Low Pressure CO₂ Storage of Raw Milk: Microbiological Effects

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INTRODUCTION

ABSTRACT

The effects of holding raw milk under carbon dioxide pressures of 68 to 689 kPa at temperatures of 5, 6.1, 10, and 20°C on the indigenous microbiota were investigated. These pressure-temperature combinations did not cause precipitation of proteins from the milk. Standard plate counts from treated milks demonstrated significantly lower growth rate compared with untreated controls at all temperatures, and in some cases, the treatment was microcidal. Raw milk treated with CO₂ and held at 6.1°C for 4 d exhibited reduced bacterial growth rates at pressures of 68, 172, 344, and 516 kPa; and at 689 kPa, demonstrated a significant loss of viability in standard plate count assays. The 689-kPa treatment also reduced gram-negative bacteria and total *Lactobacillus* spp. The time required for raw milk treated at 689 kPa and held at 4°C to reach 4.30 log₁₀ cfu/mL increased by 4 d compared with untreated controls. Total coliform counts in the treated milk were maintained at 1.95 log₁₀ cfu/mL by d 9 of treatment, whereas counts in the control significantly increased to 2.61 log₁₀ cfu/mL by d 4 and 2.89 log₁₀ cfu/mL by d 9. At d 8, *Escherichia coli* counts had not significantly changed in treated milk, but significantly increased in the control milk. Thermotolerant bacteria counts after 8 d were 1.32 log₁₀ cfu/mL in treated milk and 1.98 log₁₀ cfu/mL in control milk. These data indicated that holding raw milk at low CO₂ pressure reduces bacterial growth rates without causing milk protein precipitation. Combining low CO₂ pressure and refrigeration would improve the microbiological quality and safety of raw milk and may be an effective strategy for shipping raw single strength or concentrated milk over long distances.

(Key words: carbon dioxide, raw milk, microbiology, shelf life)

Abbreviation key: SPC = standard plate count assay.

Raw milk serves as an excellent growth medium for microorganisms, and the numbers and types of organisms that can be found in milk directly affect safety and shelf life (Rowe, 1989; Espie and Madden, 1997). Gram-negative pseudomonads predominate in raw milk and are major contributors to milk spoilage; other types of bacteria, including pathogens, survive and grow (Muir et al., 1979; Griffiths et al., 1987). Most milk-borne vegetative organisms are generally inactivated by pasteurization but their lipolytic and proteolytic enzymes can survive and cause undesirable lipolysis and proteolysis. Additionally, thermotolerant microorganisms that are potential pathogens or cause milk spoilage may survive the pasteurization process. The major strategy to extend shelf life of unpasteurized (raw) milk has been to provide rapid refrigeration. For example, decreasing the storage temperature from 6 to 2°C increases the time for the psychrotrophic count to reach 10⁶ cfu/mL from 2.9 to 5 d (Griffiths et al., 1987). Benefits in distribution and pasteurized milk quality could be derived from reducing microbial growth in raw milk.

Several authors have reported on the use of CO₂ as an antimicrobial agent in foods including dairy products (Dixon and Kell, 1989; Haas et al., 1989). In raw milk, bacterial growth was reduced by 50% after addition of CO₂ and storage at 6.7°C for 48 h (Shipe et al., 1978). King and Mabbitt (1982) demonstrated an extension in storage life of both poor and good quality milks by the addition of 30 mM CO₂. Carbon dioxide is effective at reducing the rate of growth of organisms detected in aerobic plate count assays (Roberts and Torrey, 1988). Compared with control milk, the standard plate count (SPC) of milk containing 20 to 30 mM dissolved CO₂ was 3 log₁₀ cfu/mL lower after 4 d of storage at 7°C (Mabbitt, 1982). In the presence of CO₂, the time for SPC to reach 7 log₁₀ cfu/mL was extended from 3 to 9 d at 7°C and 6 to 11 d at 4°C, whereas in the control milk, this level was reached in 5 d at 7°C and 8 d at 4°C (Hotchkiss, 1996). Coliforms and psychrotrophs were also significantly reduced compared with control milk under the same conditions (Roberts and Torrey, 1988). Generally, gram-negative psychrotrophs are more susceptible to the effects of CO₂, whereas gram-

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positive bacteria and spores are more resistant; *Lactobacillus* spp. are relatively CO₂ resistant, and their growth may be enhanced by a CO₂-enriched environment (Hendricks and Hotchkiss, 1997). Excessive growth of *Lactobacillus* spp. in raw milk may lead to spoilage or development of off-flavors due to fermentation. Treatments that reduce microbial populations may result in outgrowth of thermotolerant spore-forming bacteria due to reduced competition, increasing the likelihood of postpasteurization spoilage or reduced food safety.

The addition of CO₂ has been shown to increase the lag phase of growth and decrease the growth rate of microorganisms (Martin et al., 2003). In CO₂-treated milk, extension of the lag phase increased the generation times of *Pseudomonas* spp. (Roberts and Torrey, 1988). Increasing concentrations of CO₂ increased lag phases and extended growth rates. King and Mabbitt (1982) demonstrated an extension in storage life of poor quality milk (10⁵ cfu/mL) by 1.2 d and of good quality milk (10³ cfu/mL) by 3 d with the addition of 30 mM/L CO₂. The extension of keeping quality of milk due to CO₂ was maximized when the initial counts in the milk were low. Low-level carbonation of bulk tank milk inhibits the increase in microbiota for 3 to 4 d. The reduction in counts would thus reduce the thermotolerant lipases and proteases secreted into the milk postpasteurization (Espie and Madden, 1997).

Several theories explaining the mechanism of CO₂ action on microorganisms have been proposed. The exclusion of oxygen by replacement with CO₂ may contribute to the overall effect by slowing the growth rate of aerobic bacteria (Daniels et al., 1985). Carbon dioxide can readily pass through cell membranes and form carbonic acid within the cell with a resultant decrease in intracellular pH, which slows intracellular enzyme activities (Wolfe, 1980). Carbon dioxide has been demonstrated to be inhibitory of certain enzymes, especially decarboxylating enzymes (Gill and Tan, 1979). Carbon dioxide can also accumulate in membrane lipid bilayers, altering membrane properties and inhibiting membrane functions (Enfors and Molin, 1978). The effect of CO₂ is enhanced at lower temperatures (Gill and Tan, 1979). The increasing solubility of CO₂ at lower temperatures increased the relative inhibitory effect of CO₂ on *Pseudomonas fragi* (Enfors and Molin, 1981).

Under specific combinations of pressure and temperature, CO₂ effectively precipitates the proteins from milk. For example, at 38 and 50°C, and pressures above 5514 kPa, complete precipitation of casein from milk results (Tomasula and Boswell, 1999). Calvo and Balcones (2001) demonstrated that the amount of casein aggregation increased when increased pressure was applied to milk for increasing residence times. Carbon

dioxide pressure treatments of 294 to 735 kPa applied at 20, 30, 40, and 50°C resulted in casein aggregation. Clearly, any storage strategy that uses CO₂ must consider the potential adverse effects of protein precipitation.

Most of the work cited above has focused on the addition of low levels of dissolved CO₂ into raw milk held at atmospheric pressure. Our goal was to investigate the effect on raw milk spoilage and pathogenic microbiota of holding raw milk under positive CO₂ pressures that do not result in precipitation of milk solids. We examined changes in the following groups of typical milk microbiota as indicators of potential quality and safety: total *Lactobacillus* spp., lactose-fermenting, and nonlactose-fermenting gram-negative bacteria, *Escherichia coli*, thermotolerant bacteria, and SPC.

MATERIALS AND METHODS

Test System Design

The apparatus for pressurizing and holding raw milk samples consisted of two 13-mL stainless steel 1.27-cm o.d. cylindrical vessels; one vessel was pressurized and the other served as a control (Figure 1, A and B). Compressed and filtered CO₂ from a high-pressure tank (Figure 1C) was used (Empire Airgas, Inc., Elmira, NY). The system consisted of pressure regulator (Figure 1D), a fine metering valve (Figure 1E) (NUPRO Company, Willoughby, OH), an on-off valve (Figure 1F) (Circle Seal, Anaheim, CA), and a check valve (Figure 1G) (NUPRO Co.). The fine metering valve controlled gas flow such that the time to reach desired pressure was <5 s. The gas entered the vertically positioned treatment vessel from the bottom and was thus bubbled through the milk until the set pressure was reached. A check valve was placed immediately before the inlet to the pressure vessel to prevent the backward flow of the fluid milk into the gas inlet line. The outlet of the vessels consisted of a pressure gauge (Figure 1H) and a high-pressure release valve (Figure 1I) (High Pressure Equipment, Erie, PA). The release valve was kept tightly closed during treatment. The control vessel was closed off from both ends but not connected to the carbon dioxide line inlet and outlet lines.

The apparatus was cleaned and sanitized before and after each treatment as follows: water rinse, Conquest sodium hydroxide (Ecolab Inc., St. Paul MN) soak (20 min, 23°C), warm tap water rinse (50°C), Monarch CIP phosphoric acid bath immersion (Ecolab) (20 min, 23°C), warm tap water rinse (50°C), Trichloro-o-cide XP (Ecolab) soak (30 min, 23°C), and sterile water (50°C) rinse (3×). This protocol was validated by testing swab samples of critical control points in the dismantled apparatus for microbial load, and by testing equipment

Figure 1. Carbon dioxide batch pressurization system.

rinse water pH and residual chlorine content (Hach Company, Loveland, CO). All pH measurement was performed using an Accumet model 925 pH meter and Accumet polymer body combination electrode with silver/silver chloride reference (Fisher Scientific, Pittsburgh, PA). Temperature was controlled by a circulating water bath (Figure 1J) (VWR 1145 Refrigerated Temperature Constant Circulator, Westchester, PA), which circulated hot/cold water through copper coils (Figure 1K) immersed into water in a vacuum Dewar flask that held the treatment and control vessels. Copper-constantan thermocouples (Figure 1L) measured the temperature of the treatment and control vessels, and were continuously logged onto a temperature recorder (Figure 1M) (Omega Engineering Inc., Stamford, CT).

Milk Sampling, Treatment, and Analysis

Whole, unhomogenized, raw milk was obtained from 2 sources. Commingled milk samples were obtained from the Northeast DHIA (Ithaca, NY), a dairy analytical consulting laboratory. These samples were comingled bulk milks from 236 farms from New York, Pennsylvania, and New Jersey; and thus, could be considered

representative of a wide range of milk flora. Milk was also obtained from the Cornell University Teaching and Research Center bovine herd (T&R Center; Dryden, NY). All milk was stored at 6°C until use. Raw milk from the T&R Center was received less than 12 h after milking in sterile bottles and held on ice until it could be moved to a 6°C cooler.

Milk samples were mixed and 5 mL added into the treatment and control vessels. The treatment vessel was connected to the apparatus and the control vessel closed off. Both vessels were placed in the waterbath. When the desired temperatures were attained in both treatment and control vessels, CO₂ was introduced through the bottom of the treatment vessel until the set pressure was reached. The CO₂ pressure was maintained throughout the test period. When the desired time was reached, the CO₂ inlet was turned off, the pressure release valve on the outlet line opened, and the pressure released in <1 min. After depressurization, the treatment and control vessels were removed from the water bath and their external surfaces were wiped dry, sanitized with 95% (vol/vol) ethanol, detached from the apparatus, and the contents transferred into sterile containers for dilution and plating.

The effect of CO₂ pressure/temperature combinations on protein precipitation was measured at CO₂ pressures of 344, 689, 1378, 2067, 2757, and 3446 kPa at temperatures of 20, 10, and 5°C for 5, 15, 30, and 60 min. The amount of protein precipitation was quantified and expressed as percentage precipitated solids by the method of Tomasula (1995).

Short (<1 h) and long-term (1, 4, and 9 d) experiments were conducted. Raw milk (Northeast DHIA) in 5-mL aliquots was treated at each of the following combinations of CO₂ pressure, temperature, and time (kPa/°C/min): 1378/5/15, 2757/5/5, 3446/5/5. In the long-term studies, raw milk from the T&R Center was first stored at 6°C/48 h so that the SPC were at detectable levels at treatment initiation. Five milliliters of milk was treated with CO₂ pressures of 0 (control), 68, 172, 344, 516, and 689 kPa for 1 to 9 d at 4.1 to 10°C.

Raw milk from the T&R Center was monitored for changes in aerobic bacteria, gram-negative bacteria, and total *Lactobacillus* spp. as follows: CO₂ pressures of 0 (control), 68, 172, 344, 516, and 689 kPa, at 6.1°C for 4 d. Standard plate count, gram-negative bacteria, and total *Lactobacillus* spp. were enumerated before (d 0) and after (d 4) treatment. The time to reach an SPC of 2×10^5 cfu/mL was determined using raw milk (T&R Center) without a 2-d storage time. Equal volumes were transferred into treatment and control vessels and held at 0 and 689 kPa CO₂ and 4.1°C.

The progression of these counts (total, coliform/*E. coli*, and thermophilic bacteria) in the treatment and control samples was tracked by conducting checks on the total aerobic counts (SPC) on treatment d 4 and 6. Based on the levels of total counts on d 4 and 6, analyses of total coliforms/*E. coli*, and thermophilic bacteria after d 6 were conducted either at 1- or 2-d intervals. The control sample final count was measured on d 4 and 6.

Microbiological Methods

For all microbiological assays, milk sample aliquots of 1 mL were used in dilution series. Standard plate counts were performed by the method described in Standard Methods for the Examination of Dairy Products (Houghtby et al., 1992). Gram-negative bacteria were enumerated on MacConkey agar (Difco Manual, Becton Dickinson & Co., Sparks, MD) after spread plating and incubation at 30°C for 48 h. MacConkey agar (Difco Manual, Becton Dickinson & Co., Sparks, MD), a selective and differential media, can be used to discriminate between lactose-fermenting and nonlactose-fermenting gram-negative bacteria. Thus, use of this media allows a one-step method of obtaining estimates of both coliform and noncoliform gram-negative bacteria. Coliform bacteria may include species of *Esche-*

richia, *Klebsiella*, and *Enterobacter*, potential pathogens and spoilage organisms. Noncoliform gram-negative bacteria may include spoilage organisms such as pseudomonads or potential pathogens such as *Salmonella* spp. or *Shigella* spp. *Lactobacillus* spp. were estimated by pour plating in acidified (adjusted to pH 5.5 with glacial acetic acid) Lactobacillus de Man, Rogosa, and Sharpe agar (Difco Manual, Becton Dickinson & Co.), incubated at 32°C for 48 h under anaerobic conditions. Representative and distinctive suspect colonies were gram-stained, and confirmed gram-positive bacilli colonies were counted as an estimate of total *Lactobacillus* spp.

Initial total, coliform, and thermophilic counts were each determined for control and treated samples. Thermophilic organisms were enumerated by the laboratory pasteurization count method described in the Standard Methods for the Examination of Dairy Products (Houghtby et al., 1992). The 3M Petrifilm count plate (3M Microbiology Products, St. Paul, MN) was used to enumerate total coliforms and *E. coli* in the raw, treated, and control milk samples.

Statistical Analyses

MINITAB Release 13.1 (Minitab Inc., State College, PA) was used for statistical analyses of the data. Experiments were conducted in duplicate using 2 different milk samples ($n = 2$); each sample was tested in triplicate. Significant difference among samples was determined at $P \leq 0.05$. Analysis of variance was used to determine the effect of CO₂ pressure, and the interaction effects of pressure and temperature.

RESULTS AND DISCUSSION

Effect of Low Pressure CO₂ on Milk Protein Precipitation and pH

Application of CO₂ pressures greater than 1378 kPa for 15 to 60 min resulted in more than 1% precipitation of milk solids at 20°C (data not shown). Treatment for 30 min at 2067 kPa resulted in 2.6% (wt/wt) solids, which approached the maximum (2.8%) produced by sulfuric acid precipitation (Southward, 1986). However, lowering the holding temperature reduced the amount of precipitation; at 5°C and pressures of less than 2067 kPa, precipitation could not be detected, even after 60 min. Treatment combinations of 689 kPa for 60 min, 1378 kPa for 30 min, 2757 kPa for 5 min, and 3446 kPa for 5 min did not cause detectable precipitation at 5°C.

The pH of the treated and control milk samples (as measured at atmospheric pressure) was 6.6 to 5.9 at CO₂ pressures ≤ 516 kPa and 5.7 when treated at pressures ≥ 516 kPa and 20°C. The pH of the treated and

control milk samples, when treated at 10°C, was 5.5 at CO₂ pressures ≤516 kPa, and 5.8 when treated at pressures >516 kPa.

These results generally agree with previous reports including Jordan et al. (1987), Tomasula (1995), and Calvo and Balcones (2001), who independently investigated the precipitation of caseins from raw skim milk using pressurized CO₂. Tomasula (1995) found that CO₂ pressures between 2757 and 5514 kPa and temperatures between 38 and 49°C caused complete casein precipitation. Calvo and Balcones (2001) precipitated 85% of raw skim milk caseins by applying CO₂ pressures above 1998 kPa for 3 h at 40°C. Jordan et al. (1987) obtained 99% precipitation of skim milk casein by treatment with 3515 kPa at 50°C.

Protein precipitation occurs when the pH of the milk has been reduced below the isoelectric point of the casein (pH 4.6). The addition of CO₂ to milk leads to the formation of carbonic acid and a decrease in pH; however, pressurization with CO₂ can cause precipitation of caseins at a pH higher than its isoelectric point (Tomasula and Boswell, 1999). Ma and Barbano (2003) found that increasing CO₂ concentration and pressure decreased the pH of skim milk; the pressure effect was greater as CO₂ concentrations increased. These researchers also determined that increasing temperature influenced the solubility of milk colloidal calcium phosphate, resulting in a decrease in milk pH. Jordan et al. (1987) found that precipitation of casein occurred between 40 and 70°C, and that the yield at any specific temperature was dependent upon a minimum pressure; this minimum pressure was inversely related to temperature. Thus, specific pressure/time/temperature treatment combinations can be manipulated that will not cause precipitation of proteins from raw milk.

Effect of Low Pressure CO₂ on Raw Milk Microflora

Changes in gram-negative lactose-fermenting and nonlactose-fermenting bacteria, *Lactobacillus* spp., and SPC in raw milk treated under different temperatures and pressures is illustrated in Figure 2. All time/pressure combinations significantly reduced the SPC of the raw milk compared with untreated controls, even at a low pressure/high temperature combination of 68 kPa and 20°C. At 1378 kPa, the control SPC was 7.89 log₁₀ cfu/mL, whereas the treated milk SPC was reduced by 0.33 log₁₀ after 15 min and 0.39 log₁₀ after 30 min. Twenty-four-hour treatments at 20°C and pressures ≥344 kPa resulted in microbial inactivation. The SPC of milk treated at 344, 516, and 689 kPa was significantly reduced from initial SPC by 0.39, 0.62, and 0.82 log₁₀, respectively, whereas the SPC of the control milks significantly ($P < 0.05$) increased by as much as

2.06 log₁₀ cfu/mL. The SPC in milk held at 68 and 172 kPa significantly increased by 1.07 and 0.59 log₁₀ cfu/mL, respectively; however, this population increase was significantly less than that exhibited by the control milk.

Carbon dioxide pressure treatments of 68 and 172 kPa at 10°C applied over 24 h were more effective at curtailing growth than similar pressure/time treatments at 20°C. As found at 20°C, there was a loss in viability at pressures ≥344 kPa, and the differences between control and test counts increased with increasing holding time; significant decreases in counts of 0.31, 0.56, and 0.71 log₁₀ cfu/mL at 344, 516, and 689 kPa CO₂, respectively, were achieved. The difference in SPC between control and test milks at 689 kPa was 2.68 log₁₀ cfu/mL. These data indicate that holding raw milk under CO₂ not only slowed the growth of the microorganisms in the raw milk but also, in some cases, resulted in a loss in viability.

Others have shown inactivation of microbiota in raw and pasteurized milk with CO₂ at significantly higher pressures (Erkman, 1997, 2000; Calvo and Balcones, 2001). Calvo and Balcones (2001) found a decrease in bulk raw milk microbiota of 2 log₁₀ cfu/mL upon treatment with 3997 kPa CO₂ at temperatures ≥40°C for 30 min. Erkman (2000) demonstrated a reduction in aerobic microorganisms in whole milk of 6 log₁₀ cfu/mL after a 24-h treatment under 6044 kPa CO₂ pressure at 45°C. Erkman (1997) also demonstrated a reduction of 8 log₁₀ cfu/mL after a 5-h 14,598 kPa CO₂ treatment at 25°C. However, the use of such pressures would, in our experience, result in complete precipitation of the caseins and would require the use of specially designed equipment. Calvo and Balcones (2001) reported that pressures of 3997 kPa caused precipitation, whereas Erkman (1997, 2000) made no mention of the state of the milk. We are not aware of any reports testing the effects of low pressures.

Lowering the holding temperature to 6.1°C significantly reduced microbial growth compared with control milks when CO₂ pressures of 68, 172, 344, 516, and 689 kPa were applied for 4 d. For example, the SPC of milk held at 689 kPa was 0.89 log₁₀ cfu/mL lower than initial counts and 3.48 log₁₀ cfu/mL lower than controls. After 9 d storage under 689 kPa CO₂ at 4°C, the ratios of treated to untreated SPC, thermotolerant, coliform, and *E. coli* counts were consistently lower than the ratios of control to untreated counts for the comparable groups (log₁₀ cfu/mL) (Table 1). Milks treated at 68, 172, 344, and 516 kPa significantly increased from an initial SPC of approximately 3.30 log₁₀ cfu/mL by 1.28, 1.10, 0.94, and 0.82 log₁₀ cfu/mL, respectively, whereas the control SPC increased by 2.86, 2.85, 2.86, and 2.93 log₁₀ cfu/mL, respectively. Milk held at 689 kPa and 6.1°C for 4 d

Figure 2. Changes in gram-negative lactose fermenting (□) and nonlactose fermenting bacteria (▒), *Lactobacillus* spp. (■), and standard plate count (■) in raw milk treated at (A) 68 kPa, (B) 172 kPa, (C) 344 kPa, (D) 516 kPa, and (E) 689 kPa CO₂ pressure and 6.1°C for 4 d; n = 2, each sample plated in triplicate. ^{a-i}Counts with different letters are significantly different ($P \leq 0.05$).

exhibited greater inactivation than that observed after treatments at 10 or 20°C for 24 h ($P < 0.05$). The pH decreased from 6.6 (before treatment) to 5.5 in milks treated at 516 kPa, to 5.8 at 344 kPa, and 5.9 at 68 kPa.

In addition to SPC, there were significant differences in gram-negative lactose-fermenting and nonlactose-

fermenting bacteria and *Lactobacillus* spp. between CO₂-treated and control milks (Figure 2). For example, levels of gram-negative fermenters and nonfermenters were reduced at all pressures compared with untreated controls. Likewise, *Lactobacillus* spp. counts were approximately 1 to 2 log₁₀ cfu/mL lower in the test milks

Table 1. The effect of 689-kPa CO₂ pressure at 4°C after 4-, 6-, 8-, and 9-d treatments on standard plate count, thermophilic bacteria, coliforms, and *Escherichia coli* counts in untreated, treated, and control raw milks.¹

	Count, log ₁₀ cfu/mL								
	Untreated	Treated				Control			
	d 0	d 4	d 6	d 8	d 9	d 4	d 6	d 8	d 9
Standard plate count	3.48 ^a	2.89 ^b	4.64 ^c	4.99 ^d	5.37 ^e	5.15 ^e	6.08 ^f	6.86 ^g	6.99 ^h
Thermophilic bacteria	ND ²	ND	0.95 ^a	1.32 ^b	1.57 ^c	ND	1.91 ^d	1.98 ^e	2.00 ^f
Coliforms	2.04 ^a	2.00 ^a	2.00 ^a	1.97 ^a	1.95 ^a	2.61 ^a	2.63 ^b	2.74 ^b	2.89 ^c
<i>Escherichia coli</i>	1.30 ^a	1.23 ^a	1.23 ^a	1.23 ^a	1.18 ^a	1.56 ^b	1.59 ^b	1.79 ^b	1.87 ^b

a,b,c,d,e,f,g,h Means in the same row with different superscript letters are significantly different ($P \leq 0.05$).

¹Experiment conducted in duplicate, n = 2 (2 milk samples analyzed), each sample plated in triplicate.

²ND = Not detectable.

compared with control milk. At 689 kPa, gram-negative lactose-fermenting and nonlactose-fermenting bacteria exhibited significant decreases of 0.80 and 0.64 log₁₀ cfu/mL, respectively, compared with initial counts. Under 516 kPa CO₂ pressure, SPC of treated samples were not significantly different from initial untreated samples, whereas SPC of control samples increased by 2.95 log₁₀ cfu/mL. Reductions in total microbial populations as well as reductions in gram-negative and *Lactobacillus* spp. populations would result in improved quality of the raw milk. Ruas-Madiedo et al. (1996) found that lower levels of volatile compounds (ethanol, 2-propanone, and 2-butanone, which are microbial metabolites) were produced in carbonated milk during storage and that higher sensory scores were achieved than in untreated milks. In a later study, Ruas-Madiedo et al. (2000) found a direct association between reduced microbial growth and reduced levels of microbial glucosidases in raw milk stored with CO₂; degradation of milk glucose was subsequently reduced in the treated milks. It has also been found that levels of fat-soluble vitamins (retinol, β -carotene, and α -tocopherol) in milk treated with CO₂ and stored at 4°C for 7 d were higher than that measured for untreated raw and pasteurized milks (Ruas-Madiedo et al., 1998a,b).

In the current study, populations of *Lactobacillus* decreased after CO₂ treatment. Others have found that treatment with CO₂ at concentrations between 0 and 2000 mg/L had no impact on the lag phase of *Lactobacillus sake* when grown at 7°C, and influences on the maximum specific growth rate was least affected compared with species of *Pseudomonas*, *Aeromonas*, *Bacillus*, *Brochothrix*, and *Shewanella* (Devlieghere and Debevere, 2000). Espie and Madden (1997) reported no effect of 30 and 45 mM CO₂ on the growth of *Lactobacillus* spp. Neither of these investigations, however, incorporated pressures above atmospheric in their treatments. Reductions in populations of *Lactobacillus plantarum* of more than 6 logs was achieved after treatment with

CO₂ pressures of 13 MPa at 30°C for 30 min (Hong et al., 1999). In subsequent studies, these researchers found that high-pressure CO₂ treatment of *L. plantarum* resulted in irreversible cellular membrane damage and reduced activity of some intracellular enzymes, physiological changes that could result in microbial inactivation (Hong and Pyun, 2001). Combined or enhanced effects of low pressures and CO₂ treatments could thus explain why we observed reductions in total *Lactobacillus* populations.

The effect of 689 kPa CO₂ at 4°C on the time to reach an SPC of 1×10^5 cfu/mL was investigated. Pasteurized Milk Ordinance Grade A regulations specify that the SPC for raw milk should be $<1 \times 10^5$ cfu/mL before pasteurization (US Department of Health and Human Services, 1999). The treated milks reached 10^5 cfu/mL after 8 d of treatment, whereas the control milk reached this level after just 4 d (Figure 3). Treatment at 689 kPa and 4°C extended the treatment holding time by at least 4 d compared with the control. At the end of 4 d, treated milk SPC had decreased to 2.89 from 3.48 log₁₀ cfu/mL, whereas control milk SPC increased by nearly 2 log₁₀ cfu/mL. This reduction in SPC in treated milk agrees with the trend observed in the 4-d experiments conducted at 6.1°C (Figure 2). Milk SPC increased to 4.64, 4.99, and 5.37 log₁₀ cfu/mL after 6, 8, and 9 d, respectively (Figure 3). Neither *E. coli* nor total thermophilic bacteria counts increased in the treated milk but both significantly increased in the controls. The pH of the treated milk samples changed from an initial value of 6.6 to 5.5 at the end of d 4, 6, 8, and 9 of treatment.

CONCLUSIONS

Under selected pressure and temperature combinations, CO₂ resulted in lethality of both total aerobic bacteria and several important classes of milk-borne bacteria, whereas milder conditions decreased the rate

Figure 3. Total counts (□), thermophilic bacteria (▒), total coliforms (■), and *Escherichia coli* (■) counts in milk treated at 4°C and 689 kPa CO₂ pressure after 4, 6, 8, and 9 d of storage. Experiments were conducted in duplicate, with 2 milk samples per experiment, and each sample plated in triplicate. ^{a-r}Counts with different letters are significantly different ($P \leq 0.05$).

of growth of these organisms. After 4 d treatment at a CO₂ pressure of 689 kPa and temperature of 6.1°C, a significant decrease in SPC was observed. Between 4 and 9 d, the SPC of the CO₂-treated milk increased slowly to population levels that were always significantly lower than that observed for the control SPC. Carbon dioxide-treated milk SPC was within Pasteurized Milk Ordinance Grade A raw milk limits up to 8 d of storage, whereas untreated milk reached the Grade A limits before 4 d. Growth of spoilage organisms, coliforms, thermophilic bacteria, or *E. coli* was not enhanced by any pressurized CO₂ treatment. These data suggest that low positive CO₂ pressure might be an effective strategy for holding and shipping raw milk, providing shelf-life extension and maintenance of safety. Although CO₂ would need to be removed before further processing of raw milk, such methodology and necessary equipment is standard and readily available in the dairy industry. Potential financial benefits of extended bulk storage and low associated costs make this application attractive to commercial use.

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