J. Dairy Sci. 89:872–881

Continuous Flow Nonthermal CO_2 Processing: The Lethal Effects of Subcritical and Supercritical CO_2 on Total Microbial Populations and Bacterial Spores in Raw Milk

B. G. Werner and J. H. Hotchkiss[1]
Department of Food Science, Cornell University, Ithaca, NY 14853

ABSTRACT

The effect of pressurized (<50 MPa) CO_2 as a nonthermal process for bacterial reduction in raw skim milk was examined using a unique pressurized continuous flow system. The lethal effects of subcritical and supercritical CO_2 applied at different temperatures and pressures toward total native psychrotrophic microbial populations, total inoculated *Pseudomonas fluorescens*, and total inoculated spore populations were studied and compared. Pressures between 10.3 and 48.3 MPa; temperatures of 15, 30, 35, and 40°C; and CO_2 concentrations of 0, 3, 66, and 132 g/kg of milk were studied. For both native populations and inoculated *P. fluorescens*, greater total microbial lethality was observed under supercritical CO_2 conditions than under subcritical CO_2 conditions. At 30°C, there was no effect on total microbial lethality of increasing pressure up to 20.7 MPa with either 66 or 132 g/kg of CO_2; at 35°C, there was a positive relationship between pressure and lethality at CO_2 levels of 132 g/kg, but no relationship at 66 g/kg of CO_2. For total microbial populations and *P. fluorescens*, CO_2 applied at 132 g/kg at 30°C and pressures of 10.3 to 20.7 MPa resulted in an average standard plate count reduction of 3.81 and 2.93 log, respectively; at 35°C and 20.7 MPa, maximum reductions achieved were 5.36 and 5.02 log, respectively. For both total microbial populations and inoculated *P. fluorescens*, CO_2 exhibited a greater overall lethal effect at 132 g/kg than at 66 g/kg and a greater effect at 35°C than at 30°C. At 24.1 and 48.3 MPa and 40°C, microbial lethality in raw aged milk treated with 3 g/kg of CO_2 was not significantly different than that observed for uncarbonated milk; lethality achieved in milk treated with 132 g/kg of CO_2 was significantly higher than that achieved in these 2 low-level CO_2 treatments. No treatment studied had any significant impact on spore populations. Our work shows that, using the studied system, pressurized CO_2 results in greater microbial lethality in milk above critical temperatures than below and suggests that a critical concentration threshold level of CO_2 is required for lethal effects. Our work also suggests that supercritical CO_2 processing in a continuous flow system can achieve reductions in some microbial populations equal to or better than that typically achieved during high-temperature, short-time pasteurization.
Key words: supercritical carbon dioxide, carbon dioxide, milk, lethality

INTRODUCTION

Pressurized CO_2 as a potential nonthermal method for microbial reduction in raw foods and food products has gained interest particularly within the past decade. Carbon dioxide as a processing aid has several advantages, including low cost, ease of removal from the product after use, and status in the United States as an FDA GRAS (Generally Recognized As Safe) substance. Carbon dioxide can be used to achieve microbial reduction in foods without use of high temperatures; this poses an attractive processing alternative over currently used thermal processes such as pasteurization, which result in a range of undesirable texture and flavor changes in processed fluid milk. Pasteurization regimes for fluid milk are selected to primarily produce a safe product while secondarily optimizing shelf-life and minimizing degradative and organoleptic changes. As a result of this balance, pasteurized milk typically has a maximum refrigerated shelf-life of ≤21 d and possesses altered organoleptic qualities, such as cooked flavors, caused by thermal processing (Boor, 2001). If nonthermal CO_2 processing can result in microbial reduction equal to or greater than that achieved during pasteurization, such an alternative process could result in a greatly enhanced product in terms of shelf-life, organoleptic properties, or both. Nonthermally processed raw milk can potentially also be used to produce a broader range of cheeses than is currently feasible in the United States using pasteurized milk, opening up new markets within this important industry.

At and above the critical temperature of 31°C and critical pressure of 7.35 MPa (critical point), CO_2 exists

Received May 7, 2004.
Accepted December 19, 2005.
[1]Corresponding author: jhh3@cornell.edu

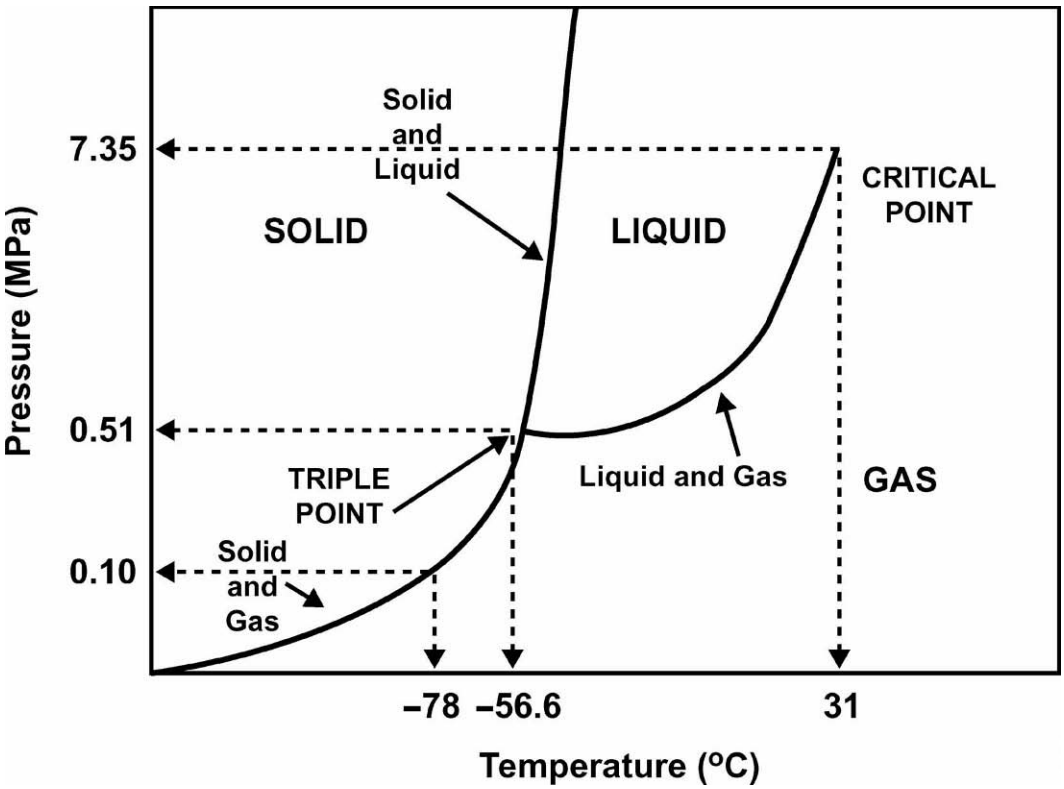

Figure 1. Phase diagram of pressure (MPa) vs. temperature (°C) for CO_2. Indicated are the regions where solid, liquid, gas, and supercritical phases exist. The critical point, or pressure and temperature above which gas and liquid becomes indistinguishable, is indicated. The triple point, the pressure and temperature at which solid, liquid, and gas exist in equilibrium, is indicated.

as a gas and liquid in equilibrium; increasing temperature or pressure above this critical point does not result in any further phase changes. Below the critical point and above the triple point of –56.6°C and 0.52 MPa, CO_2 exists as a liquid. As pressure increases within these temperature and pressure boundaries, a transition curve is reached where CO_2 exists as a solid and liquid in equilibrium (Figure 1; Weast et al., 1986). Liquid (subcritical) and supercritical forms of CO_2 have been investigated as processing aids; both forms have been shown to be antimicrobial, thought in part to be due to solubility properties of CO_2. In particular, supercritical CO_2 has been investigated for its extraction capabilities based on its excellent solubility in lipids. Thus, CO_2 could readily permeate across cell membranes, potentially also disrupting membrane integrity by dissolving lipids. Membrane-bound enzyme activities may be negatively impacted, and membrane potentials may be altered by concentrations of CO_2 within the membrane. Hong and Pyun (2001) observed that cells of *Lactobacillus plantarum* exposed to the subcritical temperature 30°C exhibited a high level of release of intracellular ions; cells were rendered nonviable by the treatments but remained intact. Those investigators hypothesized that the lethal effect of CO_2 in these treatments was due to the good solvent properties of the phase tested. In their work examining the effects of both supercritical and subcritical CO_2 on yeast cells, Isenschmid et al. (1995) found similar results in viability loss with no associated loss in cell integrity. Cell lysis was observed only at temperatures <18°C.

Much work has been completed to investigate commercial applications and lethal effects of supercritical CO_2 in batch systems; considerably fewer applications have investigated semicontinuous or continuous flow systems (Kamihira et al., 1987; Arreola et al., 1991; Spilimbergo et al., 2002). Supercritical CO_2 is generally recognized for its effectiveness in reducing microbial populations, including bacteria, yeast, and mold, to the extent that sterilization can be achieved. Kamihira et al. (1987) used a supercritical CO_2 extraction apparatus to successfully achieve sterilization conditions ($\geq 10^6$-cfu/g reduction of treated bacterial and yeast cells) after a 2-h treatment at 35°C and 20.3 MPa. Other studies have examined the effectiveness of subcritical CO_2 treatments, hypothesized to be potentially less severe in nature than supercritical CO_2 treatments (Haas et al., 1989; Ballestra et al., 1996; Ballestra and Cuz, 1998;

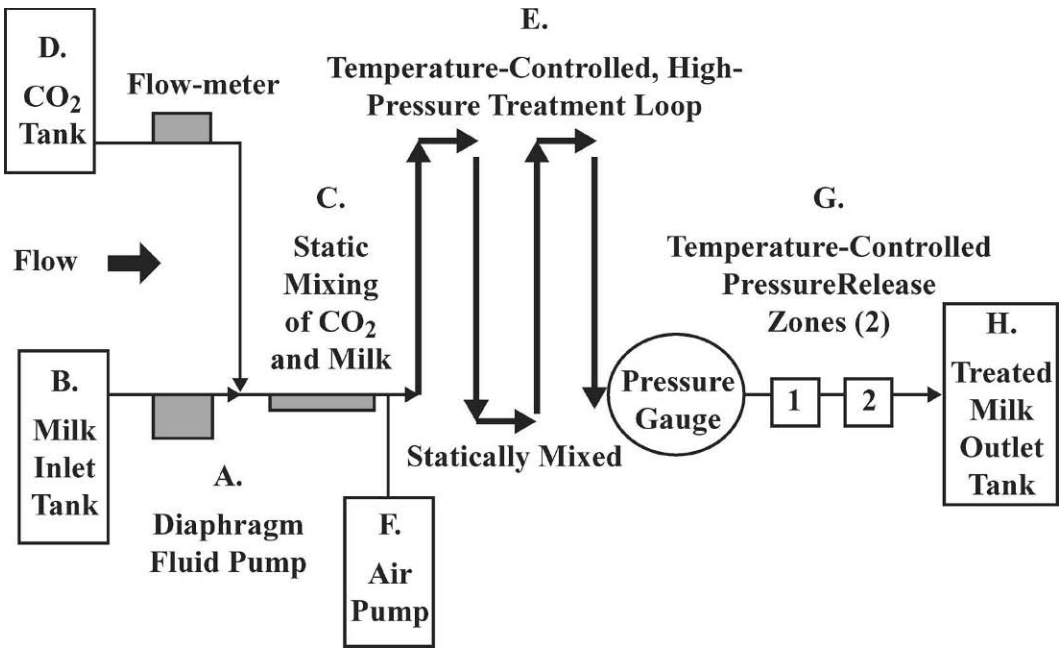

Figure 2. Schematic of the Praxair high-pressure CO_2 milk pilot plant processing unit.

Hong et al., 1999; Calvo and Bacones, 2001; Erkmen, 2001). Only a few known studies have compared the effectiveness of subcritical and supercritical CO_2 systems in reducing microbial populations in food (Kamihira et al., 1987; Lin et al., 1992; Isenschmid et al., 1995; Ishikawa et al., 1995; Sirisee et al., 1998; Dillow et al., 1999). Hong et al. (1999) and Lin et al. (1992) both used static systems to examine inactivation of yeast by supercritical and subcritical CO_2 (pressures of 6.8 to 20.4 MPa and temperatures of 25 and 35°C). They similarly found greater impacts on cell lethality of supercritical CO_2 than subcritical CO_2 treatments. Few studies have examined continuous flow systems, as depicted in a recent review of nonthermal microbial inactivation using pressurized CO_2. A comprehensive listing and description of the applications to date are given; batch operations represent the vast majority of those illustrated (Spilimbergo and Bertucco, 2003). Additionally, this application list indicates that only a few research groups have investigated effects of high-pressure CO_2 on microbial inactivation in milk; all such applications cited involved batch, not continuous or semicontinuous systems.

The goal of this study was to determine the potential of pressurized CO_2 as a nonthermal processing aid for bacterial reduction or pasteurization of raw fluid milk using a unique flow through pressurized system (Wildasin et al., 2000, 2002). Specifically, we wanted to compare the antimicrobial effectiveness of subcritical and supercritical CO_2 applied in a continuous flow system on indigenous psychrotrophic vegetative cells, on *Pseudomonas fluorescens* (a common milk spoilage microorganism), and on spores. Further, we wanted to determine the influence of CO_2 concentration, flow rate, temperature, and pressure on the process. It is not our intent to report on the effects of the process on the resulting analytical properties of milk; this comprised a separate study by our group and will be reported elsewhere.

MATERIALS AND METHODS

Bulk raw skim milk was obtained from the Cornell University Training and Research Center (Dryden, NY). Milk was obtained from a bulk tanker in individual 38- to 54-kg lots and was stored at 4°C for 6 to 8 d to obtain bacterial levels of $\geq 10^6$ cfu/g or for a maximum of 2 d when inoculated with bacterial spores or with *P. fluorescens*. All tests were performed using 2 different lots of milk obtained during different times of the year. Each lot of milk was split for duplicate testing. The standard plate count (**SPC**) assay (Houghtby et al., 1992), a standard method, was performed in duplicate to measure total microbial viability for all treated and untreated milk samples.

A high-pressure CO_2 milk pilot plant processing unit (Praxair, Inc., Joliet, IL; Figure 2) was used to treat milk at temperatures of 15, 30, 35, and 40°C; pressures of 10.3, 13.8, 17.2, 20.7, 24.1, and 48.3 MPa; and CO_2 levels of 0, 66, and 132 g/kg. Uncarbonated milk and

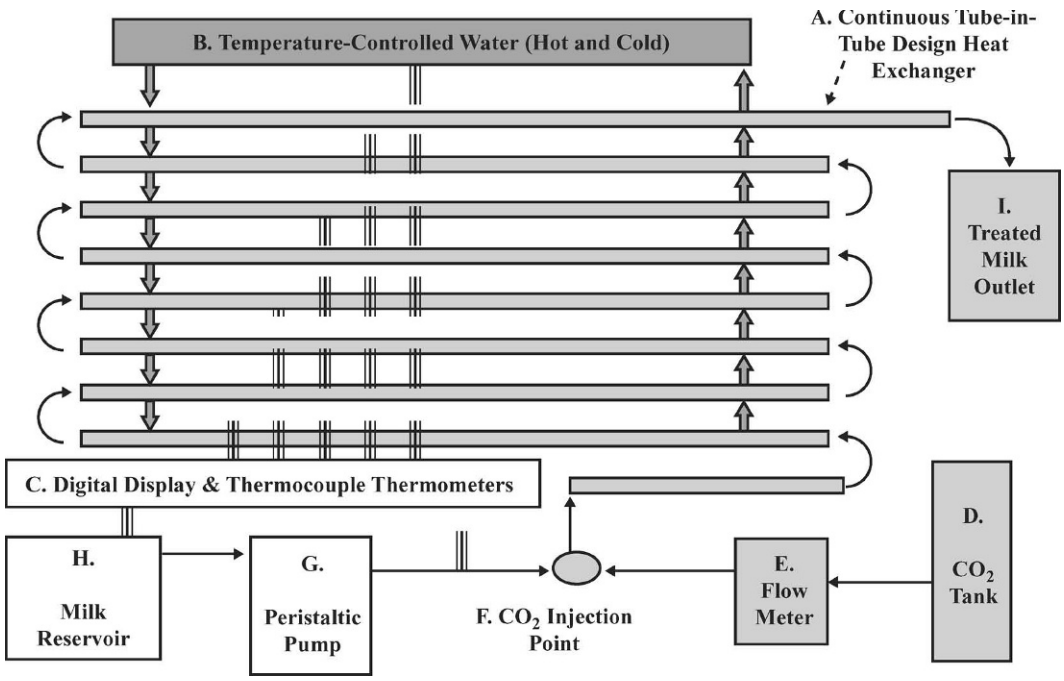

Figure 3. Schematic of the laboratory-scale bench-top pasteurizer. To provide a simplified illustration, only 9 of 19 treatment loops are shown.

milk precarbonated to 3 g/kg were also processed at the same pressure and temperature with the unit, but without further inline CO_2 addition. This flow through system uses a diaphragm pump (Figure 2A) to feed milk from an inlet sample tank (Figure 2B) through a holding tube (Figure 2C), where the milk meets and statically mixes with an inlet line of pressurized CO_2. A bottom-siphon liquid CO_2 pressurized tank (Figure 2D) is fed through at regulated pressures ≥4.8 MPa. Milk can also be introduced into the system and processed without inline CO_2 if needed. Carbonated milk then enters a 600-mL volume temperature-controlled treatment holding tube (Figure 2E) that can be pressurized to preset levels of up to 55.2 MPa. A pressurized air-driven pump (Figure 2F) cycles or controls the movement of milk in and out of this treatment loop. After the treatment loop, the carbonated milk passes through 2 successive temperature-controlled, pressure-release areas (Figure 2G) that serve to prevent product freezing upon pressure drop and allow for evacuation of CO_2 as gas from the product. The degassed milk is then released through an exit port (Figure 2H), where it can be collected in bulk or in small sample containers as needed. The range of process duration achieved with this unit (or total residency time) was 10 min. Milk flow rates achieved were 125 to 195 g/min.

Milk with CO_2 added at 3 g/kg was done so prior to further treatment, as this level was below the minimum achievable inline. A bench-top scale, gas-injection modified pasteurizer was used to precarbonate 38- to 54-kg lots of raw skim milk (Figure 3). This system incorporates a tube-in-tube design (6.35-mm stainless steel tubing within a 12.7-mm copper tube) heat exchanger with nineteen 132-cm jacketed continuous sections (Figure 3A). Water from temperature-controlled water baths (Figure 3B) is pumped through 8 inlet sections for cooling, heating, or both as needed for process conditions; thermocouple thermometers at 19 locations throughout the sections allow for continuous monitoring of temperature conditions via a digital display reader (Figure 3C). Carbon dioxide from a pressurized tank (Figure 3D), regulated by a microneedle valve and flow meter (Figure 3E), was injected through a sparging stone at a rate of 145 cc/min into the milk fluid flow within the first 5 cm of the first jacketed section (Figure 3F). A laboratory-scale peristaltic pump (Figure 3G) was used to introduce milk (Figure 3H) directly into this section and through the extent of the unit at a flow rate of 60 cc/min. Ice water was used to maintain jacket temperature throughout the system at 4°C during carbonation. The carbonation level of the pooled milk was measured using the methods of Glass et al. (1999). Further processing of this precarbonated milk was accomplished using the high-pressure CO_2 milk pilot plant processing unit with no added inline CO_2 during the test processes used.

Comparison of Subcritical and Supercritical CO_2 Treatments on Microbial Lethality (Native Psychrotrophic Populations)

To determine the effects of supercritical vs. subcritical CO_2 phases on total native milk psychrotrophic microbial populations, aged raw milk was processed using the high-pressure CO_2 milk pilot plant processing unit at temperatures of 30°C (subcritical temperature) and 35°C (supercritical temperature); pressures of 10.3, 13.8, 17.2, and 20.7 MPa; and CO_2 concentrations of 0, 66, and 132 g/kg of CO_2.

Comparison of Subcritical and Supercritical CO_2 Treatments on Microbial Lethality (P. fluorescens)

Pseudomonas fluorescens R1-232, a strain isolated from raw milk and a potential milk spoilage organism (Wiedmann et al., 2000), was used to inoculate raw milk to levels of 10^6 to 10^7 cfu/g using procedures previously described by our group (Martin et al., 2003). To determine the effects of supercritical vs. subcritical CO_2 phases on *P. fluorescens* populations, inoculated raw milk was processed using the high-pressure CO_2 milk pilot plant processing unit at temperatures of 30°C (subcritical temperature) and 35°C (supercritical temperature); pressures of 10.3, 13.8, 17.2, and 20.7 MPa; and CO_2 concentrations of 0, 66, and 132 g/kg of CO_2.

Influence of CO_2 Concentration, CO_2-to-Product Ratio, Temperature, and Pressure on the Process

To determine the effect of CO_2 concentration during the process on microbial lethality (native psychrotrophic populations), milk was processed at CO_2 levels of 0, 3, 66, and 132 g/kg. The effect of milk flow rate and treatment temperature on the process was examined at 15 and 40°C, 48.3 MPa, 3 g/kg of CO_2, and milk flow rates of 190 and 160 g/min. Alterations in flow rate of the precarbonated milk were examined to determine the effect of extending the treatment time within the processing loop. The effect of pressure on the process was examined at 24.1 and 48.3 MPa, 40°C, and 0, 3, and 132 g/kg of CO_2.

Impact of CO_2 Treatment Process on Lethality of Bacterial Spores

Four isolates of *Bacillus cereus* were used to inoculate milk at levels of 10^1 and 10^6 spores/g. Three isolates were obtained from the New York Milk Quality Improvement Program Voluntary Shelf-Life Program at Cornell University (original source milk), and the fourth isolate was ATCC 14579, a type strain (American Type Culture Collection, Manassas, VA). Spore preparation protocols followed were as previously described (Werner and Hotchkiss, 2002), and a composite strain spore suspension was used as inoculum. The Spore Test (Frank et al., 1992) was performed in duplicate to measure the concentration of total viable spores of inoculum and of treated or untreated aged and inoculated milk samples. All spore viability testing was performed immediately after treatment. Aged raw milk was tested for bacterial spore viability after treatment at 15, 30, 35, and 40°C; pressures of 10.3, 24.1, and 48.3 MPa; and CO_2 levels of 0, 3, 66, and 132 g/kg. Raw milk inoculated to levels of 10^1 and 10^6 spores/mL was tested for bacterial spore viability after treatment at 40°C, 48.3 MPa, and 132 g/kg of CO_2.

CO_2 as a Nonthermal Processing Aid for Pasteurization of Raw Fluid Milk

The bench-top scale pasteurizer (Ma and Barbano, 2003) was used to pasteurize 2 lots of aged raw skim milk. Pasteurization was performed at 74.5°C for 15 s and 76°C for 45 s, parameters that meet the regulatory definition of pasteurization of 72°C for 15 s (U.S. Department of Health and Human Services, 1999) and that we considered typical of parameters used within the industry. Pasteurized samples were used as a baseline for microbial reductions achieved typically during pasteurization; these results were compared with microbial reductions achieved during CO_2 treatments as measured by SPC and Spore Test.

Statistical Analysis

Statistical analysis of data was performed using Minitab Statistical Software, (Release 13.30, Minitab, Inc., State College, PA). Analysis of variance was performed to detect significant differences ($P \leq 0.05$) in SPC or spore counts between different CO_2, pressure, and temperature treatments.

RESULTS AND DISCUSSION

Comparison of Subcritical and Supercritical CO_2 Treatments on Microbial Lethality (Native Psychrotrophs and P. fluorescens)

The microbial counts of aged raw milk treated with 0, 66, and 132 g/kg of CO_2 at 30°C (subcritical temperature) and 35°C (supercritical temperature) and at 0, 10.3, 13.8, 17.2, and 20.7 MPa are recorded in Table 1; data for similarly treated raw milk inoculated with *P. fluorescens* are recorded in Table 2. At 30°C, CO_2 existed in the liquid or subcritical phase at all pressures tested; similarly, at 35°C and all pressures tested, CO_2 existed in the supercritical phase. For both aged and inocu-

Table 1. Standard plate count (log_{10} cfu/g; mean and standard deviation) of aged raw milk treated with 0, 66, and 132 g/kg of CO_2 at temperatures of 30°C (subcritical) and 35°C (supercritical) and pressures of 0, 10.3, 13.8, 17.2, and 20.7 MPa

CO_2	Temperature	0 MPa	10.3 MPa	13.8 MPa	17.2 MPa	20.7 MPa
0 g/kg	30°C	6.87 (0.49)	—	—	—	—
	35°C	7.28 (0.20)	—	—	—	—
66 g/kg	30°C	—	5.68 (0.11)	5.73 (0.18)	5.60 (0.18)	5.53 (0.40)
	35°C	—	4.17 (0.04)	3.73 (0.33)	3.73 (0.14)	3.65 (0.07)
132 g/kg	30°C	—	3.26 (0.20)	3.04 (0.37)	2.99 (0.16)	2.95 (0.48)
	35°C	—	3.51 (0.41)	2.55 (0.21)	2.10 (0.14)	1.92 (0.08)

lated milks, all CO_2 treatments significantly reduced the number of total viable cells as compared with the untreated samples and at all pressures tested ($P < 0.05$). At 66 g/kg of CO_2 at 30 and 35°C, increasing pressure had no added effect on lethality. At 132 g/kg of CO_2, there was no relationship between pressure and lethality at 30°C, but a positive relationship existed at 35°C. At 66 g/kg of CO_2, aged and inoculated milks treated at 30°C resulted in an average 1.24- and 1.11-log_{10} cfu/g reduction, respectively; at 35°C, the average reduction was significantly higher at 3.26 and 3.32, respectively. At 132 g/kg of CO_2, raw and inoculated milk treated at 30°C resulted in an average 3.81- and 2.93-log_{10} cfu/g reduction, respectively; at 35°C, this reduction was 3.77- and 3.67-log_{10} cfu/g, respectively, at the lowest pressure of 10.3 MPa, and the reduction was 5.36- and 5.02-log_{10} cfu/g, respectively, at the highest treatment pressure of 20.7 MPa. For both aged and inoculated milks, CO_2 had a greater overall lethal effect at 132 g/kg than at 66 g/kg and a greater effect at 35 than at 30°C. Watanabe et al (2003), using a continuous flow system, similarly found that supercritical CO_2 treatments resulted in greater reductions of *Escherichia coli* than treatments with liquid CO_2 and that both increasing temperature and pressure increased lethality under supercritical conditions.

We tested raw milk that had been aged at 4°C, resulting in microbial populations that were predominantly psychrotrophic and that could feasibly contribute to spoilage of stored raw milk; psychrotrophic genera commonly found in raw milk include *Acinetobacter-Moraxella*, *Flavobacterium*, *Enterobacter*, *Alcaligenes*, *Bacillus*, *Pseudomonas*, and *Arthrobacter* (Chambers, 2003). Gram-negative bacteria are the most commonly found psychrotrophs in raw milk, and *Pseudomonas* spp. account typically for ≥50% of this population; within the pseudomonads, *P. fluorescens* is the species that has been reported to dominate (Chambers, 2003). We did not attempt to identify the psychrotrophs present in the aged raw milk tested, but selected a spoilage organism commonly found in raw milks, *P. fluorescens*, to study as a model organism. As previously discussed and as illustrated in Tables 1 and 2, microbial populations in aged milk and *P. fluorescens* in inoculated milk responded similarly to subcritical vs. supercritical CO_2 pressure treatments. However, although overall trends in lethality for each treatment were similar, reductions in *P. fluorescens* in response to subcritical treatments at 132 g/kg of CO_2 were generally lower overall than those measured for aged milk populations (Tables 1 and 2). When supercritical conditions were applied, bacterial reductions were similar for both inoculated and aged milks.

Our findings of a greater lethal effect of CO_2 at 132 g/kg than at 66 g/kg, particularly as temperature and pressure increase, can be explained by the high affinity of CO_2 for membrane phospholipids and the increasing solubility of CO_2 at increasing temperatures and pressures (Isenschmid et al., 1995; Spilimbergo et al., 2002). Carbon dioxide can accumulate within cellular mem-

Table 2. Standard plate count (log_{10} cfu/g; mean and standard deviation) of raw milk inoculated with 6 to 7 log_{10} cfu/mL of *Pseudomonas fluorescens* and treated with 0, 66, and 132 g/kg CO_2 at temperatures of 30°C (subcritical) and 35°C (supercritical) and pressures of 0, 10.3, 13.8, 17.2, and 20.7 MPa

CO_2	Temperature	0 MPa	10.3 MPa	13.8 MPa	17.2 MPa	20.7 MPa
0 g/kg	30°C	6.57 (0.24)	—	—	—	—
	35°C	6.80 (0.37)	—	—	—	—
66 g/kg	30°C	—	5.25(0.03)	5.55 (0.21)	5.57 (0.44)	5.48 (0.11)
	35°C	—	3.55 (0.21)	3.60 (0.13)	3.47 (0.29)	3.31 (0.34)
132 g/kg	30°C	—	3.85 (0.21)	3.71 (0.34)	3.42 (0.22)	3.51 (0.14)
	35°C	—	3.13 (0.05)	2.60 (0.18)	2.65 (0.22)	1.78 (0.31)

branes, resulting in reordering of lipid structures and changes in cell permeability and fluidity (Isenschmid et al., 1995). Hong and Pyun (2001), in their direct examination of cellular effects in *Lactobacillus plantarum* as a result of pressurized CO_2 treatments, found an increased release of intracellular ions, increased proton permeability, disruptions in glycolysis, and changes in membrane-bound enzymatic activities; loss of membrane integrity was a key effect. Work by Tomasula and Boswell (1999) showed that increasing pressure has a greater influence on the solubility of CO_2 in milk than increasing temperature; temperatures of 25, 38, and 50°C and pressures of up to 6.9 MPa were examined. Ishikawa et al. (1995) found no real effect of pressure increases on viability of yeast and lactic acid bacteria treated at 25°C with CO_2 pressures of 8, 15, 20, or 25 MPa; at 35°C, above the critical temperature of CO_2, increasing pressure did enhance lethality. Work with supercritical CO_2-treated orange juice (Arreola et al., 1991) showed that D-values decreased as pressure treatments increased between 8.3 and 33.1 MPa. Our findings of generally greater lethality at CO_2 treatment temperatures of 35°C (above the critical temperature of 31°C) than at near critical treatments of 30°C can be explained by the effects of increased temperature on membrane fluidity and permeability, which can increase the amount of CO_2 able to pass through into the cytoplasm; subsequent reductions in cell buffering capacity can result in abnormalities in cell metabolic processes. Differences in membrane composition between bacterial genera may explain the differences observed in lethality of *P. fluorescens* compared with total psychrotrophic populations; these differences might have become apparent under subcritical treatment where the solubility of CO_2 in lipid-rich membranes is lower than under supercritical conditions and where temperature may differently affect membrane fluidity.

Our results and data from these investigations suggest that supercritical CO_2 is more effective in achieving cell lethality than subcritical CO_2, that the phase condition of CO_2 is responsible for the increased effect at above critical temperatures, and that increases in pressure enhance the effects of supercritical CO_2, perhaps by facilitating permeation into cells.

Influence of CO_2-to-Product Ratio, Flow Rate, Temperature, and Pressure

At 40°C, milk carbonated to 132 g/kg of CO_2 resulted in a 3.32-$\log_{10}$ cfu/g reduction at 24.1 MPa and a 4.36-$\log_{10}$ cfu/g reduction at 48.3 MPa. At this same temperature, milk treated with 3 g/kg of CO_2 resulted in a 0.79-$\log_{10}$ cfu/g reduction at 24.1 MPa and a 2.51-$\log_{10}$ cfu/g reduction at 48.3 MPa. The reductions at the 3-g/kg of CO_2 level were not significantly different than those achieved in similar temperature- and pressure-treated milk without added CO_2, suggesting that CO_2 was present below threshold lethal levels and that effects were due to combined influences of temperature and pressure. These data are depicted in Figure 4 as changes in SPC from initial ($\log_{10}$ cfu/g).

We used 2 treatment flow rates at the bottom and top of the range feasible with the milk processing unit to determine whether increased contact time (reduced flow rate) resulted in greater microbial lethality. There was no significant difference between flow rates of 190 and 160 g/min at either treatment temperature; the level of CO_2 (present or absent at low levels) did not influence treatment results. Shimoda et al. (1998) found that in a continuous flow system, the flow rate affected inactivation of *Saccharomyces cerevisiae*; however a doubling of the rate was required to see an effect, and flow rates studied were one-tenth of that examined in our study. The flow rates tested in our study were most likely not low enough to result in measurable differences in lethality effects; it would be anticipated that, in a flow through system, the effects of pressurized CO_2 would be enhanced at some increased contact time achieved at some reduced flow rate. The flow rates we tested were close to the minimum rates possible with the equipment used. Spilimbergo et al. (2003) were able to regulate flow rates between 0 and 25 g/min using a semicontinuous system that applied CO_2 pressures of 5.5 to 8 MPa between 38 and 40°C; by being able to reduce the flow rate to 0, these researchers could test much greater treatment contact times (0 to 30 min) than were feasible in our study. However, even at the maximum flow rate and minimum contact times tested, significant lethality was measured.

Standard plate counts of uncarbonated and carbonated (3 g/kg) milk treated at 24.1 MPa were not significantly different at 15 or 40°C and were not significantly different from similarly carbonated milk treated at 48.3 MPa at 15°C. At higher CO_2 levels, all milks treated at 40°C and 48.3 MPa resulted in significantly lower SPC than any milk treated at 15°C. Calvo and Balcones (2001) found that microbial reductions achieved in milk with CO_2 pressurized to up to 3 MPa were only significant when treatment temperatures were >30°C. Isenschmid et al. (1995) observed that at treatment temperatures >27°C, there was no cell lysis in response to any pressurized CO_2 treatment, although viability dropped to about 1%. The greatest loss of cell integrity, as measured by lysis, was observed at a temperature of 13°C. Isenschmid et al. (1995) hypothesized that loss of cell viability at temperatures >18°C was influenced primarily by the concentration of dissolved CO_2 and the temperature; at temperatures ≤18°C, loss of cell

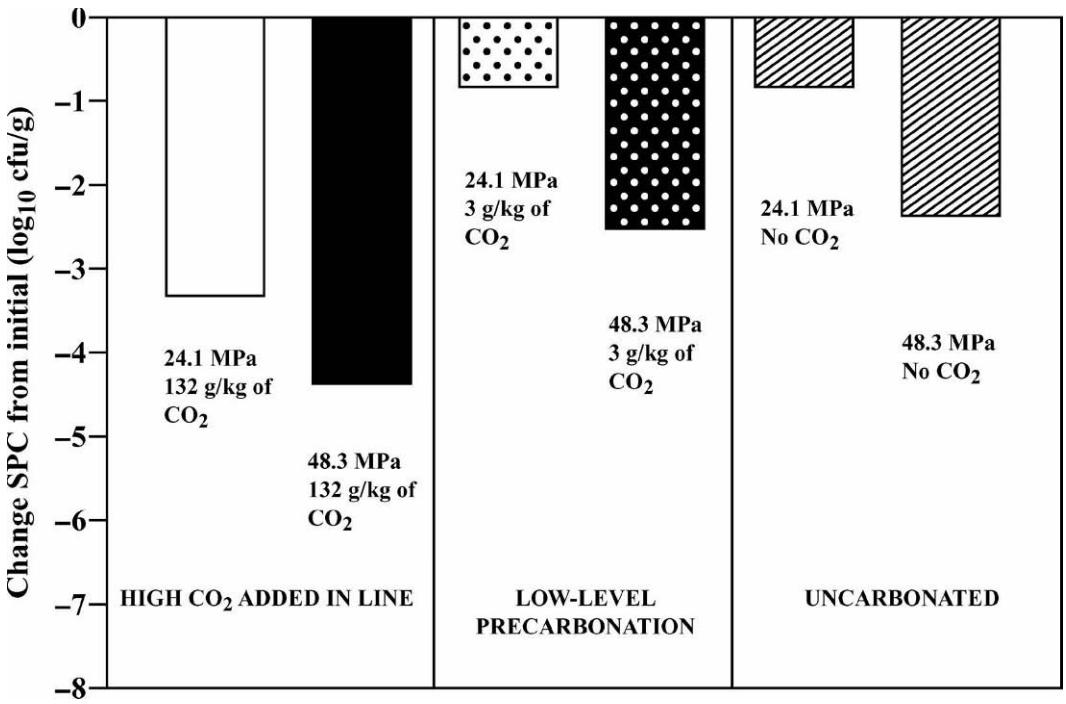

Figure 4. Change in standard plate count (SPC) from initial count (log_{10} cfu/g) of aged raw skim milk, carbonated inline to 132 g/kg, precarbonated to 3 g/kg, or uncarbonated, and treated at 40°C and 24.1 or 48.3 MPa.

viability was due primarily to the solvent characteristics of CO_2 and resulting loss of cell density. Our results show only a 1-log reduction in viable microbial cells at 15°C under 48.3 MPa of pressure, but at 40°C, the reduction was approximately 2.4 log cycles. At 40°C and 24.1 MPa of pressure, lethality was not significantly different than what was measured at 15°C; however, as previously discussed, the level of CO_2 was thought to be below lethal threshold levels; thus, effects were due to pressure and temperature alone. To test the hypothesis of Isenschmid et al. (1995), microbial lethality in response to CO_2 levels above lethal threshold levels would need to be examined at the 2 treatment temperatures (15 and 40°C) below and above their temperature threshold of 18°C.

Impact of CO_2 Treatment Process on Lethality of Bacterial Spores

No treatment applied to any inoculated level or aged raw milk resulted in a significant reduction in bacterial spore numbers ($P < 0.05$). These results were not unexpected, as more extreme pressures and temperatures applied during much greater time periods have been found to only achieve minimal spore lethality. Enomoto et al. (1997) reported a 7-log reduction in total viable spores of *Bacillus megaterium* after a static pressurized CO_2 treatment of 5.9 MPa for 30 h at 60°C. Ballestra and Cuz (1998) reported no effect on *Bacillus subtilis* spore viability after a static pressurized CO_2 treatment of 5 MPa for 1 h when temperatures were <80°C; at 80°C, a 3.5-log reduction in viable spores was measured after a 1-h treatment, and the same reduction was observed at 90°C after 50 min. Kamihira et al. (1987), using treatment parameters closer to those used in our study, found no effect on *B. subtilis* spore viability after a maximum 2-h semicontinuous flow CO_2 treatment at 20 or 35°C and 10.1 or 20.3 MPa. Ishikawa et al. (1997) reported a 3-log reduction of *Bacillus polymyxa*, *B. cereus*, and *B. subtilis* spores after a static pressurized CO_2 treatment for 30 min, 30 MPa, and 40°C. Although our study incorporated a similar treatment temperature and pressure, we used a flow through system, where a 30-min treatment was not feasible. Additionally, the method these researchers used to isolate spores from a mixed vegetative and spore cell culture (heat treatment at 80°C for 20 min to kill vegetative cells) would most likely have initiated spore germination, as prolonged heat can serve as a germination trigger. Spores undergoing germination would be expected to be more susceptible to further lethal treatments than dormant spores (Smoot and Pierson, 1982). Unpublished research from our lab suggests that high-pressure CO_2 in a continuous flow system results in initiation of spore germination rather than spore lethality; these results are confirmed in part by Furukawa et al.

(2004), who found that applying CO_2 in a hydrostatic system at 6.5 MPa and 35°C initiated germination of bacterial spores to between 40 and 70%; spore death, however, was not observed.

CO_2 as a Nonthermal Processing Aid for Pasteurization of Raw Fluid Milk

Milk pasteurized at 74.5°C for 15 s resulted in a 2.00-$\log_{10}$ reduction in SPC and no effect on total viable spores, which remained at a level of 1.90 $\log_{10}$ spores/mL after treatment. Milk pasteurized at 76°C for 45 s resulted in a 4.5-$\log_{10}$ reduction in SPC and no effect on total viable spores, which remained at a level of 2.10 $\log_{10}$ spores/mL after treatment. These 2 pasteurization treatments represent a low- and high-level range of treatment severity. The Pasteurized Milk Ordinance regulatory bacterial limit for grade "A" raw milk for pasteurization is ≤300,000/mL as commingled raw milk; the limit for grade "A" pasteurized milk is ≤20,000/mL. Thus, we may infer that at the maximum bacterial limits, the Pasteurized Milk Ordinance only requires that pasteurization achieve a reduction of slightly >1 $\log_{10}$ cfu/mL. To achieve optimum refrigerated shelf-life and to reduce pathogens to a nondetectable level, greater reductions in microbial levels are usually obtained through pasteurization protocols above recommended parameters. We would expect that any alternative processing treatment would need to parallel these reductions.

We can compare bacterial reductions achieved using the pressurized CO_2 process with the implied Pasteurized Milk Ordinance minimum pasteurization reduction and reductions achieved using the laboratory pasteurizer and standard pasteurization parameters described previously. At carbonation levels of 66 and 132 g/kg, temperatures of 30 and 35°C, and pressures of 10.3, 13.8, 17.2, and 20.7 MPa, total psychrotrophic microbial reductions ranged from 1.14 to 5.36 $\log_{10}$ cfu/g (Table 1). Treatments at 35°C, above the critical temperature of 31°C, generally resulted in greater lethality than 30°C subcritical temperature treatments. At 132 g/kg of CO_2, 40°C, and 48.3 MPa, a mean reduction of 4.36 $\log_{10}$ cfu/g was achieved, but at 24.1 MPa, a mean reduction of 3.31 $\log_{10}$ cfu/g was achieved (Figure 4). These reductions were greater than the minimum reduction anticipated based on Pasteurized Milk Ordinance regulations (≥1 $\log_{10}$ cfu/g), as discussed in the previous paragraph. Milk treated at temperatures ≥35°C with 132 g/kg of CO_2 resulted in reductions within the anticipated pasteurization target reduction range of 2.00 to 4.50 $\log_{10}$ cfu/g; reductions achieved at 132 g/kg of CO_2 and 30°C were also within this range.

Not all treatments tested resulted in a minimum 1-$\log_{10}$ cfu/g reduction. At 15 and 40°C, 3 g/kg of CO_2, and 24.1 MPa, microbial reductions were <1 $\log_{10}$ cfu/g at 0.29 and 0.86 $\log_{10}$ cfu/mL, respectively. Microbial reductions >1 $\log_{10}$ cfu/g were only achieved for this CO_2 treatment level at the higher pressure of 48.3 MPa; at 15 and 40°C, microbial reductions were 1.25 and 2.51 $\log_{10}$ cfu/mL, respectively. However, reductions achieved for any treatment were not significantly different between milk treated at 3 and 0 g/kg of CO_2; reductions were due to pressure and temperature effects. Our data suggest that an effective threshold or critical concentration of dissolved CO_2 is required and that 3 g/kg is below the threshold for the system studied. Isenschmid et al. (1995) suggested that a threshold temperature existed for a constant dissolved CO_2 concentration where cell death suddenly increases beyond an initial low plateau. In their study of yeast cell viability, 90% of the cells remained viable after a 3-g/kg of CO_2 treatment even when the temperature was increased from 8 to 43°C. This research group similarly suggested a threshold CO_2 concentration at a set temperature upon which a similar sudden increase in lethality would occur. At the maximum temperature they tested, 43°C, this critical concentration was approximately 10 g/kg; at 66 g/kg of CO_2, the critical temperature was about 28°C.

Our work shows that greater microbial lethality can be achieved in raw milk treated with supercritical phase CO_2 than with subcritical CO_2 and that the effects of supercritical CO_2 can be enhanced by increasing pressure and temperature. Supercritical CO_2 treatment using the flow through high-pressure CO_2 milk pilot plant processing unit can achieve bacterial reductions equal to or greater than those achieved by a range of possible pasteurization treatments. Our work with different levels of pressurized CO_2 suggests that there is a CO_2 concentration threshold required for lethality. Although vegetative cells are affected by the process, bacterial spores are not. The effects of the process on analytical qualities of treated milk were undertaken as an investigation separate from this study and will be presented as a second part to this research paper.

ACKNOWLEDGMENTS

The authors express their appreciation to Dairy Management, Inc. (Rosemont, IL) for their generous financial support of this project. We would also like to thank Praxair, Inc. (Joliet, IL) for the use of the high-pressure CO_2 milk pilot plant processing unit during the course of this study.

REFERENCES

Arreola, A. G., M. O. Balaban, C. I. Wei, A. Peplow, M. Marshall, and J. Cornell. 1991. Effect of supercritical carbon dioxide on microbial populations in single strength orange juice. J. Food Quality 14:275–284.

Ballestra, P., A. Abreu Da Silva, and J. L. Cuq. 1996. Inactivation of *Escherichia coli* by carbon dioxide under pressure. J. Food Sci. 61:829–836.

Ballestra, P., and J. Cuz. 1998. Influence of pressurized carbon dioxide on the thermal inactivation of bacterial and fungal spores. Food Sci. Technol. 31:84–88.

Boor, K. J. 2001. ADSA Foundation Scholar Award—Fluid dairy product quality and safety: Looking to the future. J. Dairy Sci. 84:1–11.

Calvo, M. M., and E. Balcones. 2001. Inactivation of microorganisms and changes of proteins during treatment of milk with subcritical carbon dioxide. Milchwissenschaft 56:366–369.

Chambers, J. V. 2003. The microbiology of raw milk. Pages 39–90 in Dairy Microbiology Handbook: The Microbiology of Milk and Milk Products. 3rd ed. R. K. Robinson, ed. John Wiley and Sons, Inc., New York, NY.

Dillow, A. K., F. Dehghani, S. Krkach, N. R. Foster, and R. Langer. 1999. Bacterial inactivation by using near- and supercritical carbon dioxide. Proc. Natl. Acad. Sci. USA 96:10344–10348.

Enomoto, A., K. Nakamura, M. Hakoda, and N. Amaya. 1997. Lethal effect of high-pressure carbon dioxide on a bacterial spore. J. Ferm. Bioeng. 83:305–307.

Erkmen, O. 2001. Effects of high-pressure carbon dioxide on *Escherichia coli* in nutrient broth and milk. Int. J. Food Microbiol. 65: 131–135.

Frank, J. F., G. L. Christen, and L. B. Bullerman. 1992. Tests for groups of microorganisms. Pages 271–286 in Standard Methods for the Analysis of Dairy Products. 16th ed. T. R. Marshall, ed. Am. Public Health Assoc., Washington, DC.

Furukawa, S., T. Watanabe, T. Tai, J. Hirata, N. Narisawa, T. Kawarai, H. Ogihara, and M. Yamasaki. 2004. Effect of high pressure gaseous carbon dioxide on the germination of bacterial spores. Int. J. Food Microbiol. 91:209–213.

Glass, K. A., K. M. Kaufman, A. L. Smith, E. A. Johnson, J. H. Chen, and J. H. Hotchkiss. 1999. Toxin production by *Clostridium botulinum* in pasteurized milk treated with carbon dioxide. J. Food Prot. 62:872–876.

Haas, G. J., H. E. Prescott, E. Dudley, R. Dik, C. Hintlain, and L. Keane. 1989. Inactivation of microorganisms by carbon dioxide under pressure. J. Food Safety 9:253–265.

Hong, S., W. Park, and Y. Pyun. 1999. Non-thermal inactivation of *Lactobacillus plantarum* as influenced by pressure and temperature of pressurized carbon dioxide. Int. J. Food Sci. Technol. 34:125–130.

Hong, S., and Y. Pyun. 2001. Membrane damage and enzyme inactivation of *Lactobacillus plantarum* by high pressure CO_2 treatment. Int. J. Food Microbiol. 63:19–28.

Houghtby, G. A., L. J. Maturin, and E. K. Koenig. 1992. Microbiological count methods. Pages 213–246 in Standard Methods for the Examination of Dairy Products. 16th ed. R. T. Marshall, ed. Am. Public Health Assoc., Washington, DC.

Isenschmid, A., I. W. Marison, and U. von Stockar. 1995. The influence of pressure and temperature of compressed CO_2 on the survival of yeast cells. J. Biotechnol. 39:229–237.

Ishikawa, H., M. Shimoda, H. Shiratsuchi, and Y. Osajima. 1995. Sterilization of microorganisms by the supercritical carbon dioxide micro bubble method. Biosci. Biotechnol. Biochem. 59:1949–1950.

Ishikawa, H., M. Shimoda, K. Tamaya, A. Yonekura, T. Kawano, and Y. Osajima. 1997. Inactivation of *Bacillus* spores by the supercritical carbon dioxide micro-bubble method. Biosci. Biotechnol. Biochem. 61:1022–1023.

Kamihira, M., M. Taniguchi, and T. Kobayashi. 1987. Sterilization of microorganisms with supercritical carbon dioxide. Agric. Biol. Chem. 51:407–412.

Lin, H., Z. Yang, and L. Chen. 1992. Inactivation of *Saccharomyces cerevisiae* by supercritical and subcritical carbon dioxide. Biotechnol. Prog. 8:458–461.

Ma, Y., and D. M. Barbano. 2003. Effect of temperature of CO_2 injection on the pH and freezing point of milks and creams. J. Dairy Sci. 86:1578–1589.

Martin, J. D., B. G. Werner, and J. H. Hotchkiss. 2003. Effects of carbon dioxide on bacterial growth parameters in milk as measured by conductivity. J. Dairy Sci. 86:1932–1940.

Shimoda, M., Y. Yamamoto, J. Cocunubo-Castellanos, H. Tonoike, T. Kawano, H. Ishikawa, and Y. Osajima. 1998. Antimicrobial effects of pressured carbon dioxide in a continuous flow system. J. Food Sci. 63:709–712.

Sirisee, U., F. Hsieh, and H. E. Huff. 1998. Microbial safety of supercritical carbon dioxide processes. J. Food Proc. Preserv. 22:387–403.

Smoot, L. A., and M. D. Pierson. 1982. Inhibition and control of bacterial spore germination. J. Food Prot. 45:84–92.

Spilimbergo, S., and A. Bertucco. 2003. Non-thermal bacteria inactivation with dense CO_2. Biotechnol. Bioeng. 84:627–638.

Spilimbergo, S., N. Elvassore, and A. Bertucco. 2002. Microbial inactivation by high-pressure. J. Supercrit. Fluids 22:55–63.

Spilimbergo, S., N. Elvassore, and A. Bertucco. 2003. Inactivation of microorganisms by supercritical CO_2 in a semi-continuous process. Ital. J. Food Sci. 15:115–124.

Tomasula, P. M., and R. T. Boswell. 1999. Measurement of the solubility of carbon dioxide in milk at high pressures. J. Supercrit. Fluids 16:21–26.

U.S. Department of Health and Human Services, P. H. S., Food and Drug Administration. 1999. Grade "A" Pasteurized Milk Ordinance. Vol. Publ. No. 229. 1999 rev. ed. U.S. Dep. Health Human Serv., Public Health Service, FDA, Washington, DC.

Watanabe, T., M. Imai, and I. Suzuki. 2003. Sterilization of *Escherichia coli* by high pressure and supercritical carbon dioxide. Kagaku Kogaku Ronbunshu 29:557–561.

Weast, R. C., M. J. Astle, and W. H. Beyer. 1986. The CRC Handbook of Chemistry and Physics. 67th ed. CRC Press, Inc., Boca Raton, FL.

Werner, B. G., and J. H. Hotchkiss. 2002. Effect of carbon dioxide on the growth of *Bacillus cereus* spores in milk during storage. J. Dairy Sci. 85:15–18.

Wiedmann, M., D. Weilmeier, S. S. Dineen, R. Ralyea, and K. J. Boor. 2000. Molecular and phenotypic characteristic of *Pseudomonas* spp. isolated from milk. Appl. Environ. Microbiol. 66:2085–2095.

Wildasin, R. E., J. Forbes, and R. J. Robey, inventors. 2000. Method and apparatus for continuous flow reduction of microbial activity in a liquid product using pressurized carbon dioxide. Praxair Technology, Inc., assignee. US EP 979657.

Wildasin, R. E., J. Forbes, R. J. Robey, and A. J. Paradis, inventors. 2002. Treating liquid food and other products using carbon dioxide. Praxair, Inc., assignee. US WO 0203816.