

Effect of Carbon Dioxide under High Pressure on the Survival of Cheese Starter Cultures

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ABSTRACT

A new processing method that rapidly forms curds and whey from milk has the potential to improve cheesemaking procedures if cheese starter cultures can tolerate the processing conditions. The survival of *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactococcus lactis* ssp. *lactis*, or *Streptococcus thermophilus* through this new process was evaluated. Inoculated milk containing 0, 1, or 3.25% fat or Lactobacillus MRS broth or tryptone yeast lactose broth (depending on microorganism used) was sparged with CO₂ to a pressure of 5.52 MPa and held for 5 min at 38°C. Broth contained 7.93 to 8.78 log CFU/ml before processing and 7.84 to 8.66 log CFU/ml afterward. Before processing, milk inoculated with *L. bulgaricus*, *L. lactis*, or *S. thermophilus* contained 6.81, 7.35, or 6.75 log CFU/ml, respectively. After processing, the curds contained 5.68, 7.32, or 6.50 log CFU/g, and the whey had 5.05, 6.43, or 6.14 log CFU/ml, respectively. After processing, the pHs of control samples were lower by 0.41 units in broth, 0.53 units in whey, and 0.89 units in curd. The pH of the processed inoculated samples decreased by 0.3 to 0.53 units in broth, 0.32 to 0.37 units in whey, and 0.93 to 0.98 units in the curd. Storing curds containing *L. lactis* at 30°C or control curds and curds with *L. bulgaricus* or *S. thermophilus* at 37°C for an additional 48 h resulted in pHs of 5.22, 5.41, 4.53, or 4.99, respectively. This study showed that milk inoculated with cheese starter cultures and treated with CO₂ under high pressure to precipitate casein-produced curds that contained sufficient numbers of viable starter culture to produce lactic acid, thereby decreasing the pH.

Lactic acid starter cultures play complex roles in cheesemaking and it is desirable that portions of the starter culture population survive the cheesemaking process to help the curd develop into quality cheese. Recent research describes laboratory-scale batch processing of milk using CO₂ under high pressure to precipitate the casein (7, 18). Milk is placed in a sealed chamber and CO₂ is sparged through the milk to obtain the desired pressure. After depressurization when CO₂ is vented off, an easily separated casein curd and whey mixture is left behind. If cheese starter cultures survive this processing step using CO₂ at high pressure, inoculated cheese milk could be processed into cheese curd within minutes rather than the hours currently required in cheesemaking.

Tomasula et al. (18) reports that when CO₂ is sparged through the milk (maintained at 38°C) to a pressure of 5.52 MPa and held for 5 min, the whey has a pH of 6.0 and the resulting casein mass is granular, moist, friable, and contains high levels of solids, ash, and calcium. In standard cheesemaking, milk ripening or setting steps are done at 30 to 35°C to avoid heat shocking the cheese starter cultures (9). The CO₂ processing environment should not be detrimental to the facultative anaerobic lactic acid bacteria commonly used in cheesemaking, although CO₂ has strong antimicrobial effects on psychrotrophic bacteria in the milk (1, 8, 11-13). When degassed, carbonated milk maintains

satisfactory sensory qualities (1, 13) and makes cheese with lower psychrotrophic bacterial counts (3, 10, 11, 19) that requires less rennet (10, 11) and develops satisfactory flavor (11, 19).

High hydrostatic pressure damages microbial cells (5); cell morphology is affected at pressures as low as 0.6 MPa, hydrophobic interactions are disrupted at pressures below 100 MPa, and biochemical reactions and membrane integrity are altered at pressures above 100 MPa. Compared to buffer, milk provides some protection to microorganisms when exposed to high pressure (17). When inoculated into milk, *Vibrio parahaemolyticus* is inactivated at pressures over 165 MPa and *Listeria monocytogenes* is killed at pressures over 300 MPa.

Because the use of CO₂ to precipitate casein offers a novel technique to reduce significantly the time required to produce casein curd suitable for making cheese, the goal of this study was to determine the survival rate of typical dairy starter cultures after CO₂ treatment at high pressure.

MATERIALS AND METHODS

Microorganisms. Frozen (-60°C) cheese starter cultures, *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842, *Lactococcus lactis* ssp. *lactis* ATCC 11955, and *Streptococcus thermophilus* ATCC 19258 were obtained from Dr. George A. Somkuti, ARS, ERRC, Wyndmoor, Pa. Ultra high temperature (UHT) skim milk (Parmalat, Mooachie, N.J.), used only in preparation of mother culture, and pasteurized nonhomogenized whole and skim milk (Chrome Dairies, Oxford, Pa.), used only for CO₂

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treatments, were purchased locally. Pasteurized whole and skim milks were tested for fat content using the Babcock assay (2) and blended to obtain 0, 1.0, or 3.25% fat milks for treatment. For this study, stock cultures of *L. bulgaricus* were maintained in Lactobacillus MRS broth (Difco Laboratory, Inc., Detroit, Mich.) and *L. lactis* and *S. thermophilus* were maintained in tryptone yeast lactose broth (TYL), consisting of tryptone, yeast, and beef extract (Difco) and lactose and K₂HPO₄ (J. T. Baker, Phillipsburg, N.Y.) adjusted to pH 6.5 using 1 N NaOH (4). The inoculum was prepared by transferring 0.1 ml of stock culture to test tubes containing 20 ml of the appropriate broth or UHT skim milk and incubated either at 30°C for *L. lactis* or 37°C for *L. bulgaricus* or *S. thermophilus*. The entire overnight culture (≥ 16 h) was used to inoculate 500 ml of TYL, MRS, or nonhomogenized pasteurized milk containing 0, 1.0, or 3.25% fat in a 1-liter Erlenmeyer flask. The inoculated media were incubated with shaking (100 rpm) for 3 h at either 30 or 37°C to insure adequate distribution of the cells without causing air bubble formation. All samples were incubated for 3 h after inoculation to allow for sufficient recovery and growth of the microorganisms in their new environment. Control samples were removed at 0 and 3 h for plate counting and pH determination, but only the 3-h samples were used for the before-processing CFU/ml. Each procedure was repeated three times.

Equipment. A 4521 316SS Parr reactor (Parr Instrument Co., Moline, Ill.) with a 1-liter capacity was modified as described by Tomasula et al. (18). The lid of the chamber was fitted with a three-blade propeller stirrer, a CO₂ sparger made of a modified 2- μ m porous metal filter (Supelco, Inc., Bellefonte, Pa.) on a 0.6-cm tube, and a metal filter frame fitted with a piece of silk. The chamber could be tilted and the height adjusted to allow the chamber to be filled and then raised to seal and secure the lid for pressurization.

Before each sample was processed, the sparger and stirrer were removed and the CO₂ chamber and inlet and outlet lines were rinsed with a quaternary sanitizer (Lysol I.C., National Laboratories, Montvale, N.J.) and then purged with steam for 30 min. The sparger was flamed to remove all particulate material. The sparger, stirrer, and silk filter were soaked in the sanitizer and rinsed in distilled water before being reinserted into the chamber.

On each processing day, three processing trials were conducted with all three trials using the same type of media and each trial containing a different cheese starter culture (*L. bulgaricus*, *L. lactis*, or *S. thermophilus*). On the day that broth study was scheduled, the *L. lactis* and *S. thermophilus* were processed in TYL broth and the *L. bulgaricus* was processed in MRS broth.

Approximately 440 ml of inoculated sample were placed in the batch chamber and prewarmed to 38°C. The sample was stirred during prewarming and for the first 4 min of processing to ensure adequate mixing and heat transfer. Chilled CO₂ (BOC Group, Inc., Murray Hill, N.J.) was slowly sparged into the chamber and usually took under 2 min to reach the processing pressure. Processing conditions of 5.52 MPa and 38°C were held for 5 min before pressure was released. Liquids (TYL or MRS broth or whey) were collected through the outlet line, and the casein curd was collected after the chamber was opened.

Microbiological analysis. After processing, the broth or the resulting curds and whey were collected aseptically and placed on ice for transport to the laboratory. The analysis of the broth began within 5 min from collection. The curds and whey took up to 15 min to collect and begin analysis. Whey had to settle for 10 min due to excessive foaming during collection. After the chamber was opened, the casein curd was removed and placed on sterile cheese

cloth suspended within a beaker to allow the excess whey to drain. The beaker was covered with aluminum foil.

Standard pour plate technique (6) was used to determine CFU/ml or CFU/g. Samples were serially diluted using 0.1% peptone water (Difco). The curd was prepared for plate count by first making a 1:10 dilution in 0.1% peptone water and stomached for 1 min at medium speed (Stomacher 400, Teckmar, Cincinnati, Ohio). *L. bulgaricus* was plated on MRS agar and *L. lactis* and *S. thermophilus* were plated on TYL agar. The MRS and TYL agars were prepared by adding 1% Bactoagar (Difco) to the broths. Plates were incubated for 48 h at either 30°C (for *L. lactis*) or 38°C (for *L. bulgaricus* or *S. thermophilus*) before hand counting. Triplicate plates were made for each dilution.

Chemical analysis. After portions of sample were removed for microbiological analysis, the pH was obtained for all broths, milks, whey, and drained curds. A portion of the aseptic curds was placed in a sterile container and incubated at 30°C (for *L. lactis*) or 37°C (for *L. bulgaricus* and *S. thermophilus*); curd pH was obtained at 24 and 48 h.

Statistical analysis. Processing trials were scheduled and analyzed using a split-plot experimental design. Milk or broth was blocked randomly for each processing day within a 4 processing day grouping and each of the three starter cultures randomly ordered for each processing day. Triplicate processing days were scheduled for a total of 12 processing days and a total of 36 trials. Controls consisted of trials using uninoculated milk or broth. Broth data were analyzed separately from the milk data. General linear model statistical analysis (14) was used to evaluate pH and microbiological responses. Data from milk trials were evaluated using both whole-plot and subplot analysis.

RESULTS

Microbiological: broth. Means for the number of viable starter cultures in the broth, before and after processing, are presented in Figure 1a. General linear model analysis of the CFU/ml indicated that there was more variation among the replications than among the starter cultures. Broth inoculated with *L. bulgaricus*, *L. lactis*, or *S. thermophilus* contained 7.93, 8.78, or 8.42 log CFU/ml prior to processing and 7.84, 8.16, or 8.66 log CFU/ml after processing, respectively; differences between microorganisms and between before and after processing were not significant ($P < 0.05$).

Milk. Means for the number of viable starter cultures in the milk containing 0, 1, or 3.25% fat, before and after processing, are presented in Figure 1b. Split-plot analysis of the CFU data indicated significant ($P < 0.05$) influence from the type of starter culture but not from the level of fat in milk. Milk (all fat levels combined) inoculated with *L. bulgaricus*, *L. lactis*, or *S. thermophilus* contained 6.81, 7.35, or 6.75 log CFU/ml, respectively, before processing. After processing, the curd contained 5.68, 7.32, or 6.55 log CFU/g and the whey contained 5.05, 6.43, or 6.14 log CFU/ml, respectively. *L. bulgaricus* showed the greatest decrease in numbers after processing with almost 1-log reduction in the curd and almost 2-log reductions in the whey. *L. lactis* and *S. thermophilus* decreased 0.9 and 0.6 log CFU/ml in the whey, respectively, from the starting CFU/ml in the milk. There was no significant difference between the counts in the inoculated milk and those found in the curd.

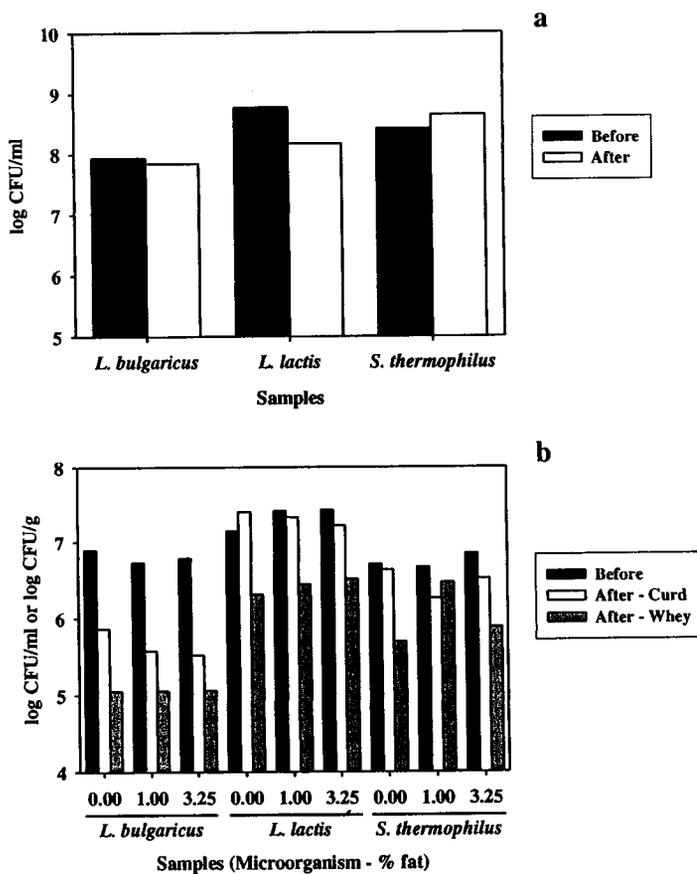


FIGURE 1. Comparison of CFU/ml (before, after, after-whey) or CFU/g (after-curd) for *L. bulgaricus*, *L. lactis*, or *S. thermophilus* before and after high-pressure CO_2 processing in (a) broth or (b) milk containing 0, 1, or 3.25% fat.

Processing characteristics: processing parameters.

Processing temperatures ranged from 35.1 to 40.7°C during individual processing trials due to the introduction of chilled CO_2 into the chamber. The trial means were not significantly ($P < 0.05$) different and averaged 37.9°C for

the broth runs and 38.2°C for the milk runs. Processing pressure ranged from 5.42 to 5.82 MPa for individual trials and the trial means were not significantly ($P < 0.05$) different. The mean average was 5.58 MPa for the broth runs and 5.59 MPa for the milk runs.

Changes in pH. A summary of the pH of broth or milk samples before and after processing is in Table 1. Before inoculation, both buffered TYL and MRS broth had pH values of 6.5, whereas after inoculation and a 3-h incubation, sufficient lactic acid was produced to lower the pH of *L. bulgaricus*, *L. lactis*, or *S. thermophilus* samples to 5.73, 6.03, or 6.22, respectively. After processing, the pH had decreased by 0.30, 0.40, and 0.53, respectively. The pH of controls was 0.41 units lower after processing.

Before inoculation, milk samples had an average pH of 6.68, whereas after inoculation and a 3-h incubation, the milk pHs were 6.59, 6.55, or 6.60, for *L. bulgaricus*, *L. lactis*, or *S. thermophilus*, respectively. Compared to the pH of milk before processing, the pH was 0.32 to 0.37 units lower in the whey and 0.93 to 0.99 units lower in the casein curd. In the control samples, the pH also decreased 0.53 ± 0.11 in the whey and 0.89 ± 0.01 in the curd. Casein curds were incubated for 2 days at the starter culture's optimum temperature to determine if the microorganisms that had survived the CO_2 process were still capable of producing sufficient amounts of lactic acid to lower the pH of the curd. After 2 days of incubation, the pH of the curds inoculated with *L. bulgaricus*, *L. lactis*, or *S. thermophilus* had significantly ($P < 0.05$) decreased another 1.12, 0.40, or 0.62 units, respectively.

DISCUSSION

During the ripening and coagulation of milk, many complex physical and chemical changes occur that are essential to obtain quality cheese. The changes begin with the inoculation of the milk with specific starter culture(s) and their subsequent growth and production of lactic acid. In

TABLE 1. Means of the pH of broth and milk (all fat levels averaged) samples inoculated with *L. bulgaricus*, *L. lactis*, and *S. thermophilus*^a

	pH			
	Control	<i>L. bulgaricus</i>	<i>L. lactis</i>	<i>S. thermophilus</i>
Broth				
Before processing	6.50 A	5.73 A	6.03 A	6.22 A
After processing	6.09 A	5.43 A	5.63 A	5.69 A
Milk				
Before processing	6.68 A	6.59 A	6.55 A	6.60 A
After processing				
Whey	6.15 B	6.24 B	6.23 B	6.23 B
Casein curd				
0 day	5.79 C	5.65 C	5.62 CD	5.61 CD
1 day	5.64 CD	4.94 CD	5.40 F	5.10 F
2 day	5.41 DE	4.53 DE	5.22 F	4.99 F

^a Controls were broth or milk that had not been inoculated. Means with different letters are significantly ($P < 0.05$) different. Broth and milk means were analyzed separately.

our study, milk samples were inoculated with *L. bulgaricus*, *L. lactis*, or *S. thermophilus*, which were representative of the many starter cultures used in fermented dairy foods (9).

The new process used in this study was based on high-pressure dissolution of CO₂ into the aqueous phase of the sample that resulted in a decrease in the pH. When the pressure was released, the majority of CO₂ returned to the gaseous phase and was vented from the chamber. In broth samples, CO₂ processing resulted in reduced pH in the broths, from 0.30 to 0.53 units, and had not significantly altered the CFU/ml, either between microorganisms or between, before, and after processing samples. This reduction in pH was due to the release of H⁺ as CO₂ dissolved in the samples and not due to significant acid production by the starter cultures.

When milk was processed with CO₂ under pressure, the pH of the milk decreased to a point where the casein precipitated from the solution. When the pressure was released, the CO₂ reformed and left precipitated casein and whey. The pHs of our curds and whey were similar to those reported in earlier CO₂-precipitated casein studies (7, 16, 18). In our study, CO₂ treatment reduced the pH in the liquid phases (either broth or whey) by 0.3 to 0.5 units, while the curd pH decreased 0.9 to 1.0 units. The amount of residual CO₂ in the curd should not hinder the cheese-making process. Earlier studies reported that milk, acidified to pH 6 to 6.5 with CO₂ and then inoculated with starter culture, had lower lactic acid levels during initial incubation (3, 11) that was due to the reduced metabolic activity of the culture (3). After 1 month of aging, cheese made from this milk had lactic acid levels similar to nonacidified control cheese (11). Milk containing low levels of CO₂ also required 50% (10) or 75% (11) less rennet for coagulation. This suggests that reduced amounts of rennet could be used, not so much for milk coagulation, as for adequate proteolysis of casein during processing and aging. Compared to control cheeses, cheeses made with carbonated milk had lower numbers of psychrotrophs (3, 10), higher final pH (3), higher moisture content (10), less proteolysis and lipolysis during aging (10, 11, 19), and, after 1 or 2 months of aging, no significant differences in sensory attributes (11, 19).

A processing temperature $\geq 38^{\circ}\text{C}$ was critical for good curd formation because processing at 32°C resulted in curds that were watery and slushy (18). The processing temperature of 38°C used in this study was above the typical 30 to 35°C used for ripening and setting of cheese milk but near the low range for cooking the curd. Thermophilic and mesophilic starter cultures should be able to tolerate short, i.e., 5-min, exposures at 38°C .

As in traditional cheesemaking, the inoculated milk was incubated for a time prior to coagulation to ensure recovery of the starter culture and encourage growth and acid production. In our study, a 3-h incubation resulted in a decrease in pH of 0.1 and a microbial population of 6.75 to 7.35 log CFU/ml. The effect of CO₂ processing reduced the *L. bulgaricus*, *L. lactis*, or *S. thermophilus* from 6.81, 7.35, or 6.75 log CFU/ml in the milk to 5.68, 7.32, or 6.50 log CFU/g in the curd, respectively. In traditional cheese

manufacturing, the fresh curd should have about 9.0 log CFU/g to ensure adequate flavor and texture development by enzymes of the microorganisms (15). Knowing the magnitude of the reduction in CFU due to CO₂ processing, further research should be able to determine ideal inoculation levels.

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