Effect of CO₂ Addition to Raw Milk on Proteolysis and Lipolysis at 4°C¹

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ABSTRACT

Fresh raw milks, with low (3.1×10^4 cell/ml) and high (1.1×10^6 cells/ml) somatic cell count (SCC), were standardized to 3.25% fat, and from each a preserved (with 0.02% potassium dichromate) and an unpreserved portion were prepared. Subsamples of each portion were carbonated to contain 0 (control, pH 6.9) and 1500 (pH 6.2) ppm added CO₂, and HCl acidified to pH 6.2. Milk pH was measured at 4°C. For the preserved low- and high-SCC milks, two additional carbonation levels, 500 (pH 6.5) and 1000 (pH 6.3) ppm, were prepared. Milks were stored at 4°C and analyzed on d 0, 7, 14, and 21 for microbial count, proteolysis, and lipolysis. The addition of 1500 ppm CO₂, but not HCl, effectively delayed microbial growth at 4°C. In general, in both the low- and high-SCC unpreserved milks, there was more proteolysis and lipolysis in control and HCl acidified milks than in milk with 1500 ppm added CO₂. Higher levels of proteolysis and lipolysis in the unpreserved milks without added CO₂ were related to higher bacteria counts in those milks. In preserved low- and high-SCC milks, microbial growth was inhibited, and proteolysis and lipolysis were caused by endogenous milk enzymes (e.g., plasmin and lipoprotein lipase). Compared with control, both milk with 1500 ppm added CO₂ and milk with HCl acidification had less proteolysis. The effect of carbonation or acidification with HCl on proteolysis in preserved milks was more pronounced in the high SCC milk, probably due to its high endogenous protease activity. Plasmin is an alkaline protease and the reduction in milk pH by added CO₂ or HCl explained the reduction in proteolysis. No effect of car-

bonation or acidification of milk on lipolysis was observed in the preserved low- and high-SCC milks. The CO₂ addition to raw milk decreased proteolysis via at least two mechanisms: the reduction of microbial proteases due to a reduced microbial growth and the possible reduction of endogenous protease activity due to a lower milk pH. The effect of CO₂ on lipolysis was mostly due to a reduced microbial growth. High-quality raw milk (i.e., low initial bacteria count and low SCC) with 1500 ppm added CO₂ can be stored at 4°C for 14 d with minimal proteolysis and lipolysis and with standard plate count $<3 \times 10^5$ cfu/ml.

(Key words: carbon dioxide, raw milk storage, proteolysis, lipolysis)

Abbreviation key: CC = coliform count, CHM = chloroform-heptane-methanol, CN/TP = casein as a percentage of true protein, LPL = lipoprotein lipase, NCN = noncasein nitrogen, NPN = nonprotein nitrogen, PBC = psychrotrophic bacterial count, PMO = Pasteurized Milk Ordinance, SPC = standard plate count, TN = total nitrogen, TP = true protein.

INTRODUCTION

The dairy industry has relied on refrigeration to maintain raw milk quality during storage and transportation. Limited by the growth of psychrotrophic bacteria, the normal refrigerated storage life of raw milk is usually less than 5 d. Psychrotrophic bacteria produce extracellular enzymes, which can cause extensive proteolysis and lipolysis in milk (Law, 1979; Cousin, 1982). Dissolved CO₂ increases both the lag phase and the generation time in the growth cycle of microorganisms (King and Mabbitt, 1982; Daniels et al., 1985), and in low levels it can control the growth of psychrotrophic bacteria in milk (King and Mabbitt, 1982; Rashed et al., 1986). It appears the inhibition mechanism of CO₂ is directly associated with CO₂ and is not the indirect effect of pH reduction or O₂ replacement (King and Mabbitt, 1982). Some of the direct effects exerted by

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CO₂ include its ability to change microbial membrane properties (Rowe, 1988), to lower intracellular pH (Hong and Pyun, 1999), and to interfere with cellular enzymatic reactions (King and Nagel, 1975). Commercially, CO₂ has been added to cottage cheese to extend product shelf life (Hotchkiss and Lee, 1996). The dairy industry is interested in expanding the use of CO₂ technology. To find new opportunities, it is important to thoroughly understand the impact of CO₂ addition on the chemistry of milk components.

Many studies on the effect of CO₂ on milk quality have focused on the microbiological quality of milk. However, low bacteria count does not guarantee high milk quality. The dairy industry is concerned about the damage to milk components due to proteolysis and lipolysis. Increased proteolysis reduces the economic value of milk by its negative impact on protein functionality, especially CN. Proteolysis can reduce cheese yield (Barbano et al., 1991; Klei et al., 1998) and cause bitter off-flavors (Ma et al., 2000) in dairy foods. Development of high levels of FFA due to lipolysis imparts a rancid off-flavor in dairy products, making them unacceptable (Ma et al., 2000).

Enzymes present in refrigerated raw milk are either endogenous (e.g., originating from the cow) or from psychrotrophic bacteria growing in the milk. The two most important endogenous enzymes are plasmin (EC 3.4.21.7) and lipoprotein lipase (LPL, EC 3.1.1.34). Plasmin and LPL are active at refrigeration temperatures and can cause slow degradation of milk protein (mainly CN) and lipid (mainly triglycerides), respectively. Enzymes of somatic cell origin in milk increase during mastitis. The extent of increase depends on the severity of infection (de Rham and Andrews, 1982) and becomes especially significant when SCC is high, above 1 million cells/ml (Saeman et al., 1988). The lipolytic and proteolytic activities contributed by psychrotrophic bacteria, in general, are not significant, unless the bacteria count has exceeded 10⁶ cfu/ml (Cousin, 1982). The types of microorganisms present and whether or not they are capable of producing active proteolytic and lipolytic enzymes are also important (Cousin, 1982).

The objective of this study was to determine the effect of CO₂ addition to raw milk on proteolysis and lipolysis during 21 d of storage at 4°C. To achieve this objective, the following questions guided the design of the experiments: 1) Does the addition of CO₂ affect the extent of proteolysis and lipolysis by the combination of both endogenous (related to increasing milk SCC) and microbial enzymes in raw milk during storage at 4°C? 2) Does the addition of CO₂ affect the extent of proteolysis and lipolysis in raw milk caused by endogenous proteases and lipases during storage at 4°C? 3) If CO₂ inhibits the activity of endogenous milk protease and lipase,

Figure 1. Experimental design flow chart. Numbers (1), (2), and (3) designate the treatments used in experiments 1, 2, and 3, respectively, as described in the Materials and Methods section. The milk pH was measured at 4°C.

how much of the effect is due to a pH reduction by CO₂ vs. a direct effect of CO₂? and 4) What is the effect of CO₂ concentration on proteolysis and lipolysis by endogenous milk enzymes?

MATERIALS AND METHODS

Experimental Design and Statistical Analysis

Three experiments were designed to answer the four questions raised above. Figure 1 is a flowchart showing the design of each experiment. Measuring the impact of added CO₂ on proteolysis and lipolysis in a high-quality raw milk (i.e., raw milk with low bacterial count and low SCC) is challenging. This is because in high-quality raw milk, the endogenous enzyme activities are very low, especially when milk is stored at low temperatures, and there is almost no proteolysis and lipolysis by enzymes of microbial origin. Because of its elevated endogenous protease and lipase activities, using raw milk with a high SCC is a good strategy for testing the effect of CO₂ on endogenous milk enzyme activities. If the addition of CO₂ has an effect on milk endogenous enzyme activities, then the effect will be more pronounced or more easily observed in a raw milk with high SCC. All experiments in this study were replicated two times using different batches of raw milk collected from the Cornell Teaching and Research Farm. Fresh raw milks with low (3.1×10^4 cell/ml) and high (1.1×10^6 cells/ml) SCC were obtained.

Experiment 1: Unpreserved milks. To answer the first question, fresh unpreserved low- and high-SCC milks were carbonated to contain approximately 0 ppm (control, pH 6.9 at 4°C) and 1500 ppm (pH 6.2 at 4°C)

Table 1. ANOVA models used for data analysis for experiments 1, 2, and 3.

Independent variables	df for experiments 1 and 2	df for experiment 3	Analyzed as	Error term
Whole-plot factors				
Replicate	1	1	Block	
SCC	1	1	Fixed effect	E(a)
Treatment	2	3	Fixed effect	E(a)
Treatment $\times$ SCC	2	3	Interaction	E(a)
E(a) = Replicate $\times$ SCC + replicate $\times$ treatment + replicate $\times$ treatment $\times$ SCC	5	7	Whole-plot error	
Repeated measure factors				
Day	3	3	Repeated measure factor	E(b)
Day $\times$ SCC	3	3	Interaction (fixed)	E(b)
Day $\times$ treatment	6	9	Interaction (fixed)	E(b)
Day $\times$ SCC $\times$ treatment	6	9	Interaction (fixed)	E(b)
E(b)	18	24	Error	
Corrected total	47	63		

added CO₂, and HCl acidified to pH 6.2 (4°C), which matched the pH of the unpreserved milk with 1500 ppm added CO₂ (Figure 1). Milks were stored in glass containers and analyzed for proteolysis and lipolysis on d 0 (day of sample preparation), 7, 14, and 21. Differences in the extent of proteolysis and lipolysis between the control and milk with 1500 ppm added CO₂ would be due to an effect of CO₂ addition on the combination of both endogenous milk enzymes plus enzymes contributed by the growth of bacteria during 21 d of storage at 4°C. Upon CO₂ addition, there was a decrease in milk pH. Addition of an inorganic acid, such as HCl, does not inhibit microbial growth (King and Mabbitt, 1982; Daniels et al., 1985) but does reduce milk pH. Therefore, the milk acidified with HCl would act as a positive control to explain whether the effect of CO₂, if any, was simply a consequence of pH reduction.

The ANOVA model used for data analysis is shown in Table 1. The analysis compared the effects among the three treatments: control, milk with 1500 ppm added CO₂, and milk acidified with HCl. Replicate is a block effect. The whole-plot factors were SCC, treatment, and treatment $\times$ SCC. The repeated measure factors were day and the interactions terms: day $\times$ SCC, day $\times$ treatment, and day $\times$ SCC $\times$ treatment. The main interest was to examine whether, during storage, treatment types affected the extent of proteolysis and lipolysis in the low- and high-SCC raw milks. Analyses were done using SAS (2001).

Experiment 2: Preserved milks. To answer the second and third questions, separate portions of the low- and high-SCC standardized milks from experiment 1 were preserved (with 0.02%, wt/wt, potassium dichromate). The preservative inhibits microbial growth and thus eliminates the production of proteases and lipases by psychrotrophic bacterial in raw milk during storage

at 4°C. The preservative does not inhibit endogenous milk enzymes activities (Senyk et al., 1985).

Similar to the unpreserved milks, the preserved low- and high-SCC milks were carbonated to contain approximately 0 ppm (control, pH 6.9 at 4°C) and 1500 ppm (pH 6.2 at 4°C) added CO₂, and HCl acidified to pH 6.2 (4°C), which matched the pH of the unpreserved milk with 1500 ppm added CO₂ (Figure 1). Milks were analyzed for proteolysis and lipolysis on d 0, 7, 14, and 21. Differences in proteolysis and lipolysis between the preserved control milk and preserved milk with 1500 ppm added CO₂ would be due to an effect of CO₂ addition on milk proteolysis and lipolysis by endogenous milk enzymes. Comparison between preserved milks of the same pH, one acidified by CO₂ and one by HCl, would indicate if the effect of CO₂ on endogenous protease and lipase activities, if any, might be a consequence of pH reduction. The ANOVA model used for data analysis was the same as the one used for data from experiment 1 (Table 1).

Experiment 3: Preserved milks with different carbonation level. To answer the fourth question, preserved low- and high-SCC milks were carbonated to contain approximately 0 ppm (control, pH 6.9 at 4°C), 500 ppm (pH 6.5 at 4°C), 1000 ppm (pH 6.3 at 4°C), and 1500 ppm (pH 6.2 at 4°C) added CO₂ (Figure 1). Milks were analyzed for proteolysis and lipolysis on d 0, 7, 14, and 21. The ANOVA model used for data analysis was similar to the one used for data from experiments 1 and 2, except in this case the treatments were the four carbonation levels (treated as a category variable): 0 ppm (control), 500, 1000, and 1500 ppm (Table 1).

Milk Collection

To obtain milk with low and high SCC, 3 d before milk collection, milks from 60 Holstein cows from the

Cornell Teaching and Research Farm were screened for milk fat, protein, and SCC using a Milk-Scan Combi 4000 (Integrated Milk Testing; A/S N. Foss Electric, Hillerød, Denmark) by a commercial laboratory (Dairy One, Ithaca, NY) licensed for milk payment testing in New York state. Eight cows that produced milk with low SCC (<100,000 cells/ml) and nine cows that produced milk with high SCC (>800,000 but <1,200,000 cells/ml) were selected. Cow selection was done to ensure that the expected levels of protein and fat in the commingled low- and high-SCC raw milks would be similar. Cows were on a thrice-daily milking schedule, and they averaged 18 ± 120 DIM and 1.7 ± 1.2 in parity. Procedures of milk collection and commingling were similar to those described by Ma et al. (2000).

Milk Treatments

Low- and high-SCC raw milks were separated at 50°C into a skim and a cream portion using a lab-scale cream separator (model 100, Delaval, Poughkeepsie, NY). Percentage of fat of the separated cream (AOAC, 2000; method number 995.18; 33.3.18) and the skim milk (Marshall, 1993; method number 15.8B) was determined by the Babcock method. High- and low-SCC milks were standardized to 3.25% fat, and their fat contents were confirmed using the Mojonnier method (AOAC, 2000; method number 989.05; 33.2.26). For both the low- and high-SCC milks, one portion of the milk was preserved with potassium dichromate (0.02%, wt/wt).

Raw preserved and unpreserved milks were sparged with CO₂ (beverage grade) in a sealed 15-L cylindrical stainless steel tank (Zahm and Nagel Co., Inc., Buffalo, NY) at <4°C to contain 500, 1000, and 1500 ppm added CO₂ (Ma et al., 2001). The HCl-acidified milks were prepared by adding 2 N HCl dropwise to milk at 4°C with gentle stirring. Milk pH was determined at 4°C using an electrode (model HA 405 DXK-58/120 combination pH probe; Mettler Toledo, Columbus, OH), calibrated with pH 3.99 and 7.10 buffers (Fisher Scientific, Fair Lawn, NJ) at 4°C. During milk collection and preparation, efforts were made to minimize bacterial contamination. Milk was stored at 4°C in 240-ml glass jars fitted with metal lids (Alltrista Co., Muncie, IN) with approximately 7% headspace. Milks were analyzed on d 0 (day of sample preparation), 7, 14, and 21.

Microbial Testing

On each of the storage days, milk from one jar was used for all the microbial and chemical tests, with samples for microbial tests removed first aseptically, prior to sampling for chemical analysis. The other containers

from the same treatment were left unopened for testing at later storage days to avoid microbial contamination. Microbial count was performed on both unpreserved (d 0, 7, 14, and 21) and preserved (d 21 only) milks. Microbiological testing included standard plate count (SPC), coliform count (CC), and psychrotrophic bacteria count (PBC; Marshall, 1993; method numbers 6.2, 7.8, and 8.1, respectively).

Chemical Analysis

All chemical analyses were performed in duplicate. Milk pH (at 4°C), CO₂ concentration (Ma et al., 2001), CN as a percentage of true protein (CN/TP), and FFA were measured at 0, 7, 14, and 21 d of storage.

Proteolysis. Total nitrogen (TN; AOAC, 2000; method number 991.20; 33.2.11), nonprotein nitrogen (NPN; AOAC, 2000; method number 991.21; 33.2.12), and non-CN nitrogen (NCN; AOAC, 2000; method number 998.05; 33.2.64) were determined by the Kjeldahl method. All nitrogen results were expressed as a protein equivalent using a conversion factor of 6.38. Calculations for content of true protein (TP) and CN were $(TN - NPN) \times 6.38$ and $(TN - NCN) \times 6.38$, respectively. CN/TP was calculated as $(CN/TP) \times 100\%$. Decrease in CN/TP was used as an index of proteolysis.

Lipolysis. The FFA content was determined using the copper soap method (Shipe et al., 1980) with modification, and results were expressed in meq FFA/kg milk. Increase in FFA was used as an index of lipolysis. Reagents used in the analysis were prepared as described by Shipe et al. (1980) unless specified otherwise. On d 0, 7, 14, and 21, aliquots of each of the milk samples were quick-frozen to -70°C and subsequently stored at -40°C. Frozen milks were analyzed in sets of 20 samples. On the first day of an analysis cycle, 20 samples were thawed in a microwave oven. During microwave thawing, the sample temperature was kept below 10°C at all times. Immediately after each sample was thawed and mixed, a 1.0-ml aliquot was pipetted into a weighed (to 0.0001 g) screw-top centrifuge tube (Nalgene Oak Ridge Teflon FEP tube, 50 ml; Fisher Scientific) that contained 0.2 ml of 0.7 N HCl. The tube was immediately capped, weighed, and vortexed to allow thorough mixing of the acid and the milk. Mixing HCl with milk stopped further lipolysis. Acidified samples were stored at 4°C until the next morning.

On the second day, after the prepared milk-acid mixture was warmed to room temperature, 0.2 ml of 1% (vol/vol) Triton-X 100 solution was added, and the mixture was vortexed. The Triton-X solution was added to help prevent the formation of emulsion during the shaking step later in the procedure. The copper soap reagent (4 ml) was added, and the mixture was vortexed

again. Next, 12 ml of chloroform-heptane-methanol (CHM [49:49:2, vol:vol:vol, HPLC grade]) solvent were added to each tube without vortexing. The mixture had two distinct layers: the deep blue aqueous layer on the bottom and the colorless CHM solvent layer on the top.

Next, the centrifuge tubes containing the reagents plus milk samples were shaken for 30 min in a basket that was attached to a Babcock shaker (Garver Shaker, Union City, IN). The tubes were in horizontal position on the shaker. The shaking speed for the Babcock shaker was 470 rpm (dial setting at 60). During shaking, the deep blue aqueous copper soap layer breaks into pea-sized "beads," and the "beads" were continuously in contact with the colorless solvent. When shaking was stopped, two distinct layers were quickly reformed. Occasionally, emulsion was formed after shaking. If emulsion occurred, the particular milk sample was prepared again. After shaking, the tubes were centrifuged at 5000 rpm ($2500 \times g$) for 10 min in a Sorvall Superspeed centrifuge (RC2-B, Sorvall SA-600 rotor; DuPont Instruments, Wilmington, DE). The top colorless solvent layer (3.5 ml) was carefully removed from the centrifuge tube using a disposable glass Pasteur pipette and transferred into an acid-washed test tube (10×75 mm) containing 0.1 ml of the color reagent. After mixing, absorbance was measured immediately at 440 nm in a cuvette with 1-cm path-length using a Spec 20 spectrophotometer (20 Genesys; Spectronic Instruments, Rochester, NY). Two blanks (1.0 ml of deionized water instead of 1.0 ml of milk) were also prepared and analyzed with the milk samples.

With each batch of 20 milk samples, a standard curve was constructed using palmitic acid (GC grade crystal, MW = 256.43; Alltech Associates, Inc., Deerfield, IL). Six concentrations of palmitic acid were prepared: 0, 60, 120, 180, 240, and 300 μg of palmitic acid/g of hexane. The standards were stored at -20°C in 2-ml GC glass vials (12×32 mm) that were tightly sealed with PTFE-lined screw caps (National Scientific, Lawrenceville, GA). On the first day of the analysis cycle, 1 ml of the standard was added to a screw-top centrifuge tube, and its weight was recorded. The hexane solvent was evaporated with nitrogen (high purity) under the hood. After the solvent was completely removed, 0.2 ml of 0.7 N HCl and 1.0 ml of deionized water was added to the tube. The standards were analyzed with each group of 20 milk samples and the two blanks.

Absorbance readings of standards were corrected by subtracting the average of the two blank readings, and a regression line was constructed, correlating the corrected absorbance with micrograms of palmitic acid. A record of slopes and intercepts of standard curves was kept to monitor and ensure consistency of method performance. Under normal conditions, the absorbance

reading of the blank was <0.01 . If readings of both blanks were high, the CHM solvent was tested for contamination using the following method: mix 3.5 ml of CHM solvent used in the analysis directly with the color reagent in an acid-washed test tube, and then measure absorbance at 440 nm. If the absorbance of the CHM and color reagent mixture was high like that of the blanks, it indicated problems with the CHM solvent, the color reagent, the test tubes, or the spectrophotometer. When the above situation occurred, minor impurities in solvent, due to different manufacturer production batch or unclean test tubes, was the reason for high blank reading. If the absorbance of the direct mixture from the CHM solvent and the color reagent was much lower than that of the high blanks, then each of the analysis steps and reagents was evaluated to identify where the problem occurred. The level of FFA in milk, in micrograms of FFA, was calculated from the standard curve. The final result was expressed in units of meq FFA/kg of milk and was calculated as: $[(\mu\text{g of FFA} \times 10^{-3} \text{ mg}/\mu\text{g})/(256.43 \text{ mg/meq})]/(\text{g of milk} \times 10^{-3} \text{ kg/g}) = \text{meq FFA/kg of milk}$.

RESULTS AND DISCUSSION

Fresh Raw Milk Quality

Mean ($n = 2$) SCC of the low- and high-SCC milks were 3.1×10^4 cell/ml and 1.1×10^6 cells/ml, respectively. The fat contents of the standardized low (3.30%) and high (3.26%) SCC fresh raw milks were similar, as planned in the experimental design. The high-SCC milk had a higher pH (6.89 at 4°C), a higher FFA (0.21 meq/kg of milk), and a lower CN/TP (79.79%) than the low-SCC milk (6.85 at 4°C , 0.15 meq/kg milk, and 82.27%, respectively). In general, differences in the initial milk composition between the low- and the high-SCC milks observed in the current study were in agreement with differences reported in the literature (Klei et al., 1998; Ma et al., 2000).

Experiment 1: Unpreserved Milks

Microbial growth. Microbial growth curves for all of the unpreserved milks are shown in Figure 2. No effect of SCC on microbial growth was observed in the current study. On d 0, for both the low- and high-SCC milks, SPC (approximately 10^4 cfu/ml) was higher than PBC (approximately 10^3 cfu/ml). During storage, SPC and PBC became similar for all treatments. This indicated that during 4°C storage, the microorganisms present in milk had become predominantly psychrotrophic bacteria.

For both the low- and high-SCC milks, the SPC and PBC in the control and the HCl-acidified treatments

Figure 2. Mean ($n = 2$) log₁₀ psychrotrophic bacteria count (PBC) and log₁₀ standard plate count (SPC) of the control (■), 1500 ppm CO₂ (○), and HCl-acidified (●) unpreserved low- (a, b) and high- (c, d) SCC standardized raw milks during 21 d of storage at 4°C.

were similar (Figure 2). The addition of 1500 ppm of CO₂ significantly decreased microbial growth in both the low- and high-SCC unpreserved milks (Figure 2). There was about a one to two logarithmic difference in PBC and SPC between the milk with 1500 ppm added CO₂ and the control or the HCl-acidified milks on d 14 and 21 (Figure 2). In general, CC were <200 cfu/ml (data not shown), and no substantial difference was observed among treatments and between the low- and high-SCC milks. Results from the current study were consistent with those previously reported (King and Mabbitt, 1982; Rowe, 1988). The antimicrobial effect of CO₂ is independent of its pH reduction effect.

The legal upper limit for SPC specified by the Pasteurized Milk Ordinance (PMO) for Grade A commingled bulk-tank milk before pasteurization is 3×10^5 cfu/ml (PMO, 1999). For SPC to reach this limit from an initial count of 10^4 cfu/ml in fresh raw milk, it took about 7 d for the control and the HCl-acidified low- and high-SCC milks, and it took approximately 14 d for the low- and high-SCC milks with 1500 ppm added CO₂ (Figure 2). From the perspective of the microbial count, the addition of 1500 ppm of CO₂ doubled the storage time of raw milk at 4°C. The storage time of raw milk based on microbial count could be extended even longer if the fresh raw milk had a lower initial bacteria count, the CO₂ concentration were further increased, and the

storage temperature were kept very low (e.g., just slightly above zero).

CO₂ level and pH. Control raw milks on d 0 contained 50 to 90 ppm of CO₂. These values are typical for fresh raw milks (Ma et al., 2001). On d 0, average CO₂ concentration in the carbonated low (1519 ppm) and high (1584 ppm) SCC milks were similar ($P > 0.05$). During storage, there was a progressive decrease in CO₂ concentration in both the low- and high-SCC carbonated milks, and the losses in the two milks were similar ($P > 0.05$, data not shown). By d 21, there were about 18 and 14% decreases in CO₂ concentration, respectively, in the low- and high-SCC carbonated unpreserved milks (data not shown).

For both the low- and high-SCC control milks, pH was similar ($P > 0.05$) between d 0 and 14 but decreased significantly ($P < 0.05$) from d 14 to 21 (Table 2). The decreases in milk pH in the unpreserved control milks were probably caused by the high level of microbial growth late in the storage period, when SPC and PBC were above 10^7 cfu/ml (Figure 2). Guinot-Thomas et al. (1995a) also observed a decrease in milk pH when microbial growth reached the end of the exponential phase.

The pH values of low- and high-SCC milks with 1500 ppm added CO₂ on d 0 were 6.18 and 6.19, respectively. The pH of the HCl-acidified low (6.18) and high (6.21)

Table 2. Mean ($n = 2$) pH of unpreserved milks at 4°C during 21 d of storage in experiment 1.

SCC level	Treatment	d 0	d 7	d 14	d 21
Low SCC	Control	6.85 ^a	6.82 ^a	6.81 ^a	6.69 ^b
	1500 ppm CO ₂	6.18 ^b	6.27 ^a	6.28 ^a	6.28 ^a
	HCl acidified	6.19 ^c	6.26 ^a	6.24 ^{ab}	6.21 ^{bc}
High SCC	Control	6.89 ^a	6.88 ^a	6.88 ^a	6.76 ^b
	1500 ppm CO ₂	6.19 ^b	6.26 ^a	6.30 ^a	6.29 ^a
	HCl acidified	6.21 ^b	6.28 ^a	6.26 ^{ab}	6.24 ^b

^{a,b,c}Means ($n = 2$) in the same row with no common superscript differ ($P < 0.05$).

SCC milks were similar ($P > 0.05$) to their carbonated counterparts on d 0. Between d 0 and 7, a similar extent of pH increase occurred in both carbonated and the HCl-acidified low- and high-SCC milks (Table 2). It is not clear what caused the increase in milk pH during the first week of storage. From d 7 to 21, the pH of the carbonated low- and high-SCC milks remained the same, but that of the HCl-acidified low- and high-SCC milks decreased slightly (Table 2). Similar to the control milks, this decrease in pH for the HCl-acidified low- and high-SCC milks could be related to high levels of microbial growth at the end of the storage period (Figure 2).

Proteolysis. The ANOVA (Table 3) found an effect of SCC, treatment, day, and day $\times$ treatment but not of day $\times$ SCC or day $\times$ SCC $\times$ treatment. The nature of the effect of different treatments on proteolysis during storage at 4°C can be seen in Figure 3. In the low-SCC control milk, significant ($P < 0.05$) proteolysis occurred on d 14 (Figure 3a), and CN/TP decreased from 82% on d 0 to 66% by d 21. A significant ($P < 0.05$) decrease

in CN/TP was also observed in the unpreserved HCl-acidified low-SCC milk (Figure 3a). The extent of proteolysis in the HCl-acidified low-SCC milk was similar ($P > 0.05$) to that of the control low-SCC milk on d 14 but was less ($P < 0.05$) on d 21. Nonetheless, the extent of decrease in HCl-acidified low-SCC milk was significant ($P < 0.05$), from 82% on d 0 to 78% on d 14 and to 73% on d 21. For the low-SCC unpreserved milk with 1500 ppm added CO₂, no significant ($P > 0.05$) proteolysis was observed over the 21-d storage period at 4°C and CN/TP was maintained at 82% (Figure 3a). Thus, 1500 ppm of CO₂ significantly reduced proteolysis in the low-SCC unpreserved milk, probably due to the inhibition of bacteria that produce proteolytic enzymes.

In the control high-SCC unpreserved milk, CN/TP decreased during storage at 4°C, and the decrease was large between d 14 and 21 (Figure 3b). A similar ($P > 0.05$) extent of proteolysis as seen in the control was observed during storage in the unpreserved high-SCC milk with HCl acidification (Figure 3b). Thus, the reduction in pH due to HCl addition did not retard proteol-

Table 3. Sum of squares (SS) and probabilities (P) from the ANOVA of proteolysis and lipolysis data in experiment 1.

Independent variables	Proteolysis SSM ¹ = 1308.39, Model R ² = 0.92		Lypolysis SSM = 0.1184, Model R ² = 0.85	
	SS ²	P	SS	P
Whole-plot factor				
Replicate	39.24		0.0133	
SCC	98.27	0.05	0.0152	<0.01
Treatment ³	183.06	<0.05	0.0133	<0.05
Treatment $\times$ SCC	5.35	NS ⁴	0.0041	NS
E (a)	78.65		0.0041	
Repeated measure factors				
Day	627.12	<0.01	0.0362	<0.01
Day $\times$ SCC	6.06	NS	0.0062	NS
Day $\times$ treatment	258.02	<0.01	0.0216	<0.05
Day $\times$ SCC $\times$ treatment	12.62	NS	0.0044	NS
E (b)	112.50		0.0203	

¹SSM = Sum squares of model.

²SS = Sum squares of individual factors.

³Treatment = unpreserved control milk, unpreserved milk with 1500 ppm CO₂, and unpreserved milk acidified with HCl.

⁴NS = Not significant, $P > 0.05$.

ysis in unpreserved high-SCC milk. In the high-SCC unpreserved milk with 1500 ppm added CO₂, CN/TP decreased significantly ($P < 0.05$) from 79.8% on d 0 to 77.5% on d 21 (Figure 3b). Even a small (e.g., 1 to 2%) decrease in CN/TP can have important economic impacts. Enzymatic damage to CN can be directly reflected in the percentage decrease in cheese yield (Barbano et al., 1991; Klei et al., 1998). In addition, a 4.04% decrease in CN/TP has been shown to cause bitter off-flavors in pasteurized fluid milk (Ma et al., 2000). By 14 d of storage, it is likely that bitter off-flavor and other off-flavors related to microbial activity would have developed in the control and HCl-acidified milks, but not in the milks containing 1500 ppm of CO₂. Addition of 1500 ppm of CO₂ significantly reduced the proteolysis in both the low- and high-SCC unpreserved milks. Lowering milk pH with HCl to the same pH as milk with 1500 ppm added CO₂ did not show the same inhibitory effect on proteolysis as the addition of 1500 ppm of CO₂ did in unpreserved low- and high-SCC milks.

Lipolysis. The ANOVA found (Table 3) significant effects of SCC, treatment, day, and day $\times$ treatment but not of day $\times$ SCC, or day $\times$ SCC $\times$ treatment. For both the control and HCl-acidified low-SCC unpreserved milks, a significant ($P < 0.05$) increase in FFA

concentration was observed between d 14 and 21 (Figure 4a). In the low-SCC milk with 1500 ppm added CO₂, FFA level remained low and showed no significant ($P > 0.05$) increase up to d 21 of storage at 4°C (Figure 4a). Thus, for unpreserved low-SCC milk, the addition of 1500 ppm of CO₂ significantly reduced lipolysis during 21 d of storage at 4°C, but acidification with HCl to the same pH as the carbonated milk did not.

Typically during mastitis, when milk SCC is high, it is expected that the extent of lipolysis is higher due to active somatic cell lipases and damaged milk-fat globule membrane (Downey, 1980; Murphy et al., 1989). However, in the current study, no significant FFA increase was observed in all of the high-SCC milks between d 0 and 14 (Figure 4b). In the high-SCC unpreserved control milk, a significant ($P < 0.05$) increase in FFA was observed between d 14 and 21 (Figure 4b), similar to what was observed in the low-SCC unpreserved control milk (Figure 4a). Both the HCl-acidified and carbonated high-SCC unpreserved milks showed no significant ($P > 0.05$) increase in FFA over 21 d (Figure 4b). Thus, the addition of 1500 ppm CO₂ to milk retarded lipolysis in high-SCC raw milk during 21 d of storage at 4°C.

Figure 3. Mean ($n = 2$) casein as a percentage of true protein (CN/TP) in the control (■), 1500 ppm CO₂ (○), and HCl-acidified (●) unpreserved low- (a) and high- (b) SCC milks during 21 d of storage 4°C. Means for the different treatments on the same storage day with no common letter differ ($P < 0.05$).

Figure 4. Mean ($n = 2$) free fatty acid (FFA) content (meq FFA/kg milk) of the control (■), 1500 ppm CO₂ (○), and HCl-acidified (●) unpreserved low- (a) and high- (b) SCC milks during 21 d of storage 4°C. Means for the different treatments on the same storage day with no common letter differ ($P < 0.05$).

Experiment 2: Preserved Milks

Microbial growth. The PBC, SPC, and CC of the preserved milks at d 21 were very low, indicating that preservative worked and that the microbial counts of preserved milks were much lower than those of unpreserved milks (Figure 2). For control, HCl-acidified, 500, 1000, and 1500 ppm of CO₂ milks, the PBC were, in general, <10 cfu/ml, the SPC were <1500 cfu/ml, and CC were <10 cfu/ml. Potassium dichromate preservative inhibited the growth of microorganisms but did not inhibit the activity of endogenous milk enzymes (Senyk et al., 1985). Therefore, any proteolysis and lipolysis that occurred in preserved milks would be caused by endogenous milk proteases and lipases and the difference in the extent of proteolysis and lipolysis between the preserved low- and high-SCC milks would be due to the difference in their endogenous enzyme activities.

CO₂ level and pH. Control raw milks on d 0 had CO₂ levels ranging from 50 to 90 ppm. On d 0, the average added CO₂ concentrations were, respectively, 1503 and 1487 ppm for the preserved low- and high-SCC carbonated milks. During storage, there was a progressive decrease in CO₂ concentration in the carbonated milks, and the extent of decrease was similar

($P > 0.05$) between the low- and high-SCC preserved milks (Figure 5). From d 0 to 21, CO₂ concentration decreased about 15.6% to 1268 ppm in the low-SCC preserved milk and about 15.3% to 1260 ppm in the high-SCC preserved milk. The extent of CO₂ loss in the preserved carbonated milks was similar to that observed in unpreserved carbonated milks in experiment 1.

The pH of the control low (6.83 to 6.86) and high (6.88 to 6.89) SCC preserved milks remained the same ($P > 0.05$) over the 21-d storage period at 4°C. In the carbonated (1500 ppm) and HCl-acidified low- and high-SCC milks, pH increased progressively with storage time, especially between d 0 and 7 (Table 4), as was observed in the unpreserved milks in experiment 1 (Table 2). On each of the storage days, pH increases were similar ($P > 0.05$) between the low- and high-SCC preserved milks of same treatment type. Compared to the unpreserved control and HCl-acidified milks in experiment 1, which all had pH decrease late in storage (Table 2), no pH decrease was observed in the preserved milks (Table 4), probably due to the lack of microbial growth in the preserved milks.

Proteolysis. The results of ANOVA comparing the decrease of CN/TP in the control, the HCl-acidified, and

Figure 5. Mean ($n = 2$) level of dissolved CO₂ content of preserved low- (a) and high- (b) SCC carbonated milks stored at 4°C for 21 d. The target levels of carbonation were 500 (□), 1000 (△), and 1500 (○) ppm. Means for the same target carbonation level across days with no common letter differ ($P < 0.05$).

1500 ppm of CO₂ preserved low- and high-SCC milks are shown in Table 5. In general, the preserved high-SCC milks had more proteolysis ($P < 0.01$) during 21 d of storage at 4°C than the preserved low-SCC milks (Figure 6), and the effects of SCC ($P < 0.01$), day $\times$ SCC ($P < 0.01$), treatment ($P < 0.01$), and treatment $\times$ SCC ($P < 0.05$) were significant (Table 5).

In the low-SCC preserved milks, the decreases in CN/TP during the 21 d of storage were relatively small (Figure 6a) compared with those for unpreserved milks (Figure 3a). Compared with the control, the decrease of CN/TP was less in the 1500 ppm of CO₂ milks on d 14 and 21 (Figure 6a). In the high-SCC preserved milks,

during the 21 d of storage at 4°C, the decrease in CN/TP was similar ($P > 0.05$) for the HCl-acidified and the 1500 ppm of CO₂ milks, and both milks had less CN/TP decrease than the control milk (Figure 6b). In the control high-SCC preserved milk, CN/TP decreased about 3% from d 0 to 21 (Figure 6b), and this was greater than the decrease in the preserved control low-SCC milk during the same period (Figure 6a), as indicated by the significant ($P < 0.05$) treatment $\times$ SCC effect (Table 5). We hypothesized that this difference was caused by the higher level of endogenous milk protease activity in the high-SCC milk, as suggested by previous research (de Rham and Andrews, 1982; Sae-

Table 4. Mean ($n = 2$) pH of preserved milks at 4°C from 0 to 21 d of storage.

SCC level	Treatment	d 0	d 7	d 14	d 21
Low SCC	Control	6.84 ^a	6.84 ^a	6.86 ^a	6.83 ^a
	500 ppm CO ₂	6.49 ^b	6.51 ^{ab}	6.55 ^a	6.56 ^a
	1000 ppm CO ₂	6.30 ^c	6.36 ^b	6.42 ^a	6.42 ^a
	1500 ppm CO ₂	6.18 ^b	6.26 ^a	6.29 ^a	6.29 ^a
	HCl acidified	6.19 ^c	6.25 ^b	6.28 ^{ab}	6.29 ^a
High SCC	Control	6.89 ^a	6.88 ^a	6.89 ^a	6.88 ^a
	500 ppm CO ₂	6.52 ^b	6.55 ^{ab}	6.59 ^a	6.60 ^a
	1000 ppm CO ₂	6.31 ^c	6.40 ^b	6.44 ^{ab}	6.46 ^a
	1500 ppm CO ₂	6.20 ^b	6.28 ^a	6.30 ^a	6.33 ^a
	HCl acidified	6.20 ^c	6.28 ^b	6.29 ^b	6.31 ^a

^{a,b,c}Means ($n = 2$) in the same row with no common superscript differ ($P < 0.05$).

Table 5. Sum of squares (SS) and probabilities (*P*) from the ANOVA of proteolysis and lipolysis data in experiment 2.

Independent variables	Proteolysis SSM ¹ = 183.09, Model R ² = 0.99		Lipolysis SSM = 0.0649, Model R ² = 0.93	
	SS ²	<i>P</i>	SS	<i>P</i>
Whole-plot factors				
Replicate	17.73		0.0036	
SCC	132.70	<0.01	0.0482	<0.01
Treatment ³	8.46	<0.01	0.0037	NS ⁴
Treatment × SCC	1.27	<0.05	0.0000	NS
E (a)	0.55		0.0032	
Repeated measure factors				
Day	14.76	<0.01	0.0032	<0.05
Day × SCC	5.03	<0.01	0.0009	NS
Day × treatment	1.82	NS	0.0010	NS
Day × SCC × treatment	0.77	NS	0.0011	NS
E (b)	2.64		0.0047	

¹SSM = Sum squares of model.²SS = Sum squares of individual terms.³Treatment = preserved control milk, preserved milk with 1500 ppm CO₂, and preserved milk acidified with HCl.⁴NS = not significant, *P* > 0.05.

man et al., 1988; Verdi and Barbano, 1991). For preserved high-SCC milks, the two treatments, addition of 1500 ppm of CO₂ and acidification with HCl, both reduced milk pH to a similar extent, and both also

reduced proteolysis to a similar extent (Figure 6b). This is in contrast to the HCl treatment not demonstrating any inhibitory effect on proteolysis in unpreserved milks (Figure 3). Therefore, the inhibitory effect of CO₂

Figure 6. Mean (*n* = 2) casein as a percentage of true protein (CN/TP) of the control (■), 1500 ppm CO₂ (○), and HCl-acidified (●) preserved low- (a) and high- (b) SCC milks during 21 d of storage 4°C. Means for the different treatment on the same storage day with no common letter differ (*P* < 0.05).

Figure 7. Mean ($n = 2$) free fatty acid (FFA) content (meq FFA/kg milk) of the control (■), 1500 ppm CO₂ (○), and HCl-acidified (●) preserved low- (a) and high- (b) SCC milks during 21 d storage at 4°C.

on proteolysis by endogenous milk protease appears to be due to the pH reduction, not a direct interaction of CO₂ with the endogenous proteolytic enzymes.

Lipolysis. Results of the ANOVA comparing lipolysis in the control, the HCl-acidified, and 1500 ppm of CO₂ preserved low- and high-SCC milks are shown in Table 5. Only the effects of SCC and day were significant

(Table 5). Milk FFA concentration increased ($P < 0.05$) with days of storage in both the low- and high-SCC preserved milks (Figure 7). Similar extent of fat degradation occurred in milks of different treatments in the low- and high-SCC preserved milks. No significant effect of carbonation or acidification on the FFA content of preserved low- and high-SCC milks was observed

Table 6. Sum of squares (SS) and probabilities (P) from the ANOVA of proteolysis and lipolysis data in experiment 3.

Independent variables	Proteolysis SSM ¹ = 239.42, Model R ² = 0.99		Lypolysis SSM = 0.0944, Model R ² = 0.93	
	SS ²	P	SS	P
Whole-plot factors				
Replicate	24.89		0.0065	
SCC	169.75	<0.01	0.0609	<0.01
Treatment ³	11.42	<0.01	0.0035	NS ⁴
Treatment × SCC	1.49	NS	0.0001	NS
E (a)	1.32		0.0083	
Repeated measure factors				
Day	22.30	<0.01	0.0069	<0.01
Day × SCC	6.29	<0.01	0.0048	<0.01
Day × treatment	1.39	NS	0.0014	NS
Day × SCC × treatment	0.57	NS	0.0020	NS
E (b)	2.70		0.0075	

¹SSM = Sum squares of model.

²SS = Sum squares of individual terms.

³Treatment = CO₂ concentration, i.e., preserved milk with control level, 500, 1000, and 1500 ppm CO₂.

⁴NS = not significant, $P > 0.05$.

Figure 8. Mean ($n = 2$) casein as a percentage of true protein (CN/TP) of preserved low- (a) and high- (b) SCC milks with control level (■), 500 (□), 1000 (△), and 1500 (○) ppm CO_2 during 21 d of storage 4°C . Means for the treatments on the same storage day with no common letter differ ($P < 0.05$).

Figure 9. Mean ($n = 2$) free fatty acid (FFA) content (meq FFA/kg milk) of preserved low- (a) and high- (b) SCC milks with control level (■), 500 (□), 1,000 (△), and 1500 (○) ppm CO_2 during 21 d of storage 4°C .

over the 21-d storage period at 4°C. Thus, lowering milk pH to 6.2 or adding 1500 ppm of CO₂ did not retard lipolysis caused by endogenous milk lipases.

Experiment 3: Preserved Milks with Different Carbonation Level

Microbial growth. The PBC, SPC, and CC of low- and high-SCC preserved milks with four levels of carbonation at d 21 were very low, as described above. Similar to the results in experiment 2, during storage microbial growth was blocked by the preservative, and there was no difference in the microbial counts among the low- and high-SCC preserved milks with the four different CO₂ concentrations (data not shown).

CO₂ level and pH. On d 0, mean ($n = 2$) CO₂ concentrations of carbonated milks were 585, 1062, and 1503 ppm for the low-SCC preserved milks and 593, 1068, and 1487 ppm for the high-SCC preserved milks. During storage, there was a progressive decrease in CO₂ concentration in all of the carbonated milks, and the decreases were similar ($P > 0.05$) over time at each carbonation level for both the low- and high-SCC preserved milks (Figure 5).

The pH of the control low- and high-SCC preserved milks remained the same ($P > 0.05$) during storage. In the carbonated low- and high-SCC milks (500 to 1500 ppm), pH increased progressively with storage time, especially between d 0 and 7 (Table 4). During storage, a similar ($P > 0.05$) extent of pH increase was observed in the low- and high-SCC preserved milks at the same carbonation level.

Proteolysis. The results of the ANOVA comparing the decrease of CN/TP in milks with four levels of carbonation (approximately 0 [control], 500, 1000, and 1500 ppm added CO₂) are shown in Table 6. In general, the high-SCC milks had more proteolysis during storage at 4°C, and the effects of SCC and day $\times$ SCC were both significant ($P < 0.01$; Table 6). In the low-SCC preserved milk, the extent of CN/TP decreases were all $< 1\%$ over the 21-d period (Figure 8a). During storage at 4°C, the levels of proteolysis in the high-SCC preserved milks with 500, 1000, and 1500 ppm added CO₂ were similar ($P > 0.05$; Figure 8b) and were all less ($P < 0.05$) than that in the high-SCC preserved control milk, especially on d 14 and 21. Thus, the addition of 500 ppm of CO₂ already effectively reduced proteolysis in the preserved high-SCC milk during storage at 4°C, and a further increase in CO₂ concentration, or a further decrease in milk pH, did not further reduce proteolysis in preserved high-SCC milk.

Lipolysis. The ANOVA (Table 6) found significant effects of SCC, day, and day $\times$ SCC ($P < 0.05$). In general, on each of the storage days, there was a high level of

FFA in the high-SCC preserved milks, and the rate of FFA increase during storage at 4°C was greater in the high-SCC preserved milks (Figure 9). No significant treatment or day $\times$ treatment ($P > 0.05$) effect was detected (Table 6). This is consistent with the result from experiment 2: in preserved milks, if there was no effect of CO₂ at a concentration of 1500 ppm, then carbonation at levels below 1500 ppm would not be expected to have an effect.

Mechanisms for Reduced Proteolysis and Lipolysis in Raw Milk with Added CO₂

Experiments 1 and 2 were designed to separately determine the effect of added CO₂ in raw milk on proteolysis and lipolysis contributed by enzymes originated from spoilage bacteria growing in raw milk vs. proteolysis and lipolysis contributed by endogenous milk enzymes (e.g., plasmin and LPL). The addition of 1500 ppm of CO₂ to unpreserved raw milk reduced microbial growth (Figure 2) and dramatically reduced proteolysis (Figure 3) and lipolysis (Figure 4). Most of this reduction in proteolysis and lipolysis was attributed to the influence of CO₂ as an inhibitor of microbial growth. Because added HCl did not have the same impact, the effect on bacteria was primarily due to the CO₂ and not the reduction of milk pH caused by the CO₂.

The concentration of extracellular enzymes of microbial origin has been shown to increase exponentially when bacteria cell count reached 10⁷ cfu/ml (Rowe et al., 1990). Our results are in agreement with previous literature (Law, 1979; Downey, 1980; Grieve and Kitchen, 1985; Guinot-Thomas, et al., 1995b) and suggest that significant contributions to proteolysis and lipolysis by microbial enzymes occur when microbial cell count reaches above 10⁶ to 10⁷ cfu/ml. Once active proteases and lipases are secreted by microorganisms, the proteolysis and lipolysis by microbial enzymes are typically much more substantial than those by endogenous milk enzymes.

In unpreserved milks, proteolysis and lipolysis may not only depend on the total bacteria count but also on the types of bacteria present, the concentration the extracellular enzymes secreted, and their activities (Cousin, 1982). This may explain the differences in proteolysis between the control and the HCl-acidified unpreserved low-SCC milks on d 21 (Figure 3a), even though the total microbial counts in these two milks were similar (Figure 2). The difference in lipolysis between the control and the HCl-acidified unpreserved high-SCC milks on d 21 (Figure 4b) could also be related to the particular types of microorganisms that were present in the two milks.

For both the low- and high-SCC preserved milks, addition of 1500 ppm of CO₂ and acidification with HCl both reduced proteolysis to the same extent as compared with the control milks during storage at 4°C. This suggested that the effect of adding 1500 ppm of CO₂ on proteolysis by endogenous milk protease was related to the pH reduction caused by CO₂, not the CO₂ itself. The endogenous milk protease is plasmin. Plasmin is an alkaline serine protease, and its maximum activity occurs at pH 7.5 at 37°C (de Rham and Andrews, 1982; Grufferty and Fox, 1988). Carbonation or HCl acidification moved milk pH away from the optimum pH for plasmin activity and, thus, may have caused a decrease in plasmin activity and a decrease in proteolysis. The effect of acidification with either HCl or 1500 ppm of CO₂ was more pronounced in the high-SCC milks (Figure 6), probably due to a higher initial plasmin activity in the high-SCC milk (not measured in the current study), as suggested by previous research (de Rham and Andrews, 1982; Verdi and Barbano, 1991).

Endogenous milk LPL is the major lipolytic enzyme responsible for elevated FFA concentration in milk with low bacteria count. No significant effect of carbonation or acidification of milk on lipolysis was observed in preserved low- and high-SCC milks. This indicated that the addition of 1500 ppm of CO₂ and the lowering of milk pH did not inhibit the activity of milk LPL.

CONCLUSIONS

The addition of 1500 ppm of CO₂, but not HCl, effectively delayed the growth of bacteria in raw milk during 21 d of storage at 4°C. The CO₂ addition to raw milk decreased proteolysis via at least two mechanisms: the reduction of microbial proteases due to reduced microbial growth and the possible reduction in plasmin activity due to a lower milk pH. The effect of CO₂ on reducing lipolysis was due to a reduced microbial growth. No effect of CO₂ addition or acidification on lipolysis by native milk lipase was detected. Adding 1500 ppm of CO₂ to high-quality raw milk (i.e., low bacteria count and low SCC) will allow raw milk to be stored for 14 d at 4°C with an SPC below 3×10^5 cfu/ml and with minimal proteolysis and lipolysis.

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