INACTIVATION OF MICROORGANISMS IN SKIM MILK AND SHREDDED MOZZARELLA CHEESE USING HIGH-PRESSURE CARBON DIOXIDE AND NITROUS OXIDE

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INACTIVATION OF MICROORGANISMS IN SKIM MILK AND SHREDDED MOZZARELLA CHEESE USING HIGH-PRESSURE CARBON DIOXIDE AND NITROUS OXIDE

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Inactivation of microorganisms with high-pressure carbon dioxide (HP-CO_2) is emerging as an innovative process for the sterilization of biological materials. However, its application in milk processing poses a challenge since CO_2-induced reduction in pH may lead to casein precipitation. High-pressure nitrous oxide (HP-N_2O) has been suggested as an alternate choice for the processing of fluid milk.

Agitated supercritical carbon dioxide (Sc-CO_2) at 10.3 MPa and 35°C with 100 ppm peracetic acid (PAA) resulted in a complete 8- and 5-$\log_{10}$ inactivation of *Escherichia coli* and *Bacillus atrophaeus* spores in thin milk-films after 15 and 40 min, respectively. The treatment also resulted in partial milk-protein coagulation (55%) and thus possible applications of this approach may be in those processes where curd formation from sterile milk is beneficial.

The HP-CO_2 treatment at 10.3 MPa with 50 ppm PAA resulted in 2.6-, 5.4- and 9.2-$\log_{10}$ reductions of *E. coli* in agitated bulk milk after 120 min at 5, 15 and 25°C, respectively, whereas a 0.7-$\log_{10}$ reduction of *B. atrophaeus* spores was obtained at 25°C. The Fermi model was used to describe the inactivation kinetics of *E. coli* and *B. atrophaeus*. This strategy should be attractive for low-temperature (≤25°C) pasteurization of fluid milk.

A 20-min treatment of skim milk with added nisin (150 IU/mL) using HP-N_2O (15.2 MPa and 65°C) resulted in 8- and 8.6-$\log_{10}$ reductions of *E. coli* and *Listeria innocua,* respectively.

Meanwhile, a 2.5-$\log_{10}$ inactivation of *B. atrophaeus* spores was obtained when lysozyme (50 µg/mL) was also added and the temperature was increased to 85°C. There were no significant changes in the physico-chemical properties of the treated milk and no sub-lethally injured cells were detected following the treatment.

Agitated Sc-CO_2 at 9.8 MPa and 35°C with 100 ppm PAA synergistically resulted in the inactivation of major microbial groups in shredded Mozzarella cheese after 30-min of treatment. A >5-$\log_{10}$ reduction in the populations of *E. coli*, *L. innocua*, yeasts & molds and the total bacterial counts along with a 4-$\log_{10}$ reduction of *Geobacillus stearothermophilus* spores was achieved during storage for 21 days at 25°C.

BIOGRAPHICAL SKETCH

Adi Md Sikin was born September 2, 1972, in Johor, a state in southern part of Peninsular Malaysia. He received the Bachelor of Food Science and Technology from Universiti Putra Malaysia in 1999 and the Master of Science in Food Technology from the University of New South Wales, Australia in 2005. He held a quality control executive position with Kentucky Fried Chicken for 3 years and a production executive position with Cadbury for a year between 1999 and 2004. Adi began his academic career in 2005 as a senior lecturer at the Faculty of Applied Science, Universiti Teknologi MARA, Malaysia. In 2009, he was awarded a scholarship by the Ministry of Higher Education, Malaysia to pursue his PhD at Cornell University. He minored in Education during the course of his graduate study at Cornell. His research has focused on the application of high-pressure carbon dioxide and nitrous oxide for the microbial safety of dairy products.

Khas buat abah dan mak,

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Chapter 1: Literature review

1.1 Introduction

Thermal processing is a well-known traditional method that is used to kill food-borne pathogens and spoilage microorganisms in milk. Depending on the treatment time and the temperature applied, thermal processing methods can be classified into three categories: low-temperature long-time (LTLT) (63 ^{o}C, 30 min, batch), and high-temperature short-time (HTST) (72 ^{o}C, 15 s, continuous) or ultra-high temperature (UHT) (135-150 ^{o}C, 2-20 s, continuous). However, certain treatment conditions may not suffice for the inactivation of certain heat resistant spoilage or pathogenic microorganisms. *Mycobacterium tuberculosis* was first considered to be the most heat resistant pathogen associated with milk, followed by *Coxiella burnetti*, the agent for Q-fever which was found to be more heat resistant than the former (Cerf & Condron, 2006). These microorganisms are currently recognized as being the most heat-resistant non-spore-forming pathogens in raw milk and, as such, form part of the Codex Alimentarius (2004) definition of milk pasteurization. More recently, concerns were shifted to the occasional survival of *Mycobacterium avium subsp. Paratuberculosis* (*Map*) in pasteurized milk (Robertson et al., 2012). *Map* has been causing a significant concern to dairy industry because it has long been suspected as an aetiological agent of Crohn's disease in humans (Grant, 2005). Furthermore, the heat-resistant nature of *Map* and the ability to survive current pasteurization treatments (Grant et al., 1998; 1999) have increased this concern.

Thermoduric bacilli are significant in dairy processing due to their heat-resistant spores which may survive UHT treatment (Scheldeman et al., 2006; Silva & Gibbs 2010).

The production of heat-resistant (80–100 °C for 10–30 min), and highly heat-resistant (>106 °C for 30 min) endospores by thermophillic bacilli is an issue of particular importance to milk powder producers, as these powders are used as ingredients in other types of dairy foods, such as UHT-treated products, baby foods, etc. (Burgess et al., 2010). This impacts the industry as milk powder is one of the most widely traded dairy products globally (>3 million tonnes per annum) (IDF, 2010). In particular, infant formulas are observed to be frequently contaminated with *Bacillus cereus* spores (Shaheen et al., 2006). These spores originating from raw milk and the dairy plant equipment may survive any UHT treatment and concentration steps prior to drying. Consequently, when spores are present in infant formulas, mishandling of the reconstituted products in households can lead to proliferation and toxin production of both emetic and diarrhoeal type strains (Shaheen et al., 2006) and thus pose a threat to the consumer's safety.

Outbreaks of illness associated with milk and dairy products continue to occur although dairy production technology has advanced and strict regulations have been widely implemented for industrial processing of milk. The United States Food and Drug Administration (FDA) reported that dairy products were the fourth highest in the ranking under the FDA-regulated food categories for foodborne outbreaks and illnesses in the U.S. between 1998 and 2007 (CSPI, 2009). Of the dairy products-related outbreaks, 35.2% were associated with milk. In fact, 44.6% of fluid milk-related outbreaks in the U.S. from 1990 to 2006 were associated with the consumption of pasteurized milk and products made from such milks (Newkirk et al., 2011). This poses the risk of serious economic losses to the U.S. dairy industry which was worth $140 billion in economic output, $29 billion in household earnings, and provided more than 900,000 jobs in 2002 (DFT, 2007).

Despite the health risks associated with the consumption of unpasteurized milk, there has been a growing demand for unpasteurized milk and products made from such milk (e.g., specialty cheeses) in recent years (Buzby et al., 2013). As high temperatures are known to have detrimental, albeit small, effects on the nutritional and sensory qualities of heat sensitive products like milk, consumer demand for minimally processed and fresh-like foods has accelerated in recent years. For example, UHT sterilization can promote strong sulfurous, cooked, cabbage-like flavors in milk, thus limiting its acceptance. The intensity of these flavors is dependent on the presence of volatile sulfides liberated via heat denaturation of the protein β-lactoglobulin (Vazquez-Landaverde et al., 2006). Also, the Maillard reaction between reducing sugars (i.e., lactose) and the free amine group of protein-bound lysine residues causes sensorial changes and reduces the level of available lysine in heated milk (Erbersdobler & Somoza, 2007; Arena et al., 2010). Protein cross linking, another common feature of heated milk, can lead to the formation of aggregates and insoluble precipitates (Holland et al., 2011).

Non-thermal technologies (e.g., bactofugation, microfiltration, high hydrostatic pressure, and high pressure CO_2) have been proposed as alternative processing methods in response to the increased demand for nutritious, fresh food products with a high organoleptic quality and an extended shelf-life. Processes such as bactofugation can be used to reduce the number of spores and total bacteria in milk. Indeed, this processing step has been demonstrated to achieve a > 95% reduction in total bacterial load (Kosikowski & Fox, 1968) and to bring about 60% reductions in spore numbers (Su & Ingham, 2000). However, this process is expensive, time consuming and labor intensive (Walstra et al., 2010). Microfiltration is another processing step which can also be used. This process is

restricted to skim milk, as spores are roughly the same size as fat globules in whole milk (Rysstad & Kolstad, 2006). The requirement for milk fat separation to facilitate microfiltration makes this process labor intensive and expensive to carry out (Skanderby et al., 2009). High hydrostatic pressure processing uses conditions on the order of 600 MPa. By contrast, the CO_2 or N_2O pressures applied for preservation purposes are much lower (generally <20 MPa) hence making it easier to control, more feasible and less expensive (Gasperi et al., 2009).

1.2 Microbiological action of high pressure carbon dioxide

High pressure carbon dioxide (HP-CO_2) has been known to exert an inhibitory effect since the 1950s (Fraser, 1951). Several reviews concerning the effect of HP-CO_2 on foods have been published during the past 10 years (Spilimbergo & Bertucco, 2003; Damar & Balaban, 2006; Zhang et al., 2006; Garcia-Gonzalez et al., 2007; Hu et al., 2013; Ferrentino & Spilimbergo, 2011), which mainly focused on the microbial and enzyme inactivation following HP-CO_2 treatment. The suggested inactivation mechanisms presented in the reviews include internal acidification due to the formation of carbonic acid, cell rupture, increased permeability of cell membranes, metabolic interference due to the inactivation of key enzymes and the extraction of cell constituents. However, no single mechanism of microbial inactivation by HP-CO_2 has been generalized and documented for all the microbial strains studied. More recently, the mechanism of action of HP-CO_2 based on molecular biological evidence in microorganisms has been studied which reported that HP-CO_2 inhibits protein synthesis (András et al., 2010) and induces DNA damage (Liao et al., 2011) in microorganisms. Nevertheless, both intracellular acidification and modification of the cell membrane properties remain the two main reasons for explaining cell

deactivation, and much of its explanation is attributed to the CO_2 dissolution in the substrate (Bertucco & Spilimebrgo, 2006).

The non-thermal food processing technology of HP-CO_2 uses pressurized CO_2 in the liquid, gaseous or supercritical fluid states. Supercritical CO_2 (Sc-CO_2) is CO_2 at a temperature and pressure above its critical point values (T_c = 31.1 °C, P_c = 7.38 MPa), and exists as a single phase. Sub-critical (gaseous or liquid) CO_2, on the other hand, is CO_2 at a temperature or pressure below or close to its critical point values. However, both forms have been shown to be antimicrobial, partly due to the solubility properties of CO_2. Kamihira et al. (1987) were the first to compare the inhibitory effect of HP-CO_2 in the gaseous, liquid or supercritical state on *Escherichia coli* and *Staphylococcus aureus* cells. Lin et al. (1992) pointed out that once the concentration of CO_2 is built up to a critical level within the cells, it is able to extract constituents to an extent that is sufficient to modify the structure of the membrane or disturb the biological system. This theory was confirmed by Hong & Pyun (2001), who investigated the physiological changes of *Lactobacillus plantarum* by HP-CO_2 treatment. However, these authors only made a hypothetical association with the increase in concentration of CO_2 and pressure, thus the CO_2 extractability of lipid substances seemed to be more effective in the supercritical regions, which could be attributed to the gas-like diffusivity and liquid-like density of Sc-CO_2. As the penetration of CO_2 is believed to be the controlling step in deactivation of bacterial cells, Sc-CO_2 has been considered to be superior to subcritical CO_2 (Lin et al., 1993). Nevertheless, as the field has progressed, the effects of measured concentration of dissolved CO_2 on microbial inactivation have been reported in a few studies using a novel, low-pressure CO_2 micro-bubbling technique (Kobayashi et al., 2009; 2012a & b).

1.3 Milk pasteurization under high pressure carbon dioxide

HP-CO_2 treatments of milk have been investigated as an alternative and innovative process to replace thermal pasteurization. Early works were carried out to investigate the applications and lethal effects of sub-critical CO_2 in batch systems (see Table 1.1 below). These studies showed that liquid CO_2 at sub-critical stage was effective but required a long time. Lin et al. (1994) demonstrated that *L. monocytogenes* cells suspended in lactose-reduced non-fat milk and reduced fat milk were reduced by respectively 6.2 and 5.9 $\log_{10}$ cycles at 6.9 MPa and 45 °C after 1 h of exposure, whereas only 1 $\log_{10}$ reduction could be obtained with regular milk. Erkmen (1997) reported more than 8-$\log_{10}$ reduction of *Staphylococcus aureus* cells in whole milk following its treatment at 14.6 MPa and 25 ^{o}C for 5 h but in skim milk only 9 MPa and 25 ^{o}C for 2 h was needed. In a later study, a 6.42 and 7.24 $\log_{10}$ reduction of *E. coli* was achieved in whole and skim milk, respectively, as a result of a batch treatment carried out at 10 MPa and 30 ^{o}C for 6 h (Erkmen, 2001). These studies generally showed higher inactivation of microorganisms suspended in skim milk than in whole milk, possibly due to the protective effect exerted by the fat globules in milk on bacterial cells from the penetration of CO_2. The same effect was reported by Kim et al. (2008) who observed a substantially diminished inactivation effect when *L. monocytogenes* suspended in physiological saline were treated with HP-CO_2 at 35 °C and 10 MPa for 15 min in the presence of the lipophilic substance, oleic acid.

Table 1.1 High pressure carbon dioxide (HP-CO_2) inactivation of microorganisms in milk

Target microorganisms	Samples	Process conditions	Types of operation	Log_{10} reduction (CFU/ml)	References
L. monocytogenes	Whole milk	6.9 MPa, 45 ^oC, 1 h	Batch	1	Lin at al., (1994)
	Skim milk			6.2	
Natural aerobic flora	Whole milk	14.6 MPa, 25 ^oC, 5 h	Batch	8.72	Erkmen, (1997)
	Skim milk	9 MPa, 25 ^oC, 2 h		8.72	
S. aureus	Whole milk	14.6 MPa, 25 ^oC, 5 h		8.72	
	Skim milk	9 MPa, 25 ^oC, 2 h		8.72	
Enterococcus faecalis	Whole milk	6.05 MPa, 45 ^oC, 24 h	Batch	5.8	Erkmen, (2000a)
	Skim milk	6.05 MPa, 45 ^oC, 16 h		5.5	
Natural aerobic flora	Whole milk	6.05 MPa, 45 ^oC, 24 h		5.8	
	Skim milk	6.05 MPa, 45 ^oC, 16 h		5.8	
L. monocytogenes	Whole milk	6.05 MPa, 45 ^oC, 24 h	Batch	6.9	Erkmen, (2000b)
	Skim milk	6.05 MPa, 45 ^oC, 16 h		6.5	
Natural aerobic flora	Whole milk	6 MPa, 45 ^oC, 16 h		4.6	
E.coli	Whole milk	10 MPa, 30 ^oC, 6 h	Batch	6.42	Erkmen, (2001b)
	Skim milk			7.24	
Yersinia enterocolitica	Whole milk	6 MPa, 45 ^oC, 24 h	Batch	5.8	Erkmen, (2001a)
	Skim milk	6 MPa, 45 ^oC, 16 h		5.8	
Natural aerobic flora	Whole milk	6 MPa, 45 ^oC, 16 h		4.9	
Aerobic bacteria	Raw milk	25 MPa, 50 ^oC, 70 min	Batch	4.96	Hongmei et al., (2013)
Pseudomonas	Raw milk	7.5 MPa, 25 ^oC, 40 min	Batch	1.5	Yao et al., (2013)
Enterobacteriaceae				0.7	
S. aureus				1	
P. fluoresecens	Raw milk	20.7 MPa, 35 ^oC, 10 min	Continuous	5.02	Werner & Hotchkiss, (2006)
B. cereus spores		[CO_2] : 132 g/kg		None	

Similarly, Garcia-Gonzalez et al. (2009a) reported that increasing the concentration of sunflower oil to 10% and 30% in a brain heart infusion-medium, diminished the degree of inactivation of *Pseudomonas fluorescens* from 6 to 3.9 and 3-$\log_{10}$ cycle, respectively, when treated with HP-CO_2 at 10.5 MPa and 35 °C for 20 min, again demonstrating the antagonistic effect of fat on the HP-CO_2 efficacy. It was believed that when CO_2 is injected into the treatment vessel, it is dissolved partly in the water-phase and partly in the fat-phase of the medium (Garcia-Gonzalez et al., 2009a). As a consequence, part of the CO_2 will be "consumed" and a lesser amount of CO_2 will dissolve in the water-phase of the food, resulting in a lower CO_2 concentration in the water-phase in fatty solutions (Devlieghere et al., 1998).

Since 2001, there have been no published articles on the use of HP-CO_2 as an alternative non-thermal pasteurization technique for milk using a batch treatment system. Most recently, Hongmei et al. (2013) reported a 4.96-$\log_{10}$ colony forming unit per millimeter (CFU/mL) reduction of aerobic bacteria in milk when treated with Sc-CO_2 at 25 MPa and 50 °C for 70 min. Also, a complete inactivation of yeast and molds and coliforms (10^2 CFU/mL) was achieved when raw milk was subjected to Sc-CO_2 at 25 MPa and 40 °C for 70 min, and at 25 MPa and 40 °C for 30 min, respectively. Commercial applications of batch process is generally limited because of the low processing efficiency. In addition, large-scale batch production requires significant downtime for depressurizing, cleaning and refilling (Tomasula et al., 1997). To ensure the success of this technology, HP-CO_2 treatment in a continuous set-up is more promising, yet it has not been studied as extensively as batch systems. The really important variables in terms of improving the effectiveness of continuous treatment are the flow regime and the contact between the HP-

CO_2 and the food products (Casas et al., 2012). In this case, promoting good mixing and dispersion of CO_2 would be the most critical objective for scaling up the process. This measure would help to greatly reduce the treatment duration, to a few minutes, as already shown for orange juice (Sims & Estigarribia, 2002). It would also help reduce the temperatures required to destroy the more resistant microorganisms such as spores, and thus would allow much milder treatments that would preserve or even improve product quality. This technical solution is already being investigated, and in fact, several patents now describe effective methods of CO_2 dispersion in liquid media, for example, by microbubbles (Osajima et al., 1998) and by a membrane contactor (Sims, 2001).

Ho (2004) conducted a preliminary study on the combined effect of continuous HP-CO_2 processing and heat pasteurization using the Better-Than-Fresh ^{TM} System developed by Praxair Inc. (Burr Ridge, IL). Although the degree of inactivation was not reported, the author claimed that the samples of the combined treatments had a longer shelf-life than those pasteurized only by heat. Similarly, a continuous, 15-min Sc-CO_2 treatment (15 MPa, 35 ^{o}C $\leq$ T < 40 ^{o}C and CO_2/milk ratio equal to 0.33) was reported to extend the shelf-life of skim milk to over 35 days. Also, it was claimed that the taste of the Sc-CO_2 treated sample was better in comparison to the HTST pasteurized sample, although the result was not supported by any experimental data (Di Giacomo et al., 2009). Werner & Hotchkiss (2006) compared the effectiveness of the process utilizing CO_2 at subcritical and supercritical phases carried out in a continuous-flow system to inactivate indigenous psychrotrophic vegetative cells of *P. fluorescens* and *Bacillus cereus* spores in raw milk. Pressures between 10.3 and 48.3 MPa, temperatures of 15, 30, 35 and 40 ^{o}C and CO_2 concentrations of 3, 66 and 231 g/kg of milk were studied. At 30 ^{o}C, no effects on the total

microbial count were observed even at pressures up to 20.7 MPa with either 66 or 132 g/kg of CO_2. Upon increasing the temperature up to 35 ^oC, with CO_2 in the supercritical state, a direct proportionality was noted between lethality and pressure at a CO_2 level of 132 g/kg, both for psychrotrophic vegetative cells and *P. fluorescence*. The respective log_{10} reductions achieved were 5.36 and 5.02 in milk treated with CO_2 at 35 ^oC, 20.7 MPa. In all the treatment conditions tested, no effect on spore populations was detected. The significance of this study is that a higher microbial inactivation was achieved in milk above the CO_2 critical parameters and there is a CO_2 concentration threshold required for lethality. The quality of treated milk was not indicated in this paper, although undesirable texture and flavor changes due to such treatments have been mentioned in a review paper by Ferrentino & Ferrari (2012). However, investigations on the effect of these treatments on the organoleptic and physico-chemical properties of milk have not been reported in any of these studies.

1.4 Isoelectric precipitation of casein by HP-CO_2

A pH decrease due to the acidification by dissolved CO_2 in milk would negatively affect the protein stability since casein precipitates out at its isoelectric point of pH 4.6 (Fox, 2003). As illustrated in Figure 1.1, casein molecules associate into casein micelles aggregates of 20-200 nm in size. Casein is comprised of submicelles (10-20 nm) held together by colloidal calcium phosphate (CCP) and hydrophobic bonds. When CO_2 is sparged into milk, it hydrolyses water to form carbonic acid. With increasing pressure the solution pH drops, CCP solubilizes and casein precipitates. In fact, it was shown that precipitation of casein using CO_2 was complete at 0.1 pH units higher than it was for

precipitation with HCl, indicating that there might be slight pressure effects on the solubilization of CCP (Tomasula et al., 1999). However, other factors such as the interaction of the protein with carbon dioxide should not be neglected. For example, Gevaudan et al. (1996) and Tomasula et al. (1999) reported that milk acidification induced

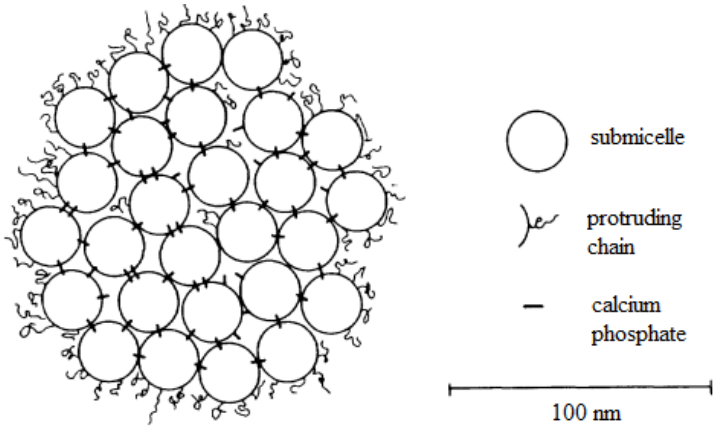

Figure 1.1 Schematic representation of the structure of a casein micelle (Walstra & Jenness, 1984).

by HP-CO_2 leads to solubilization of the inorganic calcium phosphate and calcium directly bound to casein, followed by the formation of various salt systems based on the interactions of calcium, phosphate and bicarbonate. This was indicated by two respective buffering peaks at pH 4.95 and 5.40. as opposed to a single buffering peak at pH 5.05 when milk treated with HCl (Tomasula et al., 1999). Moreover, the nature of the precipitate obtained is temperature dependent and is believed to result from the hydrophobic interactions (Fox, 1989). For example, the hysteresis observed in the pH-pressure plots suggested that the CCP dissolves at higher temperatures (40-50 ^{o}C), and the calcium and phosphate concentrations in whey after precipitation remained constant at 40 ^{o}C. This could be due to the faster kinetics of the precipitation and increased syneresis at higher temperatures which

results in a compact and inaccessible precipitate, giving the calcium phosphate little opportunity to diffuse out (Hofland et al., 1999).

Sub-micelles rich in κ-casein occupy a surface position, whereas those with less κ-casein are buried in the interior. The hydrophilic tails of κ-casein project into the milk serum as protruding chain (or carbohydrate hair) and the hydrophobic regions attach to the micelle core. Consequently, a sub-micelle with κ-casein would exhibit steric repulsion on its surface, and aggregation at such a site would be hindered. The stability of this colloidal protein suspension is also due to Coulombic repulsion of the equally charged protein molecules. As a result, the stearic repulsion becomes negligible upon acidification, as the carbohydrate hairs collapse to the surface of the micelle. Moreover, the addition of H^+ ions neutralizes the negative charges and thus the protein molecules become electrically neutral and form large protein clusters (Bringe & Kinsella, 1991).

There have been few studies related to the effects of HP-CO_2 on proteins. HP-CO_2 has been reported to change the particle size distribution (PSD) of casein in raw, whole milk and skim milk as compared to untreated controls (Tisi, 2004). Similarly, the PSD of the bovine colostrum were observed to increase after HP-CO_2 treatment (Liao et al., 2009). This change in PSD in milk was suggested to be due to HP-CO_2-induced denaturation of the above-mentioned proteins due to the decline of both the pH and the absolute value of ζ-potential (Zhou et al., 2010), resulting in protein aggregation and/or precipitation (Tisi, 2004; Liao et al., 2009). As a consequence, an accurate estimation of the microbial population reduction is difficult to make due to the phase separation in HP-CO_2 treated milk (Graham et al., 1987).

On the other hand, exploiting HP-CO_2 for pH-induced precipitation of casein has been used to develop technologies for industrial applications. Previous studies have reported that the degree of precipitation tended to instantaneously increase and then remained stable at the HP-CO_2 treatment variables (pressure and time) examined. For example, Jordan et al. (1987) showed that the same amount of precipitated casein was obtained in a run carried out for 1 min as that carried out for 5 min at pressures between 2.8 and 5.5 MPa and temperatures of 38, 49 and 60 ^{o}C. Similarly, casein yield was about the same (2.8 %, db) at 32, 38, 43 and 49 ^{o}C, and at pressures of 5.5, 6.9 and 7.6 MPa for a residence time of 1 min in a tubular reactor (Tomasula et al., 1997). However, the precipitation of casein was found to be a strong function of temperature in the range of 40-50 ^{o}C (Tomasula et al., 1997), which is in agreement with a study by Hofland et al. (1999) on the dissolution of casein phosphate in milk. The pressures required for yielding high amount of casein was greater when process was conducted at low temperatures (Jordan et al., 1987). It was highlighted that when the process was carried out at lower temperatures (< 38 ^{o}C) no precipitation occurred. Also, the physical nature of the casein curds which changed from soft to rubbery and firm with increasing pressure indicated that pressure is also an important variable to consider for quality control purposes (Jordan et al., 1987).

The above literature indicates that in order to avoid the aggregation and/or coagulation of casein, control of the amount of carbonic acid in milk via both the level of pressure and the ratio of milk-to-CO_2 should be evaluated. Also, process temperatures close to and/or slightly lower than that of the body temperature of animals (38-40 ^{o}C) should not severely disturb the natural state of emulsion in bovine milk (Di Giacomo et al., 2009). Thus, a mild treatment for a reasonable inactivation of microorganisms in milk is possible

by manipulating a contact mode between high pressure gases and liquid (Di Giacomo et al., 2009), and similar approaches in the field of Sc-CO_2 sterilization have been reviewed elsewhere (Sikin & Rizvi, 2011).

1.5 Strategies for enhanced inactivation by high pressure carbon dioxide

Pressures and temperatures also affect the characteristics of CO_2 mass transfer rates and thus biological activities of microbial cells (Kamihira et al., 1987; Taniguchi et al., 1987; Lin et al., 1993; Isenschmid et al., 1995) The acidification and solvent properties of CO_2 confer antibacterial properties on it, which are enhanced as the amount of CO_2 present in an aqueous medium increases, for example when the solubility increases with lower temperature or with higher CO_2 pressure (Enfors & Molin, 1981). However, these parameters should not be applied at an indefinite level and are limited by the saturation solubility of CO_2. The solubility of CO_2 in water is a weak function of pressure at >10 MPa (Spilimbergo et al., 2005). Similarly, a pressure increase of over 10 MPa decreased the diffusion of N_2O in the liquid phase and resulted in low biocidal efficiency of Sc-N_2O (Jou et al., 1992; Spilimbergo et al., 2007b). As for the temperature, its stimulating effect can in part be counteracted by its inhibiting effect on CO_2 solubility as the temperatures go far above the critical point. These underscore the fact that elevation of dissolved CO_2 level in the subject solution is the primary factor for antimicrobial effectiveness. Nonetheless, numerous patents have been granted in the area of pasteurization of liquid food which claim to improve the diffusivity of CO_2 by increasing the interfacial area between CO_2 and the liquid substrates (Sikin & Rizvi, 2011). These include the installation of an in-line mixer, a high-performance impeller, a membrane contactor, a CO_2 micro-bubble generator, and a CO_2 nano-bubble generator. The foods include fruit and vegetable juices, fermented

products (e.g. soy sauce, sake, beer and wine), liquid egg and milk products (Sikin & Rizvi, 2011).

Although different Sc-CO_2 treatment system designs have been studied and patented, little attention has been given to the exposure geometry such as the sample surface and volume ratio and the ratio of the sample volume and the pressure vessel volume or the working volume ratio (WVR). As bacterial inactivation relies on diffusion of Sc-CO_2 into the solution, understanding the impact of sample exposure geometry should be considered for successful application of this technology. This is more relevant in a static batch system as opposed to that in a continuous system which is dependent on flow regime as previously mentioned in this review. Nevertheless, quantification of the influence of treatment geometry could help in the design of continuous systems that take advantage of this concept.

1.5.1 Headspace and liquid agitation

Batch treatment systems usually require longer times for microbial inactivation compared to continuous systems as indicated in Table 1.1 above. Nevertheless, it is possible to increase the inactivation rate of batch systems by agitation. Headspace agitation enhances mass transfer of the CO_2 and additives by eliminating voids in the fluid such that the organisms being inactivated come into more complete contact with the fluid (Christensen et al., 2006). This principle is well-utilized in a sterilization technique patented by NovaSterilis to achieve at least a 6-$\log_{10}$ reduction of spores inoculated in various biomedical solid materials (White et al., 2006; Hemmer et al., 2007; Nichols et al., 2009; Qiu et al., 2009). Similarly, sample agitation can enhance the solubilization of CO_2 and its contact with bacterial cells, making the cellular penetration easier (Lin et al., 1992; Oulé

et al., 2006). Numerous studies have reported the importance of adequate agitation, but no specific data were given (Lin et al., 1992; Lin et al., 1994; Hong et al., 1997; Dillow et al., 1999; Oulé et al., 2006). Lin et al. (1992), Tsuji et al. (2005), and Oulé et al. (2006) observed higher reduction rates of bacterial and enzymatic inactivation with increasing rotation speed when mixing CO_2 into liquid substrates. In an agitated system, the transfer rate of CO_2 is determined by the gas flow rate, the stirring rate and the geometrical aspects (such as WVR). Garcia-Gonzalez et al. (2009b) studied the influence of agitation by varying the stirring speed in a pressure vessel (100, 200 and 400 rpm) at 35 °C, 13.0 MPa, 50% WVR during 20 min of treatment with CO_2. Generally, increasing the stirring speed accelerated the inactivation of natural microflora in the liquid whole egg samples. Without agitation, Lin et al. (1992) reported a significant decrease of inactivation rates of yeast cells. Hong et al. (1997) found that microbial inactivation appeared to depend on the sample size in the absence of agitation. They observed higher inactivation rates of *Lactobacillus sp.* with decreasing WVR.

1.5.2 Addition of antimicrobials

Hurdle technology consisting of HP-CO_2 and antimicrobial agents may result in a more efficient treatment under milder conditions and in shorter times. This may be suitable for the treatment of milk as is evident from the literature cited above that the treatment temperatures should not exceed 40 ^{o}C to avoid precipitation of casein. Also, HP-CO_2 alone cannot assure a complete elimination of all kind of microbial contaminants including spores. Addition of even a low concentration of a strong oxidant, such as hydrogen peroxide, tert-butyl hydroperoxide, peracetic acid or trifluoroacetic acid, to HP-CO_2 could

achieve high-efficacy inactivation of bacterial spores at milder temperatures (35-60 ^{o}C) (Rao et al., 2015).

Peracetic acid (PAA) was reported to increase the sensitivity of bacterial spores to heat (Marquis & Thom, 1992). It works synergistically with hydrogen peroxide (Alasri et al., 1992) and is a strong oxidant, very active at very low concentrations against bacteria (0.001%), fungi (0.003%), spores (0.3%) (Greenspan & MacKellar, 1951) and viruses (0.75%) (Baldry & French, 1989). Its antimicrobial action is presumably based on the oxidation of thiol groups in proteins (Block, 2001; Kitis, 2004), disruption of membranes (Block, 2001; Russell, 2003; Kitis, 2004), or damage to bases in DNA (Block, 2001), and with regard to consumer safety, it is worth mentioning that PAA is unstable and readily degrades into acetic acid and water, which alleviate concerns about residual toxicity in treated products after treatment (Block, 2001; Kerkaert et al., 2011). In a hurdle approach, the role of highly diffusive Sc-CO_2 fluid is to act as a vector, so that PAA can easily penetrate into the microbial cells and inactivate them (Qiu et al., 2009).

PAA is an approved sanitizer in the U.S. for food contact surfaces (21CFR178.1010) and for direct contact with fruits and vegetables (21CFR173.315) and meat, poultry and seafood (21CFR173.370) at a maximum concentration of 80, 85 and 110 ppm, respectively. In contrast, PAA application in dairy products is not known though its oxidation did not result in the formation of high molecular weight aggregates in both whey and casein (Kerkaert et al., 2011), which may lead to protein coagulation in milk. Considering that a very low dosage of the acid is required due to its strong oxidative properties, it can be hypothesized that PAA would have a minimum impact on protein

coagulation in milk while enhancing the inactivation of microorganisms when used in combination with HP-CO_2.

1.5.3 Supercritical N_2O (Sc-N_2O) as an alternative to Sc-CO_2

Nitrous oxide (N_2O), which shows critical conditions very close to CO_2 (Table 1.2) and a similar bactericidal effect does not induce any acidification of the external microbial environment. It is a non-toxic and relatively inexpensive, colorless and practically odorless gas. Although the water solubility is similar to that of CO_2, N_2O does not acidify water and hence does not affect the pH of aqueous medium to a great extent. It also has a permanent dipole moment and is known to be a better solvent than CO_2 for many solutes (Raynie 1993). Sc-N_2O has been considered as yet another interesting alternative to thermal pasteurization technology.

The mechanism by which N_2O inhibits microbial growth is still not completely understood. Due to its high density and high solubility in lipids (Table 1.2), N_2O could be easily dispersed into the phospholipid layer of cell membranes with the support of high pressure, causing the modification of the membrane structure (Spilimbergo et al., 2002). For example, the release of cell materials (nucleic acids and proteins) of *P. aeruginosa* following treatment with Sc-N_2O was 3-4 times higher than with Sc-CO_2 at the standard conditions (10 MPa, 37 ^oC, 600 rpm, and 10% working volume) (Mun et al., 2011). This indicated a greater cell or cell membrane damage due to a greater extraction capacity of Sc-N_2O without the acidic pH effect in such treatments. In another study, the amount of intracellular materials in *E. coli* was 5 times greater than that in *S. aureus* after Sc-N_2O treatment (600 rpm, 10% working volume). The difference in sensitivity to the treatments observed between these two vegetative bacteria could be attributed to the thicker

peptidoglycan layer in the cell wall structure of gram-positive *S. aureus* than that of gram-negative *E. coli*, which may have led to a lower resistance of the latter against penetration by pressurized N_2O (Mun et al., 2012). Also, experimental evidence has shown that N_2O anesthetics selectively combine with hydrophobic groups in membrane proteins, altering their ion flow. Therefore, N_2O likely acts at the microbial membrane level, inhibiting solute transport, which in turn results in growth inhibition (Spilimbergo et al., 2009).

Table 1.2 Summary of the physical properties of N_2O and CO_2. (From Mun, et al., 2011).

	Mol. weight ($gmol^{-1}$)	Critical points		Dipole Moment (D)	[a]Solubility (mole fraction)	[b]Diffusivity (cm^2s^{-1})	[c]Density (gmL^{-1})	[d]pH	
		^oC	MPa					Before	After
N_2O	44.01	36.6	7.2	0.16	3.4×10^{-4}	4.9×10^{-5}	0.732	7.2 ± 0.1	7.2 ± 0.2
CO_2	44.01	31.1	7.4	0	4.8×10^{-4}	2.5×10^{-5}	0.683	7.2 ± 0.1	3.5 ± 0.3

[a] The mole fraction solubility in water achieved in the Handbook of Chemistry and Physics (David, 2003).
[b] The diffusivity achieved in the reference Spilimbergo et al. (2007b).
[c] Density at 10 MPa and 37 °C achieved in http://webbook.nist.gov/chemistry/, NIST Standard Reference Database Number 69, June 2005 Release.
[d] The pH was measured in the Ringer solution before and after the Sc-N_2O or Sc-CO_2 treatments at 25 °C and 6 MPa for 5 min, respectively.

N_2O is also a strong oxidizing agent capable of provoking cell wall oxidization by liberation of free radicals ($N_2O + H_2O \rightarrow N_2 + OH^* + OH^*$) and consequently microbial inhibition. Moreover, these free radicals can also affect lipid, nucleic acid and protein integrity (Marquis & Thom, 1992). Thom & Marquis (1984) observed that N_2O inhibited *E. coli* growth through damage linked to cellular oxidation phenomena. N_2O can also provoke stress by acting on two genes, *sox*R and *sox*S, that govern the synthesis of oxidative stress proteins (Marquis & Thom, 1992).

Sc-N_2O offers great potential for pasteurization of highly pH-sensitive food products, but previous studies on the bactericidal action of N_2O only focused on fruit juices (Spilimbergo et al., 2007a; Spilimbergo et al., 2007b; Gasperi et al., 2009) and tomato purees (Bizzotto et al., 2009). So far there was only one study on the effect of Sc-N_2O (12 MPa; 40, 45 and 50 ^oC) on naturally occurring microorganisms in raw skim milk (Spilimbergo, 2011). It was reported that the minimum treatment time is 10 min at 50 ^oC and 12 MPa for a 5-$\log_{10}$ CFU/mL reduction. The chemical analysis confirmed stable pH of 6.8 before and after the treatment and showed a very slight mean increase in titratable acidity of 0.1 above that of the raw milk sample. However, precipitation of casein was not reported and further studies were recommended to confirm the impact of N_2O on sensorial/chemical characteristics of the treated product.

1.6 Post-processing microbial contaminants in cheese

Cheese has been implicated in 10 of 12 multistate outbreaks involving dairy products in the U.S. from 1998 to 2012 (CDC, 2013). A standard pasteurization such as HTST or equivalent treatments destroys most common pathogenic and spoilage bacteria, and it is the most important heat treatment applied to cheese milk to provide acceptable safety and quality. However, heat-resistant pathogenic and spoilage bacteria may be present in raw milk or on equipment surfaces, and even non-heat-resistant bacteria could be incidentally introduced during the cheese manufacturing process (Kikuchi et al., 1996). For example, phospholipase enzymes (lipases and proteases) excreted by *Pseudomonas* contaminating raw milk are resistant to pasteurization and UHT treatments (Koka & Weimer, 2000; 2001), which could consequently impart unacceptable cheese flavors and textures (Cabrini & Neviani, 1983). A serious health impact could also come from Stx2 shiga toxins from

enterohemorrhagic *E. coli* O157:H7, which is destroyed only by much more severe heat treatment of 100 ^{o}C for 5 min (Rasooly & Do, 2010). Other incident bacteria such as *Staphylococcus aureus* produce toxins in foods causing emetic illness upon consumption, while surviving enterotoxigenic *E. coli* in contaminated foods could produce toxins in the intestine and cause diarrhea (Weeratna & Doyle, 1991; Bowen & Henning, 1994; Delbès et al., 2007; Zhang & Sack, 2012). Fecal streptococci also enter through routes similar to *E. coli* and are tolerant to 6.5% salt and both high and low temperatures (45 and 10 °C) (Bissonnette et al., 1980; Morea et al., 1999). Although Mozzarella cooking (66 to 77 °C) and brining (4 °C for 12 h) temperatures are effective in controlling listeria, the bacteria are capable of adhering to various surfaces and forming biofilms on biotic and abiotic materials (Beresford et al., 2001; Harvey et al., 2007). *Listeria* is also a salt- and cold-tolerant pathogen commonly found in semi-hard and soft cheeses (FDA, 2009a &b; 2010).

Therefore, the application of alternative control strategies should be applied if the product is exposed to environmental contamination after the lethality treatment (e.g., pasteurization) and before packaging (FSIS, 2014). This is very crucial for shredded products like cheese as shredding greatly increase surface exposure for even airborne microbial contamination (Eliot et al., 1998). In fact, consumption of contaminated Mozzarella cheese resulted in an outbreak of *Salmonella javiana* and *S. oranienberg* infections that affected 139 people (Herdberg et al., 1992), and it was reported that the contamination occurred at the processing plants during shredding. More recently, in 2012 multiple types of cheeses had to be recalled due to the detection of *L. monocytogenes* on product contact surfaces at the shredding line used for processing these products (FDA, 2013).

1.7 Post pasteurization treatments of shredded Mozzarella cheese (SMC)

Shredded cheese is the second largest category of cheese sold in the U.S. The sale of shredded cheese is estimated to be $1.3 billion of the $8.8 billion cheese market (Anon, 1998). Convenience is the key force driving shredded cheese sales coupled with the availability of different market blends in one bag (Anon, 1998). This has influenced a steady increase in the production of Mozzarella cheese, which ranks first, with a 33.4 percent share of 11.1 billion pounds of cheese produced in the U.S. (USDA 2013). The shredded Mozzarella cheese (SMC), in particular, is characterized by its meltability and spreadibility with a non-pronounced flavor used for pizza toppings or as an ingredient in various foods (Kindstedt, 1995). Therefore, production of safe and shelf-stable Mozzarella cheese is an important issue to both the dairy and the food industry due to large group of consumers that could be impacted as well as the high interest in extending the distribution boundaries of the traditional product beyond market borders.

Several attempts have been made to develop modified atmosphere packaging (MAP) and active packaging to extend the shelf-life of different dairy products (Floros et al., 2000; Pantaleão et al., 2007). MAP has emerged as a solution that is competitive with vacuum packing. Although the vacuum technique is the most common packing employed for cheese, it is not suitable for grated or shredded cheese due to clumping of individual portions of cheeses (Pluta et al., 2005). There are few published papers on shredded Mozzarella cheese (SMC) packaged in MAP. Eliot et al. (1998) reported that SMC packaged in MAP containing 75% CO_2 was well protected from undesirable organisms and gas formation. The CO_2-based MAP stabilized lactic and mesophilic flora, and inhibited staphylococci, molds and yeasts, but not all the psychrotrophs. Alves et al. (1996) also found that microbial growth in sliced Mozzarella cheese packaged in MAP was

delayed with high concentrations of CO_2. Oyugi & Buys (2007) studied the microbiological quality of shredded Cheddar cheese packaged in different modified atmospheres with and without O_2 scavengers included in the packaging film and concluded that the film with O_2 scavengers was more effective than the control film against mold growth and 73% CO_2/27% N_2 atmosphere resulted in the cheese with the best microbiological qualities.

Another potential approach is the use of active coatings that cover the cheese surface with bio-materials containing antimicrobial compounds. For example, natamycin is an antimicrobial food additive commonly used to control mold growth on cheese surfaces. Shredded Cheddar and Mozzarella cheeses were powder coated electrostatically with a mixture of natamycin and powdered cellulose to improve the product shelf life by 15-30% (Elayedath & Barringer, 2002). Suloff et al. (2003) reported that the antimycotic activity of a semisynthetic derivative of natamycin is similar to that of natamycin in suppressing the growth of mold capable of producing *trans*-1, 3-pentadiene (i.e. a volatile compound with an unpleasant hydrocarbon-like odor) in shredded Cheddar cheese. Most recently, Han et al. (2015) showed potential for inhibition of microorganisms using essential oils as antimicrobial agents in SMC. The combination of rosemary and thyme oils (1% w/w each) inhibited *L. monocytogenes* by 1.7 $\log_{10}$ CFU/g in low fat SMC after 20 day of storage at 4 °C. The addition of sodium acetate (0.2%; w/v) with rosemary and thyme oils produced a difference of 4.2 $\log_{10}$ CFU/g in *L. monocytogenes* populations between the treated and the control SMC after 20 days.

It is important to highlight that the approaches used in MAP and active coatings are aimed primarily at treating only the surface contamination of cheese and shreds. On the

other hand, pressurized CO_2, with physical properties such as adjustable densities, low viscosities, high diffusivities and low interfacial surface tension, can penetrate into complex structures of difficult shapes (Sikin & Rizvi, 2011) and hold the possibility of the inactivation of microbes embedded in the treatment substrate such as cheese. The only work with HP-CO_2 was carried out by Haas et al. (1989) who reported a 87% reduction in standard plate counts after sub-critical CO_2 treatment (6.2 MPa, 23 °C, 16h) of Mozzarella cheese. To date no research has been conducted on treatment of SMC by Sc-CO_2. This is due to the lack of information on the bactericidal effect of HP-CO_2 in solid foods in general. Compared to liquids, the HP-CO_2 process applied to solid foods is less studied due to i) the more limited diffusion of CO_2 into the solid matrices and cells since the sample cannot be agitated, ii) much reduced levels of water which may limit the solubility of CO_2 into the food, and iii) a concern of cellular damage and therefore texture and other quality changes at the surface of solid foods (Garcia-Gonzalez et al., 2007; Balaban & Duong, 2014). Therefore, these limitations must be considered in an attempt to design an effective treatment of cheese with desirable qualities using HP-CO_2.

1.8 Thesis motivation and objectives

This study is concerned with the development of bacterial inactivation strategies for the microbial safety of milk and SMC without sacrificing quality attributes. It is hypothesized that any HP-CO_2-induced quality changes can be minimized by reducing the severity of treatment in terms of temperature, pressure and thus CO_2 content. A few approaches are proposed to increase contact efficacy between CO_2 and food substrates such as the liquid agitation of milk with CO_2 and the diffusion of CO_2 into thin-milk-films with and without headspace agitation of CO_2 in the treatment vessel. An optimal set of parameters (pressure,

temperature, CO_2-to-milk ratio and contact time) is selected through evaluation of milk protein stability and, more importantly, efficient inactivation of biological indicators. It is also proposed to use HP-N_2O as an alternative to HP-CO_2 for eliminating acidification problems while attaining similar microbial inactivation capability. Also considered is the use of antimicrobial agents at low concentrations to enhance microbial inactivation at milder HP-CO_2 and HP-N_2O conditions. This would help design a batch/continuous pasteurization process compatible with commercial processes. In this thesis, each chapter is written in a paper format for publication purposes and each chapter can thus be read independently. They are described as follows:

Chapter one: In this chapter, a literature review on the fundamental principles and application for developments of HP-CO_2 and HP-N_2O pasteurization technology, especially for milk and cheeses, is presented.

Chapter two: In this chapter, HP-CO_2 with and without added PAA is evaluated for inactivation of microorganisms in milk by its diffusion in thin films since the penetration of CO_2 through a liquid medium is often facilitated by maximizing the surface exposure of the liquid. Comparison is also made with agitated CO_2 as a means of eliminating voids in the fluid for better interfacial mixing with PAA and enhancing its mass transfer into liquids.

Chapter three: In this chapter, HP-CO_2 with and without added PAA is evaluated for the inactivation of microorganisms when liquid milk is mechanically agitated. This strategy is tested as it is hypothesized that the bulk mixing of milk would increase the contact of microbial cells with CO_2 and enhance the mass transfer of CO_2 molecules into the aqueous environment.

Chapter four: In this chapter, the synergistic effect of HP-N_2O in combination with heat and antimicrobial agents such as nisin and lysozyme on the inactivation of major bacterial groups in milk is reported. Also, the effects of such treatments on milk quality are assessed.

Chapter five: In this chapter, the synergistic effect of HP-CO_2 in combination with PAA on inactivation of major microbial groups and the storage stability of SMC at ambient temperature are reported.

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Chapter 2: Inactivation of *Escherichia coli* and *Bacillus atrophaeus* spores in skim milk using supercritical carbon dioxide with added peracetic acid

2.1 Abstract

The aim of this study was to investigate the effects of supercritical carbon dioxide (Sc-CO_2) treatment of skim milk at different pressures (10.3-24.1 MPa) and temperatures (35-70 °C) on the inactivation of *Escherichia coli* and *Bacillus atrophaeus* spores. The addition of peracetic acid (PAA) to the Sc-CO_2 at 100 ppm to increase the inactivation rate of those microorganisms in skim milk was also studied. To enhance the diffusion of Sc-CO_2, treatments were conducted by spreading the milk in thin film of 1.8 or 4.8 mm thickness, without or with headspace agitation. Treatments without agitation of Sc-CO_2 (10.3 MPa and 35 °C) for 40 min resulted in a complete 8-$\log_{10}$ inactivation of *E. coli*, while the addition of PAA with Sc-CO_2 demonstrated a more linear rate of inactivation. In contrast, a 1-$\log_{10}$ reduction of *B. atrophaeus* spores was achieved at 10.3 MPa and 70 °C for 60 min with PAA. A 54-55 % of protein in skim milk was coagulated as a result of treatment with non-agitated Sc-CO_2 at 35 °C. The coagulation was observed to occur instantaneously but remained stable as the exposure time and pressure were increased. Treatments with agitation of Sc-CO_2 (10.3 MPa and 35 °C at 650 rpm) for 15 min were more effective, resulting in a maximum 8-$\log_{10}$ inactivation of *E. coli* with Sc-CO_2 alone. A total 5-$\log_{10}$ reduction of *B. atrophaeus* spores was achieved at 10.3 MPa and 35 °C for 40 min with PAA, whereas only 1-$\log_{10}$ reduction was achieved without PAA. This offers a potential pre-treatment with PAA-containing Sc-CO_2 of milk intended for production of sterilized casein, cheese and other dairy products where acidification and curd formation from sterile milk may be beneficial.

2.2 Introduction

Milk is a complex and very conducive medium for the growth of pathogenic and spoilage microorganisms. Thermal treatment is generally sufficient to kill most vegetative bacteria, but certain bacterial spores (i.e. thermophilic bacilli) are resistant to even ultra-high temperatures (UHT). High-pressure carbon dioxide (HP-CO_2) in liquid, gaseous or supercritical fluid states can be used to inactivate microorganisms in milk. Supercritical carbon dioxide (Sc-CO_2) (T_c = 31.1 °C, P_c = 7.38 MPa) has been shown to exhibit more microbial lethality than sub-critical CO_2 due to its gas-like diffusivity and liquid-like density (Garcia-Gonzalez *et al.* 2007). This is based on the bactericidal action of CO_2 due to its dissolution in a cell by pressurization which lowers the intracellular pH of microbial cells (Dillow *et al.* 1999).

The use of Sc-CO_2 to inactivate microorganisms in milk has been reported in previous studies (Werner and Hotchkiss, 2006; Hongmei *et al*. 2013). Werner and Hotchkiss (2006) reported a 5-$\log_{10}$ inactivation of both psychrotrophic vegetative cells and *Pseudomonas fluorescens* at 20.7 MPa 35 °C for a 10-min holding time and CO_2 concentration of 132 g/kg of milk, but they did not find any effect on the spore population. Most recently, Hongmei *et al.* (2013) tested the use of Sc-CO_2 to inactivate bacteria associated with raw milk. They reported an almost 5-$\log_{10}$ reduction of aerobic bacteria at 25 MPa and 50 °C for a 70-min treatment. None of the above-mentioned studies reported any quality changes resulting from Sc-CO_2 treatments, such as protein coagulation in treated milk. A more effective processes is necessary to completely inactivate heat-resistant

spores and thereby ensure sterility of Sc-CO_2 treated milk which will subsequently be used for commercial production of dairy products.

The Sc-CO_2 alone cannot assure complete elimination of all kinds of microbial contaminants including spores. Incorporating a small amount of a co-solvent, such as peracetic acid (PAA), with Sc-CO_2 results in more efficient treatments using milder conditions and shorter times (White *et al*. 2006; Christensen *et al*. 2009; Eisenhut *et al*. 2009; Qiu *et al.* 2009; Christensen *et al*. 2011). PAA, a strong oxidant, is very effective at deactivating bacteria and spores even at concentrations as low as 0.3 wt. % (Greenspan and MacKellar, 1951). PAA is highly degradable and leaves no toxic residues in treated products (Block 2001; Kerkaert *et al*. 2011).

PAA dissolves only weakly in Sc-CO_2 (Reverchon et al. 2010). Agitating the headspace in the Sc-CO_2 reactor keeps the fluids moving constantly and maintains a homogeneous mixture of Sc-CO_2 and PAA throughout the reactor space. Agitated treatments with combined Sc-CO_2 and PAA resulted in a 6-$\log_{10}$ inactivation of *Bacillus* spores with PAA concentrations of 20 to 200 ppm (White *et al*. 2006; Christensen *et al*. 2009; Eisenhut *et al*. 2009; Qiu *et al.* 2009; Christensen *et al*. 2011). This technique has been patented to produce sterilized solid materials such as implants and biomedical devices. A similar strategy should be evaluated to make the process viable for a liquid substrate such as milk.

Protein coagulation is an apparent drawback of HP-CO_2 pasteurization of milk; however, this treatment could be advantageously applied to processes where acidification and curd formation are key. For example, HP-CO_2 has been successfully applied for

isoelectric precipitation of casein for industrial production of food protein ingredients such as caseins and whey protein concentrates and isolates (Tomasula *et al.* 1997). Production of food proteins becomes feasible if a continuous process is considered. Maximizing the yield of such a process requires a strategic design to ensure the Sc-CO_2 and milk are in optimally in contact. Tomasula *et al.* (1997) proposed the design of a continuous process consisting a reactor/precipitator in which milk was sprayed into a stagnant column filled with pressurized CO_2. They showed that the method of contacting the CO_2 and milk using this reactor was insufficient for casein precipitation and only yielded < 2.8 wt. % of protein on average at process temperatures between 32 and 49 °C at 4.1 or 5.5 MPa. To increase the yield, headspace agitation should be adapted for further mixing between the reactants as recommended by the authors. However, Tomasula *et al.* remained focused on casein production yields and thus data on microbial inactivation within the produced casein was not reported. A study by Calvo and Balcones (2001) did report that HP-CO_2 treatment (5 MPa, 40 °C, 180 min) resulted in a 2-$\log_{10}$ reduction of the micro-flora population and precipitated 85 wt. % of casein in skim milk.

Other treatments have been used to minimize the late blowing defect (LBD) in dairy products. Heat treatment of cheese-milk at higher temperatures than conventional pasteurization was applied to inactivate *Clostridium tyrobutyricum* spores and thus minimize the LBD during cheese ripening (Schreiber and Hinrichs, 2000). Although high heat treatment (HHT) is able to increase cheese yield by exploiting heat induced association of caseins with whey proteins, facilities capable of heating cheese-milk to temperatures more than 100 °C are not available; therefore, increasing pH prior to heating is necessary in order to denature any significant amount of whey protein (Banks *et al.* 1995). HHT also

suffers from a few limitations including poor coagulation and syneresis properties (Guinee *et al.* 1998; Marshall, 1986), low calcium-level products (Singh and Waungana, 2001), and reduced flavor-intensity and firmness in cheeses (Guinee *et al.* 1998). Bactofugation is also known to remove *Clostridium* spores from cheese-milk but the achieved reduction in spore numbers may be insufficient to prevent LBD (Garde *et al.* 2011). The contaminated milk (or bactofugate) is sometimes collected for subsequent UHT treatment and mixed with the bacteria-free skim milk to minimize yield loss (Griffiths *et al.* 2012).

Due to its versatility, the Sc-CO_2-PAA combination could potentially simplify a downstream processing of some dairy products. Furthermore, it may be possible to implement this strategy with very minimal adjustment to the equipment currently in use in industry since HP-CO_2 technology is already well-established, particularly for the production of casein. The present study investigates the Sc-CO_2-PAA treatment process in terms of its effectiveness at inactivating *E. coli* and *Bacillus atrophaeus* spores in skim milk. Evaluated process conditions included pressure-holding time, pressure, temperature, concentrations of CO_2, presence of agitation in the headspace, and treatment geometry. Sc-CO_2-induced coagulation was determined by measuring the protein content in the centrifuged-supernatant (or whey) obtained from treated milks.

2.3 Materials and methods

2.3.1 Milk samples preparation and inoculation

Commercially available skim milk powder (Barry Farm, OH, USA) with an average of 0.7 ± 0 % of fat and 90 ± 3 % total solid was weighed and added to autoclaved, distilled water to produce a 10 % (w/v) total solid of milk. The reconstituted skim milk was stored in a

refrigerator at 4 °C for up to 22 h to allow full hydration of the sample. Prior to any treatment, the samples were removed from the refrigerator and immediately placed in an ice bath to maintain the sample temperature at approximately 4°C. The challenge microorganism used was non-pathogenic *E. coli* ATCC 25922 obtained from the Food Safety Laboratory, Department of Food Science, Cornell University. *E. coli* ATCC 25922 was maintained on Trypticase soy agar (TSA; Becton Dickinson, Sparks, MD, USA) at 4 °C. Prior to the Sc-CO_2 treatments, a single colony was transferred onto a TSA plate and incubated for 22 ± 2 h at 37 ± 1 °C. A single colony was then transferred into Trypticase soy broth (TSB; Becton Dickinson) and incubated at 37 ± 1 °C for 20 ± 2 h on a shaker (at 230 rpm). An aliquot of 6 mL of inoculum was transferred into 54 mL of the milk sample, resulting in a starting population (N_0) of approximately 10^8-10^9 colony forming unit per milliliter (CFU/mL). A commercially available *B. atrophaeus* spore suspension (10^6 CFU/10µL in aqueous solution) (ATCC #9372, Mesa Laboratories Inc. Omaha, USA) was used as a biological indicator (BI) to validate the efficiency of Sc-CO_2 treatments. The spore suspension was kept refrigerated (2-8 °C) before use to maintain the spore viability. One mL of the spore suspension was inoculated into 99 mL sterile milk to give a final concentration of about 10^5 CFU/mL.

2.3.2 Sc-CO_2 treatments of thin-milk-films (TMF) without headspace agitation

A custom-built Sc-CO_2 system (Figure 2.1 below) with a 500-mL stainless steel pressure vessel (22.2 cm length, 5.6 cm internal diameter) (No. 10) was used. The system's temperature was monitored with both internal and external thermocouples. A 100 ppm PAA (NovaKillGen2) was transferred by a syringe onto the surface of a tarred glass-wool ball and inserted in a treatment vessel (No. 10) prior the vessel's closure. The loaded, sealed

vessel was then pressurized with a high-pressure gas compressor (No.6) (Newport Scientific, Jessup, MD, USA), and the pressure was controlled using a back-pressure regulator (No. 7) (Tescom, Elk River, MN). Once operating pressure was achieved, the vessel was held under pressure for the allotted treatment time. At the end of each treatment, CO_2 was slowly vented through an open, heated depressurization valve (No. 14).

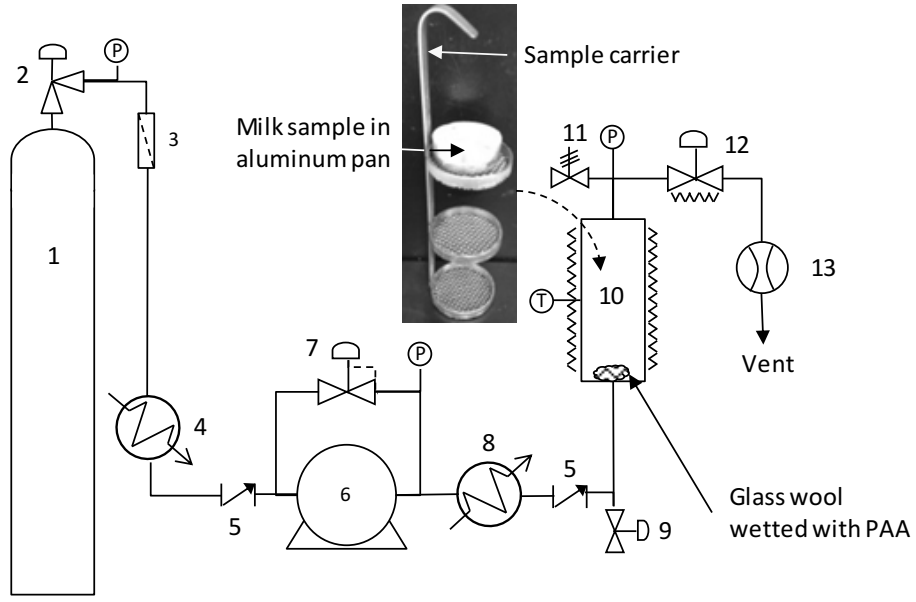

Figure 2.1 Schematic diagram of non-agitated supercritical carbon dioxide (Sc-CO_2) system.

1) CO_2 tank 2) tank valve 3) filter 4) sub-cooler 5) check valve 6) high pressure gas compressor 7) back pressure regulator 8) pre-heater 9) drain valve 10) high pressure treatment vessel (heated) 11) safety release valve 12) depressurization valve 13) flow meter. P: Pressure gauge, T: Thermocouple; Insert is a stainless sample carrier used for loading milk into 10)

To evaluate the influence of treatment geometry on the inactivation of *E. coli*, the milk's surface-area-to-volume ratio was adjusted by varying the radius of the aluminum pan to 10, 13 and 19 mm. A 2-mL sample of milk was placed into each of the three sized

pans to create a TMF with an estimated thickness of 6.4, 3.8 and 1.8 mm, respectively. Thus, the milk's surface-area-to-volume ratio in the pans was calculated as 1:2, 2:2, and 4:2, respectively. Prior to treatments, the pan was placed on a custom-made stainless steel sample carrier with three mesh platforms (diameter: 50.8 mm) (inset of Figure 2.1), which was subsequently lowered into the treatment vessel (No. 10).

The ratio of milk-to-CO_2 was varied to evaluate its effect on *E. coli* populations in treated skim milk. Glass beads were added to the vessel (No.10) to reduce its volume and treatments were performed on 2-mL samples in a 19-mm radius aluminum pan with vessel-volumes 100, 300 and 500 mL. The initial milk-to-CO_2 volume ratio was then converted into a mass ratio and calculated as 0.005, 0.01 and 0.03, respectively.

The treatments were performed at 10.3 MPa, 35 ^{o}C for 60 min without PAA in the evaluation of the influence of treatment geometry and the amounts of CO_2 on the inactivation of *E. coli*. The optimal surface-area-to-volume and milk-to-CO_2 ratios were then used in further experiments to investigate the effect of Sc-CO_2 on the inactivation of *E. coli* and *B. atrophaeus* spores in skim milk. Treatments for *E. coli* were performed at 10.3 MPa and 35 ^{o}C with and without PAA for 10, 20, 30 and 40 min. Treatments for *B. atrophaeus* spores were performed at different levels of pressure (10.3, 17.3 and 24.1 MPa) and at 50 ^{o}C for 60 min. Also, various temperatures (50, 60 and 70 ^{o}C) were evaluated for the inactivation of *B. atrophaeus* spores at 10.3 MPa for 60 min and treatment times (60, 120,180 min) at 10.3 MPa and 50 ^{o}C.

2.3.3 Sc-CO_2 induced coagulation of skim milk in TMF

A 19-mm aluminum pan containing a 4-ml milk sample was placed on the top mesh platform of the sample carrier (inset of photograph Figure 2.1) and the milk was exposed to Sc-CO_2 (non-agitated) in the treatment vessel (No.10 in Figure 2.1) for different treatment times (1, 10 and 20 min) and pressures (7.6, 10.2 and 13.8 MPa) at 35 °C. A sample from each trial (1 ml in 1.5 ml Eppendorf tube) was centrifuged at 14,000 revs/min (25,000g average) for 30 min at 20°C, and the obtained supernatant was diluted with distilled water (1:100). The protein content of the supernatant (or whey) was determined by a modified Lowry assay according to the manufacturer's instructions (Bio-Rad, Mississauga, ON, Canada). The assay was selected because of the small volume of milk used in this study. The absorbance at 750 nm was then measured with a UV-Vis spectrophotometer (Beckman Du640, Fullerton CA, USA), and the protein content quantified using a standard curve of Bovine Serum Albumin (BSA) standards in the range 25 to 1000 μg of protein/mL. All measurements were made at 25 °C and in triplicate (three independent experiments from different milk samples). The protein yield was calculated as

$$\text{The protein yield} = \left(1 - \frac{\text{protein in whey after treatments}}{\text{protein in whey before treatments}}\right) 100\ \% \qquad (1)$$

2.3.4 Sc-CO_2 treatments of TMF with headspace agitation

A patented Sc-CO_2 system (Nova2200^{TM}) described by White *et al*. (2006) was used for the treatments (Figure 2.2). The system was comprised of a 20-L stainless steel pressure vessel (240 mm internal diameter and 420 mm height) with an impeller stiring the headspace Sc-CO_2. Unlike the non-agitated Sc-CO_2 technique, the skim milk had to be contained in a rigid and intact package which allowed diffusion of Sc-CO_2 into the sample

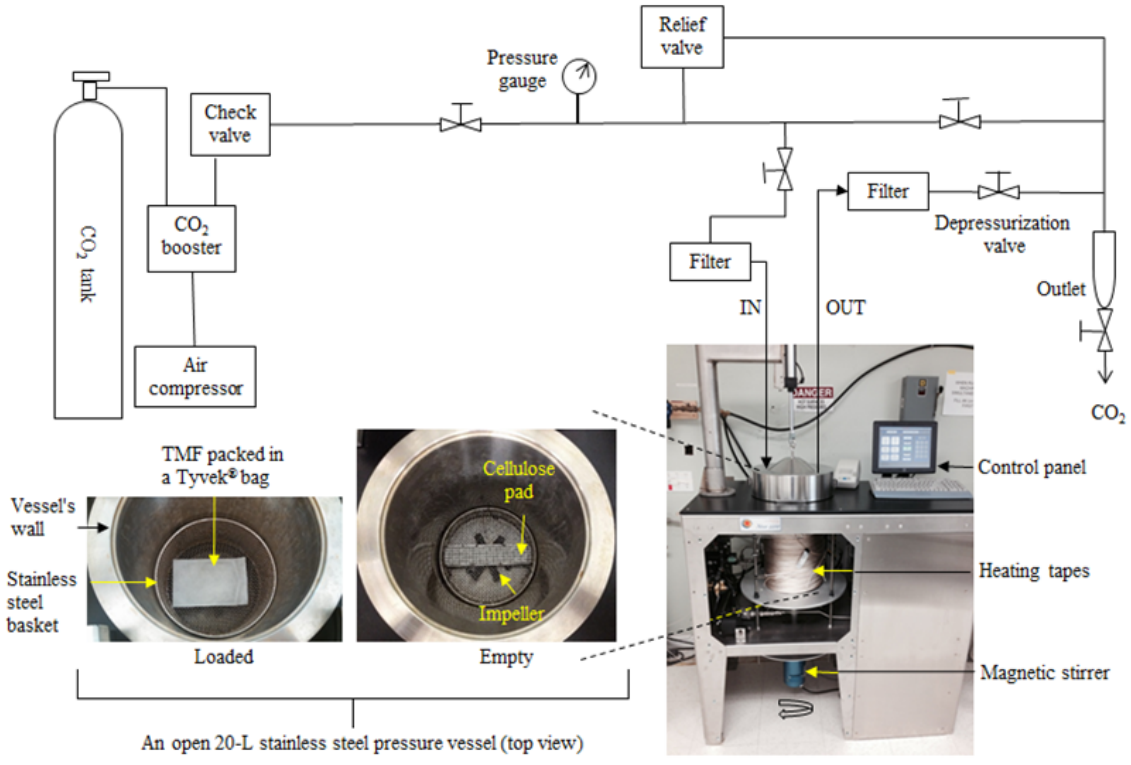

Figure 2.2 Schematic diagram of agitated supercritical carbon dioxide (Sc-CO_2) treatment system (Nova2200TM).

while simultaneously confining it to a TMF geometry. Therefore, in the present study, a 20-mL skim milk was packaged in a gas permeable Tyvek® bag (Beacon Converters, NJ), 140 mm × 30 mm and 140 mm × 10 mm (length × width) in size to create a TMF with an estimated thickness of 4.8 mm and 14.3 mm and the milk's surface-area-to-volume ratio of 7:10 and 2:10, respectively. The treatment vessel was loaded with two stainless steel baskets (23.5 cm diameter, with 17.8 and 12.7 cm height), in which the bags were laid flat with the gas permeable surface facing up. For combinations of the Sc-CO_2 treatment with PAA, a 16-mL portion of NovaKillGen2 sterilant corresponding to estimated 100 ppm of PAA in Sc-CO_2 was pipetted onto a 20 × 3.8 cm (length × height) cellulose pad, which was then secured at the bottom of the pressure vessel before closing it. In 6 min, the vessel was

charged with CO_2 from STP to a pressure of 9.8 ± 0.5 MPa and a temperature of 35 ± 3˚C with constant agitation at 680 ± 20 rpm. System parameters and pressure-holding times (10, 15, 20 and 40 min) were maintained before the vessel was depressurized over 15 min.

2.3.5 Enumeration of survivors

Following Sc-CO_2 treatments, two 1-mL samples from each treatment were transferred into two separate 9-mL aliquots; one aliquot of Butterfield's phosphate buffer (BPB) for *E. coli* enumeration and the other of distilled water for *B. atrophaeus* spore enumeration. This yielded a volume of 10 mL of each recovery liquid. The recovery liquid was serially diluted in BPB or distilled water after which 100-µL volumes was spread plated in duplicate on TSA and incubated for 22 ± 2 h at 37 ± 2 °C. Heat shock was not performed in line with previous works using *B. atrophaeus* spores as a BI (White et al. 2006; van Bokhorst-van de Veen et al. 2015) following the population assay recommended by the supplier. The spores that survived the Sc-CO_2 treatments were expected to be likely injured and the heat shock step would risk inactivating these spores as previously reported by Zhang et al. (2006). Log_{10} N/N_0 was calculated to determine the inactivation effect, where N_0 is the initial microorganism count in the untreated milk sample and N is the viable microorganism count in the milk sample after treatments using Sc-CO_2. In some cases, no colony growth was detected due to the culture assay sensitivity of <25 CFU/mL.

2.3.6 Statistical Analysis

For each treatment, the mean and standard deviation of survivor ratios were calculated. Data was evaluated by analysis of variance using JMP 10.1 statistical software (SAS Institute Inc. 2010). The Tukey-Kramer HSD test was used to determine the least

significant differences (LSD) at 5 % significance level. All treatments were performed in duplicate.

2.4 Results and discussion

2.4.1 Sc-CO_2 treatments of TMF without headspace agitation

2.4.1.1 Influence of milk surface area-to-volume ratio on *E. coli* inactivation

The resulting inactivation of *E. coli* in skim milk after exposure to Sc-CO_2 alone (10.3 MPa, 35 °C) by diffusing the fluids in TMF at different surface-area-to-volume ratios (1:2, 2:2, and 4:2) is shown in Figure 2.3. The results showed that the level of inactivation of *E. coli* in milk samples after treatments increased ($p<0.05$) with the increased surface-area-to-volume ratio. Although this result is to be expected, quantification of the influence of treatment geometry in enhancing the contact between Sc-CO_2 and liquid could help in the design of continuous systems that takes advantage of this concept. Similar studies have been reported utilizing this principle by employing a CO_2 microbubble generator (Kobayashi *et al*. 2014) and a gas-liquid metal contactor (Yuk *et al*. 2014) in a continuous system to improve the diffusion of CO_2 at low concentration and pressure.

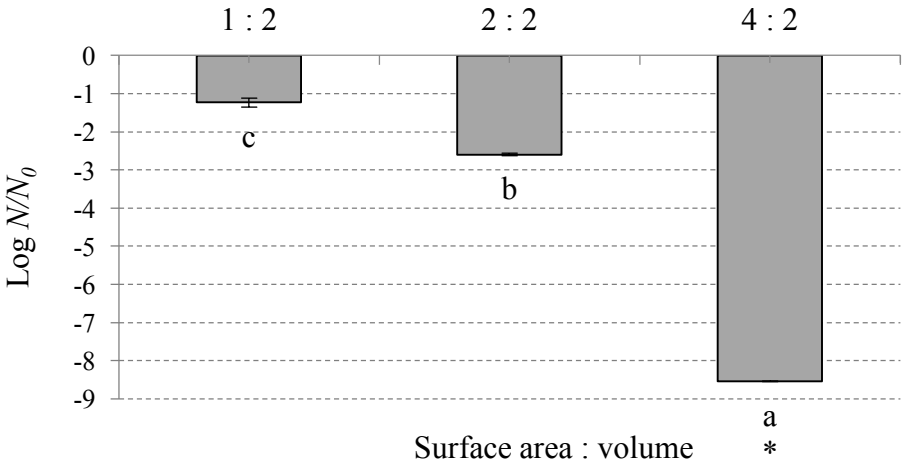

Figure 2.3 Inactivation of *Escherichia coli* in thin-milk-films (TMF) by non-agitated supercritical carbon dioxide (Sc-CO_2) (10.3 MPa, 35 ^oC, 60 min) as a function of surface area-to-volume ratio.

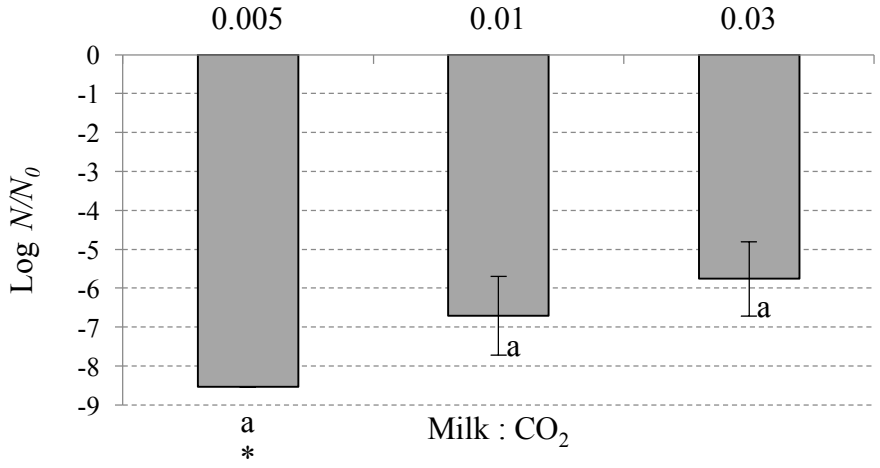

Figure 2.4 Inactivation of *Escherichia coli* in thin-milk-films (TMF) by non-agitated supercritical carbon dioxide (Sc-CO_2) (10.3 MPa, 35 ^oC, 60 min) as a function of sample-to-CO_2 ratio.

Error indicates standard deviation among replicates (n=2); *N*=number of survivors after the treatment; N_0=number of microorganisms before the treatment; Values of $\log_{10} N/N_0$ with different letter are significantly different ($p<0.05$); * indicates that the detection limit (10^2 CFU/mL) was reached. * <10: means that no colony was detected.

2.4.1.2 Influence of milk-to-CO_2 ratio on *E. coli* inactivation

The inactivation of *E. coli* in skim milk after exposure to Sc-CO_2 alone (10.3 MPa, 35 °C) by diffusing the fluids in TMF at different milk-to-CO_2 ratios (0.005, 0.01 and 0.03) (w/w) is shown Figure 2.4. The *E. coli* inactivation decreased significantly as the milk-to-CO_2 ratio increased from 0.005 to 0.03. In a biphasic system of milk and Sc-CO_2, the increasing amounts of CO_2 provided more available CO_2 diffusing into a constant volume of sample causing a higher inactivation of *E. coli.* Similar observations were reported in both batch (Garcia-Gonzalez *et al*. 2009; Mun *et al*. 2011) and continuous systems (Werner and Hotchkiss, 2006) by controlling the sample size and flow rate of CO_2, respectively.

Overall, an optimum milk's surface-area-to-volume and milk-to-CO_2 ratios were determined as 4:2 and 0.005, respectively. These ratios were used for the subsequent Sc-CO_2 treatments of skim milk in both stagnant and agitated headspace treatments for the inactivation of *E. coli* and *B. atrophaeus* spores.

2.4.1.3 Influence of pressure-holding time on *E. coli* and *B. atrophaeus* spores inactivation

The inactivation of *E.coli* in skim milk as exposed to Sc-CO_2 alone (10.3 MPa, 35 °C) and Sc-CO_2 combined with 100 ppm PAA by diffusing the fluids in TMF for different pressure-holding times up to 40 min is shown in Figure 2.5. The inactivation of *E. coli* went from 0.5- to 2-$\log_{10}$ in the first 10 min and increased progressively to achieve a complete 8-$\log_{10}$ inactivation after 40 min of treatment with non-agitated Sc-CO_2. The same level of *E. coli* inactivation was achieved after 40-min treatment of Sc-CO_2 with PAA but at a faster rate with a slightly higher degree of linearity (R^2=0.98) as compared to treatments without PAA (R^2=0.88). This finding was in agreement with the inactivation

curves reported in literature for all of the *E. coli* strains that were treated with Sc-CO_2 (Ballestra *et al*. 1996; Kim *et al*. 2007).

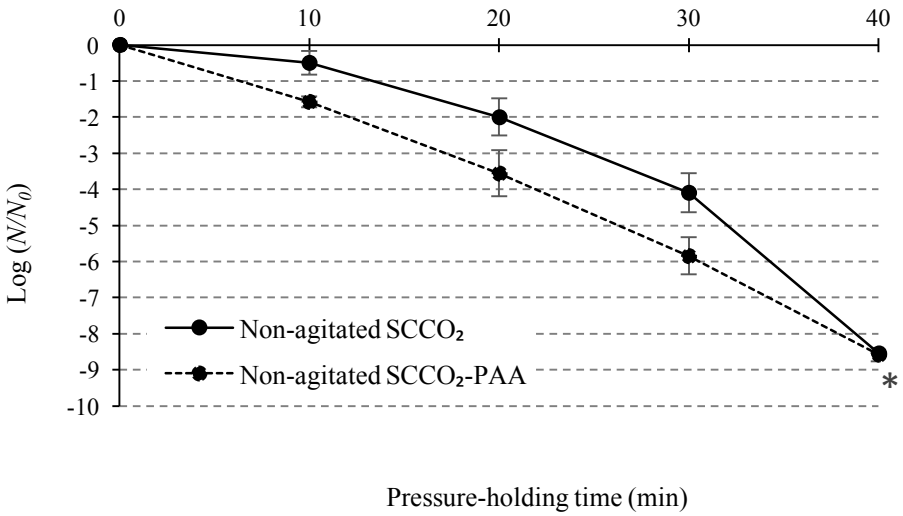

Figure 2.5 Inactivation of *Escherichia coli* in thin-milk-films (TMF) by non-agitated supercritical carbon dioxide (Sc-CO_2) (10.3 MPa, 35 ^{o}C) with and without 100 ppm peracetic acid (PAA) as a function of pressure-holding time.

N=number of survivors after the treatment; N_0=number of microorganisms before the treatment; Values of $\log_{10} N/N_0$ are the mean ± standard deviations from two replicate experiments. * <10: means that no colony was detected.

Generally, the biphasic inactivation curve consists of a 'lag phase' (i.e. an initial delay of inactivation) and a 'death phase' (i.e. decline in cell counts) as a result of HP-CO_2 treatments (Garcia-Gonzalez *et al.* 2007). This result suggests that CO_2 must first spend some amount of time dissolving into the cellular suspension and penetrating the cell membrane which time constitutes the 'lag phase'. The slow inactivation of *E. coli* indicated by a long lag phase can be partly attributed to the buffering effect of milk. Similarly, Kim *et al*. (2007) reported a longer treatment time (60 min) to achieve an 8-$\log_{10}$ reduction of generic *E. coli* in phosphate buffered saline, whereas, similar reduction of *E. coli* was achieved in phosphate saline after 30-min of Sc-CO_2 treatments at 10 MPa and 35 °C.

The same static approach without headspace agitation of Sc-CO_2 was tested at elevated temperatures and pressures and with exposure times as long as 180 min, but it was not effective for the inactivation of *B. atrophaeus* spores (Table 2.1). The highest reduction of *B. atrophaeus* spores was only 1-$\log_{10}$ after 60-min of Sc-CO_2 treatments at 10.3 MPa, 70 °C with 100 ppm PAA. Therefore, this study proceeded with the mechanical intervention approach by agitating the Sc-CO_2 and PAA to evaluate its effectiveness on the spore inactivation.

Table 2.1 Inactivation of *Bacillus atrophaeus* spores in thin-milk-films (TMF) by non-agitated supercritical carbon dioxide (Sc-CO_2) with 100 ppm peracetic acid (PAA)

Pressure (MPa)	Temperature (°C)	Time (min)	$\text{Log}_{10}\ N/N_0$
10.3			-0.5 ± 0.06^{b}
17.3	50	60	-0.6 ± 0.01^{b}
24.1			-0.8 ± 0.02^{a}
10.3	60	60	-0.9 ± 0.08^{a}
	70		-1.0 ± 0.11^{a}
10.3	50	120	-0.6 ± 0.10^{a}
		180	-0.7 ± 0.00^{a}

Error indicates standard deviation among replicates (n=2); *N*=number of survivors after the treatment; N_0=number of microorganisms before the treatment. Values of $\log_{10}\ N/N_0$ for the same parameter tested, with different letter are significantly different ($p<0.05$).

2.4.1.4 Influence of pressure and pressure-holding time on milk protein coagulation

The amount of protein in whey obtained from Sc-CO_2-treated milk samples coagulated at different levels of pressure (7.6, 10.3 and 13.8 MPa) and pressure-holding times (1, 10 and 20 min) at 35 °C is shown in Figure 2.6. These data were also converted to percentage yields by using Eq. (1). The lower the protein content in whey, the greater the percentage yield under given conditions and the higher the degree of protein coagulation in milk as previously described by Ramasubramaniam *et al.* (2012). From Figure 2.6, the Sc-CO_2

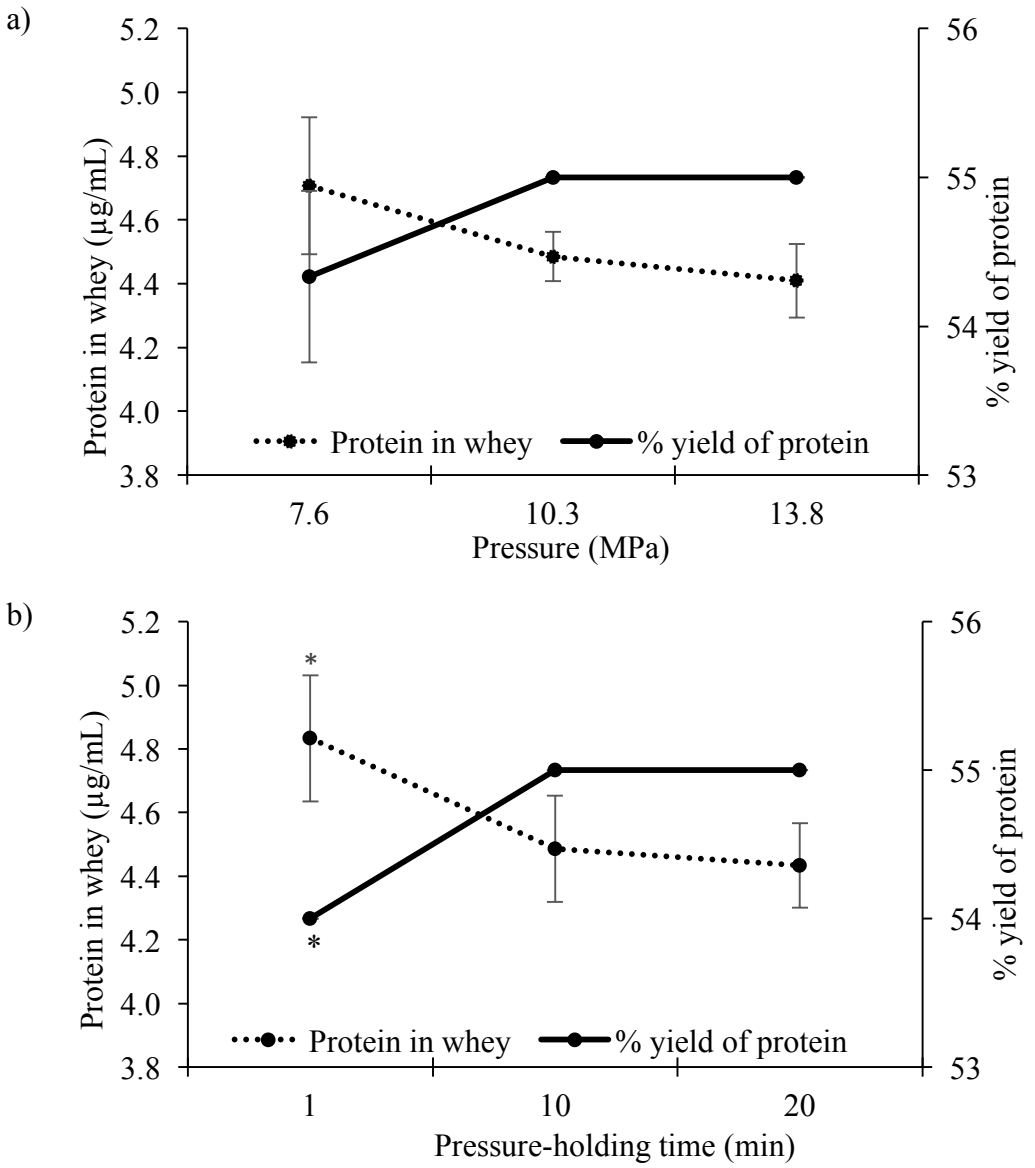

Figure 2.6 Effect of a) pressure (35 ºC for 15 min) and b) time (10.3 MPa and 35 °C) on protein content in whey and yield (%) of protein from coagulated skim milk in thin films using non-agitated supercritical carbon dioxide (Sc-CO_2); Error indicates standard deviation among replicates (n=3); The asterisk (*) represents significant differences between mean values of protein content in whey and yield of protein in coagulated milk after 1 and 10 and 20 min of treatment ($p<0.05$).

treatments were observed to also affect the coagulation of the skim milk, but the variability of the coagulation depended more on pressure-holding time (Figure 2.6a) than it did on pressure (Figure 2.6b). A 54 % of protein yield was obtained at the shortest treatment of 1 min at 10.3 MPa and 35 °C. The yield significantly increased ($p<0.05$) to 55 % for a treatment time of 10 min but remained relatively stable ($p\geq0.05$) to 20 min. The yield of 54, 55 and 55 % was observed at 7.6, 10.3 and 13.8 MPa, respectively at 35 °C after 15 min of treatment. However, the statistical analysis showed that the protein yields were not significantly different ($p<0.05$) regardless of pressures (Figure 2.6b). This pattern is in agreement with previous studies which focused on isoelectric precipitation of casein using HP-CO_2 in batch systems (Jordan *et al.* 1987; Tomasula *et al.* 1997). The sub-critical CO_2-precipitation process for casein is optimized at temperatures from 38 to 43°C and pressures of 4.1 to 6.9 MPa (Tomasula *et al*. 1995).

2.4.2 Sc-CO_2 treatments of TMF with headspace agitation

Our preliminary study indicated that the surface area of skim milk in thin films exposed to agitated Sc-CO_2 had an influence on the inactivation of *E. coli*. A complete 8-$\log_{10}$ inactivation of *E. coli* was achieved in milk with a higher surface-area-to-volume ratio of 7:10 as compared to a ratio of 2:10 which yielded 1.7 $\log_{10}$ reduction after a 15-min treatment of Sc-CO_2 (10.3 MPa, 35 °C and agitation rate of 680 rpm). A complete inactivation curve for *E. coli* was not experimentally obtained because the inactivation of *E. coli* exposed constantly agitated Sc-CO_2 was too rapid even without the addition of PAA. Nevertheless, an 8-$\log_{10}$ reduction of *E. coli* is considered very effective as bacteria counts in raw bulk tanks or raw commingled silos rarely exceeds 10^4 CFU/mL (Van Kessel *et al*. 2004; Jackson *et al*. 2012). The experiments on protein coagulation were not carried

out as we had previously established the coagulated protein yield profile in TMF due to Sc-CO_2 without headspace agitation in which the treatment was considered less severe than that with agitation. Subsequently, the focus of the agitated Sc-CO_2 treatments on thin skim milk films shifted to the inactivation of *B. atrophaeus* spores. The milk's surface-area-volume ratio of 7:10 was maintained for the *B. atrophaeus* spore trials.

The inactivation of *B. atrophaeus* spores in thin skim milk films exposed to agitated Sc-CO_2 alone (10.3 MPa, 35 °C, at 680 rpm) and agitated Sc-CO_2 with added PAA (100 ppm) for different holding times (10, 20 and 40 min) is shown in Figure 2.7. The results showed that a total 5-$\log_{10}$ reduction of *B. atrophaeus* spores was achieved after 40-min treatment of Sc-CO_2 with PAA, whereas only 1-$\log_{10}$ reduction was achieved without PAA. From Figure 2.7, a strong linear inactivation curve (R^2=0.98) for the spores was observed as a result of Sc-CO_2-PAA with no lag phase from 10 to 40 min; whereas, an almost 20-min lag phase was observed (R^2=0.91) with Sc-CO_2 alone. The former is in agreement with the linear inactivation profile for a 6-$\log_{10}$ reduction of *B. subtilis* spores (inoculated on spore strips) as a result of agitated Sc-CO_2 exposure (10.3 MPa, 35 ^{o}C, 680 rpm for 60 min) with 0.75 ppm of PAA (White *et al*. 2006). Also, Qiu *et al*. (2009) reported an 8-$\log_{10}$ inactivation of *B. atrophaeus* spores (inoculated onto an allograft tissue, e.g. porcine acellular) after Sc-CO_2 treatment (9.4-10 MPa, 35–41°C, 680 rpm for 30 min) with 55 ppm of PAA. The 5-$\log_{10}$ inactivation of *B. atrophaeus* spores observed in this present study reduces the spore populations below the level (10^4 CFU/mL) at which bacterial spore contaminations are practically found in the dairy processing industry (Burgess *et al.* 2010).

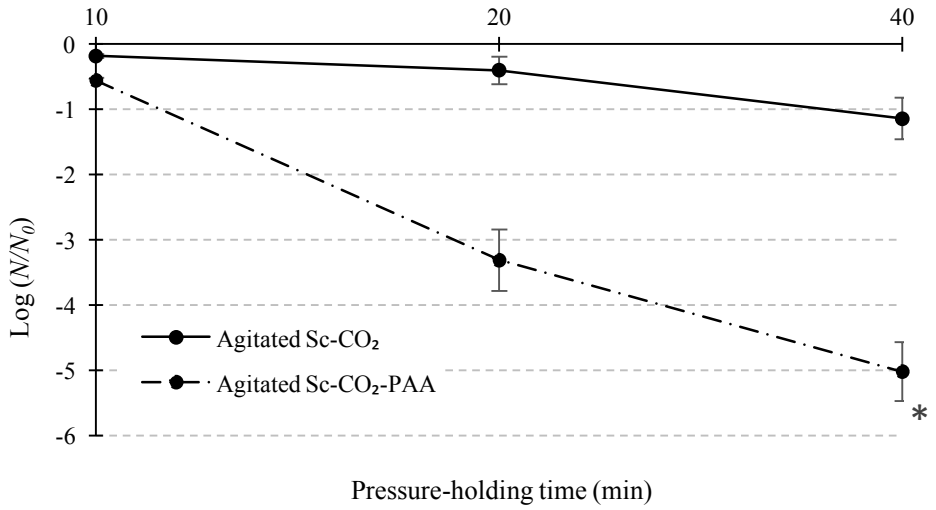

Figure 2.7 Inactivation of *Bacillus atrophaeus* spores in thin-milk-films (TMF) by agitated supercritical carbon dioxide (Sc-CO_2) (10.3 MPa, 35 ^{o}C, 650 rpm) with and without 100 ppm peracetic acid (PAA) as a function of pressure-holding time.
N=number of survivors after the treatment*; N_0*=number of microorganisms before the treatment; Values of $\log_{10} N/N_0$ are the mean ± standard deviations from two replicate experiments. * <10: means that no colony was detected.

2.5 Conclusions

A treatment combining Sc-CO_2 and PAA with headspace agitation was more effective at inactivating microbes in skim milk than the same treatments conducted in a static, non-agitated reactor. A total of 5-$\log_{10}$ inactivation of *B. atrophaeus* spores was achieved in thin-milk-films exposed to turbulent Sc-CO_2 carrying dissolved PAA. This novel treatment potentially alleviates the concerns over heat-resistant and/or spore-forming bacteria in milk. Increasing the milk's surface area by forming it into a thin, liquid film for treatment with Sc-CO_2 enhanced the coagulation of protein therein. Therefore, it may be possible to integrate antimicrobial Sc-CO_2 treatments with existing dairy processes such that milk sterilization, acidification, and curd formation occur in a single step. In terms of industrial applications, the current study offers a potentially advantageous strategy of exposing free

falling liquid films to Sc-CO_2 and PAA in a column reactor with agitated headspace for the sterilization of various foods and biological products.

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Chapter 3: Low-temperature pasteurization of skim milk by high-pressure carbon dioxide with peracetic acid

3.1 Abstract

High-pressure carbon dioxide (HP-CO_2) is effective towards microbial inactivation, but it also lowers the pH of milk which may lead to sedimentation due to casein precipitation and limit its utility. Studies were conducted to quantify the effects of pressure (7.6, 10.3 and 13.1 MPa) and temperature (5, 15 and 25 °C) of HP-CO_2 with and without added PAA (0, 50, 75 or 100 ppm) on sedimentation in skim milk. A respective pressure, temperature and concentration of PAA of 10.3 MPa, 5-15 °C and 50 ppm resulted in 0.34-0.38 g/100 mL of sediment in treated skim milk after 30-min of HP-CO_2 treatments. This amount of sediment was not significantly different from that observed in untreated skim milk (0.34 g/100mL). HP-CO_2 treatments at 10.3 MPa for 120 min resulted in a reduction of 0.3, 2.9 and 3.4 log-cycles of *Escherichia coli* at 5, 15 and 25 °C, respectively. Addition of PAA to HP-CO_2 increased the inactivation of *E. coli* to 2.6, 5.4 and 9.2 log-cycles at 5, 15 and 25 °C, respectively. HP-CO_2 treatment at 10.3 MPa and 25 °C with PAA for 120 min resulted in an inactivation of 0.7 log-cycles of *Bacillus atrophaeus* spores. Comparing the fit of literature models to the experimental data, the Fermi model was better able to describe the HP-CO_2 inactivation of *E. coli* with and without PAA. Also, the Fermi model provided a better description of the HP-CO_2-PAA inactivation kinetics of *B. atrophaeus* spores. This study demonstrates a potential for use of HP-CO_2-PAA as a non-thermal pasteurization method of skim milk and related foods.

3.2 Introduction

Milk is a complex and nutritionally rich medium for growth of many bacteria. However, thermal processing at high temperatures leads to undesirable effects in milk (e.g. flavor and nutrient loss and browning reactions) (Ramaswamy et al., 2009). Non-thermal high-pressure carbon dioxide (HP-CO_2) processes, which include subcritical and supercritical carbon dioxide (Sc-CO_2), have been widely researched as alternative techniques for food pasteurization. The principle of HP-CO_2 treatment is based on gas dissolution in microbial cells by pressurization, which consequently lowers the intracellular pH and impairs cell viability (Dillow et al., 1999). The choice of pressure and temperature thus affect the characteristics of CO_2 mass transfer rates and the biological activities of treated microbial cells (Isenschmid et al., 1995; Kamihira et al., 1987; Lin et al., 1993; Taniguchi et al., 1987). However, a pH decrease due to the acidification by dissolved CO_2 in milk negatively affects the protein stability since casein precipitates out at the isoelectric point of pH 4.6 (Fox, 2003). Furthermore, the precipitation of casein by HP-CO_2 is known to be a strong function of temperature in the range of 38-43 ^{o}C at pressures between 4.1 and 6.9 MPa (Tomasula et al., 1995). Therefore, an accurate estimate of the reduction in microbial population becomes difficult due to protein coagulation and phase separation in treated milk (Graham et al., 1987).

To minimize the amount of carbonic acid formed in the aqueous solution to avoid aggregation and/or coagulation of casein, the pressure and temperature levels need to be controlled. Thus, a milder treatment for microbial inactivation in milk is possible by manipulating the contact mode between the HP-CO_2 and the liquid to be treated, which has been previously reviewed elsewhere (Sikin & Rizvi, 2011). For example, Kobayashi's

group has developed a pasteurization technique utilizing CO_2 microbubbles at pressures lower than 2 MPa (Kobayashi et al., 2014), which is a pressure that has no bactericidal effects (Oulé et al., 2006). However, this technique faces a practical problem in the processing of high-protein foods due to rapid and high acidification and therefore coagulation. Agitation too can enhance the solubilization of CO_2 and increase the frequency of contact of bacterial cells with CO_2, making the cellular penetration of CO_2 easier. As reported in several studies (Hong et al., 1997; Lin et al., 1992; Mun et al., 2011; Oulé et al., 2006), agitation generally improves microbial inactivation.

As expected, batch systems have been reported to require longer treatment times (Erkmen 2000a, 2000b, 2001a, 2001b; Hongmei et al., 2013; Lin et al., 1994) as compared to continuous systems (Werner & Hotchkiss, 2006) for microbial inactivation in milk. Most of the studies on batch systems relied on self-diffusion of CO_2 into milk in a static environment, whereas the effectiveness of continuous treatment is attributed to flow patterns which promote good mixing and the dispersion of CO_2 in the food products (Casas et al., 2012). In the absence of agitation, the microbial inactivation has been reported to depend on the sample size (Hong et al., 1997). A low working volume ratio (WVR) (i.e., the ratio of sample volume to reactor volume, expressed as %) was often used in these studies (mostly $\leq$ 10 %) to achieve reasonable inactivation of microorganisms. However, the low WVR may not be viable for a commercial batch process. None of these studies has quantitatively measured and reported any quality parameters of milk together with bacterial inactivation as a result of HP-CO_2 treatments. As it is possible to increase the inactivation rate in various media by agitation, it is reasonable to implement similar strategies for treatment of milk with HP-CO_2 for bacterial inactivation.

Incorporation of a small amount of co-solvents such as peracetic acid (PAA) with HP-CO_2 has been shown to result in a more efficient bacterial destruction under milder conditions and shorter times (White et al., 2006; Christensen et al., 2009; Eisenhut et al., 2009; Qiu et al., 2009; Christensen et al., 2011). PAA is a strong oxidant and is very reactive at low concentrations against bacteria (0.001 wt. %) and spores (0.3 wt. %) (Greenspan & MacKellar, 1951). PAA is an approved sanitizer in the United States for food contact surfaces (21CFR178.1010) and for direct contact with fruits and vegetables (21CFR173.315), and meat, poultry and seafood (21CFR173.370) at a maximum concentration of 80, 85 and 110 ppm, respectively. However, PAA application in dairy processing is not known. Its oxidation has been shown not to result in the formation of high molecular weight aggregates in both whey and casein suspensions (Kerkaert et al., 2011). We hypothesized that PAA would have a minimum impact on milk quality while enhancing the inactivation of microorganisms when used at low dosage, in combination with HP-CO_2. The current study was designed to quantify the effects of HP-CO_2 treatment (pressure, temperature and concentration of PAA) on sediment formation and population reduction of *Escherichia coli* (ATCC 25922) and *Bacillus atrophaeus* (ATCC 9372) spores in skim milk. The data collected were also analyzed to compare selected kinetic models for their fit to effectively describe the inactivation of those microorganisms by HP-CO_2 and HP-CO_2 with added PAA.

3.3 Materials and methods

3.3.1 Milk samples preparation and inoculation

Commercially available skim milk powder (Barry Farm, OH, USA) with an average of 0.7 ± 0 % of fat and 90 ± 3 % total solid was weighed and added to autoclaved distilled water

to produce a 10 w/v % total solid containing milk. The reconstituted skim milk was stored in a refrigerator at 4 °C for up to 22 h to allow full hydration of the reconstituted sample. Prior to any treatment, the samples were removed from the refrigerator and immediately placed in an ice bath to maintain the sample temperature at approximately 4°C. The challenge microorganism used was non-pathogenic *E. coli* ATCC 25922 obtained from the Microbiology Laboratory, Department of Food Science, Cornell University. *E. coli* ATCC 25922 was maintained on Trypticase soy agar (TSA; Becton Dickinson, Sparks, MD) at 4 °C. Prior to the $HPCO_2$ treatments, a single colony was transferred onto a TSA plate andincubated for 22 ± 2 h at 37 ± 1 °C. A single colony was then transferred into Trypticase soy broth (TSB; Becton Dickinson) and incubated at 37 ± 1 °C for 20 ± 2 h on a shaker (at 230 rpm). An aliquot of 6 mL of inoculum was transferred into 54 mL of milk sample, resulting in a starting population (N_0) of approximately 10^8-10^9 colony forming unit per milliliter (CFU/mL). A commercially available *B. atrophaeus* spore suspension (10^6 CFU/10µL in aqueous solution) (ATCC #9372, Raven Biological Laboratories, Inc., Omaha, NE, USA) was used as a biological indicator to validate the efficacy of HP-CO_2 treatments. One mL spore suspension was inoculated into 99 mL sterile skim milk to give a final concentration of about 10^5 CFU/mL.

3.3.2 HP-CO_2 treatments of agitated bulk milk (ABM)

A custom-built HP-CO_2 system (Figure 3.1) with a 284-mL stainless steel pressure vessel (46 mm internal diameter, 175 mm height) (No. 13) (Autoclave Engineers, Erie, PA, USA) was used. The interior of the vessel was washed using sterile water, sanitized for 20 min with 70% ethanol and then rinsed twice with sterile water prior to each treatment. A 60-mL sample of skim milk was aseptically loaded into the vessel, giving an estimated

cylindrical geometry of ABM with a radius of 23 mm and height of 30 mm and a WVR of 21 %. An impeller (Disperimax^{TM}Turbine type) (32 mm diameter) was then immersed halfway down to allow for direct mixing of the liquid phase, which provides radial flow while drawing CO_2 down a hollow shaft which disperses through the impeller. A variable control electric motor was used to control the impeller at 60 rpm throughout the experiment. A 0.4, 0.6 and 0.8 mL portions of NovaKillGen2 sterilant (NovaSterilis, Ithaca, NY, USA), corresponding to estimated 50, 75 and 100 ppm of PAA in CO_2, respectively, were pipetted onto a weighed cotton wool, which was then placed at the base of a 500-mL pressure vessel (222 mm length, 56 mm internal diameter) (No. 10 in Figure 3.1) prior to closure. A 30-min preconditioning of the PAA at 50 ppm was carried out by allowing the PAA vapor to equilibrate with CO_2 (99.99 % purity; Airgas, Elmira, NY, USA) in the vessel (No. 10) prior to injection. The vessel was then brought up to set pressures (7.6, 10.3 and 13.1 MPa) with a high-pressure gas compressor (No. 6) (Newport Scientific, Jessup, MD, USA). The pressure was controlled using a back-pressure regulator (No. 7) (Tescom, Elk River, MN, USA). Once set pressure was achieved, the ball valve (No. 12) was opened to allow the flow of $HPCO_2$ with PAA into the treatment vessel (No. 13). The stop watch was started when the set pressure had equalized in both the vessels and the internal temperature of treatment vessel had reached the desired operating temperature (5, 15 or 25 °C). The temperature was monitored with both internal and external thermocouples (identified as T in Figure 3.1). Treatment times ranged from 10 to 120 min. At the conclusion of each treatment, the heated depressurization valve was opened and the CO_2 was released. After each treatment, the presence of residual PAA in treated milk samples was verified by immersing a PAA test strip (LaMotte, MD, USA) into the sample

for 10 s, and the color change from test strips was compared with the color chart indicating a PAA concentration range from 0 to 50 ppm. All treatments were performed in duplicate.

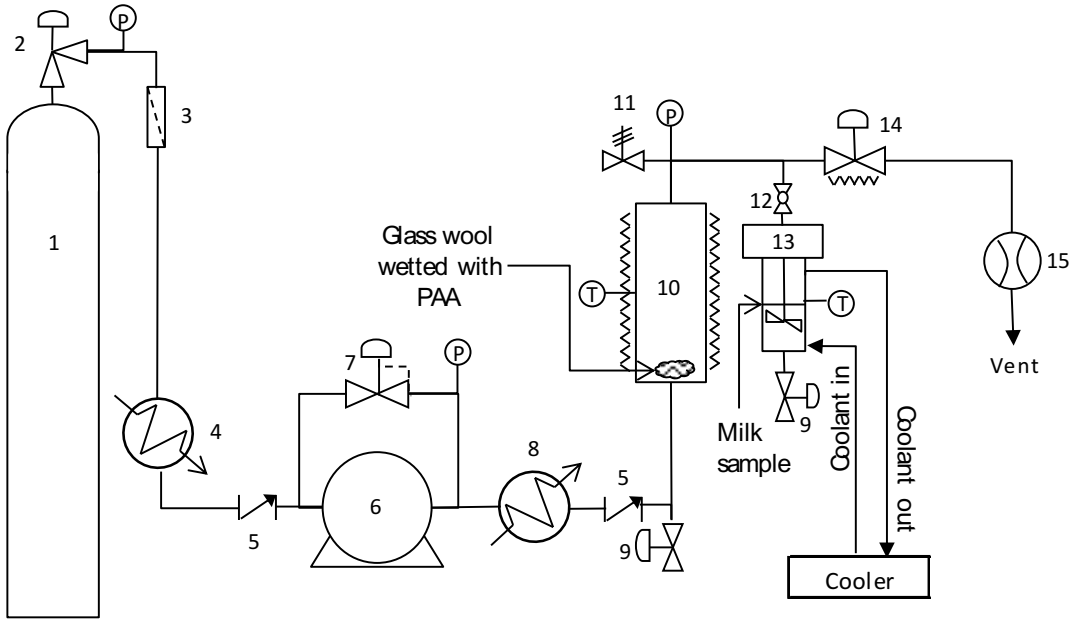

Figure 3.1 Schematic diagram of high pressure carbon dioxide (HP-CO_2) treatment system for agitated bulk milk.

1) CO_2 tank 2) tank valve 3) filter 4) sub-cooler 5) check valve 6) high pressure gas compressor 7) back pressure regulator 8) pre-heater 9) drain valve 10) high pressure treatment vessel (heated) 11) safety release valve 12) ball valve 13) high pressure treatment vessel with impeller (cooled) 14) depressurization valve (heated) 15) flow meter P: Pressure gauge, T: Thermocouple.

3.3.3 Sediment analysis of ABM

Skim milk samples were processed with HP-CO_2 under the pressures of 7.6, 10.3 and 13.1 MPa at 5, 15 or 25 °C for 30 min. Subsequent HP-CO_2 treatments at pre-determined optimized conditions (e.g. pressure and temperature) were carried out with added PAA at different concentrations of 50, 75 and 100 ppm. After each HP-CO_2 treatment with and

without added PAA, control and treated skim milk was accurately weighed and poured into a calibrated tube and centrifuged (Centaur 2 at 4200 rpm for 15 min), corresponding to 2760 g. After removing the supernatant, the wet weight of the sediment was recorded and the sediment was then oven-dried at 102 ± 1 °C to a constant weight to determine its dry weight (g/100mL). Sediment was estimated as a key indicator in optimizing the process parameters of HP-CO_2 including the concentration of added PAA in HP-CO_2 at which sedimentation was minimal.

3.3.4 Enumeration of survivors

Following HP-CO_2 treatments, the treated 1-mL sample was transferred into a solution of 9 mL of Butterfield's phosphate buffer (BPB) (for *E. coli*) and distilled water (for *B. atrophaeus* spores). This yielded a volume of 10 mL of recovery liquid. The recovery liquid was serially diluted in BPB, after which 100-µL volumes were spread plated in duplicate on TSA and incubated for 22 ± 2 h at 37 ± 2 °C.

3.3.5 Modeling of inactivation kinetics

The survival data are presented as a survival ratio $log\frac{N}{N_0}$ vs. time relationship, where N_0 is the initial microorganism counts in the untreated sample and N is the viable microorganism count in the HP-CO_2-treated sample. Experimental data were fit to the Weibull model (Peleg, 2006; McKellar & Lu, 2003) in equation (1) and Fermi model (Peleg, 2006; McKellar & Lu, 2003) in equation (2) by Microsoft Excel (version 2013).

$$log\frac{N}{N_0} = -bt^n\,, \tag{1}$$

where the constant *b* can be considered as a non-linear rate parameter and *n* is the parameter responsible for the curve shape, a concave upward semi-logarithmic survival curve will be represented by $n < 1$; a concave downward curve by $n > 1$ and a log-linear survival curve is a special case of the model where $n = 1$.

$$\log\frac{N}{N_0} = -ln[1 + \exp\{k(t - t_c)\}], \quad (2)$$

where k and t_c are kinetic constants; k is the rate constant (min^{-1}), t_c (min) is the longest treatment time in which the survival fraction equals 100%, that is, the lag phase, and t is the treatment time.

The goodness of fit of the linear and nonlinear models was compared by computing the coefficient of determination (R^2) and the root mean square error (RMSE). R^2 measures how well a linear or nonlinear model fits the data, and the higher the R^2 value, the better the adequacy of the model for describing the data (Baranyi, Pin, & Ross, 1999). RMSE measures the average deviation between the observed and fitted values. A smaller RMSE value for a model indicates a better fit of data for that model.

$$\text{RMSE} = \sqrt{\frac{\sum(\text{fitted} - \text{observed})^2}{n - p}}, \quad (3)$$

where *n* is the number of observations and *p* is the number of parameters to be estimated.

3.3.6 Statistical analyses

For each treatment, the mean and standard deviation of survivor ratios were calculated. Data were analyzed by analysis of variance using JMP 10.1 statistical software (SAS

Institute Inc. 2010). Tukey-Kramer HSD test was used to determine the least significant differences (LSD) at 5% significance level. All treatments were performed in duplicate.

3.4 Results and discussion

3.4.1 Sedimentation and pH changes

HP-CO_2 is known to induce the denaturation of protein due to a decline in pH and ζ-potential (Zhou et al., 2010), resulting in protein aggregation and/or precipitation (Tisi, 2004; Liao et al., 2009). For this reason, the dry sediment was chosen as a parameter for protein precipitation because it is considered to be the most accurate and is directly related to the stability of solids in milk (Boumpa et al., 2008). To find the optimal pressure and temperature which result in low sediment, several preliminary studies were conducted and the results indicated that the dry sediment was 2.6 ± 0.4, 2.6 ± 1.0 and 2.9 ± 1.1 g/100 mL (n=2) at 7.6, 10.3 and 13.1 MPa, respectively, for a 30-min HP-CO_2 treatment at 30 °C. The average of these values was almost 9× the amount of dry sediment in control samples (0.3 ± 0.0 g/100 mL). As an indicator of casein aggregation, Tisi (2004) reported that $HPCO_2$ changed the particle size distribution (PSD) of raw skim milk as compared to untreated controls with an increase in temperature from 15 to 40 °C at 7 or 62 MPa. He extended the study by adding a pre-carbonation step prior to HP-CO_2 treatments at 62 MPa and different temperatures (15, 20, 25, 30 and 35 °C). The treatment at 15 °C was found to leave the PSD of the sample unchanged. Based on Tisi's and our preliminary results, the treatment temperature in the range of 5 to 25 °C was used for evaluation of sediment formation in skim milk treated with HP-CO_2 at 7.6, 10.3 or 13.1 MPa.

Figure 3.2 shows the effect of temperatures and pressures on sediment formation in skim milk after HP-CO_2 at an agitation rate of 60 rpm for 30 min. As can be seen, an increase in pressure did not affect ($p \geq 0.05$) sediment formation and its amount was close to the untreated control at 5 and 15 °C. However, there was a steady increase in sediment quantity with pressure ($p < 0.05$) at 25 °C and coagulation was most noticeable at 13.1 MPa. To supplement the sediment analysis results, the pH data (Table 3.1) indicated that the $HPCO_2$ treatments reduced the initial pH of untreated skim milk from 6.8 ± 0.0 to 6.0-6.1 but an increase in pressure and temperature did not significantly change ($p \geq 0.05$) the pH of treated skim milk. This pattern was consistent with results previously reported elsewhere (Hofland et al., 1999; Tomasula et al., 1995; 1999).

This could be ascribed to the buffering capacity of milk due to the electrical properties of substances such as proteins, phosphates, carbon dioxide and citrates in milk (Walstra & Jenness, 1984). A pressure/temperature range of 10.3 MPa/5-25 °C was then used to determine the effect on sediment formation of PAA added to HP-CO_2 during the process.

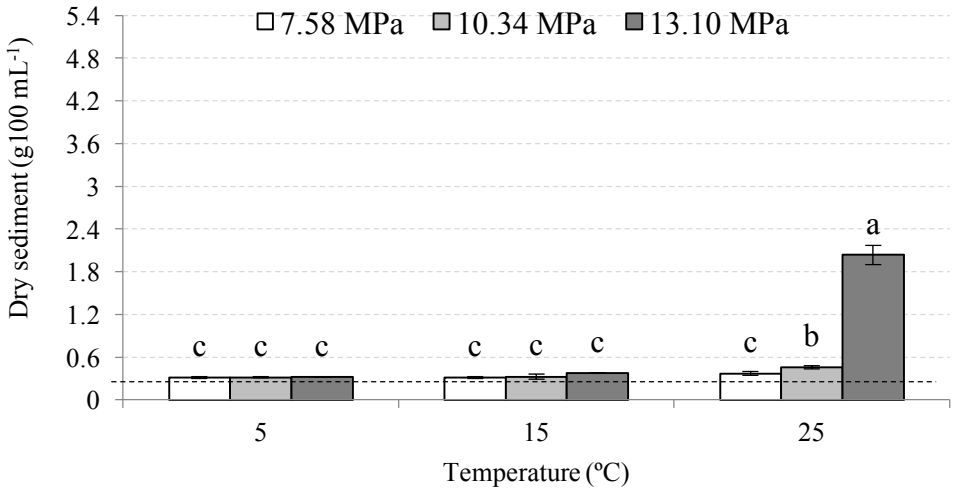

Figure 3.2 Effect of temperatures and pressures on sediment formation in agitated bulk milk (ABM) after high pressure carbon dioxide (HP-CO_2) at 60 rpm for 30 min.

Error indicates standard deviation among replicates (n=2) Values of dry sediment for the same treatment temperature, with different letter are significantly different ($p<0.05$). The dashed line (- - -) represents the average value of dry sediment for untreated sample (0.34 ± 0.02).

Table 3.1 Effect of temperatures and pressures on pH of agitated bulk milk (ABM) in high pressure carbon dioxide (HP-CO_2) at 60 rpm for 30 min.

Temperature (^oC)	pH at different treatment pressures		
	7.6 MPa	10.3 MPa	13.1 MPa
5	6.1 ± 0.0^a	6.0 ± 0.0^a	6.0 ± 0.0^a
15	6.1 ± 0.0^a	6.0 ± 0.0^a	6.0 ± 0.0^a
25	6.1 ± 0.0^a	6.0 ± 0.0^a	6.0 ± 0.0^a

Error indicates standard deviation among replicates (n=2); Within a row, means with different letters are significantly different ($p<0.05$). The average value of pH for untreated sample was 6.8 ± 0.0

Figure 3.3 shows the effect of PAA concentrations on dry sediments in skim milk after HP-CO_2 treatments (10.3 MPa, 60 rpm and 30 min) at 5, 15 and 25 ^oC. It is observed that an increase in the concentration of PAA increased the sediment formation progressively ($p<0.05$) at all temperatures. Furthermore, a spike of PAA in HP-CO_2 seems to be more effective at disrupting the buffering behavior of milk as compared to HP-CO_2 treatment alone. The addition of PAA in CO_2 streams significantly reduced the pH of skim milk from 6.8 (control) and 6.0 (HP-CO_2-treated without PAA) to 5.3 and 4.7 with the addition of 50, 75 and 100 ppm, respectively (Table 3.2) ($p<0.05$) at all temperatures tested.

As shown in Figure 3.4 (photographs), there is no visible sign of protein coagulation in skim milk treated with 50 ppm PAA at 10.3 MPa and 15 ºC for 30 min, but the coagulation gradually became apparent when the concentration of PAA was increased from 75 to 100 ppm PAA and the pH decreased from 5.0 to 4.7. These changes are in agreement with those of Gastaldi & Lagaude (1996) who observed the onset of milk gelation when pH changed from 5.2 to 4.7. Our preliminary study also indicated that the concentration of PAA used also affects the quantity of residual PAA in skim milk. The strip test indicated a complete release of PAA after 0.5 and 24 h from skim milk treated with 50 and 75 PAA, respectively, while the concentration of residual PAA remained approximately at 50 ppm for skim milk treated with 100 ppm PAA. It is known that PAA is highly unstable and readily degrades into acetic acid and water, which helps alleviate concerns about residual toxicity.

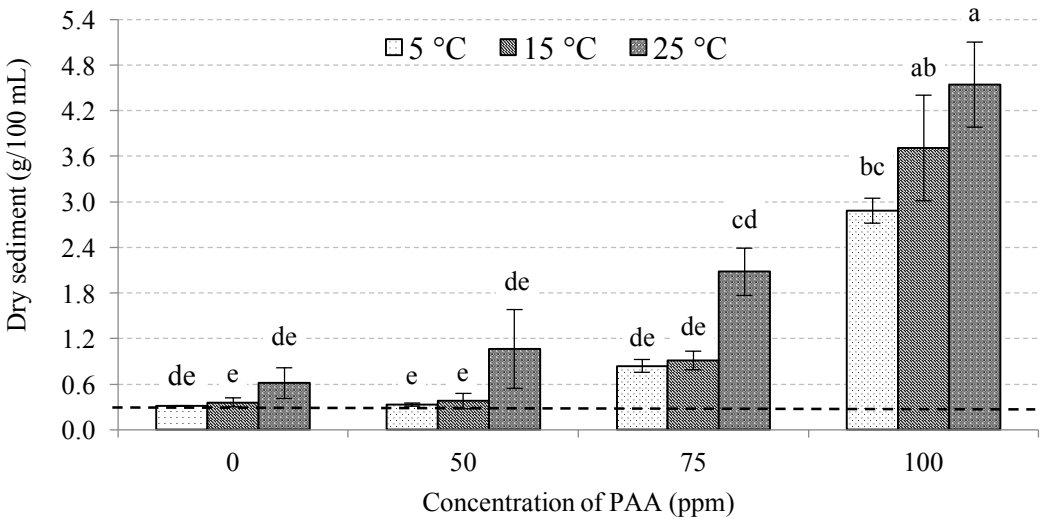

Figure 3.3 Effect of peracetic acid (PAA) concentrations (ppm) on dry sediment in agitated bulk milk (ABM) after high pressure carbon dioxide (HP-CO_2) treatments (10.3 MPa, 60 rpm and 30 min) at 5, 15 and 25 ^{o}C.

Error indicates standard deviation among replicates (n=2); Under the same treatment temperature, means with different letter are significantly different ($p<0.05$). The dashed line (- - -) represents the mean value of dry sediment for untreated samples (0.34 ± 0.02).

Table 3.2. Effect of peracetic acid (PAA) concentrations (ppm) on pH of agitated bulk milk (ABM) after high pressure carbon dioxide (HP-CO_2) treatments at 10.3 MPa and 60 rpm for 30 min

Temperature (°C)	pH at different concentration of PAA			
	0 ppm	50 ppm	75 ppm	100 ppm
5	6.0 ± 0.1^{a}	5.4 ± 0.1^{b}	4.8 ± 0.1^{cd}	4.8 ± 0.1^{cd}
15	6.0 ± 0.1^{a}	5.4 ± 0.0^{b}	5.0 ± 0.1^{bcd}	4.7 ± 0.2^{d}
25	6.0 ± 0.1^{a}	5.3 ± 0.0^{bc}	4.9 ± 0.1^{cd}	4.8 ± 0.1^{d}

Error indicates standard deviation among replicates (n=2); Within a row, means with different letters are significantly different ($p<0.05$). The average value of pH for untreated sample was 6.8 ± 0.0.

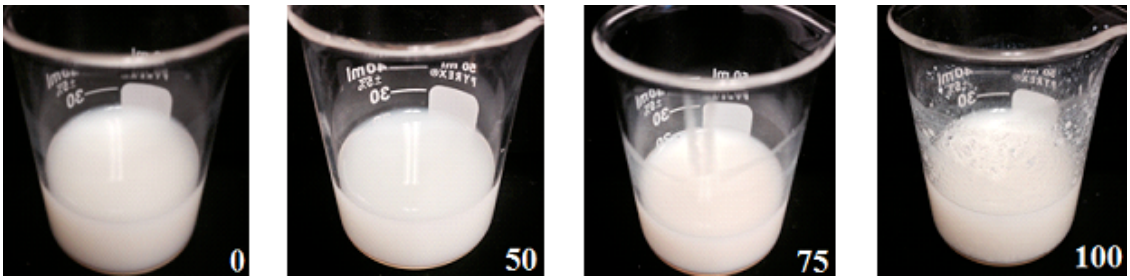

Figure 3.4 Appearance of agitated bulk milk (ABM) samples after high pressure carbon dioxide (HP-CO_2) treatments at different concentration of peracetic acid (PAA) (indicated at the bottom right as ppm) at 10.3 MPa, 15 °C, 60 rpm for 30 min. A 20-ml milk sample was swirled gently in the beaker before photograph was taken for each treatment.

Overall, an optimum pressure, temperature, and concentration of PAA for the HP-CO_2 process was found to be 10.3 MPa, 5-25 °C and 50 ppm at which the sedimentations were not significantly ($p \geq 0.05$) different from the untreated control. These process parameters of HP-CO_2 were therefore chosen for studies on the inactivation kinetics of *E. coli* and *B. atrophaeus* spores in skim milk.

3.4.2 Inactivation of *E. coli*

Figure 3.5 a) and b) show the respective inactivation of *E. coli* in skim milk by HP-CO_2 alone (10.3 MPa, 60 rpm, 21 % WVR) and HP-CO_2 combined with 50 ppm PAA at 5, 15 and 25 °C. The results indicate that there was no inactivation of *E. coli* observed for the first 60 min irrespective of the temperatures used and a 0.3-, 3.0- and 3.4-$\log_{10}$ reduction was achieved at 5, 15 and 25 °C, respectively, after 120 min of treatment with HP-CO_2 alone. Meanwhile, the application of HP-CO_2 with PAA showed a gradual trend toward higher inactivation and a 2.5-, 5.4- and 9.2-log of *E. coli* were inactivated after 120 min at 5, 15 and 25 °C, respectively. Generally, the biphasic inactivation curve consists of a lag (i.e. an initial delay of inactivation) and death (i.e. decline in cell counts) phase as a result

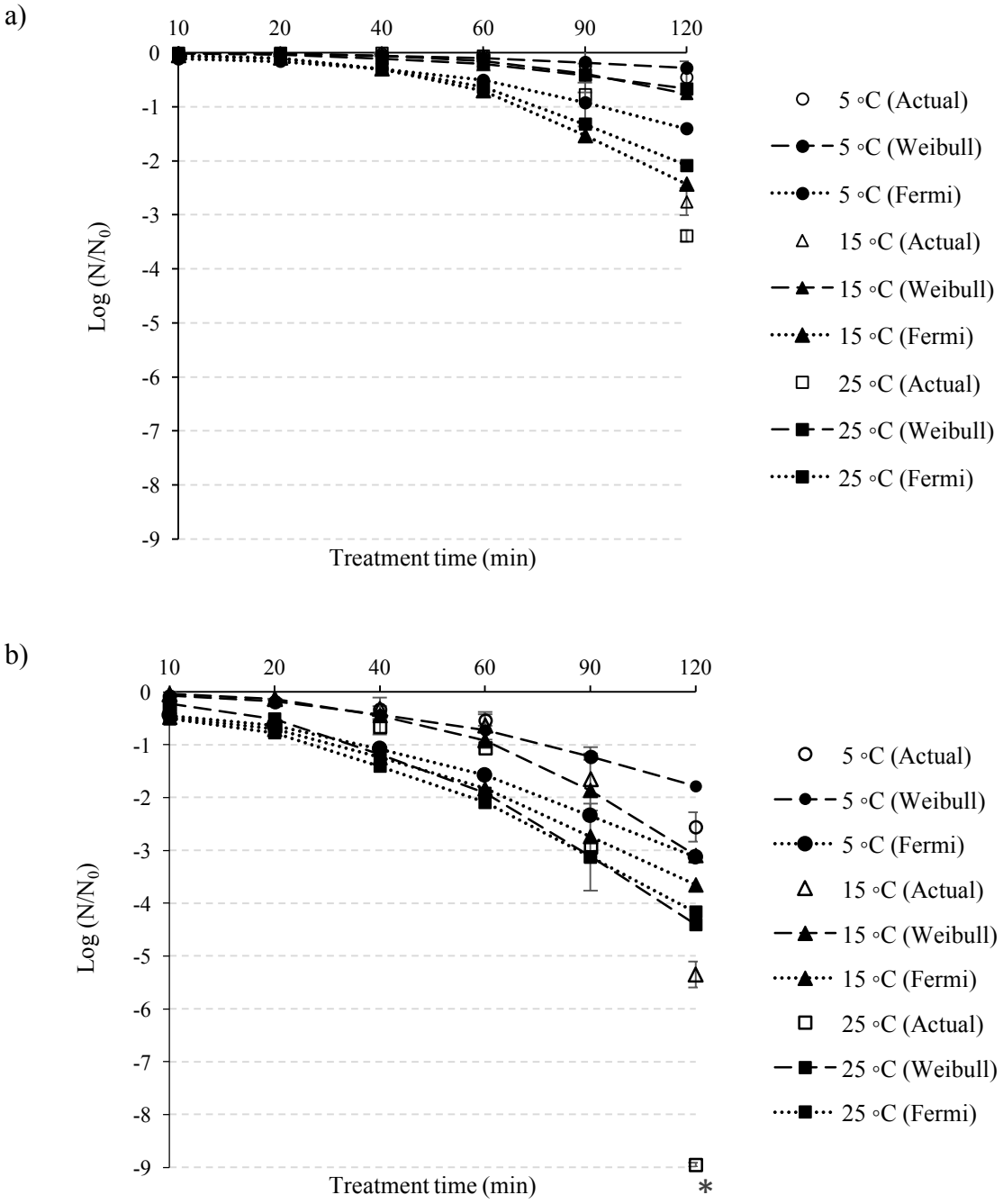

Figure 3.5 Data fitting of the survival curves of *Escherichia coli* in agitated bulk milk (ABM) treated by high-pressure carbon dioxide (HP-CO_2) (10.3 MPa, agitation rate of 60 rpm) without a) and with 50 ppm PAA b) at different treatment times and temperatures. Data were fitted with the Weibull and Fermi models.

N=number of survivors after the treatment*; N_0*=number of microorganisms before the treatment; Values of $\log_{10} N/N_0$ are the mean ± standard deviations from two replicate experiments. * <10: means that no colony was detected.

of HP-CO_2 treatments. In this study, the two distinctive phases as previously described were observed in the inactivation curve of *E. coli* in skim milk by HP-CO_2 alone (Figure 3.5) that was not observed when PAA was added (Figure 3.6).

As a diffusive process, surface exposure of a liquid to HP-CO_2 during agitation didnot substantially enhance the inactivation of *E. coli* in this study. Although previous studies have reported that agitation generally improves microbial inactivation during HP-CO_2 treatments (mostly in supercritical phase), the treatment substrates were mostly simple media with very low WVR and subjected to high intensity mixing (at ≥ 100 rpm) (Garcia-Gonzalez et al., 2009; Mun et al., 2011). Due to the delicate nature of milk, a low mixing speed is often maintained, which is not effective for microbial inactivation. For example, Yao et al., (2013) reported a small reduction of *Pseudomonas* (2.9 logs), Enterobacteriaceae (2.5 logs), *Staphylococcus aureus* (1.9 logs), total bacterial count (1.8 logs) and lactic acid bacteria (0.8 log) in raw milk following HP-CO_2 treatments (7.5 MPa, 25 °C) for 60 min at agitation rate of 100 rpm with an estimated WVR of 50 %.

3.4.3 Inactivation of *B. atrophaeus* spores

Figure 3.7 shows a 0.7-$\log_{10}$ reduction of *B. atrophaeus* spores in skim milk by HP-CO_2 (10.3 MPa, 60 rpm, 21% WVR) with 50 ppm PAA at 25 °C after a 120-min treatment. At a cellular level, the delay in inactivation as shown by the lag phase of up to 90 min (Figure 3.7) can be attributed to the barrier of a thick envelope of hard-to-penetrate material around bacterial spores (Driks, 1999; Madigan et al., 2001). Furthermore, a high inoculum load of spores (10^5 CFU/mL) used in the study could have hindered the sporicidal effect of the treatments as spores are more susceptible to aggregation. Previous studies have suggested that inner spores in a spore aggregate are protected by killed spores on the top, forming

passive or active barriers which the HP-CO_2 must diffuse through to be effective (Checinska et al., 2011; Checinska et al., 2012; Enomoto et al., 1997).

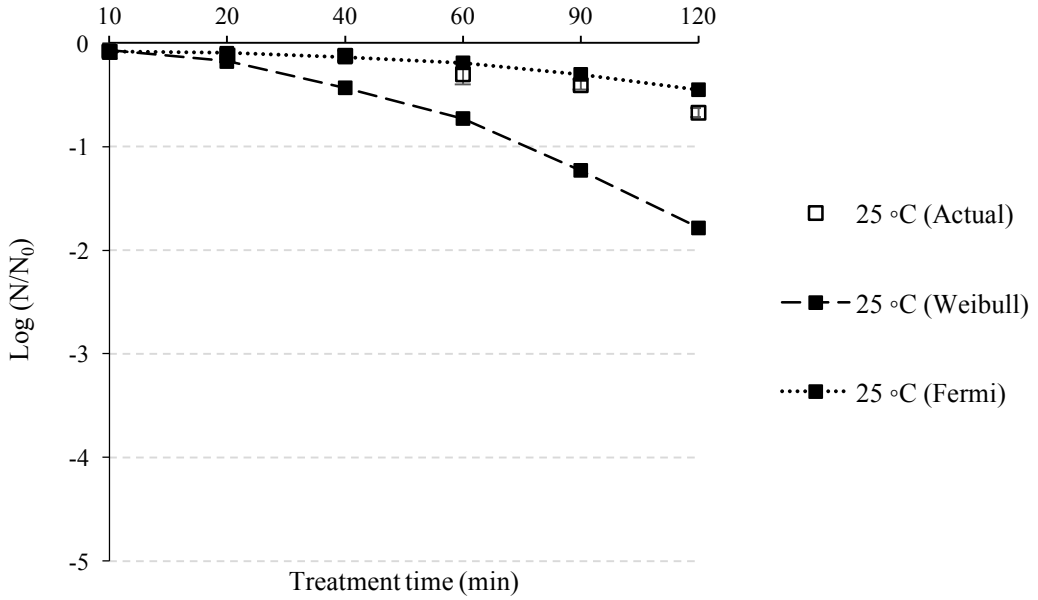

Figure 3.6 Data fitting of the survival curves of *Bacillus atrophaeus* spores in agitated bulk milk (ABM) treated by high-pressure carbon dioxide (HP-CO_2) (10.3 MPa, 25°C and agitation rate of 60 rpm) with added peracetic acid (PAA) at 50 ppm.

Data were fitted with the Weibull and Fermi models. N=number of survivors after the treatment; N_0=number of microorganisms before the treatment; Values of $\log_{10} N/N_0$ are the mean ± standard deviations from two replicate experiments.

It is important to highlight that carbon dioxide is not a good solvent for polar and high molecular compounds such as PAA due to the lack of dipole polarity (Tang et al., 2014). Thus, a homogenous mixture could not be formed between the two compounds even at a supercritical state for an effective delivery of the antimicrobial agent to the bacterial cells (Heldebrant et al., 2006; Kordikowski et al., 1995; Reverchon et al., 2010). For this reason, headspace agitation which creates a constant movement of Sc-CO_2 fluid in the reactor and maintains homogeneity of Sc-CO_2 and PAA was reported to achieve a 6-$\log_{10}$

inactivation of *Bacillus* spores with PAA concentrations of 20 to 200 (White et al. 2006; Christensen et al. 2009; Eisenhut et al. 2009; Qiu et al. 2009; Christensen et al. 2011). In this study, the 30-min residence time of the PAA in the stagnant headspace before mixing with CO_2 prior to the treatments was found to have little impact on the inactivation of *B. atrophaeus* spores in the skim milk.

3.4.4 Modeling the inactivation of *E. coli* and *B. atrophaeus* spores

Table 3.3 and Table 3.4 show the statistical parameters for the fit of the two kinetic models to inactivation data of *E. coli* and *B. atrophaeus* spores. The regression coefficients (R^2) values ranging from 0.81 to 0.96 indicate that, overall, a reasonably good fit was obtained with Weibull and Fermi models for the HP-CO_2 and HP-CO_2 with added PAA treatments, with one exception: the fit of the *E. coli* survival data to Weibull model for HP-CO_2 treatment at 25 °C was not as good (R^2 = 0.50). For the treatments at all temperatures with HP-CO_2 alone, the Fermi model produced a better fit as indicated by its higher R^2 value (0.91 vs. 0.75) and lower RMSE (0.58 vs. 0.67) than Weibull model, see Table 3.3. A very useful way to judge the performance of a model is to examine the residuals (i.e. the difference between model predictions and measured values) and for a better fit, they should be randomly distributed. An analysis of the residual plots of these two models suggests that they are equally applicable since the residuals obtained for both the models showed almost similar distribution patterns. Nonetheless, visual inspection of the inactivation curve indicated that the Fermi model was more appropriate in describing the survival curve of *E. coli* as shown in Figure 3.5.

Table 3.3 Estimated model parameters for the inactivation kinetics of *Escherichia coli* using high-pressure carbon dioxide (HP-CO_2) at 10.3 MPa for 120 min with and without added 50 ppm PAA.

Treatment	T	Weibull				Fermi			
	(°C)	b (min^{-1})	n	R^2	RMSE	k (min^{-1})	t_c (min)	R^2	RMSE
HP-CO_2	5	0.00	1.47	0.88	0.08	0.04	40	0.97	0.53
	15	0.00	2.31	0.86	0.82	0.07	40	0.95	0.58
	25	0.00	1.66	0.50	1.12	0.06	40	0.81	0.64
HP-CO_2-PAA	5	0.00	1.29	0.96	0.33	0.06	0	0.96	0.77
	15	0.00	1.75	0.96	0.93	0.07	0	0.95	1.07
	25	0.00	1.20	0.83	1.90	0.08	0	0.84	2.02

Table 3.4 Estimated model parameters for the inactivation kinetics of *Bacillus atrophaeus* spores using high-pressure carbon dioxide (HP-CO_2) at 10.3 MPa for 120 min with added 50 ppm PAA

Treatment	T	Weibull				Fermi			
	(°C)	b (min^{-1})	n	R^2	RMSE	k (min^{-1})	t_c (min)	R^2	RMSE
HP-CO_2-PAA	25	0.00	1.29	0.96	0.61	0.02	90	0.96	0.11

b; rate parameter; n: shape factor; k: rate constant; t_c: lag phase duration; R^2: regression coefficient based on linearized equation of the respective model; RMSE: root mean square error; significance level, p=0.05.

It is noteworthy that, based on the lowest R^2 and the highest RMSE, the poorest fit for the two models was at 25 °C for both HP-CO_2 and HP-CO_2 with PAA treatments. As shown in Table 3.4, R^2 indicates that, overall, a good fit was obtained with both models for the inactivation data of *B. atrophaeus* spores in skim milk following the HP-CO_2-PAA treatments at 25 °C. However, the lower RMSE value confirmed that the inactivation kinetics of the spores was better fitted by the Fermi than the Weibull model.

The *n* values of the Weibull model representing the shape factor were higher than 1 in all cases indicating that all survival curves were concave downward, as shown in Figure 3.5, 3.6 and 3.7. In physiological terms, n >1 indicates that the remaining bacterial cells become increasingly weaker or damaged when treatment time increases. Conversely, n < 1 indicates that the surviving cells have the ability to adapt to the applied stress (van Boekel, 2002). However, the effect of temperature on the shape factor in the inactivation curve of *E. coli* could not be detected because the n values fluctuated with increasing temperatures in both HP-CO_2 and HP-CO_2 with PAA treatments. This is consistent with the argument by Fernandez et al., (2002) and van Boekel, (2002) that the curvature measurement as indicated by the n parameter only described the kinetic pattern of the mechanism controlling the process studied and, therefore, should be independent of external factors.

The *k* value of the Fermi model describing the inactivation (or death) phase of *E. coli* increased from 0.04 to 0.06 min^{-1} and from 0.06 to 0.08 min^{-1} with an increase in temperature from 5 to 25 °C following the HP-CO_2 and HP-CO_2 with PAA, respectively. The duration of the lag phase denoted by t_c in the inactivation of *E. coli* was 40 min after HP-CO_2 treatment irrespective of the temperatures used. With an increase in temperature

from 5 to 25 °C, the duration of the lag phase t_c was not changed. Although the bactericidal effect of HP-CO_2 generally increases with pressure and temperature the penetration of CO_2 provokes cell stress while the bacterial cells are still intact at the sub-critical conditions (Oulé et al., 2006). The penetration of CO_2 into the cells is slow during this stress phase and does not significantly modify their morphology (Hong et al., 1997). Oulé et al., (2006) observed the perforation of the cellular envelope of *E. coli* cells in nutrient broth only after 30 min of HP-CO_2 treatment at 12 MPa, 25 °C and 200 rpm through scanning electron microscopy and transmission electron microscopy images. Also, the addition of PAA to $HPCO_2$ diminished the lag phase (t_c=0) regardless of temperatures tested. As is to be expected, the longest duration of lag phase was 90 min for the inactivation of *B. atrophaeus* spores by HP-CO_2 with added PAA treatment at 25 °C. Comparing the Eq. (1) with (2), the Weibull and Fermi model consider the kinetic rate constants *(b* vs. *k*) whereas only the Fermi model takes into account a lag phase in describing how the microbial inactivation occurs. Therefore, the Fermi model was considered more effective as the former in describing the inactivation kinetics of *E. coli* in which the lag phase was more distinguishable. For the same reason, the model provides a better fit than the Weibull model for the inactivation data of *B. atrophaeus* spores.

3.5 Conclusion

HP-CO_2 with added PAA was able to achieve 5-$\log_{10}$ reductions of *E. coli* in skim milk at 10.3 MPa and 15 °C for 120 min, whereas an almost 1 $\log_{10}$ reduction of *B. atrophaeus* spores was obtained at 25 °C. Although effective with no visible sign of protein coagulation at temperatures lower than 25 °C, this 2-h treatment is considered long. Therefore, this technique may find its application in tandem with low temperature storage of raw milk

which normally take 72 h before processing. Comparatively, the Fermi model was more appropriate than the Weibull model to describe inactivation kinetics of *E. coli* in skim milk that had been treated with HP-CO_2 and HP-CO_2-PAA. Likewise, the Fermi model fitted the inactivation data of *B*. *atrophaeus* spores better than Weibull model as a result of HP-CO_2-PAA at 10.3 MPa and 25 °C.

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Chapter 4: Synergistic processing of skim milk with high pressure nitrous oxide, heat, nisin and lysozyme to inactivate vegetative and spore-forming bacteria

4.1 Abstract

Individual and combined effects of high pressure nitrous oxide (HP-N_2O), heat, and antimicrobials on the inactivation of *Escherichia coli*, *Listeria innocua*, and *Bacillus atrophaeus* endospores in milk were evaluated after 20-min treatments. Respective $\log_{10}$ reduction of 8.0 and 8.6 for *E. coli* and *L. innocua* in milk were achieved through a combination of HP-N_2O (15.2 MPa), heat (65 °C) and nisin (150 IU/mL). A 2.5-$\log_{10}$ inactivation of spores was obtained by HP-N_2O, nisin (at both 50 and 150 IU/mL) and lysozyme (50 µg/mL) at 85 °C. Combining HP-N_2O, heat, and antimicrobials resulted in significantly greater microbial inactivation than the sum of the individual reductions achieved from each treatment alone, indicating synergy. HP-N_2O irrespective of temperatures (25, 45 and 65°C) did not cause any occurrence of sub-lethally injured cells or disruption in colloidal stability of milk at 65 and 85 °C. Minimal changes were observed in pH and color of milk following treatment with HP-N_2O, nisin (150 IU/mL) and lysozyme (50 µg/mL) at 85 °C.

4.2 Introduction

With a complex food matrix milk provides a conducive environment for the growth and survival of many pathogenic and spoilage microorganisms. Thermal treatment has been the predominant method to ensure its microbial safety, but processing conditions commonly

applied in the dairy industry (70 to 120 °C) could lead to heat-related damages such as flavor and nutrient loss as well as browning reactions (Siciliano et al., 2000). High pressure carbon dioxide (HP-CO_2) has emerged as one of the most promising preservation techniques for heat-sensitive foods, however, with limitations such as pH change due to solubilization of CO_2 in milk, which may cause an isoelectric precipitation of casein (at pH 4.6), as well as HP-CO_2 efficacy based on acidification and extraction (Lin et al., 1994; Hong & Pyun, 1999; Kim et al., 2008), which could be hindered by high buffering capacity and the presence of fat in milk, respectively (Mun et al., 2012).

Nitrous oxide (N_2O) has been suggested as an alternative fluid to CO_2 at supercritical state, because it does not decrease the pH in aqueous media, is colorless and non-flammable at room temperature, and has critical properties (T_c=36.4 °C and P_c=7.25 MPa) close to that of CO_2. High pressure N_2O (HP-N_2O) offers great potential for the preservation of highly pH-sensitive protein-based foods, but previous research on the bactericidal action of N_2O focused almost exclusively on fruit juices (Spilimbergo et al., 2007a; Spilimbergo et al., 2007b; Gasperi et al., 2009; Spilimbergo & Ciola, 2010) and tomato purees (Bizzotto et al., 2009), except for a study by Spilimbergo et al. (2011) that investigated the effect of HP-N_2O on natural microflora in raw skim milk. However, to the best of our knowledge, the application of HP-N_2O for the inactivation of spores in milk and dairy products has not been explored previously. It is also noteworthy that no studies concerning the HP-N_2O bactericidal application have been carried out for both gram-positive and -negative bacteria in a single food system.

Nisin, a bacteriocin produced by *Lactococcus lactis* spp. *lactis*, is normally active against gram-positive bacteria (Delves-Broughton, 1990). Lysozyme is an enzyme that

catalyzes the hydrolysis of the peptidoglycan in cell walls of only gram-positive bacteria, because gram-negative bacterial cell walls are shielded by an outer membrane (Proctor & Cunningham, 1988). Both of these antimicrobials have been granted generally recognized as safe (GRAS) status by the U.S. Food and Drug Administration (FDA) (CFR, 2006; 1998). Their spectrum of activity can be extended to gram-negative bacteria when used in combination with other agents or treatments. For example, high hydrostatic pressure (HHP) inflicts sub-lethal injury in both gram-positive and gram-negative bacterial cells, making them more susceptible to antibacterial compounds such as nisin and/or lysozyme among others (Hauben et al., 1996; López-Pedemonte et al., 2003). The CO_2 or N_2O pressures applied for preservation purposes are much lower (generally <20 MPa) as compared to the hydrostatic pressures employed in HHP (300-600 MPa), making it easier to control, more feasible and a less expensive process (Gasperi et al., 2009).

Combining these different decontamination methods, each of them known as a hurdle in so-called hurdle technologies for the preservation of foods was an approach first introduced by Leistner (1985). Hurdle-based strategies have subsequently been adopted by various researchers (Masschalck et al., 2001; Rodríguez-González et al., 2011; Walkling-Ribeiro et al., 2009; Wordon et al., 2012) for minimal processing of foods. The latter refers to microbiologically safe and gentle processing of foods that allows for a maximum quality retention, thus constituting a compromise between adequate safety and highest possible quality of foods. In addition to determining the recovery from sub-lethal injury of bacterial cells which is a well-known approach to assess efficacy of the processing strategy, synergistic effects of two or more hurdles have proven to be valuable for the evaluation of the effectiveness of several treatments in a previous work (Sikin et al., 2015).

The objective of the present research was to investigate the synergistic effects of heat and HP-N_2O with and without nisin for the inactivation of gram-negative and -positive bacteria in milk. Possible occurrence of sub-lethal cell damage inflicted by HP-N_2O on *E. coli* and *L. innocua* was evaluated at 25, 45 and 65 °C. In order to optimize the efficacy of our hurdle strategies we also utilized the differences in modes of action between lysozyme and nisin for an enhanced inactivation of *B. atrophaeus* spores at 85 °C. Finally, the impact of HP-N_2O at 25, 45, 65 and 85 °C on pH, particle size, ζ-potential and color of milk was also assessed.

4.3 Materials and methods

4.3.1 Milk sample preparation and inoculation

The appropriate amount of skim milk powder (with an average of 0.7 ± 0 % of fat and 90 ± 3 % total solid) (Barry Farm, OH, USA) was weighed and added to autoclaved distilled water to produce a 10% (w/v) level of total solid. The reconstituted samples were then stored in a refrigerator at 4 °C for up to 22 h to allow full hydration. Prior to the treatments, the samples were removed from the refrigerator and immediately placed in an ice bath to maintain the sample temperature at approximately 4 °C. The challenge microorganisms used were non-pathogenic *E. coli* ATCC 25922 and *L. innocua* FSL C2008 obtained from the Laboratory of Food Safety, Department of Food Science, Cornell University. These *E. coli* and *L. innocua* strains were maintained on Trypticase soy agar (TSA; Becton Dickinson, Sparks, MD, USA) at 4 °C. Prior to the HP-N_2O treatments, a single colony was transferred onto a TSA plate and incubated for 22 ± 2 h at 37 ± 1 °C. A single colony was then transferred into Trypticase soy broth (TSB; Difco, BD, Sparks, MD, USA) and incubated at 37 ± 1 °C for 20 ± 2 h, under shaking (at 230 rpm). An aliquot of 0.5 mL of

the inoculum was transferred into 5 mL of milk sample, resulting in a starting population (N_0) of approximately 10^8-10^9 colony forming units per milliliter (CFU/mL). Similarly, 0.5 mL of *B. atrophaeus* spore suspension (10^6 CFU/10µL in aqueous solution) (ATCC #9372, Raven Biological Laboratories, Inc., Omaha, NE, USA) was inoculated into 5 mL sterile milk to give a final concentration of about 10^5 CFU/mL.

4.3.2 Preparation of antimicrobials

A stock solution of 500 and 1500 IU/ml nisin was prepared by dissolving respective 0.005 and 0.015 g of a commercial 2.5% nisin powder (Sigma-Aldrich) in 10 ml of 0.02 N HCl containing 0.75% NaCl. For a 500 µg/mL lysozyme stock solution 0.05 g of hen-egg white lysozyme powder (Sigma-Aldrich) (≥90% protein, ≥40,000 unit/mg protein) were dissolved in 100 mL of potassium phosphate buffer (10mM, pH 7). These stock solutions were then refrigerated until use. Final working concentrations were 50 or 150 IU/ml nisin (N50 or N150) and 50 µg/mL lysozyme (L). Both antimicrobials were added to the samples immediately before the treatments.

4.3.3 Heat and high pressure N_2O treatments of milk

A custom-built HP-N_2O system (Figure 4.1) with a 50-mL stainless steel high-pressure vessel (No. 13, Autoclave Engineers, Erie, PA, USA) was used in this study. The interior of the vessel (9 cm length, 2.6 cm internal diameter) was washed using sterile water, sanitized for 20 min with 70% ethanol and then rinsed twice with sterile water prior to each treatment. Bacteria-inoculated milk samples (5 mL) were loaded in the vessel,

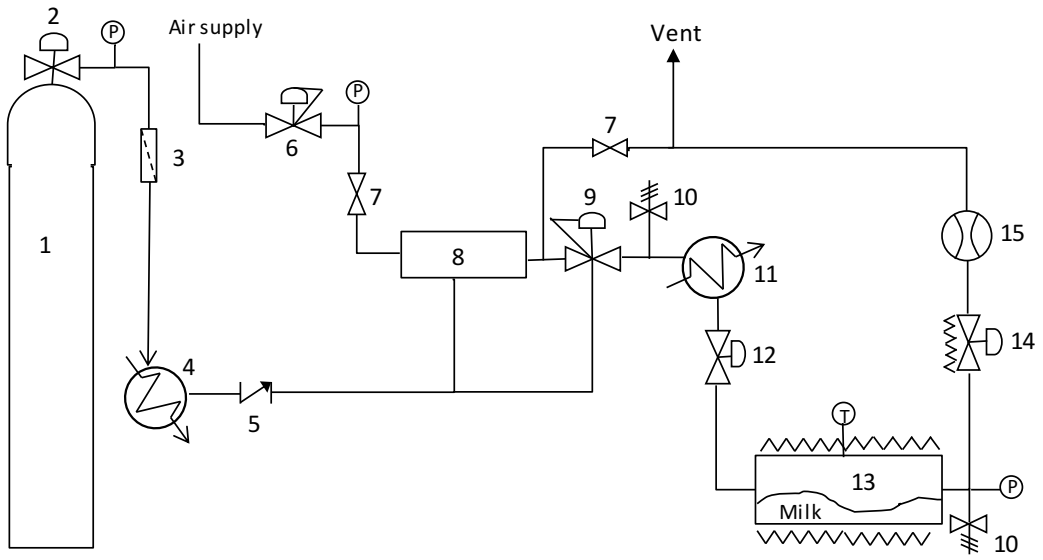

Figure 4.1 Schematic diagram of high pressure nitrous oxide (HP-N_2O) treatment system for milk.

1) N_2O tank 2) tank valve 3) filter 4) sub-cooler 5) check valve 6) pressure regulator 7) hand valve 8) pump 9) back pressure regulator 10) pressure release valve 11) heat exchanger 12) ball valve 13) high pressure treatment vessel (heated) 14) depressurization valve (heated) 15) flow meter. P and T stand for pressure gauge and thermocouple, respectively.

either with or without the addition of antimicrobials, while maintaining and monitoring its temperature using heating tape (Briskheat, Columbus, OH, USA) and both internal and external thermocouples, respectively. The pre-heated vessel was sealed and flooded with liquid N_2O (99.99% purity; Airgas, Elmira, NY, USA). The vessel was brought up to operating pressure of 15.2 MPa at 25, 45, 65 and 85 °C with an air driven high pressure pump (Haskel, Burbank, CAL, USA) (No. 8), and the pressure was controlled using a back-pressure regulator (Tescom, Elk River, MN, USA) (No. 9). Once operating pressure was achieved, the vessel was held under pressure for 20 min. At the end of each treatment cycle the heated depressurization valve (No. 14) was opened and the N_2O was released. Thermal

control treatments at the same temperature range were also performed for 20 min but without the addition of N_2O and under atmospheric pressure using the above-mentioned HP-N_2O system.

4.3.4 Enumeration of survivors and sub-lethally injured cells

Following HP-N_2O treatments, the treated 1-mL sample was transferred into 9 mL of Ringer's solution (BR0052G, Oxoid Ltd., Basingstoke, UK) for *E. coli*, *L. innocua*, and *B. atrophaeus* spores. This yielded a volume of 10 mL of recovery liquid. The latter was serially diluted in Ringer's solution, after which 0.1 mL volumes were spread-plated in duplicate on non-selective TSA (standard preparation), selective TSA (supplemented with 3% NaCl), MacConkey agar (MCA; Difco) and Listeria selective agar (LSA; Oxoid). TSA plates were incubated at 37 °C for 24 h, while plates with NaCl-supplemented TSA, MCA and LSA were incubated at 37 °C for 48 h. Log_{10} reductions were calculated as the difference between the logarithmic counts of colonies in untreated (N_0) and treated (N) samples ($\log_{10}N_0$-$\log_{10}N$). In some cases, no colony growth was detected due to the culture assay sensitivity of $\geq 10^2$ CFU/mL (i.e. the detection limit). Sub-lethal injury was evaluated by comparing the CFU counted on non-selective and on selective TSA, MCA and LSA, respectively.

4.3.5 Determination of pH and color

To further characterize the effect of HP-N_2O treatments at different temperatures (25, 45, 65 and 85 °C) on milk quality, pH and color were determined. The pH was determined using a basic pH meter (Denver Instruments, Bohemia, NY, USA) and the color was determined using a Minolta CM-2002 spectrophotometer (Minolta Camera Co., Osaka,

Japan) in the reflection mode and calibrated with a standard white plate (Y = 94.00, x = 0.3158, y = 0.3322). The net color difference was evaluated with the following equation (Chugh et al., 2014), using the parameters L*(lightness), a* (green chromaticity) and b*(yellow chromaticity) coordinates, and comparing the HP-N_2O treated milk samples with the untreated milk:

$$\Delta E=\sqrt{(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2} \quad (1)$$

4.3.6 Determination of particle size and ζ-potential

The mean particle sizes of the control and HP-N_2O treated milk samples were measured using the method described by Beliciu & Moraru (2009). Dynamic light scattering was used to evaluate the particle size and measurements were made at 20 °C using a scattering angle of 90° and laser with a wavelength of 658 nm. Data collection and analysis was performed using the BIC software (Brookhaven Instruments Corp., Holtsville, NY), which converted the experimental data into size distributions of each sample by using a viscosity of 1 mPa.s and a refractive index of n = 1.343. The ζ-potential of samples was measured by using the ZetaPlus option of the 90Plus Nanoparticle Size Analyzer (Brookhaven Instruments Corp., Holtsville, NY). Measurements were performed using a 35 mW solid state laser, λ = 660 nm, in the "high precision" mode at 20 °C, and setting "water" as solvent. Milk dilutions were adjusted in order to achieve an optimum ratio (0.1–0.5) between the instrument and the reference count (1454 kcps) rates. The measurement consisted of 30 cycles/run, with an inter-cycle delay of 5 s.

4.3.7 Statistical Analysis

For each treatment, the mean and standard deviation of survivor ratios translated as inactivation were calculated. The synergistic effects of the treatment were determined when the inactivation of the individual treatment added up to the same inactivation as achieved by their combination or when the latter exceeded the inactivation of the former. The analysis of variance (one-way ANOVA) was performed to compare treatment mean values using the Tukey's test. Significance was based on $p \leq 0.05$. The data were processed using the JMP 10.0 (SAS Institute Inc., Cary, NC, USA). All treatments were performed in duplicate.

4.4 Results

4.4.1 Effect of single hurdle processing strategies on *E. coli* and *L. innocua*

The responses of *E. coli* and *L. innocua* in milk to individual heat treatments at 45 (H45) and 65 (H65) °C, nisin additions at 50 (N50) and 150 (N150) IU/mL, and high pressure nitrous oxide (HPN2O) processing at 15.2 MPa for 20 min are shown in Figure 4.2 below. The initial concentrations of *E. coli* and *L. innocua* in the milk were in the range of 10^8-10^9 CFU/mL on average. The results demonstrated that the highest reduction of 2 $\log_{10}$ cycles was achieved for both microorganisms by H65 applied alone, which was in contrast to reductions obtained for 0.1 and 0.3 $\log_{10}$ in the respective *E. coli* and *L. innocua* cell population with the single H45 treatment ($p<0.05$). *L. innocua* showed higher susceptibility to nisin than *E. coli* ($p<0.05$) as demonstrated by inactivation at both the N50 (0.7 vs. 0.07 $\log_{10}$, respectively) and N150 (1.7 vs. 0.14 $\log_{10}$, respectively), also indicating that resistance of *L. innocua* to nisin was dose-dependent.

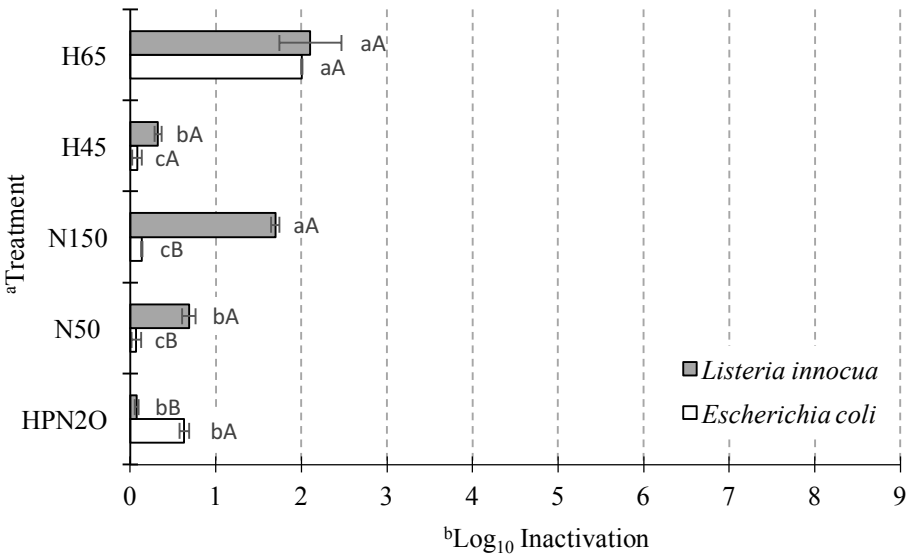

Figure 4.2 Inactivation of *Escherichia coli* and *Listeria innocua* in milk obtained with a single high pressure nitrous oxide (HP-N_2O), nisin, or heat treatments for 20 min.

Error bars represent standard deviation among replicates of the treatment (*n*=2). Different capital letters next to the bars indicate, that inactivation of different bacteria was significantly different based on the application of the same decontamination technique ($p<0.05$). The same lower-case letters next to the bars indicate that inactivation was not significantly different between the varying decontamination treatments and conditions applied to the same bacterium ($p\geq0.05$). Abbreviations used: H45 and H65 abbreviate heat treatment at 45 and 65 °C, respectively; HPN2O indicates high pressure nitrous oxide at 15.2 MPa; N50 and 150 denote nisin addition at 50 and 150 IU/mL, respectively. Log_{10} inactivation = $Log_{10}N$-$Log_{10}N_0$; N=number of survivors after the treatment; N_0=number of microorganisms before the treatment.

In addition, it can be noted that nisin at 150 IU/mL alone was similarly effective as the single thermal treatment at 65°C ($p\geq0.05$). On the contrary, HP-N_2O applied individually exhibited higher bactericidal effect ($p<0.05$) on *E. coli* than *L. innocua* (0.63 log_{10} vs. 0.08 log_{10} reduction, respectively).

4.4.2 Effect of double hurdle processing strategies on *E. coli* and *L. innocua*

The inactivation of *E. coli* and *L. innocua* in milk using heat at 45 and 65 °C in conjunction with HP-N_2O (H45/HPN2O and H65/HPN2O, respectively), in combination with nisin at 50 (H45/N50 and H65/N50, respectively) and 150 (H45/N150 and H65/N150, respectively) IU/mL, and resulting from simultaneous processing with HP-N_2O and either of the nisin concentrations (HPN2O/N50 and HPN2O/N150, respectively) at room temperature are presented in Figure 4.3 below.

A reduction of 3.7 and 6.0 $\log_{10}$ cycles in *E. coli* cells in milk exposed to HP-N_2O was reached at 45 and 65 °C, respectively. Similarly, with increasing temperature, the level of inactivation for *L. innocua* cells suspended in milk was increased ($p<0.05$) from 3.6 (H45/HPN2O) to 5.1 $\log_{10}$ cycle (H65/HPN2O). At 45 °C, *E. coli* cells were resistant to nisin and showed no sensitivity ($p\geq0.05$) to either H45/N50 or H45/N150 milk treatments ($p>0.05$). Moreover, the amount of nisin added did not affect the degree of inactivation achievable for *L. innocua* processed at 45 °C as evidenced by 2.2 (H45/N50) and 2.5 (H45/N150) $\log_{10}$ cycle reductions. However, at 65 °C the extent of inactivation was significantly dependent ($p<0.05$) on nisin concentration for both microorganisms. Application of H65/N50 and H65/N150 led to respective $\log_{10}$ reductions of 1.5 and 3.2 in *E. coli* and 2.7 and 3.5 in *L. innocua* cells indicating that increased heat intensity enhanced the bactericidal action of nisin.

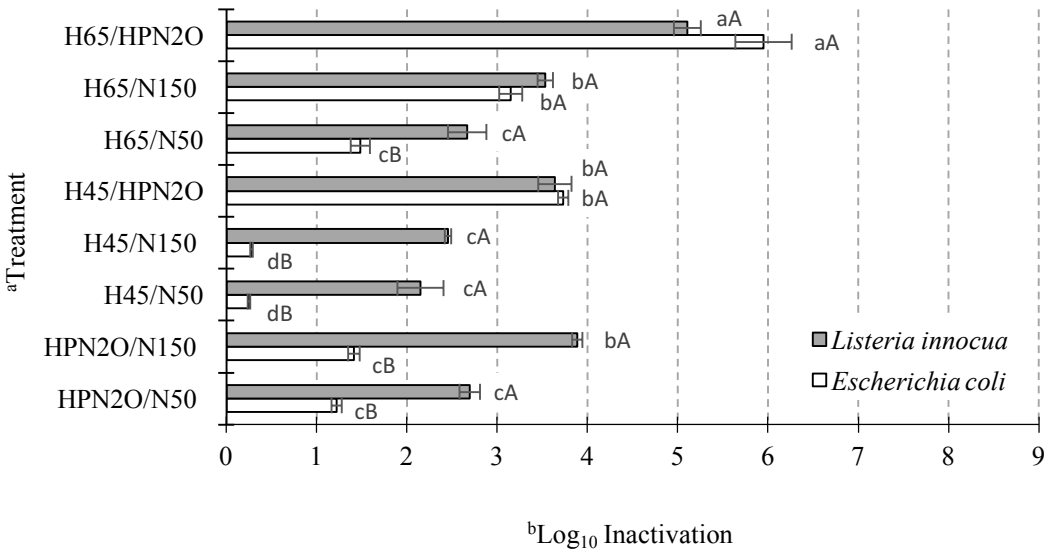

Figure 4.3 Inactivation of *Escherichia coli* and *Listeria innocua* in milk obtained with double hurdle treatments combining high pressure nitrous oxide (HP-N_2O), nisin, and heat for 20 min.

Error bars represent standard deviation among replicates of the treatment (n=2). Different capital letters next to the bars indicate, that inactivation of different bacteria was significantly different based on the application of the same decontamination technique ($p<0.05$). The same lower-case letters next to the bars indicate, that inactivation was not significantly different between the varying decontamination treatments and conditions applied to the same bacterium ($p\geq0.05$). Abbreviations used: H45 and H65 abbreviate heat treatment at 45 and 65 °C, respectively; HPN2O indicates high pressure nitrous oxide at 15.2 MPa; N50 and 150 denote nisin addition at 50 and 150 IU/mL, respectively. Log_{10} reductions = $Log_{10}N$-$Log_{10}N_0$; N=number of survivors after the treatment; N_0=number of microorganisms before the treatment.

Utilizing nisin together with HP-N_2O in a hurdle technology led to a higher inactivation of both vegetative bacteria ($p<0.05$) than their exposure to nisin alone. It was observed that *E. coli* was more resistant to these combined processing approaches than *L. innocua* resulting in lower reductions ($p<0.05$) following HPN2O/N50 (1.2 vs. 2.7 log_{10}, respectively) and HPN2O/N150 (1.4 vs. 3.9 log_{10}, respectively) treatments. Similar to the individual nisin treatment, a dose-dependent pattern for *L. innocua* inactivation was determined, accounting for a difference of 1.2 log_{10} cycles between HPN2O/N50 and

HPN2O/N150 treatments. In contrast, nisin exhibited very slight increase ($p \leq 0.05$) in inactivation of *E. coli* population (by 0.2 $\log_{10}$ cycles) with an increase in its concentration when applied in combination with HP-N_2O. By contrasting juxtaposition, the non-thermal HPN2O/N150 (3.9 $\log_{10}$) treatments resulted in comparable ($p \geq 0.05$) or even higher inactivation level of *L. innocua* ($p < 0.05$) with some of the heat-assisted dual treatments such as H65/N50 (2.7 $\log_{10}$), H65/N150 (3.5 $\log_{10}$) and H45/HPN2O (3.6 $\log_{10}$).

4.4.3 Effect of triple hurdle processing strategies on *E. coli* and *L. innocua*

The efficacy of simultaneous milk treatments for bacterial decontamination using heat (H45 and H65), HPN_2O at 10.5 MPa (HPN2O) and nisin (N50 and N150) over 20 min is shown in Figure 4.4 below. The highest inactivation of *E. coli* and *L. innocua* was achieved when the triple hurdle technology H65/HPN2O/N150 was applied, resulting in a reduction of 8.0 and 8.6 $\log_{10}$ cycles respectively, which indicates significant ($p < 0.05$) increases of 4.2 (*E. coli*) and 3.0 (*L. innocua*) $\log_{10}$ cycles compared to those obtained for H45/HPN2O/N150. Similarly, the reduction of *E. coli* and *L. innocua* populations following H65/HPN2O/N50 processing of milk were 7.4 and 7.5 $\log_{10}$ cycles, respectively, which accounts for respective increases of 3.7 (from 3.7) and 2.1 (from 3.8) $\log_{10}$ cycles in comparison to treating both vegetative bacteria with H45/HPN2O/N50. These findings point out that higher processing temperatures substantially enhanced the microbial inactivation of these hurdle strategies and, in addition, that the treatment temperature effect decreased the gap between gram-positive *L. innocua* and gram-negative *E. coli* ($p \geq 0.05$) in their response to antibacterial action of nisin. Interestingly, the inactivation of *E. coli* in milk subjected to H45/HPN2O (3.7 $\log_{10}$ cycles) was almost equivalent ($p \geq 0.05$) to

H45/HPN2O/N50 (3.7 $\log_{10}$ cycles) and H45/HPN2O/N150 (3.8 $\log_{10}$ cycles), thereby revealing that nisin did not increase the sensitivity of *E. coli* cells to HP-N_2O at 45 °C.

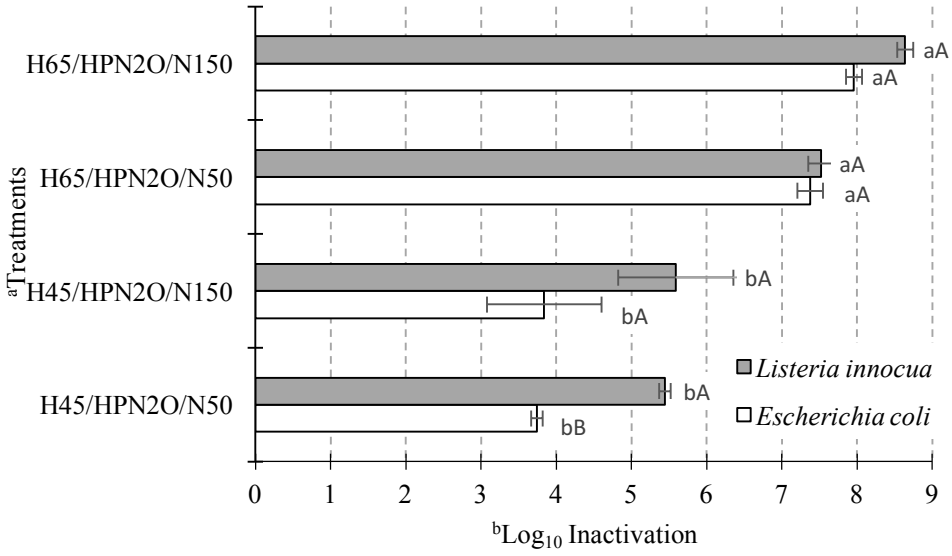

Figure 4.4 Inactivation of *Escherichia coli* and *Listeria innocua* in milk obtained with triple hurdle treatments combining high pressure nitrous oxide (HP-N_2O), nisin, and heat for 20 min.

Error bars represent standard deviation among replicates of the treatment (n=2). Different capital letters next to the bars indicate, that inactivation of different bacteria was significantly different based on the application of the same decontamination technique ($p<0.05$). The same lower-case letters next to the bars indicate, that inactivation was not significantly different between the varying decontamination treatments and conditions applied to the same bacterium ($p\geq0.05$). Abbreviations used: H45 and H65 abbreviate heat treatment at 45 and 65 °C, respectively; HPN2O indicates high pressure nitrous oxide at 15.2 MPa; N50 and 150 denote nisin addition at 50 and 150 IU/mL, respectively. Log_{10} reductions = $\text{Log}_{10}\text{N}-\text{Log}_{10}\text{N}_0$; N=number of survivors after the treatment; N_0=number of microorganisms before the treatment.

4.4.4 Effect of individual and combined treatments on *B. atrophaeus* spores

The initial concentrations of *B. atrophaeus* spores in the milk were in the range of 10^5-10^6 CFU/mL on average. Preliminary trials indicated that the inactivation of *B. atrophaeus* spores was almost negligible when processing conditions with a maximum treatment temperature of 65 °C were used. This would have enabled a direct comparison to the results obtained for the vegetative bacteria strains. Since increasing the processing temperature of the quadruple hurdle technology combining heat, HP-N_2O, nisin (150 IU/mL) and lysozyme (50 µg/mL) by another 10 °C had a limited impact, resulting in 0.5 ± 0.3 (n=3) $\log_{10}$ reduction of spores and a decrease in a bacterial population below 90%, the processing temperature was further increased to 85 °C which is still within pasteurization standards.

As in Figure 4.5 below, the additions of nisin (at 50 and 150 IU/mL) and lysozyme (50 µg/mL) applied individually in milk were not effective against *B. atrophaeus* spores, showing no detectable antimicrobial effect. Similarly, the population of *Bacillus* spores in milk, subjected to heat at 85 °C (H85) and HP-N_2O alone at 15.2 MPa (HPN2O), exhibited very limited prospect of reduction at 0.04 and 0.16 $\log_{10}$, respectively. The addition of 50 (H85/N50), 150 (H85/N150) IU/mL nisin and 50 µg/mL lysozyme (H85/L) at 85 °C did not significantly decimate the number of spores as indicated by $\log_{10}$ cycle reductions of 0.02, 0.13 and 0.10, respectively (Figure 4.5) ($p>0.05$). Also, a triple combination of H85/N50/L and H85/N150/L did not bring about considerable inactivation of *B. atrophaeus* endospores (0.1 and 0.2 $\log_{10}$, respectively). Nevertheless, non-thermal application of HPN_2O with 50 (HPN2O/N50) and 150 (HPN2O/N150) IU/mL nisin and

lysozyme (HPN2O/L) induced a slight sporicidal effect, reducing the load of endospores by 0.21, 0.33 and 0.27 $\log_{10}$, respectively.

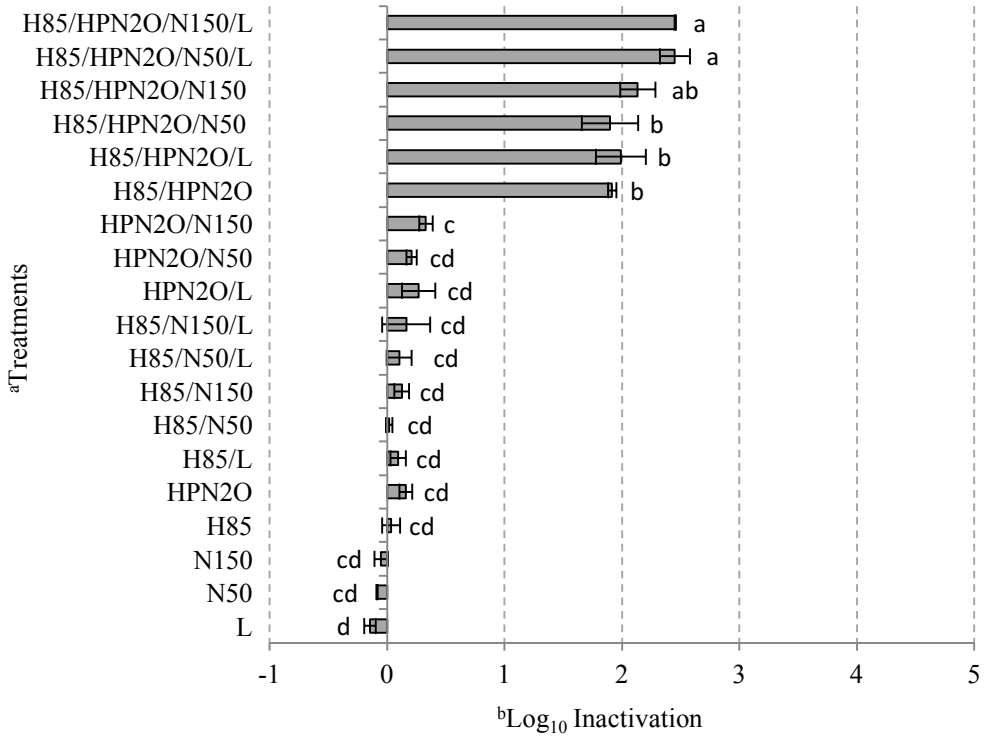

Figure 4.5 Inactivation of *Bacillus atrophaeus* endospores in milk obtained with quadruple hurdle treatments combining high pressure nitrous oxide (HP-N_2O), heat, nisin and lysozyme for 20 min.

Error bars represent standard deviation among replicates of the treatment (n=2). Different capital letters next to the bars indicate, that inactivation of different bacteria was significantly different based on the application of the same decontamination technique ($p<0.05$). The same lower-case letters next to the bars indicate, that inactivation was not significantly different between the varying decontamination treatments and conditions applied to the same bacterium ($p\geq0.05$). Abbreviations used: H45 and H65 abbreviate heat treatment at 45 and 65 °C, respectively; HPN2O indicates high pressure nitrous oxide at 15.2 MPa; L is lysozyme at 50 µg/mL; N50 and 150 denote nisin addition at 50 and 150 IU/mL, respectively. $\log_{10}$ reductions = $\log_{10}N$-$\log_{10}N_0$; N=number of survivors after the treatment; N_0=number of microorganisms before the treatment.

The combined treatments proved to be more successful when HP-N_2O and heat (H85/HPN2O) were carried out simultaneously, amounting to a 1.92 $\log_{10}$ reduction of spores. Subsequently, the addition of nisin at 50 and 150 IU/mL and lysozyme in conjunction with conjunction with HP-N_2O treatments at 85 °C further enhanced the sporicidal effect inactivating 1.90, 2.14 and 1.99 $\log_{10}$ cycles of spores, respectively. The largest *B. atrophaeus* spore reduction of 2.4 and 2.5 $\log_{10}$ cycles was achieved, when the milk was subjected to H85/HPN2O/N50/L and H85/HPN2O/N150/L, respectively.

4.4.5 Determination of treatment synergies

A comparison of each individual treatment component to their combined application provides an insight on interactions between them, which are typically synergistic or additive in nature. Synergistic interaction is defined as a combination effect observed, that is greater than the sum of the effects with two or more treatment components independently (Barry, 1976). The findings on interactions between heat, pressurized N_2O, nisin, and lysozyme for decontamination of *E. coli*, *L. innocua* and *B. atrophaeus* spores in milk are listed in Table 4.1 and Table 4.2, respectively.

Table 4.1 Occurrence of synergistic treatment effects regarding the reduction of *Escherichia coli* and *Listeria innocua* in milk processed with different techniques for 20 min.

Processing Methods[a]					Log_{10} Reductions[b]					
					Escherichia coli			*Listeria innocua*		
H45	H65	HPN2O	N50	N150	∑I[c]	CS[d]	Synergy[e]	∑I[c]	CS[d]	Synergy[e]
		•	•		0.76	1.16	+	0.83	2.59	+
		•		•	0.81	1.35	+	1.82	3.83	+
•			•		0.21	0.24	+	1.08	1.90	+
•				•	0.25	0.27	+	2.07	2.42	+
•		•			0.77	3.46	+	0.44	3.56	+
•		•	•		0.84	3.55	+	1.15	5.37	+
•		•		•	0.89	3.73	+	2.14	4.83	+
	•		•		2.08	1.38	-	3.08	2.46	-
	•			•	2.15	3.02	+	4.02	3.45	-
	•	•			2.65	5.57	+	2.62	4.96	+
	•	•	•		2.72	7.17	+	3.15	7.35	+
	•	•		•	2.79	7.48	+	4.09	8.53	+

[a]Processing methods applied: H45 and H65 stand for heat treatment at 45 and 65 °C, respectively; HPN2O abbreviates pressurized nitrous oxide treatment at 15.2 MPa; N50 and N150, denote use of nisin at 50 and 150 IU/mL, respectively.

[b]Log_{10} reductions are expressed as $Log_{10}N$-$Log_{10}N_0$, where N and N_0 indicate the number of surviving microorganisms after the treatment and the initial number of microorganisms before the treatment, respectively.

[c]∑I stand for the sum of Log_{10} reductions obtained from the processing methods (•) applied individually, plus their mean standard deviation.

[d]CS designates the experimental Log_{10} reduction obtained from processing methods (•) combined simultaneously, minus its standard deviation

[e]Synergistic (+) and additive (-) treatment effects ($P \leq 0.05$) occurred when CS>∑I and CS ≤ ∑I, respectively.

Table 4.2 Occurrence of synergistic treatment effects regarding the reduction of *Bacillus atrophaeus* spores in skim milk processed with different techniques for 20 min.

Processing Methods[a]					Log_{10} Reductions[b]		
H85	HPN2O	N50	N150	L	∑I[c]	CS[d]	Synergy[e]
•				•	-0.07	0.03	+
•		•			-0.09	-0.01	+
•			•		-0.01	0.06	+
	•			•	0.14	0.13	-
	•	•			0.19	0.17	-
	•		•		0.17	0.27	+
•	•				0.26	1.88	+
•		•		•	-0.17	0.00	+
•			•	•	-0.11	-0.04	+
•	•			•	0.14	1.78	+
•	•	•			0.21	1.66	+
•	•		•		0.21	1.99	+
•	•	•		•	-0.15	2.32	+
•	•		•	•	0.00	2.45	+

[a]Processing methods applied: H85 stands for heat treatment at 85 °C; HPN2O abbreviates pressurized nitrous oxide treatment at 15.2 MPa; N50 and N150, denote use of nisin at 50 and 150 IU/mL, respectively; L abbreviates the addition of lysozyme at 50 µg/mL.

[b]Log_{10} reductions are expressed as $Log_{10}N$-$Log_{10}N_0$, where N and N_0 indicate the number of surviving microorganisms after the treatment and the initial number of microorganisms before the treatment, respectively.

[c]∑I stand for the sum of Log_{10} reductions obtained from the processing methods (•) applied individually, plus their mean standard deviation.

[d]CS designates the experimental Log_{10} reduction obtained from processing methods (•) combined simultaneously, minus its standard deviation

[e]Synergistic (+) and additive (-) treatment effects ($P \leq 0.05$) occurred when CS>∑I and CS ≤ ∑I, respectively.

A significant positive interaction ($p<0.05$) was observed between treatment components in all hurdle strategies employed against both *E. coli* and *L. innocua*, exhibiting synergistic behaviour, except for H65/N50. However, H65/N150 was found to be additive for *L. innocua*, indicating that listeria cells are relatively prone to single processing with H65 or N150. Most hurdle combinations were also found to act synergistically ($p<0.05$) with regard to the inactivation of *B. atrophaeus* spores except for HPN2O/L and HPN2O/N50.

4.4.6 Effect of HP-N_2O on sub-lethal injury of *E. coli* and *L. innocua*

The effectiveness of HP-N_2O treatments applied alone at 25 °C (HPN2O), as well as heat-assisted at 45 (H45/HPN2O) and 65 (H65/HPN2O) °C for the inactivation of *E. coli* and *L. innocua* in milk is reflected in the sub-lethal injury data of Figure 4.6 and Figure 4.7, respectively.

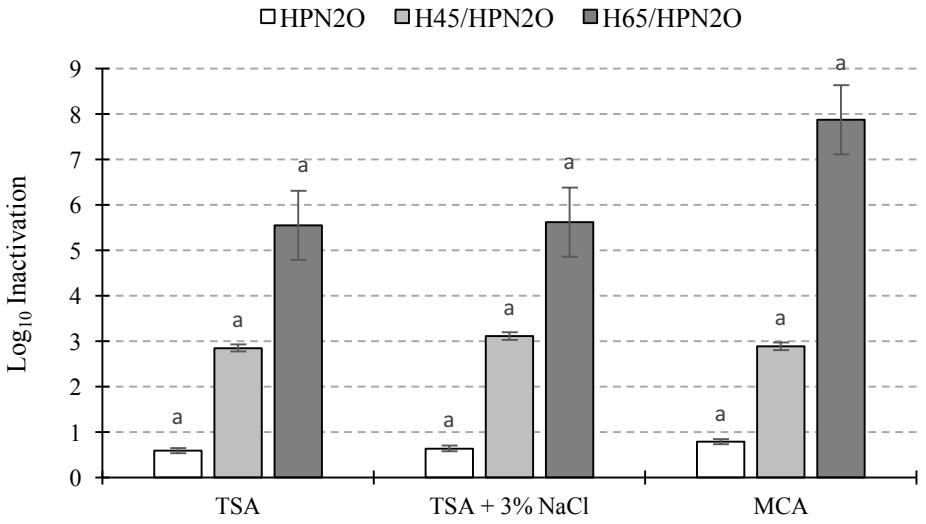

Figure 4.6 Sub-lethal cell injury based on *Escherichia coli* inactivation obtained in milk after treatment with high pressure nitrous oxide at 25 (HPN2O), 45 (H45/HPN2O) and 65 (H65/HPN2O) °C using 15.2 MPa for 20 min and subsequent enumeration on Trypticase soy agar (TSA), TSA supplemented with 3% sodium chloride (TSA + 3% NaCl), and MacConkey agar (MCA).

The same letters above the bars indicate, that inactivation of *Escherichia coli* was not significantly different ($p \geq 0.05$) under the same treatment conditions but grown on different recovery media; error bars indicate the standard deviations of the treatment means (n=2).

Reduction of *E. coli* cell counts in milk (see Figure 4.6 above) varied between 0.6 (both TSA types) and 0.7 (MCA) $\log_{10}$ cycles following stand-alone HP-N_2O processing, from 2.9 (both TSA and MCA) to 3.1 (TSA+3% NaCl) following H45/HPN2O treatment, and from 5.6 (both TSA types) to 7.9 (MCA) following exposure to H65/HPN2O. Based on the comparability of these findings on different growth media ($p \geq 0.05$), no indication of sub-lethal damage was detected for the *E. coli* strain.

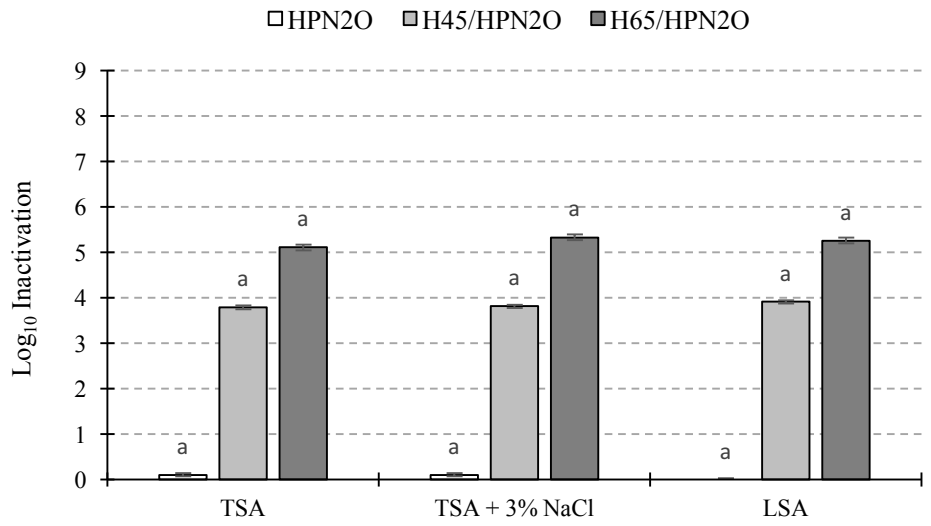

Figure 4.7 Sub-lethal cell injury based on *Listeria innocua* inactivation obtained in milk after treatment with high pressure nitrous oxide at 25 (HPN2O), 45 (H45/HPN2O) and 65 (H65/HPN2O) °C using 15.2 MPa for 20 min and subsequent enumeration on Trypticase soy agar (TSA), TSA supplemented with 3% sodium chloride (TSA + 3% NaCl), and Listeria selective agar (LSA).

The same letters above the bars indicate, that inactivation of *Escherichia coli* was not significantly different ($p \geq 0.05$) under the same treatment conditions but grown on different recovery media; error bars indicate the standard deviations of the treatment means (n=2).

With regard to the survival of *L. innocua* cells in milk (see Figure 4.7 above), reduction in counts ranging between 0.1 (both TSA types) and 0.0 (LSM) $\log_{10}$ cycles after stand-alone HPN2O, from 3.8 (both TSA types) to 3.9 (LSM) after H45/HPN2O, and from 5.1 (TSA) to 7.3 (TSA+3% NaCl and LSM) after H65/HPN2O were observed. Altogether, analyses of sub-lethal cell damage for listeria yielded similar results ($p \geq 0.05$) independent of the recovery medium and confirmed the absence of sub-lethal cell injury after treatment with HPN_2O alone and in combination with heat at 45 and 65 °C.

4.4.7 Effect of HP-N_2O on physico-chemical properties of milk

Analytic results of selected physico-chemical properties such as pH, mean particle size, and ζ-potential in HP-N_2O-treated milk at 25, 45, 65, and 85 °C are summarized in Table 4.3.

Table 4.3 Effect of high pressure nitrous oxide (HP-N_2O) (15.2 MPa, 20 min) at 25, 45, 65, and 85 °C on physicochemical properties of milk

Temperature (°C)	pH	Particle size (nm)	Polydispersity	ζ- potential (mV)
Control	6.7 ±0.0^{ab}	203±3.0^{a}	0.09±0.02^{a}	-25.9±0.6^{a}
25	6.7±0.0^{a}	204±6.9^{a}	0.09±0.02^{a}	-26.5±1.7^{a}
45	6.7±0.0^{ab}	204±1.0^{a}	0.07±0.01^{a}	-26.4±1.2^{a}
65	6.6±0.0^{bc}	204±1.6^{a}	0.09±0.01^{a}	-27.8±0.5^{a}
65$^{\infty}$	6.4±0.0^{d}	204±4.6^{a}	0.08±0.05^{a}	-28.1±0.0^{a}
85	6.5±0.0^{c}	214±2.8^{a}	0.10±0.03^{a}	-28.0±1.0^{a}
85$^{\mu}$	6.3±0.0^{d}	214±2.0^{a}	0.08±0.03^{a}	-27.9±0.4^{a}

$^{\infty}$ designates the addition of nisin at 150 IU/mL in milk before treatment.
$^{\mu}$ indicates the addition of nisin at 150 IU/mL and lysozyme at 50 µg/mL in milk before treatment.
The results were expressed as mean ± standard deviation (*n*=2). Within columns, treatment means not followed by the same superscripted letter are significantly different ($p<0.05$)

The application of HP-N_2O alone at different temperatures did not result in drastic changes in the pH of milk that ranged from 6.5 to 6.7 as compared to controls. The addition of antimicrobials in milk before HP-N_2O treatments at 65 (H65/HPN2O/N150) and 85 (H85/HPN2O/N150/L) °C, caused a slight decrease of the pH ($p<0.05$). For the particle size, no change ($p\geq0.05$) was detected with increasing temperatures at pressurization, but the particle size increased by 4.5% of its initial size when the processing temperature was set to 85 °C with (H85/HPN2O/N150/L) and without (H85/HPN2O) the addition of antimicrobials in milk. Similarly, the polydispersity index (PI) remained relatively unchanged ($p>0.05$) when compared with the untreated samples, indicating that the width of the size distribution was not markedly affected by the treatment. In addition, instrumental color attributes have been analysed (Table 4.4) and measurements of the latter

confirmed, that milk samples did not show any important changes in color parameters L* and b* at different temperatures. Slight changes in a* (shifting towards negative direction or more green) and ΔE occurred between the control and treated milk at 65 and 85 °C. Likewise, there were no major changes in color observed in treated milk after HP-N_2O applications with antimicrobials at 65 (H65/HPN2O/N150) and 85 (H85/HPN2O/N150/L) °C.

Table 4.4 Color attributes of milk after high pressure nitrous oxide (HP-N_2O) treatments (15.2 MPa, 20 min) at different temperatures

Temperature (°C)	L*	a*	b*	ΔE^{α}
Control	64.99±0.55^{a}	-4.75±0.03^{a}	-1.34±0.03^{ab}	-
25	65.23±0.12^{a}	-4.82±0.01^{ab}	-1.33±0.10^{ab}	0.1
45	64.90±0.35^{a}	-4.94±0.06^{abc}	-1.53±0.28^{ab}	0.2
65	65.50±0.80^{a}	-5.10±0.03^{c}	-1.15±0.16^{ab}	0.6
65$^{\infty}$	65.68±0.03^{a}	-5.02±0.04^{c}	-1.92±0.29^{b}	1.2
85	65.88±0.05^{a}	-5.05±0.04^{c}	-0.97±0.08^{a}	1.0
85$^{\mu}$	65.86±0.03^{a}	-4.96±0.08^{bc}	-1.23±0.30^{ab}	1.0

$^{\infty}$ designates the addition of nisin at 150 IU/mL in milk before treatment.
$^{\mu}$ indicates the addition of nisin at 150 IU/mL and lysozyme at 50 µg/mL in milk before treatment.
L*: lightness (ranging from 0 to 100), a*: green to red (ranging from -60 to +60) and the b*: blue to yellow (ranging from -60 to + 60).
$^{\alpha}$ΔE: calculated color differences evaluated as not noticeable (0-0.5), slightly noticeable (0.5-1.5), noticeable (1.5-3) and well visible (3-6) as described by Cserhalmi et al. (2006).
The results were expressed as mean ± standard deviation (*n*=2). Within columns, treatment means not followed by the same superscripted letter are significantly different ($p<0.05$)

4.5 Discussion

In this study, HP-N_2O alone exhibited a stronger bactericidal effect on *E. coli* than on *L. innocua*, which is in agreement with previous works on the application of both pressurized CO_2 (Zhang et al., 2006; Kim et al., 2008; Garcia-Gonzalez et al., 2010) and N_2O (Mun et al., 2012). This observation can be attributed to the thicker peptidoglycan layer in the cell wall structure of gram-positive *L. innocua* than that of gram-negative *E. coli* which may

have led to a higher resistance of the former against penetration by N_2O at a supercritical state. However, *E. coli* was more resistant than *L. innocua* to nisin, and similar observations were reported in the case of the combined application of HHP (Ponce et al., 1998) and dimethyl dicarbonate (Yu et al., 2014) with nisin in liquid whole egg and lychee juice, respectively. The extent of immediate lethality due to heat application alone was low for both *E. coli* and *L. innocua* (max. 0.3 and 2.1 $\log_{10}$ cycles reduction at 45 °C/20 min and 65 °C/20 min respectively), indicating that the thermal treatment used here was relatively mild.

The inactivation of *E. coli* and *L. innocua* using heat and nisin was slightly enhanced with increasing temperature. This could be due to temperature-induced changes in the membrane fluidity, caused by alteration of fatty acid components of the lipids (Russell et al., 1995), which may have potentiated the lethal effect of the peptide. Previous studies have reported that physical treatment, such as heating, could affect outer membrane permeability and lead to nisin sensitivity (Tsuchido et al., 1985; Boziaris et al., 1998). However, nisin did not display a synergistic effect on the inactivation of both *E. coli* and *L. innocua* when combined with heat treatment at 65 °C, except for H65/N150 treatment of *E. coli*, likely because the cells already exhibited a very high sensitivity to thermal treatment alone.

Inactivating bacteria by HP-N_2O was primarily in response to a combination of its chemical properties and physical factors. The chemical nature of HP-N_2O fluids due to its high solubility in lipids enable it to be easily dispersed into the phospholipid layer of cell membranes with the support of high pressure, particularly, above its supercritical state (Spilimbergo et al., 2002). However, a pressure raise over 10 MPa decreased the diffusion

of N_2O inside the liquid phase and resulting low biocidal efficiency of supercritical N_2O (Jou et al., 1992; Spilimbergo et al., 2007b). This was not reported in the inactivation of *Pseudomonas aeruginosa* by supercritical N_2O as the pressure increased from 10 to 20 MPa (Mun et al., 2011), and thus, a single operating pressure of 15.2 MPa was considered appropriate and used in this study. It is note-worthy that non-thermal HPN2O/N150 treatments achieved a noticeable inactivation of *E. coli* (1.7 $\log_{10}$ cycle) and *L. innocua* (3.9 $\log_{10}$ cycle) (Figure 4.3), which indicates that pressurized N_2O alone could still have caused the permeabilization of cells necessary for further action of nisin in a synergistic manner (Table 4.1).

With reference to its chemical nature, the hypothetical explanation for the bactericidal effect of HP-N_2O could be that the pressurized N_2O dissolved into the microbial membrane, increasing the membrane fluidity and affecting its integrity, promoting the extraction of vital constituents from the cytoplasm and inhibiting the transport of solute and proteins through the membrane. This mode of action was evidenced through UV-absorbance analysis (Mun et al., 2012), which confirmed the release of intracellular substances (e.g. nucleic acid and protein) in *E. coli* and *S. aureus* and scanning electron microscopy images (Vo et al., 2013; Mun et al., 2012), which showed some lysis and rough surfaces mainly on *E. coli* cells in response to HP-N_2O treatments.

Clearly, the inactivation of *E. coli* and *L. innocua* by HP-N_2O and heat in this study was greater than any combination of heat and nisin. Heat enhanced the inactivation by HP-N_2O and this combination resulted in a greater reduction in bacterial counts than that with HP-N_2O and nisin. For example, Mun et al. (2011) reported that increasing temperature from 37 and 42 °C at 10 MPa on both supercritical N_2O and CO_2 treatments resulted in

enhancing the bactericidal efficiency against *P. aeruginosa* by 16% and 25%, respectively. Likewise, Spilimbergo (2011) observed an inactivation of 2, 4 and 5-$\log_{10}$ cycles in microflora counts in raw milk as a result of HP-N_2O treatments (12 MPa, 10 min) at 40, 45 and 50 °C, respectively. Hence, we suggest that increasing temperature can stimulate not only the diffusivity of N_2O, but also the fluidity of the cell membrane to make penetration easier, like to the mechanism known for HP-CO_2 inactivation.

A higher dosage of nisin, when combined with heat in a double hurdle approach at 45 °C, did not enhance the inactivation of both *E. coli* and *L. innocua*, presumably, because the 20-min treatment time was not sufficient for nisin to affect the bacteria at this moderate temperature. However, the response of both bacteria was proportional to the nisin concentration at 65 °C. Interestingly, once heat was introduced together with nisin and HP-N_2O and implemented in a triple hurdle processing strategy, it showed less reliance on nisin dosage to be more effective in killing of both *E. coli* and *L. innocua*. Moreover, combining heat-assisted HP-N_2O treatments with nisin for *E. coli* inactivation were as effective as those for the decontamination of nisin-sensitive *L. innocua* cells when the processing temperature was raised from 45 to 65 °C. This could coincide with the findings of a study by Liao et al. (2010) suggesting that HP-CO_2 (10-30 MPa, 5-75 min) and mild heat treatment at 37, 42 and 47 °C disrupted the cell wall of *E. coli* cells without damage to the cytoplasmic membrane, while treatment at 57 °C led to damage of the cytoplasmic membrane.

The occurrence of injured cells after an adverse environmental stress is of great concern for pathogenic and spoilage microorganisms, because under favorable conditions these cells can recover in food and cause food poisoning or spoilage (Jay et al., 2005). In

addition, milk possesses a higher pH value compared with vegetable and fruit juices, and is conducive to the recovery of sub-lethally injured microbial cells during storage. The analysis of sub-lethal cell damage for *E. coli* and *L. innocua* cells as a result of HPN2O, H45/HPN2O and H65/HPN2O treatments revealed that the vegetative bacteria grown on the selective media for an extended incubation time produced an almost equivalent ($p>0.05$) $\log_{10}$ reduction as compared to that on non-selective media. These findings consistently prove that no sub-lethal cell injuries occurred as a result of the applied treatments. To date, mixed results analyzing sub-lethal cell injury and recovery of bacterial cells following HP-CO_2 treatments have been obtained, with positive (Sirisee et al., 1998; Erkmen, 2000a) and negative (Erkmen, 2000b; Erkmen, 2000c; Hong & Pyun, 2001) outcomes as well as on both counts (Yuk & Geveke, 2011). To our knowledge, a direct comparison with other HP-N_2O studies on the emergence of injured cells is currently not possible, since the present research appears to be the first one that investigated sub-lethal damage of bacterial cells after exposure to pressurized N_2O.

Based on our observations that any double hurdle treatment except for H85/HPN2O did not inactivate spores of *B. atrophaeus* beyond 1 $\log_{10}$ cycle (Figure 4.5), we can deduce that HP-N_2O in combination with high temperature (85 °C) is almost as effective as a triple hurdle strategy consisting of HP-N_2O and nisin or lysozyme at the same temperature ($p>0.05$). These findings manifest the generally-known resistance of *Bacillus* spores to many bactericidal agents, which has been attributed to the structure and chemical composition of the spore cortex, core and, more importantly, the spore coat. The spore coat, a proteinaceous multi-layered structure composed of intricate crosslinks of over 30 different polypeptides, is primarily responsible for resistance against chemicals and lytic

enzymes (Henriques & Moran, 2000; Lai et al., 2003). The quadruple combination of HP-N_2O with thermal treatment at 85 °C and the addition of nisin regardless of concentration and lysozyme acted synergistically and provided the highest lethality value of about 2.5 $\log_{10}$ cycles. Synergistic processing of *B. atrophaeus* spores using heat, HP-N_2O, nisin, and lysozyme and the resulting inactivation of the microorganism could be ascribed to diverse mechanisms that may occur simultaneously in complex and interrelated ways. Nevertheless, we speculate that pressurized N_2O at high temperature may have resulted in sufficient permeabilization of the spore coat, thereby rendering the underlying cortex susceptible to lysozyme, to initiate germination. The germinated spores were more vulnerable to free permeation of lysozyme and nisin than dormant spores, leading to a relatively high lethality in the spore population in milk.

The near-neutral values of pH in all samples after application of HP-N_2O at different temperatures suggested that cell death was not caused by acidity or alkalinity of the milk. Similarly, Spilimbergo (2011) reported an average pH of 6.75 ± 0.06 in raw milk treated with HP-N_2O at 12 MPa, 60 min at different temperature conditions (40, 45 and 50 °C). It is worth mentioning, that the acid solution dissolving nisin rather than nisin itself may have played a role in the inactivation of microorganisms in its combination effect with HP-N_2O as previously reported with regard to HP-CO_2 treatments of *E. coli* in physiological saline buffer (Bi et al., 2014). However, a pH reduction in treated milk with addition of acidic nisin solution was very low even at 65 and 85 °C (Table 4.3), suggesting that nisin itself was the factor for inactivation of microorganisms in this hurdle approach.

It is known that κ-casein, colloidal calcium phosphate (CCP), and hydrophobic interactions play key roles in maintaining the stability and integrity of casein micelles

(CMs). It is thus appropriate that either pH or the soluble calcium level (indirectly related to the solubility of CCP) was monitored to indicate any disruption to the CMs (Anema & Klostermeyer, 1997; Orlien et al., 2010). In this study, the pH data was used as an indicator for the integrity of CMs upon HP-N_2O treatments as we were unable to locate any references discussing the mechanism of changes in the colloidal stability of milk following treatments with HP-N_2O. As shown in Table 4.3, pH in the treated samples did not show any drastic change at any temperature tested, which is in agreement with our particle size data. Hence, it can be concluded that the CCP in casein micelles remains stable and the micelles are held intact by the CCP and have not been disrupted by pressurization of N_2O. Another indicator that reflects this theory are near-zero values of PI, indicating a very narrow size distribution of particles in all the treated samples according to Santos & Castanho (1996). These combined results suggest that neither disintegration nor aggregation of casein micelles occurred as a consequence of exposure to HP-N_2O at the temperatures studied in this work.

In this study, ζ-potential was used as a measure of the electrical charge of CMs and as a relative indicator of the colloidal stability of the HP-N_2O-treated milk. A large negative or positive ζ-potential value indicates that the particles in a disperse system repel each other, thus hindering aggregation and maintaining the stability of the system. It is known that with an absolute value approaching or above 25 mV, the system will be readily stable (Gülseren et al., 2010); if, however, particles have low absolute values of ζ-potential, they will agglomerate and the dispersion will become unstable. In this study, all the treated samples did not show signs of aggregation maintaining a stable ζ-potential value after HP-N_2O treatments at all temperatures as compared to control (-25.9 mV) with an average ζ-

potential value of -27.2 ±1.1 mV. Typically, a ζ-potential of about −20 mV has been reported at the natural pH of milk (pH 6.7-6.8) (Fox & McSweeney, 1998). Color difference classification was adopted from Cserhalmi et al. (2006). Based on this classification system ΔE can be categorized as: 0 to 0.5 = "not noticeable", 0.5 to 1.5 = "slightly noticeable" and > 1.5 = "noticeable". The total color difference for milk after 20 min of heat-assisted HP-N_2O treatments at 65 and 85 °C caused slightly higher color differences with a ΔE value of 0.6 and 1.0, respectively, suggesting that the HP-N_2O application resulted in slightly noticeable instrumental color differences in milk samples. Similar observations were obtained in treated milk after HP-N_2O applications with antimicrobials at 65 (H65/HPN2O/N150) and 85 (H85/HPN2O/N150/L) °C.

4.6 Conclusion

Overall, the present study highlighted concurrent application of HP-N_2O, heat, and natural antimicrobials, such as nisin and lysozyme, in hurdle technologies, thereby, synergistically inactivating representatives of the major bacteria groups (gram-positive and gram-negative vegetative, and spore-forming) in milk. The absence of sub-lethal injuries at cellular level for the supercritical N_2O-treated milk as well as the fact that pH stability or other physical and chemical quality characteristics of milk were not adversely affected, the efficacy and adequacy of these hurdle strategies for use in commercial milk processing has been substantiated. Although N_2O is known for promoting greenhouse effect, a minimal processing approach such as that used in this research as well as the recycling of the gas in the process, means it can be used in a limited way, while preventing its release and impact on the environment.

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Chapter 5: Synergistic effect of supercritical carbon dioxide and peracetic acid on microbial inactivation in shredded Mozzarella-type cheese and its storage stability at ambient temperature

5.1 Abstract

Supercritical carbon dioxide (Sc-CO_2) in combination with peracetic acid (PAA) could represent an effective decontamination technique for microorganisms in shredded cheese. Preservation of shredded Mozzarella-type cheese (SMC) was assessed at 25 °C for 21 d, using Sc-CO_2 (9.8 MPa, 35 °C, 30 min) individually and combined with PAA at concentrations of 50 (Sc-CO_2/PAA50), and 100 (Sc-CO_2/PAA100) ppm with optional 30-min pre-conditioning time (Sc-CO_2/PAA100PC). Process efficacy was assessed based on achievable inactivation and treatment synergism reflected in counts of inoculated *Escherichia coli*, *Listeria innocua*, *Geobacillus stearothermophilus* spores and indigenous microflora such as total bacteria (TBC) and total yeasts and molds (TYMC). Complete inactivation of *E. coli* cells (≥7.0 $\log_{10}$) in SMC was achieved with any of the combined treatments, whereas initial reduction of *L. innocua* was lower when Sc-CO_2/PAA50 and Sc-CO_2/PAA100 combinations were applied (2.9 and 4.6 $\log_{10}$, respectively). *G. stearothermophilus* spores exhibited the highest resistance, allowing for a reduction of up to 3.8 $\log_{10}$ (Sc-CO_2/PAA100) and indicating no advantage of pre-conditioning. However, PAA concentration significantly affected microbial inactivation, comparing Sc-CO_2/PAA100 to Sc-CO_2/PAA50 in TBC (minimum of 6.6 vs. 4.2 $\log_{10}$, respectively) ($P<0.05$) and TYMC (minimum of 7.7 vs. 1.1 $\log_{10}$, respectively) ($P<0.05$). The TBC and TYMC were not significantly decreased by stand-alone decontamination techniques ($P\geq0.05$); however, synergistic treatment effects ($P<0.05$) occurred for all microorganisms

except for *E. coli*. Overall, the findings demonstrate great potential for a Sc-CO_2/PAA hurdle technology as an alternative to post-processing decontamination strategies for production of shelf-stable SMC at ambient temperatures.

5.2 Introduction

Over the last decades, production of Mozzarella and Mozzarella-type cheeses has steadily increased, reflecting their growing popularity among consumers. In 2013 Mozzarella ranked first, with a 33.4 percent share of 11.1 billion pounds of cheese produced in the United States (USDA, 2013). Its commercial relevance is apparent from preferences shown by mainstream consumers, food services or fast food chains, which use them, for instance, as topping in baked preparations such as pizza and gratin dishes or as an ingredient in other foods (Mastromatteo et al., 2014). However, food safety concerns regarding microbial contamination of dairy products post processing exist especially, with a scale-up of production and if the effectiveness and dimension of intervention methods remain at the same level as prior to the scale-up (Griffiths & Walkling-Ribeiro, 2012). This is crucial for shredded products like cheese as shredding greatly increases surface exposure for even airborne microbial contamination (Eliot, Vuillemard, & Emond, 1998). Growing consumer demand for reduced- or low-sodium processed cheese also increases the water activity, thus making the product more sensitive to microbial spoilage and potentially affecting the storage stability (Taylor et al., 2013). While product spoilage associated with native aerobic bacteria and yeasts and molds could cause economic loss (Corbo et al., 2001; Eliot, Vuillemard, & Emond, 1998), a far more serious health risk comes from contamination with major pathogens such as *E. coli* (Spano et al., 2003), *L. monocytogenes* (Stecchini,

Aquili, & Sarais, 1995), and *Bacillus cereus* (Bonerba et al., 2010). These pathogens could be introduced in the absence of an effective system to ensure safety during cheese processing (i.e. good manufacturing practice (GMP), hazard analysis and critical control point (HACCP)) or incidentally, beyond the control and detection of a food safety system.

In order to address these food microbiological challenges an innovative hurdle strategy, combining supercritical carbon dioxide (Sc-CO_2) and peracetic acid (PAA), could allow for safe preservation of shredded Mozzarella-like cheese (SMC) stored at ambient temperatures, thereby also meeting the consumer trend for a convenient (i.e. long, non-refrigerated shelf stability) and environmentally sustainable (i.e. not requiring additional energy for cold storage) food supply chain (Schmidt Rivera et al., 2014). To date, a few approaches have been proposed to inactivate the pathogenic and spoilage microorganisms in Mozzarella-type cheeses such as high pressure processing (Sheehan et al., 2005), ozone (Segat et al., 2014) and irradiation (Huo et al., 2013). Sc-CO_2 at 7.3 MPa and 31 °C or higher has unique properties which render it effective as a non-thermal decontamination technique for foods. The chemical nature of Sc-CO_2 fluids due to its molecular behaviour in water (i.e. low intracellular pH due to formation of carbonic acid) brings about inhibitory action on microbial cells, making the treatment not entirely dependent on pressure (Sikin, Zoellner, & Rizvi, 2013). Physical properties such as adjustable densities, low viscosities, high diffusivities and low interfacial surface tension facilitate its penetration into various matrices (Sikin & Rizvi, 2011). Practically, Sc-CO_2 does not affect the stability of most food matrices and it can also be easily handled at industrial scales. It is also noteworthy that Sc-CO_2 is nontoxic, non-flammable, chemically inert and a benign solvent with

generally recognized generally regarded as safe (GRAS) status, leaving no residue in the treated food products upon depressurization (Clifford & Williams, 2000).

Unlike liquid foods, application of Sc-CO_2 for microbial inactivation in solid foods suffers a few limitations such as limited diffusion of CO_2 into solid matrices and bacterial cells since the sample cannot be agitated and much reduced levels of free water at the surface, which may limit the solubility of CO_2 into the food (Ferrentino & Spilimbergo, 2011; Balaban & Duong, 2014). In order to overcome this limitation, Sc-CO_2 is often combined with a co-solvent or an antimicrobial agent as a hurdle technology to achieve better microbial inactivation under milder treatment conditions (≤ 10 MPa, ≤ 40°C) and in shorter times (≤ 60 min). Peracetic acid (PAA) is approved for use as a sanitizer in the United States on food contact surfaces (21CFR Part 178.1010) and for direct food contact with fruits and vegetables (21CFR Part 173.315) and meat, poultry and seafood (21CFR Part 173.370) at a maximum concentration of 80, 85 and 110 ppm, respectively. For combined treatments, the role of highly diffusive Sc-CO_2 fluid is to act as vector, so that PAA can easily penetrate into the microbial cells and inactivate them. Thus, a rapid penetration of PAA into microbial cells and the release of oxygen and free radicals, critical for the oxidation and destruction of cellular enzymes, are likely associated with its efficacy (Pruss et al., 2001).

Hence, the aim of this work was to investigate the use of Sc-CO_2 and PAA, alone and in combination, to reduce the microbial load in SMC and to monitor its storage stability at 25 °C over a period of 21 d. In addition to the analysis of spoilage by native bacteria, yeasts and molds, non-pathogenic surrogates, such as *E. coli*, *L. innocua*, and *G.*

stearothermophilus spores were used for inoculation in a selective approach to study their resistance and survival following single or combined Sc-CO_2 treatments.

5.3 Materials and Methods

5.3.1 Inoculation and sample preparation

Shredded low-moisture part-skim Mozzarella cheese (C&S Wholesale Grocers Inc., Keene, NH, USA) was purchased from a local supermarket and stored under refrigeration (4±1 °C) before treatments. *E. coli* American Type Culture Collection # 25922 (*E. coli* ATCC25922) and *L. innocua* Food Safety Laboratory # C2-008 (*L. innouca* FSL C2-008) were obtained from the -80 °C stock culture collection of the Food Microbiology and Safety Laboratory at Cornell University. Non-pathogenic *E. coli* and *L. innocua* strains were selected as surrogates, commonly used for challenge studies in the food industry, to simulate cheese contamination with respective gram-negative and -positive pathogens of great relevance (pathogenic *E. coli* and *L. monocytogenes*, respectively) to food product safety. A commercially available *Geobacillus stearothermophilus* (ATCC 9372, NAMSA Products, Northwood, OH, USA) endospore suspension of 10^6 - 10^7 colony forming units per ten mililiter (CFU/10mL) was used as biological sterilization indicator and as a possible non-pathogenic surrogate for *Bacillus anthracis* (Guan et al., 2013). This is regarded as a pathogenic surrogate for *Bacillus cereus* due to its genotypical and phenotypical resemblance (Greenberg et al., 2010). Prior to experiments, the culture was streaked onto trypticase soy agar (TSA; 236920 Difco™, BD, Sparks, MD, USA) and incubated for 24 ± 2 h at 37 ± 2 °C. For each of the vegetative challenge bacteria, a single isolated colony was transferred into trypticase soy broth (TSB; 296264 BBL™, BD, Sparks, MD, USA)

and incubated for 24 ± 2 h at 37 ± 2 °C, under agitation at 225 rpm. A subsequent loop transfer into fresh TSB and incubation for 24 ± 2 h at 37 ± 2 °C, shaken at 225 rpm, was performed to produce an initial inoculum of about 10^9 - 10^{10} CFU/mL. A 10-mL culture or spore suspension was used for inoculation of 90 mL distilled water (1:10 (v/v) dilution) to prepare a final concentration of 10^8 - 10^9 CFU/mL for both *E. coli* ATCC 25922 and *L. innocua* FSL C2-008, and 10^5 - 10^6 CFU/mL of *G. stearothermophilus* ATCC 9372 spores.

For the inoculation with selective microorganisms (*E. coli* ATCC 25922, *L. innocua* FSL C2-008 and *G. stearothermophilus* spores), an aliquot of 80 g of cheese was submerged into 100 mL inoculum, that was previously 1:10 diluted with sterile, quarter-strength Ringer's solution (BR0052G, Oxoid Ltd., Basingstoke, UK) (v/v) for 15 min. For contamination with native microorganisms (total aerobic bacterial counts and total yeast and mold counts), cheese was spoiled at 25 °C for 72 h and, subsequently, immersed in a 100 mL suspension, made up of sterile TSB that was 1:10 diluted with sterile Ringer's solution (v/v). In the same manner negative control samples were drenched in sterile, uninoculated TSB diluted with Ringer's solution in order to assess microbial contamination of the commercial product prior to inoculation or spoilage. After a 15 min dwell period, 2 g of soaked cheese sample were weighed and dried for 15 min in weighing dishes (08-732-112 Fisherbrand, Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) before aseptic transfer to gas-permeable 279.5 × 179.7 × 0.3 mm (length × depth × height) Tyvek bags (NovaSterilis, NY). Each bag was segmented into 8 pouches of equal size using a vacuum sealer (AGW Multivac Vakuum-Verpackungsmaschine, Sepp Haggenmüller KG, Wolfertsschwenden, Germany), allowing for 2 g of cheese sample to be vacuum-sealed in each pouch. Based on common practice in high pressure processing, bags were double-

sealed (Ahmadi et al., 2015) to ensure that sample migration and cross-contamination did not occur, and then labelled appropriately, to separate untreated positive control SMC samples from those destined for subsequent PAA or high pressure treatments, and refrigerated prior to non-thermal processing.

5.3.2 Non-thermal processing with Sc-CO_2 and PAA

A patented Sc-CO_2 system (Nova2200™ ,Nova Sterilis, Lansing, NY) described by White, Burns, & Christensen (2006) was used for the treatments (see Figure 5.1 below).

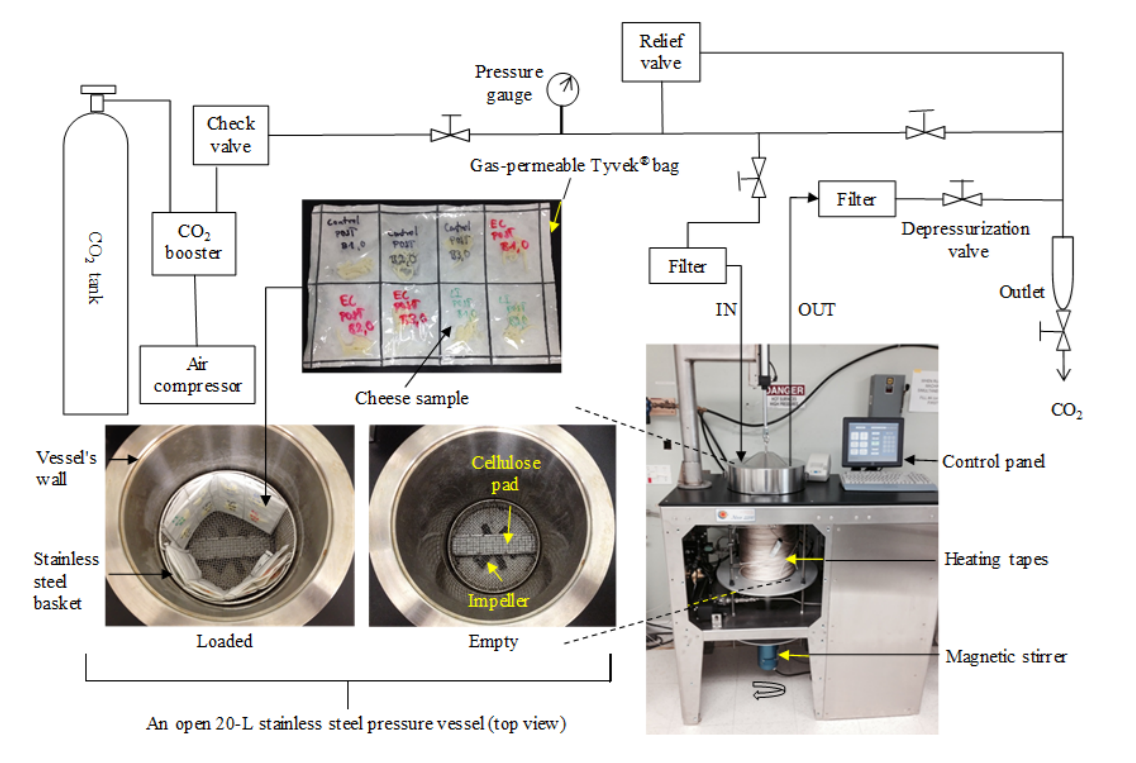

Figure 5.1 Schematic diagram of a supercritical carbon dioxide (Sc-CO_2) processing apparatus (Nova2200™ , Nova Sterilis, Lansing, NY).

The system comprises a 20-L stainless steel pressure vessel (24 cm internal diameter and 42 cm height) with an impeller for internally stirring the Sc-CO_2 fluid. The vessel can be loaded with two stainless steel baskets (23.5 cm diameter, with 17.8 and 12.7

cm height), in which the 8 sealed Tyvek bags with cheese samples were arranged concentrically. For combinations of the high pressure carbon dioxide treatment with PAA, 8 and 16 mL portions of NovaKillGen2 sterilant, corresponding to an estimated concentration of 50 and 100 ppm, respectively, were pipetted onto a 3.8 length × 20 cm depth cellulose pad, which was then secured by the vessel's lower 2.5 cm section of the stainless steel basket with a holder, before closing the pressure vessel. Water has been shown to facilitate inactivation of microbes with Sc-CO_2 (Dillow et al., 1999). A 25-mL portion of water was nebulized to humidify the interior of the vessel before each treatment and thereby facilitate the inactivation of the investigated microorganisms. In 6 min the vessel was charged with CO_2 from ambient conditions to a pressure of 9.8 ± 0.5 MPa and a temperature of 35 ± 3˚C with constant stirring at 680 ± 20 rpm. System parameters and run times were maintained as specified before the vessel was depressurized over 15 min. When pre-conditioning was applied, samples were exposed to 100 ppm PAA vapour circulating in the vessel for 30 min, in order to extend sample exposure to the anti-microbial agent before the high pressure treatment cycles. This additional approach was selected to possibly enhance treatment efficacy based on a longer exposure of the pathogen surrogates to 100 ppm PAA at unpressurized conditions. For the purpose of this article, stand-alone Sc-CO_2 and 100 ppm PAA treatments and the combined treatments of Sc-CO_2 with 50 ppm PAA, 100 ppm PAA, and preconditioned 100 ppm PAA were abbreviated as Sc-CO_2, PAA100, Sc-CO_2/PAA50, Sc-CO_2/PAA100 and Sc-CO_2/PAA100PA, respectively.

5.3.3 Microbiological analysis

Microbiological analysis after Sc-CO_2 processing of cheese was carried out by enumerating viable cells before and after the treatments as well as on day 7, 14 and 21 of storage at 25 ± 1 °C. Untreated and treated samples (1 g each) were aseptically removed from the Tyvek bags and then transferred into sterile 15 mL centrifuge tubes (05-539-5 Fisherbrand, Thermo Fisher Scientific, Pittsburgh, PA, USA) containing 4 sterile 6 mm diameter glass beads. The samples were then diluted 1:10 (w/v) using sterile Ringer's solution and each centrifuge tube's content was subsequently vortexed (K-550-G Vortex-Genie, Scientific Industries Inc., Bohemia, NY, USA) for 30 s to allow for adequate disintegration of the cheese. These neat suspensions or their ten-fold serial dilutions were then spread-plated (100 µl) on selective and non-selective media in duplicate at appropriate conditions prior to colony counting of: *E. coli* ATCC 25922 on MacConkey agar (MCA; 211387 BBL™ BD, Sparks, MD, USA) incubated at 37 °C for 24 h, *L. innocua* FSL C2-008 on *Listeria* Selective Agar (LSA; CM0856 Oxoid Ltd., Basingstoke, UK) incubated at 37 °C for 24 h, *G. stearothermophilus* spores on TSA incubated at 55 °C for 24 h, total aerobic bacteria on TSA incubated at 30 °C for 48 h, and total yeasts and molds on potato dextrose agar (PDA; 213400 Difco™, BD, Sparks, MD, USA) incubated at 25 °C for 5 d. Following the incubation periods counts of untreated and treated cheese samples were enumerated. The degree of inactivation was determined by evaluating the $\log_{10}(N/N_0)$ versus storage time, where N_0 (CFU/g) was the number of microorganisms initially present in the untreated sample and N (CFU/g) was the number of survivors in the treated samples at 0, 7, 14 and 21 d. In some cases, no colony growth was detected due to the culture assay sensitivity, enabling microbial enumeration up to 10^2 CFU/g (i.e., the detection limit).

5.3.4 pH determination

The pH value was recorded using a BASIC pH meter (Denver Instrument Co., USA) equipped with a glass electrode that was immersed directly into the sample suspension. At each sampling day (0, 7, 14 and 21) three samples were analyzed per treatment.

5.3.5 Statistical analysis

For each treatment, the mean and standard deviation of survivor ratios translated as inactivation were calculated over storage time. The synergistic effects of the treatment were determined when the inactivation of the individual treatment added up to the same inactivation as achieved by their combination or if the latter exceeded the inactivation of the former. The analysis of variance (one-way ANOVA) was performed to compare treatment mean values using the Tukey's test. Significance was based on $p < 0.05$. The data were processed using the JMP (John's Macintosh Project) 10.0 (SAS Institute Inc., Cary, NC, USA).

5.4 Results

In the negative control Mozzarella cheese samples the initial level of natural microflora in SMC before inoculation between 10^3 and 10^4 CFU/g was determined. The responses of the different microorganism groups (*E. coli*, *L. innocua*, *G. stearothermophilus*, total aerobic bacteria and total yeasts and molds) to Sc-CO_2, PAA100, Sc-CO_2/PAA50, Sc-CO_2/PAA100 and Sc-CO_2/PAA100PC treatments for the preservation of SMC are shown in Figure 5.2, Figure 5.3, Figure 5.4, Figure 5.5, and Figure 5.6, respectively. The plate counts of untreated *E. coli*, *L. innocua*, *G. stereothermphilus*, total aerobic bacteria and

total yeasts and molds ranged from 1.1 to 8.1 × 10^9, 9.7 × 10^8 to 1.2 × 10^9, 4.2 × 10^5 to 1.3 × 10^7, 3.3 to 3.7 × 10^9, and 2.9 × 10^8 to 5.5 × 10^9 CFU/mL on average, respectively.

Maximum inactivation of *E. coli* up to the detection limit (7.0 to 7.9 $\log_{10}$) was observed instantly after all Sc-CO_2 treatments were applied in conjunction with PAA (day 0) ($p \geq 0.05$) and maintained until the end of the study (day 21) ($p \geq 0.05$) (Figure 5.4-Figure 5.6). By contrast, Sc-CO_2 alone (Figure 5.2) was not able to reduce *E. coli* effectively in the SMC, inactivating 6.5 $\log_{10}$ of *E. coli* cells initially, but allowing for cell survival and recovery (-1.6 and -0.9 $\log_{10}$ at 14 and 21 d, respectively). For PAA100 treatments (Figure 5.3), a 2.2 $\log_{10}$ inactivation of *E. coli* cells was achieved at day 0, whereas, individual application of the antimicrobial showed significantly higher inactivation than that of single Sc-CO_2 during 21 days of storage. *L. innocua* cells in SMC were more resistant than the cells of *E. coli* to Sc-CO_2 alone or when combined with PAA as indicated by lower inactivation ($P<0.05$) of 0.9, 2.9, and 4.6 $\log_{10}$ following exposure to Sc-CO_2, Sc-CO_2/PAA50, and Sc-CO_2/PAA100, respectively. Although Sc-CO_2/PAA100PC led to a maximum reduction of *L. innocua* (7.0 $\log_{10}$) ($P \geq 0.05$) initially, the listeria were not kept consistently below the detection level (6.6 $\log_{10}$, day 14). Overall, inhibition of *L. innocua* contaminated cheese samples during ambient temperature storage was more challenging, as indicated by its irregular pattern, than for *E. coli* for which the inactivation was consistent over the storage duration. Lower response of *L. innocua* occurred when the detection limit of the bacteria was not reached before day 14 and 7 for Sc-CO_2/PAA50 and Sc-CO_2/PAA100 treatments, respectively. However, inactivation of *L. innocua* cells below the detection level was achieved at day 14 and 21 following a stand-alone PAA100

treatment, which indicated that *L. innocua* cells responded better to the PAA100 treatment as compared to the Sc-CO_2 alone.

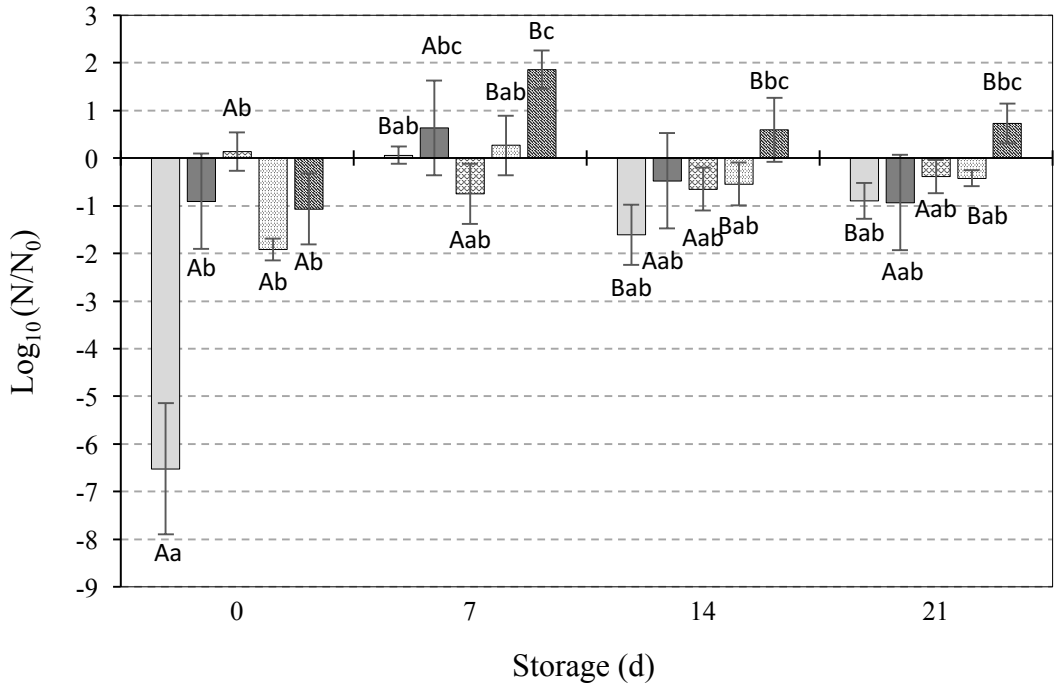

Figure 5.2 Inactivation of *E. coli* (□), *L. innocua*(■), *G. stearothermophilus* spores (▨), total aerobic bacteria (▩) and total yeasts and molds (▧) obtained in shredded Mozzarella cheese (SMC) following supercritical carbon dioxide (Sc-CO_2) treatment at 9.9 MPa and 35°C for 30 min and subsequent storage at 25°C for up to 21 d.

N = number of survivors after the treatment; N_0 = number of microorganisms before the treatment. Presented data are the mean values of three different samples ± standard deviation. Bars indicate standard deviations. Different capital letters and lower-case letters on the bars indicate statistical differences in microbial inactivation based on product storage time and belonging to a specific microorganism group, respectively ($p<0.05$).

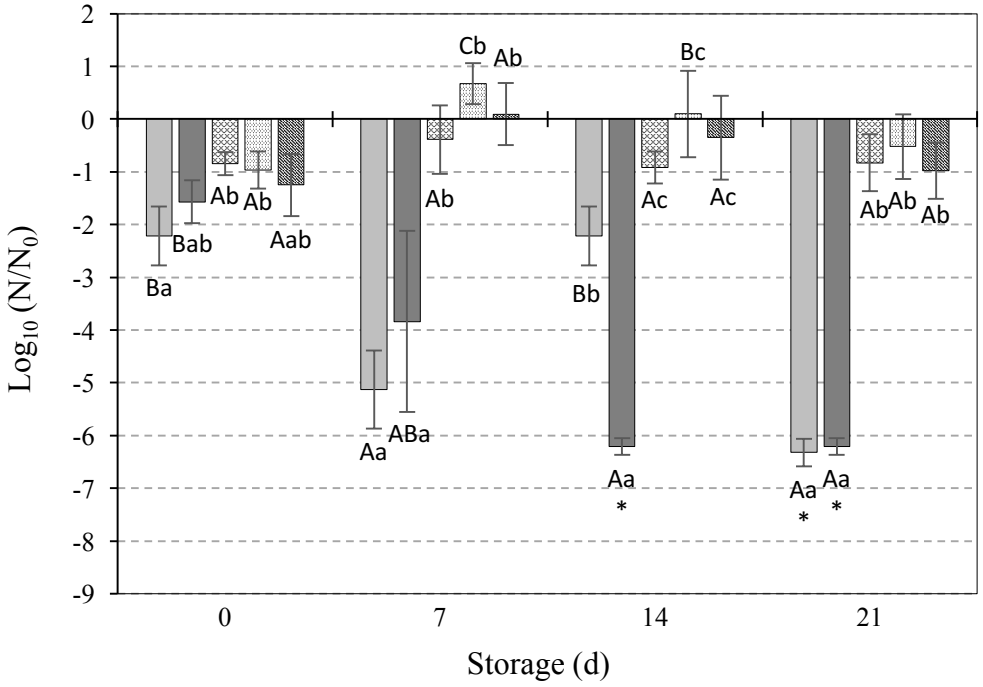

Figure 5.3 Inactivation of *E. coli* (), *L. innocua*(), *G. stearothermophilus* spores (), total aerobic bacteria () and total yeasts and molds () obtained in shredded Mozzarella cheese (SMC) following peracetic acid treatments (100 ppm) (PAA100) at atmospheric pressure, 35 °C for 30 min and subsequent storage at 25°C for up to 21 d.

N = number of survivors after the treatment; N_0 = number of microorganisms before the treatment. Presented data are the mean values of three different samples ± standard deviation. Bars indicate standard deviations based on the minimum inactivation achieved when reaching the detection limit. * indicates that the detection limit (10^2 CFU/g) was reached. Different capital letters and lower-case letters on the bars indicate statistical differences in microbial inactivation based on product storage time and belonging to a specific microorganism group, respectively ($p<0.05$).

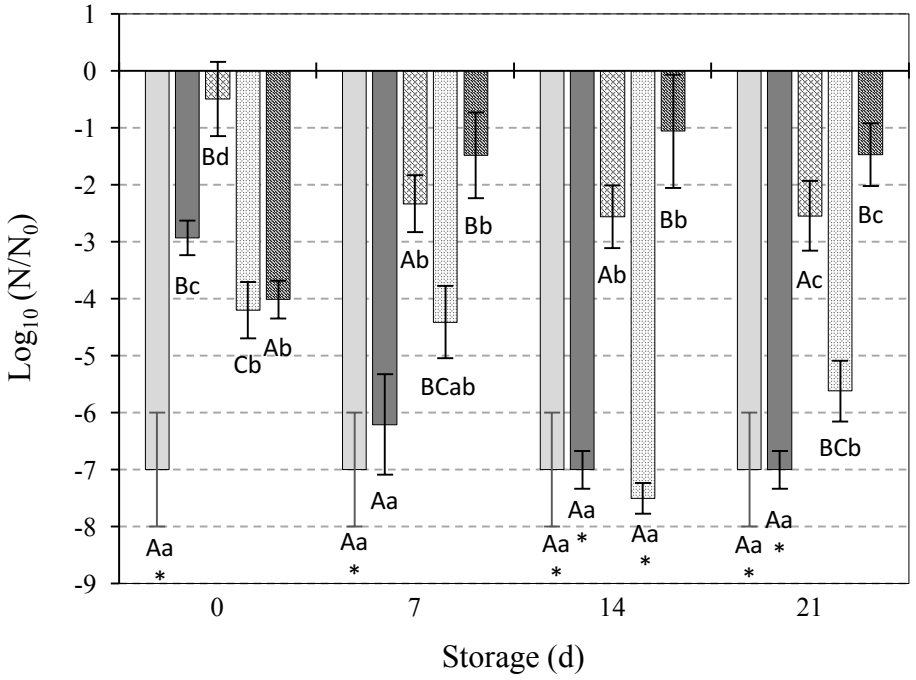

Figure 5.4 Inactivation of *E. coli* (□), *L. innocua*(■), *G. stearothermophilus* spores (▨), total aerobic bacteria (▫) and total yeasts and molds (▩)obtained in shredded Mozzarella cheese (SMC) following combined treatment with supercritical carbon dioxide at 9.9 MPa and 35°C for 30 min and with 50 ppm peracetic acid (Sc-CO_2/PAA50) and subsequent storage at 25 °C for up to 21 d.

N = number of survivors after the treatment; N_0 = number of microorganisms before the treatment. Presented data are the mean values of three different samples ± standard deviation. Bars indicate standard deviations based on the minimum inactivation achieved when reaching the detection limit. * indicates that the detection limit (10^2 CFU/g) was reached. Different capital letters and lower-case letters on the bars indicate statistical differences in microbial inactivation based on product storage time and belonging to a specific microorganism group, respectively ($p<0.05$).

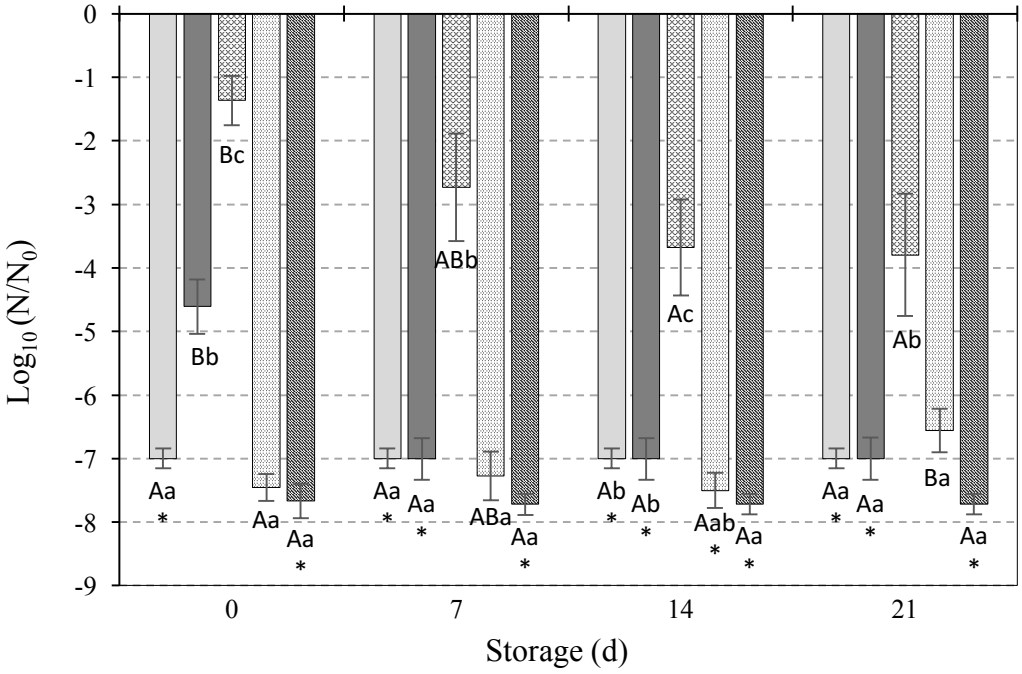

Figure 5.5 Inactivation of *E. coli* (□), *L. innocua*(■), *G. stearothermophilus* spores (▩) total aerobic bacteria (▨) and total yeasts and molds (▧) obtained in shredded Mozzarella cheese (SMC) following combined treatment with supercritical carbon dioxide at 9.9 MPa and 35°C for 30 min and with 100 ppm peracetic acid (Sc-CO_2/PAA100) and subsequent storage at 25°C for up to 21 d.

N = number of survivors after the treatment; N_0 = number of microorganisms before the treatment. Presented data are the mean values of three different samples ± standard deviation. Bars indicate standard deviations based on the minimum inactivation achieved when reaching the detection limit. * indicates that the detection limit (10^2 CFU/g) was reached. Different capital letters and lower-case letters on the bars indicate statistical differences in microbial inactivation based on product storage time and belonging to a specific microorganism group, respectively ($p<0.05$).

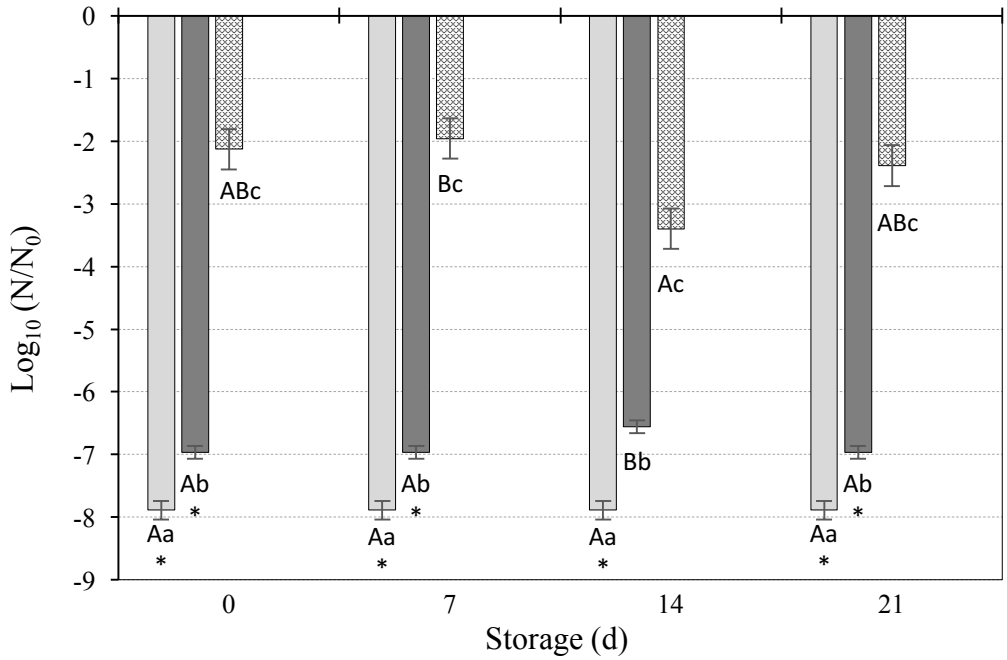

Figure 5.6 Inactivation of *E. coli* (□), *L. innocua* (■), and *G. stearothermophilus* spores (▨) obtained in shredded Mozzarella cheese (SMC) following 30-min pre-conditioning using peracetic acid (100 ppm) at unpressurized conditions as well as combined treatment with supercritical carbon dioxide at 9.9 MPa and 35°C for 30 min and with peracetic acid at 100 ppm (Sc-CO_2/PAA100PC) and subsequent storage at 25 °C for up to 21 d.

N = number of survivors after the treatment; N_0 = number of microorganisms before the treatment. Presented data are the mean values of three different samples ± standard deviation. Bars indicate standard deviations based on the minimum inactivation achieved when reaching the detection limit. * indicates that the detection limit (10^2 CFU/g) was reached. Different capital letters and lower-case letters on the bars indicate statistical differences in microbial inactivation based on product storage time and belonging to a specific microorganism group, respectively ($p<0.05$).

Endospores of *G. stearothermophilus* were most resistant to Sc-CO_2 processing of SMC, indicating no significant effect over 21 d ($P \geq 0.05$) when the spores were exposed to Sc-CO_2 alone, but showed reductions of up to 2.6, 3.8, and 3.4 $\log_{10}$ when subjected to Sc-CO_2/PAA50, Sc-CO_2/PAA100, and Sc-CO_2/PAA100PC treatments. It was also observed that spore inactivation plateaued ($P \geq 0.05$) after 7 and 14 days of storage for respective PAA dosages of 50 and 100 ppm used in conjunction with Sc-CO_2, while the inactivation obtained with the pre-conditioned hurdle approach peaked on day 14 (3.4 $\log_{10}$) but declined subsequently (2.4 $\log_{10}$) ($P < 0.05$). An almost 1 $\log_{10}$ inactivation of *G. stearothermophilus* spores was consistently observed during storage following PAA100 treatment with an exception of 0.4 $\log_{10}$ at day 7.

Total aerobic bacteria count (TBC) in SMC decreased by 1.9 $\log_{10}$ and 1.0 $\log_{10}$, following Sc-CO_2 and PAA100 treatments, respectively. However, a full recovery and growth of the bacteria was indicated at day 7 during ambient temperature storage for both treatments. Combined treatments proved to be more successful in keeping the counts of TBC low ($P < 0.05$), inactivating between 4.2 (day 0) and 7.5 (day 14) $\log_{10}$ in Sc-CO_2/PAA50-treated cheese and between 6.6 (day 21) and 7.7 (day 14) $\log_{10}$ in Sc-CO_2/PAA100-processed SMC. Similar results were obtained for TYMC with an initial inactivation of 1.1 and 1.2 $\log_{10}$ as a result of Sc-CO_2 and PAA100 treatments, respectively. It was also observed that total yeasts and molds were most resistant to Sc-CO_2 alone with a subsequent growth after treatment each week during storage. In contrast, PAA100 treatment showed a gradual increase in inactivation from day 14 (0.4 $\log_{10}$) to 21 (1.0 $\log_{10}$) after experiencing a full recovery of total yeasts and molds at day 7. Higher inactivation in the yeasts and molds in Mozzarella was observed for Sc-CO_2/PAA50 and Sc-

CO_2/PAA100, ranging from 1.1 (day 14) to 4.0 $\log_{10}$ and remaining constant at 7.7 $\log_{10}$, respectively.

Table 5.1 pH of treated shredded mozzarella cheese (SMC) after treatment (day 0) and during storage.

Treatments	pH of cheese at different storage time (days)			
	0	7	14	21
Sc-CO_2[1]	5.8±0.0aA	5.7±0.0aA	5.7±0.1aA	5.9±0.2aA
PAA100[2]	5.1±0.0bA	4.7±0.0bB	4.7±0.1bB	4.7±0.0bB
Sc-CO_2/PAA50[3]	4.9±0.1bA	4.7±0.0bcAB	4.6±0.1bcB	4.6±0.1bB
Sc-CO_2/PAA100[4]	4.6±0.0cA	4.6±0.1cdA	4.4±0.0cdA	4.5±0.1bA
ScCO_2/PAA100PC[5]	4.5±0.1cB	4.5±0.0dB	4.5±0.1dB	4.7±0.0bA

[1]Sc-CO_2, stand-alone supercritical carbon dioxide treatment at 9.9 MPa, 35°C for 30 min.
[2]PAA100, stand-alone peracetic acid (100 ppm) treatment at atmospheric pressure, 35°C for 30 min.
[3]Sc-CO_2/PAA50, simultaneous treatment of Sc-CO_2 and 50 ppm peracetic acid at 9.9 MPa, 35 ºC for 30 min.
[4]Sc-CO_2/PAA100, simultaneous treatment of Sc-CO_2 and 100 ppm peracetic acid at 9.9 MPa, 35 ºC for 30 min.
[5]Sc-CO_2/PAA100PC, simultaneous treatment of Sc-CO_2 and 100 ppm peracetic acid at 9.9 MPa, 35 ºC for 30 min with 30-min pre-conditioning of peracetic acid prior to pressurization.
Within each column, means sharing at least one common lower-case letter are not significantly different at a P-value of < 0.05.
Within each row, means sharing at least one common upper-case letter are not significantly different at a P-value of < 0.05.
Data are mean values ± standard deviations (n = 3).

Analysis of the pH data, presented in Table 5.1 above, indicated that stand-alone Sc-CO_2 treatment did not affect ($p \geq 0.05$) the pH of cheese sample as compared to the untreated control (5.9 ± 0.2). However, the pH of treated cheese samples (5.1 ± 0.0) was significantly decreased by PAA100 treatments. Sc-CO_2/PAA100 treatments further reduced ($p < 0.05$) the product pH (4.6 ± 0), but Sc-CO_2/PAA50 did not (4.9±0.1). However, Sc-CO_2/PAA100PC did not indicate a significant ($p \geq 0.05$) change in the pH level as compared to Sc-CO_2/PAA100. Overall, the pH of cheese slightly decreased on day 7 of storage and remained consistently low thereafter regarding PAA100 and Sc-CO_2/PAA50 samples. The low pH, monitored for Sc-CO_2/PAA100 and Sc-CO_2/PAA100PC during the

entire storage period, potentially provided a prolonged stress to any treatments in which microorganisms survived.

Table 5.2 Synergistic effects of supercritical carbon dioxide (Sc-CO_2) and peracetic acid (PAA) on inactivation of microorganisms on shredded mozzarella cheese (SMC).

Microorganisms	Log_{10} Reductions[b]		
	∑I[c]	CS[d]	Synergy[e]
Listeria innocua	2.86	4.18	+
Escherichia coli	9.9	8.84	-
Geobacillus stearothermophilus spores	0.9	1.03	+
Total aerobic bacteria	3.17	7.24	+
Yeast and molds	2.98	7.39	+

[a]Processing methods applied: PAA100, stand-alone peracetic acid (100 ppm) treatment at atmospheric pressure, 35 ºC for 30 min; Sc-CO_2, stand-alone supercritical carbon dioxide treatment at 9.9 MPa, 35°C for 30 min.
[b]Log_{10} reductions are expressed as $Log_{10}N_0$-$Log_{10}N$, where N and N_0 indicate the number of surviving microorganisms after the treatment and the initial number of microorganisms before the treatment, respectively.
[c]∑I stand for the theoretical sum of Log_{10} reductions obtained from Sc-CO_2 and PAA100 applied individually (or Sc-CO_2+PAA100), plus their mean standard deviation.
[d]CS designates the experimental Log_{10} reduction obtained from Sc-CO_2 and PAA100 combined simultaneously (or Sc-CO_2/PAA100) , minus its standard deviation.
[e]Synergistic (+) and additive (-) treatment effects ($P \leq 0.05$) occurred when CS>∑I and CS ≤ ∑I, respectively.

A comparison of Sc-CO_2 and PAA100 stand-alone treatments to their combined applications provides an insight into the effect of the individual processing approaches added up to the same inactivation as achieved by their combination, or whether the latter exceeded the inactivation of the former with treatments acting synergistically when applied simultaneously (see Table 5.2 above). It was observed that there is a significant positive interaction ($p<0.05$) between Sc-CO_2 and PAA100 for all microorganisms, exhibiting synergistic behavior, except for *E. coli*.

5.5 Discussion

Pathogens such as *L. monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus* and enteropathogenic *E. coli* are known to pose great risks to the safety of cheese (Donnelly, 2004). In this study, *E. coli* ATCC25922 and *L. innocua* FSL C2-008 were used as respective gram-negative and gram-positive model organisms. There have been many investigations with non-pathogenic *E. coli* ATCC25922 as a challenge microorganism (Melo Silva et al., 2013; Meujo et al., 2010; Tamburini et al., 2014) and few specifically reported equal or higher resistance of the strain to Sc-CO_2 treatments in comparison to *E. coli* O157:H7 (Choi et al., 2009; Kim et al., 2007). Although the use of *L. innocua* FSL C2-008 has never been reported in the field of Sc-CO_2 inactivation technology, the non-pathogenic *L. innocua* is the *Listeria* species most closely related to *L. monocytogenes* (Paillard et al., 2003) and considered a suitable biological indicator of *L. monocytogenes* in the food industry, facilitating trials and studies that produce comparable findings under safer operating conditions (Kamat & Nair, 1996; Margolles et al., 2000; Piyasena, Lious, & McKellar, 1998).

With regard to this study, Sc-CO_2 exhibited a stronger bactericidal effect on *E. coli* than on *L. innocua*. This trend is consistent with previous works on the application of both supercritical carbon dioxide (Dillow et al., 1999; Garcia-Gonzalez et al., 2010; Kim et al., 2008; Zhang et al., 2006) and nitrous oxide (Mun et al., 2012), which reported higher sensitivity of gram-negative bacteria to these supercritical fluids. The difference in resistance to the treatments observed between vegetative bacteria in the present study could be attributed to the thicker peptidoglycan layer in the cell wall structure of gram-positive *L. innocua* than that of gram-negative *E. coli*, which may have led to a higher resistance of the former against penetration by pressurized CO_2. In accordance with our findings,

Garcia-Gonzalez et al. (2010) also observed that *E. coli* was the organism most sensitive to Sc-CO_2 exposure (10.5 MPa, 35 °C) as compared to *L. monocytogenes* and *S. cerevisiae*, and based on their analyses of membrane susceptibility through spectrofluorometry and transmission electron microscopy (TEM) and cell viability on growth media. Similarly, Kim et al. (2008) also reported negligible changes in the TEM images of *L. monocytogenes* cells following Sc-CO_2 treatment (10 MPa, 35 °C, 30 min), which further confirmed the influence of cell morphology on inactivation effectiveness of Sc-CO_2.

Spore-formers are important contaminants in the dairy industry because they can significantly affect food quality and safety. One third of spore-forming isolates (n=467) in the dairy industry was found to be heat-resistant (surviving 100 °C, 20 min), with *B. subtilis* and *G. stearothermophilus* being the prevalent species (Lücking et al., 2013). However, the U.S. Federal and Drug Administration (FDA)'s recommended pasteurization treatments (63 °C for 30 min or 72 °C for 15 s) (CFR Part 133.3) for raw milk in cheese making are not sufficient for the inactivation of bacterial endospores. In addition to being a suitable surrogate for *B. cereus*, *G. stearothermophilus* was therefore chosen for this study due to its highest resistance to heat treatments among *Bacillus* species (Burgess, Lindsay, & Flint, 2010; Feeherry, Munsey, & Rowley, 1987; Hemmer et al., 2007; López et al., 1997). Furthermore, the capacity of this bacterium to adhere to stainless steel and grow in biofilms appears to be a likely cause of contamination of manufactured dairy products (Flint et al., 2001). According to previous studies, Sc-CO_2 treatments can only inactivate *G. stearothermophilus* spores substantially when a combination of high pressures (≥35 MPa) and elevated temperatures (≥50 °C) are applied together with co-solvents (Furukawa et al., 2009; Hemmer et al., 2007; Watanabe et al., 2003). A milder Sc-

CO_2-based sterilization process (9.6 MPa, 35 °C, 1 h) using a low concentration of PAA (20 ppm) was developed by White, Burns, & Christensen. (2006) to achieve a 6-$\log_{10}$ inactivation of *G. stearothermophilus* spores. Although effective, this study used growth media and test strips which represent less complex and challenging treatment substrates than that of an actual food matrix. While an individual application of PAA and Sc-CO_2 showed little to no effect on the microorganisms, their efficacy was observed to increase when applied in combination (i.e. Sc-CO_2/PAA50 and Sc-CO_2/PAA100) in this study. Similarly, a synergistic treatment effect was previously demonstrated by White, Burns, & Christensen. (2006), who reported that PAA in combination with Sc-CO_2 was nearly 100 times more effective than PAA with pressurized air for the inactivation of *G. stearothermophilus* endospores. Other studies, which looked into the effect of Sc-CO_2-PAA sterilization on various medical polymers and vaccines, reported an inactivation of 6 $\log_{10}$ of *Bacillus* spores at PAA concentrations ranging from 20 to 200 ppm (Howell et al., 2012; Nichols, Burns, & Christopher, 2009; Qiu et al., 2009; White, Burns, & Christensen, 2006). These studies further support the PAA data obtained in this study. The effect of pre-conditioning, which extended the exposure time of the cheese to PAA, did not substantially alter the antimicrobial effect on *L. innocua*, except for *E. coli* which was least resistant and, thus, completely inactivated.

The maximum reduction of *G. stearothermophilus* endospores obtained (1.4 ± 0.4-$\log_{10}$ CFU/g) after treatment with Sc-CO_2/PAA100 may seem insufficient to ensure the safety of cheese. However, initial cell concentrations used in the present study was high (5-7 $\log_{10}$ CFU/g) and the reduction level of spores was observed to steadily increase to 3.8 $\log_{10}$ during 21-day storage. It is worth noting that the mesophilic spore counts in raw

milk usually ranges from 2.6 to 3 $\log_{10}$ CFU/mL (White, Marth & Steele, 2001) and spore contamination in the dairy food production chain has been reported to rise up to 10^4 CFU/mL (Burgess, Lindsay, & Flint, 2010). With reference to *B. cereus* spores, the minimum dosage required for causing enteric intoxication is normally around 6-$\log_{10}$ CFU/g of food (Granum & Lund, 1997). Also, a high inoculum load of spores used in this study could have hindered the sporicidal effect of the treatments as spores are more susceptible to aggregation. Previous studies have suggested that inner spores deposited in a spore aggregate are protected by killed spores on the top, forming passive or active barriers which the Sc-CO_2 must diffuse through (Checinska et al., 2011; Checinska, Burbank, & Pasczynski, 2012; Enomoto et al., 1997) to be effective. Nonetheless, the relatively high initial load of microorganisms used in the present study was deemed necessary to enable a comparison of the effects of the different hurdles applied, so that additive or synergistic treatment effects could be evaluated and for the assessment of the efficacy and potential of the different techniques used.

The Sc-CO_2/PAA100 treatment proved to be more consistent in inhibiting microbial growth over the duration of the study in contrast to Sc-CO_2/PAA100PC, for which higher inactivation was found initially for *L. innocua* and *G. stearothermophilus*. However, bacterial inhibition during storage was reduced, with the growth of *L. innocua* increasing beyond the detection limit at 14 d of storage and higher counts for *G. stearothermophilus* on day 21 of storage. A possible explanation for the recovery of the gram-positive bacteria during the extended treatment could be that part of the PAA was used up during the 30 min pre-conditioning period. This would have a more pronounced antibacterial effect on the outlying structure of the bacteria or spores, accounting for a

stronger short-time effect on the external cell layers, but at the same time reducing the amount of PAA available for impacting on the internal and vital components of the cell under subsequent supercritical treatment. For this reason, internal cell damage may have been greater after Sc-CO_2/PAA100, preventing or slowing down repair of bacterial cells, whereas Sc-CO_2/PAA100PC could have inflicted sub-lethal damage that allowed for their recovery after two weeks of ambient-temperature storage.

In this study, the complexity of the mesophilic microbiota was highlighted as none of the applied treatments achieved complete inactivation in TBC, thereby suggesting limited susceptibility to Sc-CO_2, PAA or both in SMC as compared to the single vegetative bacteria strains used. In addition to the greater diversity of microbial species the findings could be explained by the ability of sub-lethally injured cells to repair themselves and recover following Sc-CO_2/PAA50 and Sc-CO_2/PAA100 treatments. It is also understood that microorganisms can exist in a state where they are viable but non-culturable (VNC) (Oliver, 2005) and thus we consider that a transformation of vegetative microbial cells to a VNC state takes place following the different treatments applied in this study. Similar observations of cellular recovery were made for the TYMC with the exception of the Sc-CO_2/PAA100 treatment, which yielded no indication of surviving yeasts and molds, thereby demonstrating its efficacy. The resistance of yeasts and molds could be attributed to the mechanical strength of a rigid cell wall, which is a complex structure consisting of glucan cross-linked with chitin and cell wall proteins (Dielbandhoesing et al., 1998). However, to our knowledge no mechanistic study regarding the ineffectiveness of Sc-CO_2 and PAA on yeasts and molds has been conducted to date. Qiu et al. (2009) reported the difference in resistance between microorganisms to Sc-CO_2/PAA treatments at conditions

identical to our study. They reported that the molds (e.g. *Penicillum*, *Aspergillus* and *Verticillium*) were reduced by approximately 6 $\log_{10}$ after a 30-min Sc-CO_2/PAA treatment (9.9 MPa, 35 ^{o}C and 55 ppm), which was found to be lower than the inactivation obtained for bacteria (>10 $\log_{10}$) and yeast (8.4 $\log_{10}$), despite the fact that a longer treatment time was used. This could explain the findings of low reduction in TYMC following Sc-CO_2/PAA50 treatment and during the subsequent storage period applied in this study. Moreover, the overall higher resistance of yeasts and molds in comparison to aerobic bacteria could be reasoned by the low pH of the cheese and the growth temperature that seemed to favor the optimum growth of native yeasts and molds due to better adaptation to the storage conditions.

Typically, the pH analysis is important in determining a decontamination technique and/or system capabilities of killing microorganisms (Gurol et al., 2012). However, the bactericidal action predominantly caused by a proposed technique alone could be ascertained if the cell death was not caused by acidity or alkalinity of the treatment substrates following its application. The microbial safety of cheese in the present study should not be exclusively ascribed to low pH because proliferation of most microorganisms in cheese after Sc-CO_2/PAA50 was higher ($p \geq 0.05$) than that after Sc-CO_2/PAA100 although the pH values of cheese from both treatments were very close during storage ($p < 0.05$). In contrast, the proliferation of microorganisms in cheese under acidic pH environment during storage was reported in a previous work by Sinigaglia et al. (2008). With regard to consumer safety, it is worth mentioning that PAA is unstable and readily degrades into acetic acid and water, which alleviates concerns about residual toxicity (Block & Seymour, 2001; Kerkaert et al., 2011). Since the presence of PAA residue was

not detected in cheese after treatments (data not shown), we hypothesize that the decrease in pH observed could be the consequence of a mass exchange, between the cheese and the "covering" by-products of PAA that takes place during storage.

The determination of additive or synergistic treatment effects facilitates the evaluation of the efficacy of a hurdle technology. However, only few references have been found in the literature assessing synergistic effects of combined treatments in regard to inactivation by coupling Sc-CO_2 with pulsed electric field (Pataro et al., 2010; Spilimbergo et al., 2014b) and high power ultrasound (HPU) (Cappelletti, Ferrentino, & Spilimbergo, 2014; Ortuño et al., 2013) with cell suspension and liquid foods as substrates, respectively. With regards to solid foods, a combination of Sc-CO_2 (12 MPa and 35 °C for 30 min) and organic acids (acetic acid and lactic acid) had no synergistic effect on the reduction of a non-pathogenic *E. coli* and three pathogenic bacteria (*L. monocytogenes, Salmonella Typhimurium* and *E. coli* O157:H7) in fresh pork. A synergistic effect of Sc-CO_2 (12 MPa, 35 °C, 5 min) and HPU (10 W) was reported with a complete inactivation (10^9 CFU/g) of *L. monocytogenes* on dry cured ham, but it was not extended to any other microorganisms (Spilimbergo et al., 2014a). Similar effects were also obtained for Sc-CO_2 (10 MPa, 35 °C, 3 min) and HPU (10 W), which resulted in a complete inactivation of *E. coli* (10^8 CFU/g) on fresh cut carrots. In addition, maximum inactivation level of total coliforms (10^6 CFU/g) and yeasts and molds (10^5 CFU/g) was observed with Sc-CO_2 + HPU in 5 min at 12 MPa, 35 °C and 10 W (Ferrentino & Spilimbergo, 2014).

In this study, many synergisms between Sc-CO_2 and PAA100 were achieved for Sc-CO_2/PAA100-treated SMC contaminated with *L. innocua*, total aerobic bacteria, and total yeasts and molds and *G. stearothermophilus*. A plausible explanation for the additive

effects observed for *E. coli* could be that the cells already exhibited a very high sensitivity to permeabilization by Sc-CO_2 treatment alone, which suggests that cell sensitization to PAA by Sc-CO_2 may account for most of the synergy observed in the present work. Overall, with the exception of *G. stearothermophilus*, both Sc-CO_2/PAA100 and Sc-CO_2/PAA100PC achieved a 4.6 $\log_{10}$ or higher for all microorganism groups in SMC, which is broadly in agreement with the 5.0 $\log_{10}$ reduction food safety requirement for pasteurized foods stipulated by the FDA (FDA 2013; FDA, 2010).

5.6 Conclusion

A combination of Sc-CO_2 and PAA can be regarded as an effective approach for cheese preservation at ambient temperatures based on the initial microbial load in the commercial SMC of 10^3 to 10^4 CFU/g and the fact that much higher initial cell concentrations were studied. However, use of either of these methods for stand-alone processing of the shredded cheese did not produce a sufficient reduction of microorganisms and lacked the treatment synergism obtained when combining Sc-CO_2 and PAA. The promising findings of this study should encourage further research on Sc-CO_2/PAA hurdle strategies for use with different types of cheeses and challenges with other relevant microorganisms as well as their impact on physico-chemical and sensory product quality aspects. The data from these proposed follow-up studies could contribute to a more comprehensive understanding of Sc-CO_2/PAA efficacy and its potential for commercial applications.

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