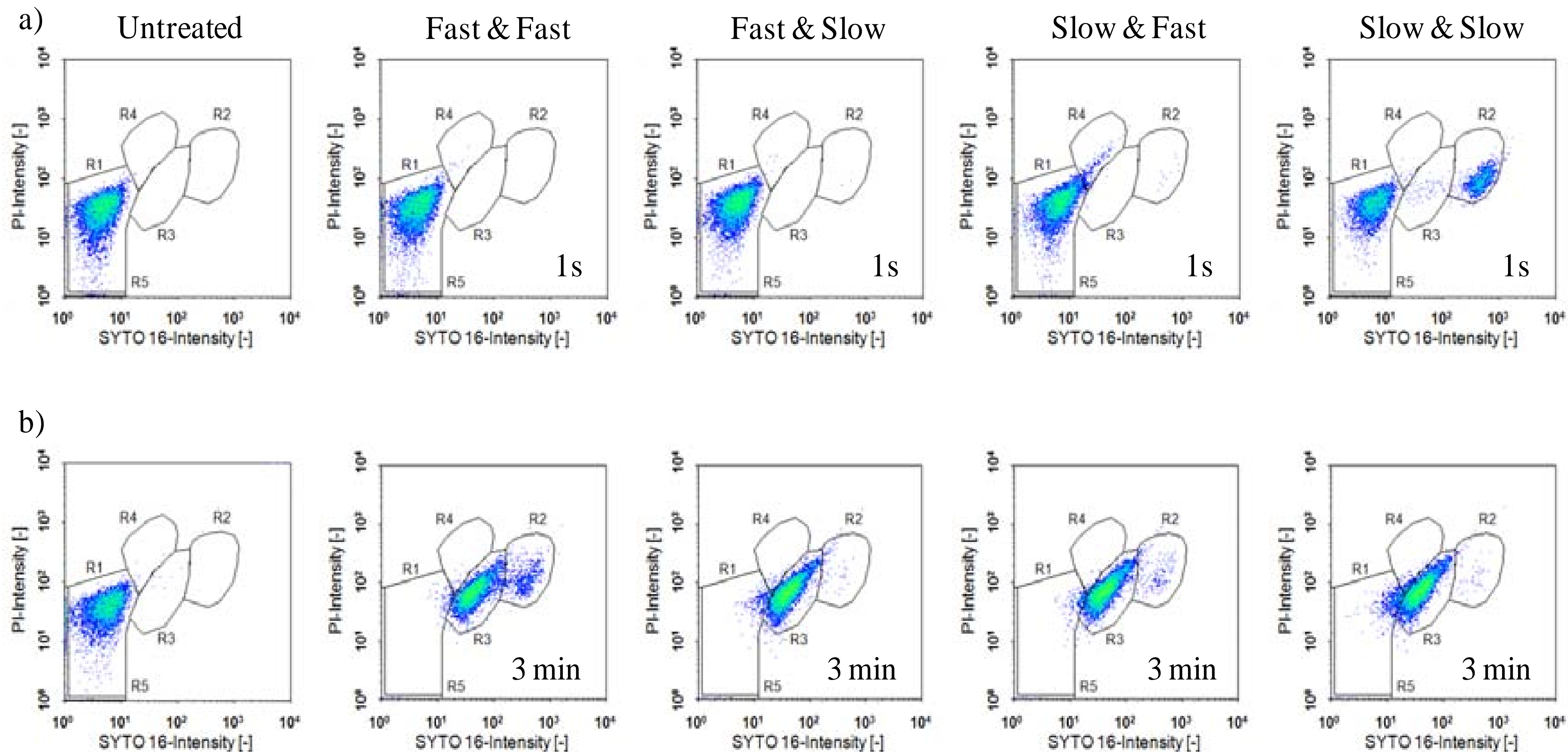


Figure 5.2. Flow cytometric density plots from *B. Subtilis* spores after (a) 1 s pressure treatment at 600 MPa, 60 °C and (b) 3 min pressure treatment at 600 MPa, 60°C in Tris buffer (pH 6.85, 0.01 M). Sub-population assignement: R1 = dormant, R2 = germianted, R3 = unkown, R4 = inactivated, R5 background signal. Increasing Syto 16 intensity is displayed on the axis of abscissa and increasing PI intensity on the axis of ordinate.

To exclude the pressure, temperature effects under isothermal isobaric conditions and to consequently lower the amount of spores in the unknown state, spores suspended in Tris buffer were treated with the different HHP treatments at 60ºC with no pressure holding time (about 1 s). The FCM data for this dynamic pressure and temperature treatment are presented in Figure 5.2a. Again, FF and SF and now additionally SF had no impact on the physiological state of the spores and they remained in the dormant state (R1). For the SS treatment, a proportion of 15 % germinated spores could be detected, which confirms that these treatment conditions would be the most lethal for an industrial scale HHP process. Similar finding were reported by Ratphitagsanti et al. (2008), who worked with *Bacillus amyloliquefaciens* spores at 90-120 ºC. They reported that for short pressure holding times (<2 minutes), HHP treatment with the slow pressurization rate provided enhanced spore reduction, however, these differences diminished with the longer holding times.

This behavior of spores during a slow pressurization could be explained by a pressure induced non-nutrient germination, in which the germination receptors are triggered at pressures between 200 and 500 MPa (Wuytack, et al., 1998). If the spores are held under these pressure conditions for a longer time, the amount of germinated spores continuously increases and consequently these spore are now sensitive to a combination of heat and pressure due to the release of DPA (Black, et al., 2006). During the pressure dwell time the additional effect of a pressure induced opening of the DPA-channels ($p < 500$ MPa) (Setlow, 2003) accelerates the spore germination, and consequently in combination with higher temperatures, the inactivation rate. Furthermore, the rate of germination is highly temperature dependant (Reineke, et al., 2011a),

which could be an explanation for the statistically not significant inactivation of spores at 20 °C, even if they were compressed very slowly.

Moreover, an increase in the final process temperature from 60 °C to 70 °C during holding time (with T max of 77 °C for the slow compression) enhanced the spore inactivation up to >4 $\log_{10}$ in contrast to maximum 1.4 $\log_{10}$ at 60 °C , which was also found by Reineke et al. (2011a) for the same spore strain and by (Mathys, et al., 2009) for *Geobacillus stearothermophilus* and by Margosch et al. (2006) for *Clostridium botulinum*.

Hence, these data support the finding that above a certain threshold temperature the HHP effectiveness can be raised up to greater extent even with little increase in processing temperature (Reineke, et al., 2011a). Higher level of spore inactivation by slow decompression ramps can be understood by the longer times the germinated spores are kept under pressure, with the result of a higher lethality of pressure and temperature even at reduced pressure levels.

CONCLUSION:

In conclusion, one could summarize that regardless of the treatment matrix, a certain threshold temperature has to be exceeded to inactivate bacterial spores under HPP. Furthermore, a significant influence on the lethality of different compression and decompression ramps as well as the type of the treatment matrix was found. The highest effectiveness for spore inactivation in Tris buffer, skimmed milk and orange juice was found for a slow compression and a slow decompression, which was attributed to a higher amount of germinated spores during the compression phase and a higher lethal impact of pressure and temperature during the slow compression on the germinated cells. This was also confirmed by FCM analyses, in which large amounts of spore in the so-

called unknown and not cultivable state were detected. These probably sub-lethal injured spores were highly sensitive presumably to the low pH (3.4) in orange juice, which could explain the continuous decrease of viable cells during a chilled storage in contrast to constant cell counts for spores in Tris buffer and skimmed milk.

CHAPTER 6

GENERAL DISCUSSION

Application of high hydrostatic pressure for food preservation is a growing concern among food scientists and food manufacturers. Although, bacterial inactivation efficiency of HHP is directly proportional to pressure level, holding time and treatment temperature, at the same time the rates of compression and decompression also determine the bacterial inactivation during HHP processing. Different compression and decompression treatments are supposed to have varying impacts on cellular bodies that can alter their ability to survive normally. Selection of these parameters is interlinked with physiological state of target bacteria i.e. vegetative or spore forms etc. The types and growth phases of microorganisms (lag, exponential or stationary phase) and type of matrix also determine the effectiveness of HHP process. The efficacy of HHP treatments in food processing is often attributed to the pressure induced pH changes. However, the knowledge of in-*situ* pH values of foods subjected to HHP is scarce, because of obvious limitations of existing pH measurement methods. Recently, Chaminda et al. (2013) developed a pH sensor capable to work under high pressure and measured pH of liquid foods (juices and milk) under 800 MPa. They reported an increase of acidity in liquid foods including water with increasing pressure. It is recommended that for efficient HHP processing, food manufacturers should be well aware of their target microorganisms as well as chemical constituents of the foods and HHP processing parameters about pressure level, holding time, temperature as well as rate of compression and decompression should be selected accordingly.

In this study, selected strains of vegetative and spore forming bacteria were subjected to varying rates of compression and decompression suspended in orange juice, skimmed milk and Tris buffer solutions. These media were selected to represent an acid food product, a low acidity food and a medium

with the same pH but without nutrients. Although Tris buffer and milk had the same pH at room pressure it is possible they had different pH under HHP, as it is known phosphate buffer is more sensible to pressure than Tris buffer (Chaminda et al 2013).

E. coli (Gram negative, vegetative), *S. aureus* (Gram positive, vegetative) and *B. subtilis* (spore forming) represent different bacterial classes. These bacteria were inoculated in selected matrices and subjected to HHP. *E. coli* were subjected to 600 MPa / 3 min pressure treatments at 4 ºC initial temperature whereas for *S. aureus* (being pressure resistant) 700 MPa / 5 min was selected at 4 ºC initial temperature. *B. subtilis* (spores) were treated at 600 MPa / 3 min at elevated temperatures of 20º, 60º and 70 ºC. The selection of pressurizing ramps were mainly dependent on HHP equipment capacity (mentioned previously in corresponding chapters)

Stressed Cells

Stressed cells have been rarely discussed in the literature. They consist of the cells which are temporarily shocked due to adverse pressure conditions and remain inactive if analysed immediately after HHP treatments. But after certain time they are able to grow just like normal healthy cells. We analysed stressed cells by differential microbial count between Day 0 and Day 1 as it is supposed that mechanically injured cells are unable to recover their injuries so rapidly especially under refrigeration storage conditions. Stressed population comprises the same level of risk as healthy cells because of there ability to activate after certain relax time in suitable environment.

Table 6.1 shows recovery of stressed cells of *E. coli* and *S. aureus* in Tris buffer and skimmed milk. In case of orange juice no recovery of stressed cells

was observed after overnight storage. Table 6.1 shows mixed trend for different HHP treatments. In Tris buffer greater number of stress cells of *E. coli* (600 MPa / 3 min) could be observed by slow and fast compression treatments as compared to medium compression. Similarly, slow and fast decompression produced greater number of stressed *E. coli* cells in Tris buffer. In contrast to Tris buffer, *E. coli* in skimmed milk were more stressed by medium treatments than fast or slow rates of compression and decompression.

Stressed cells of *S. aureus* (700 MPa / 5min) in Tris buffer were highest by fast compression rates as compared to other HHP treatments, while in skimmed milk highest stressed cells were observed by slow compression treatments (2.37 log cfu/ml) followed by slow decompression and fast compression (2.29 and 2.24 cfu/ml, respectively).

For *B. subtilis* spores (Table 5.3) higher numbers of stressed cells were observed in Tris buffer and skimmed by fast compression and slow decompression treatments (ranging from 0.14-0.29 log cfu/ml).

Table 6.1: Determination of stressed cells (log cfu/ml) after overnight storage.

		HHP Treatments				
	Matrix	**FM**	**SM**	**MM**	**MF**	**MS**
E. coli	**Tris buffer**	1.03	1.19	0.74	0.94	1.24
	Skimmed milk	0.81	0.64	0.84	0.34	0.69
S. aureus	**Tris buffer**	1.57	0.42	0.80	0.69	0.47
	Skimmed milk	2.28	2.37	1.67	1.46	2.29

Lethal and sublethal effects of HHP treatments

In general fast compression with slow decompression was observed to be more lethal for inactivation of vegetative bacteria (*E. coli* and *S. aureus*) whereas for *B. subtilis* spores slow compression with slow decompression resulted in higher inactivation (Figures 3.2, 3.3, 4.1, 4.2, & Table 5.2). In vegetative cells, compression stage causes mechanical shock resulting in physical injuries whereas in spores, compression stage cause germination of bacterial spores that make them vulnerable to adverse environmental conditions. Compression during HHP is often attributed with adiabatic heating of the compression medium and consequently heating of the tested samples. Hence for ensuring isothermal processing conditions HHP machines must be equipped with temperature controlling system. Moreover, starting HHP treatments at lower temperatures also helps to avoid overshoot of temperature during compression. In our experiments vegetative bacteria (*E. coli* and *S. aureus*) were treated at 4 ºC starting temperature, whereas *Bacillus* spores were treated at 20, 60 and 70 ºC. All the HHP equipments used in our experiments were fitted with refrigeration systems for temperature control.

The subsequent dwell time and decompression treatment are mainly concerned with inactivation of bacterial cells. Fast decompression may also cause physical injuries in bacterial cells if the decompression rate is too fast (in milliseconds) (Noma et al. 2002). Since ultra fast decompression is not practicable with HHP machines being used in most of the research centres, the slow decompression expresses higher inactivation of bacteria because of extended treatment time.

Both selected vegetative bacterial strains (*E. coli* & *S. aureus*) expressed almost the similar response to HHP treatments, i.e. fast compression resulted in

higher inactivation than slow compression and slow decompression resulted in higher inactivation than fast decompression. However *S. aureus* was found to be more pressure tolerant as compared to *E. coli*. Hence the HHP treatment of *S. aureus* was carried out at 700 MPa for 5 min and for *E. coli* the selected pressure was 600 MPa for 3 min.

Our results of microbial inactivation by different compression and decompression rates verify the findings of some researchers like Smelt (1998) who assumed that slow compression might induce stress response in bacteria and hence reduce the lethality of HHP process. On the other hand, our results do not match with few other researchers like Noma et al. (2002) and Rademacher et al. (2002). The main reasons for disagreement include different capacities of HHP equipments, and selected parameters. In our experiments we used different HHP units; however the ultra fast decompression in 1 ms was beyond practical limits. It might be one of the reasons that our results disagree with Noma et al. (2002) who reported greater lethality when fast decompression was done in 1 ms. Rademacher et al. (2002) did not found any difference in bacterial inactivation when using fast and slow rates of compression and decompression. There might be the reason that they tested only 1.7 MPa/ s and 8.3 MPa/ s compression and decompression rates, whereas in our experiments various rates were tested e.g. compression at 1.3 MPa/ s vs 3.6 MPa/ s and 11.4 MPa/ s. (for *E. coli* experiment). It might be understood that the greater is the difference between fast and slow rates, the greater is difference in bacterial inactivation. Moreover, in this study we tested three different levels of compression and decompression that helps to understand the impact of HHP treatments more effectively.

Microbial analyses for total survival count are simply determined by using non-selective agar media. But to estimate injured population of different strains need particular attention. No standard procedure has still been reported for this purpose. In our experiments, for *E. coli,* tryptone soy agar (TSA) and violet red bile green agar (VRBGA) were quite distinctive to estimate the differential count between two populations (healthy and injured), but for *S. aureus* the results obtained in selective media Baird Parker agar were not clearly distinguishable from microbial count obtained in non selective (TSA). So alternatively we used optical density measurement for determination of lag times of differently treated samples. For *B. subtilis* spores, injured cells were estimated by differential microbial count between HHP treated samples and HHP plus temperature treated samples. Further, flow cytometry analyses were carried out for deeper insights. It was observed that highest number of sublethally injured cells /germinated spores resulted by fast compression and slow decompression treatment.

Recovery of Sublethally Injured Cells

Recovery of injured cells is mainly dependant on suspension media. In our experiments it was observed that orange juice strongly inhibit the recovery of vegetative and spore forming bacteria and even slightly injured cells are unable to heal their injuries. This is mainly because of high acidity of orange juice (pH = 4). In Spanish market certain juice brands have been observed that claim additives free preservation of product just by taking advantage of low pH (e.g. ¨veritas¨ brand is producing apple, peach, pear, and carrot juices without preservatives). Skimmed milk and Tris buffer provide favourable pH medium for recovery of slightly injured cells and that is why increase in healthy cells

was observed during storage for both spore and vegetative bacteria. It has been observed that in favourable storage conditions *E. coli* tends to recover more rapidly as compared to *S.* aureus and suppress the sublethal impact of different compression and decompression rates. Figure 6.1 and 6.2 shows inactivation of *E. coli* and *S. aureus* in skimmed milk and Tris buffer respectively on D15. Comparing these findings to previously discussed results in (Figures 3.2, Figure 3.4), where fast compression and slow decompression treatments resulted in significantly higher inactivation of *E. coli* at D1, it is revealed that at D15, inactivation resulted by sublethal injuries in *E. coli* by different HHP treatments become almost similar in milk and Tris buffer, whereas in *S. aureus* even after 15 days FM and MS treatments showed highest level of inactivation similar to D1. From these findings it can be concluded that recovery capability of *E. coli* after HHP treatments is greater that *S. aureus* in skimmed milk and Tris buffer.

Figure 6.1: Inactivation of vegetative bacteria in skimmed milk at D15 after HHP treatments.

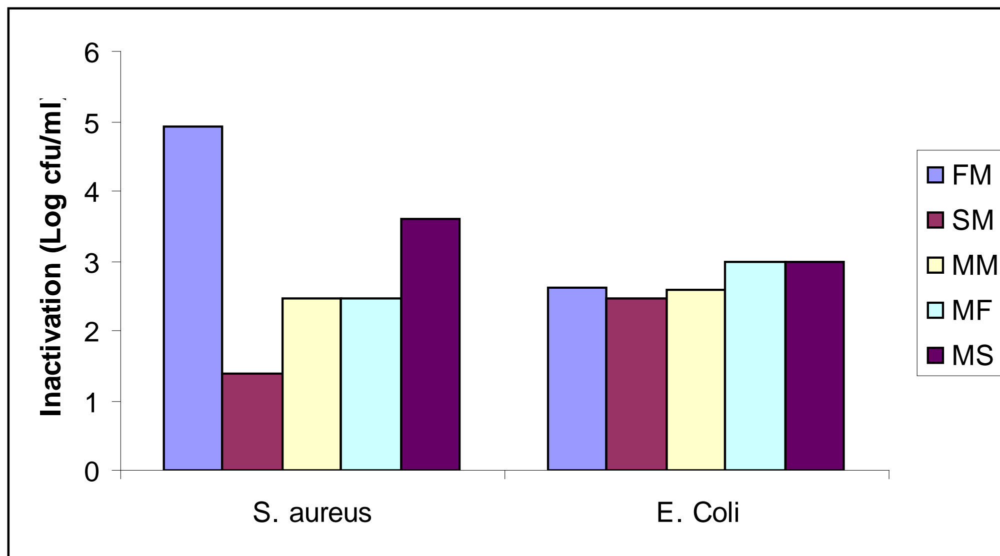
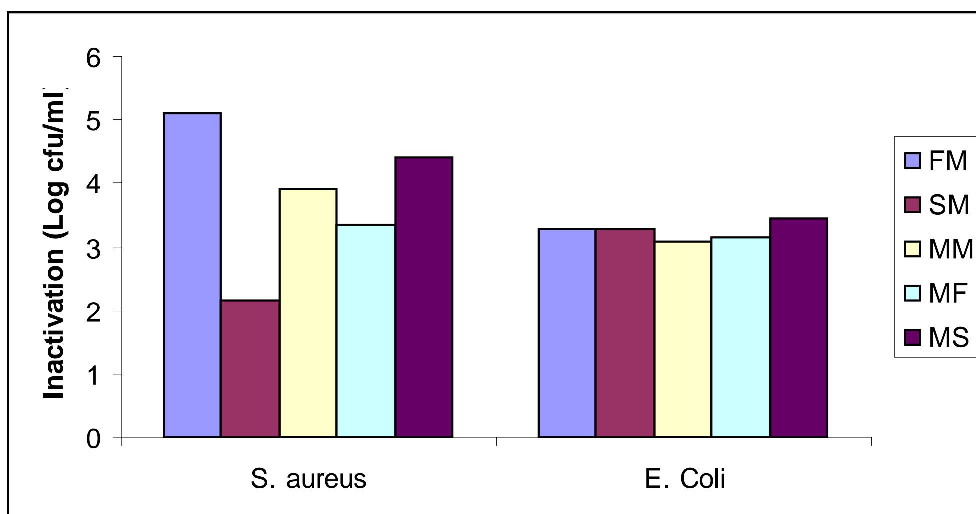


Figure 6.2: Inactivation of vegetative bacteria in Tris buffer at D15 after HHP treatments.



CHAPTER 7

CONCLUSIONS

(*CONCLUSIONES*)

Changing rates of compression and decompression may alter bacterial inactivation efficiency of HHP process

- Bacterial inactivation efficiency of high hydrostatic pressure (HHP) process can be enhanced by selecting optimum rates of compression and decompression.

- Fast compression is more lethal to inactivate *Escherichia coli* & *Staphylococcus aureus* as compared slow and medium rates of compression.

- For inactivation of *Bacillus subtilis* spores slow rates of compression are more lethal than fast compression.

- In general, slow decompression is more lethal for inactivation of vegetative as well as bacterial spores as compared to fast decompression.

Bacterial spores need high processing temperature for inactivation

- Inactivation of bacterial spores (B. subtilis) by HHP is possible in combination with elevated temperatures (60º-70 ºC).

- Vegetative cells do not need increase in HHP process temperature for inactivation.

Presence of injured and stressed cells in HHP treated samples may challenge food safety during storage and should be considered as a potential risk for food spoilage.

- Presence of stressed cells in HHP treated samples might be misleading about the inactivation efficiency of process if analysed immediately after HHP treatments.

- Fast compression produces higher number of injured cells than slow decompression in all matrices.

- Orange juice strongly inhibits the recovery of injured bacterial cells (spores as well as vegetative cells).

- The development of HHP equipments with more precise control and extreme rates of compression and decompression can enhance bacterial inactivation efficiency of the products.

CONCLUSIONES

Los cambios en las tasas de compresión y descompresión pueden alterar la eficacia de inactivación bacteriana del proceso HHP

- La eficiencia de la inactivación bacterial en el proceso de alta presión hidrostática (HHP) se puede mejorar mediante la selección de las tasas óptimas de compresión y descompresión.

- Una compresión rápida es más efectiva para inactivar *Escherichia coli* y *Staphylococcus aureus* en comparación con velocidades bajas y medianas de compresión.

- Velocidades de compresión lentas son más letales para la inactivación de esporas de *Bacillus subtilis* que la compresión rápida.

- En general, la descompresión lenta es más letal para la inactivación de células vegetativas, así como para esporas bacterianas en comparación con la descompresión rápida.

Se necesitan temperaturas elevadas de procesado para la inactivación de las esporas bacterianas

- La inactivación de las esporas bacterianas (B. subtilis) por HHP es posible en combinación con temperaturas elevadas (60º - 70 ºC).

- No se necesita de éste aumento de la temperatura del proceso de HHP para la inactivación de células vegetativas

La presencia de células lesionadas y estresadas en las muestras tratadas HHP puede cuestionar la seguridad alimentaria durante el almacenamiento y se debe considerar como un riesgo potencial para el deterioro de los alimentos.

- La existencia de células estresadas tras el tratamiento de HHP puede conducir a engaño sobre la eficiencia del proceso si las muestras son sometidas a ensayo inmediatamente después del tratamiento HHP

- La compresión rápida produce un mayor número de células dañadas que la descompresión lenta en todas las matrices estudiadas.

- El zumo de naranja inhibe fuertemente la recuperación de las bacterias lesionados, tanto esporas como células vegetativas.

- El desarrollo de equipos de HHP con un control más preciso y las tasas extremas de compresión y descompresión puede mejorar la eficiencia de inactivación bacteriana en los productos procesados con esta tecnología.

CHAPTER 8

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