J. Dairy Sci. 86:1932–1940

Effects of Carbon Dioxide on Bacterial Growth Parameters in Milk as Measured by Conductivity

J. D. Martin, B. G. Werner, and J. H. Hotchkiss
Department of Food Science,
Cornell University, Ithaca, NY 14853

ABSTRACT

Inhibition of bacterial growth by dissolved carbon dioxide (CO_2) has been well established in many foods including dairy foods. However, the effects of dissolved CO_2 on specific growth parameters such as length of lag phase, time to maximum growth rate, and numbers of organisms at the stationary phase have not been quantified for organisms of concern in milk. The effect of dissolved CO_2 concentrations of 0.6 to 61.4 m*M* on specific bacterial growth parameters in raw or single organism inoculated sterile milk was determined at 15°C by conductance. Commingled raw or sterile milks were amended to a final concentration of 0.5 mg/ml each of urea and arginine HCl. Sterile milks were inoculated singly with one of six different microorganisms to a final concentration of approximately 10^2 to 10^3 cfu/ml; raw milk was adjusted to a final indigenous bacterial population of approximately 10^3 cfu/ml. Conductivity of the milk was recorded every 60 s over 4 to 5 d in a circulating apparatus at 15°C. Conductivity values were fit to Gompertz equations and growth parameters calculated. Conductance correlated with plate counts and was satisfactory for monitoring microbial growth. Data fit the Gompertz equation with high correlation (R^2 = 0.96 to 1.00). In all cases, dissolved CO_2 significantly inhibited growth of raw milk bacteria, influencing lag, exponential, and stationary growth phases as well as all tested monocultures.
(**Key words:** milk bacteria, carbon dioxide, conductance, Gompertz model)

Abbreviation key: SPC = standard plate counts, **TSA** = tryptic soy agar.

INTRODUCTION

Dissolved carbon dioxide (CO_2) has been shown to affect the growth characteristics of several microorganisms (Dixon and Kell, 1989). The direct addition of CO_2 to fluid milk results in the reduction in the growth rate of indigenous organisms making up a standard plate count (**SPC**) in milk in a temperature and CO_2 concentration dependent manner (Hotchkiss et al., 1999). However, CO_2 delays or reduces bacterial growth and does not result in bacterial death.

King and Mabbitt (1982) demonstrated that 10 to 40 m*M* CO_2 inhibited microbial growth in raw milk stored at 4, 7, and 10°C. Both increasing the CO_2 concentration and lowering the temperature resulted in greater reductions in growth rates than either treatment alone. Roberts and Torrey (1988) inoculated sterile milk with several common proteolytic psychrotrophic bacteria isolated from milk and found 20 to 30 m*M* dissolved CO_2 inhibitory at 7°C. They found that generation times increased in the presence of dissolved CO_2 due to an apparent increase in the lag phase and that the aerobic plate counts in uninoculated raw milk were likewise reduced. Ruas-Madiedo et al. (1996) conducted a pilot-scale study in which the effect of sufficient CO_2 to lower the pH of raw milk to 6.0 and 6.2 was investigated. Neither caseins nor whey proteins were affected by CO_2 treatment followed by removal by vacuum and pasteurization. Generally, the organic acid content of the milks was not different except for lactic acid, which was slightly lower in the CO_2-treated milks, and the volatile organic compound concentration of the treated product was lower, presumably because of lower microbial activity. The major effect of CO_2 was to lower coliform, psychrotroph, proteolytic psychrotroph, and lipolytic psychrotroph counts compared with untreated raw milk after 4 d of storage. The authors concluded that CO_2 could be added to raw milk to inhibit microbiological deterioration and be completely removed during processing without detrimental effects. The additional shelf life gained by the addition of CO_2 did not affect vitamin (Ruas-Madiedo et al., 1998a, 1998b) or monosaccharide (Ruas-Madiedo et al., 2000) content of raw milk. Espie and Madden (1997) reported the effects of 30 and 45 m*M* CO_2 on the indigenous microbial populations in raw milk stored at 6°C for up to 7 d. With the exception of lactobacillus, all organisms demonstrated inhibition with the addition of CO_2.

Received September 9, 2002.
Accepted December 6, 2002.
Corresponding author: J. H. Hotchkiss; e-mail: jhh3@cornell.edu.

Conductivity has been used to estimate the microbial contamination and shelf life of milk, enumerate organisms, and monitor the activity of a specific bacterium in a mixed culture (Houghtby, 1992). However, only a limited number of studies have applied electrochemical data to model bacterial growth. Only yeast (Deak and Beuchat, 1994) and *Yersinia enterocolitica* growth have been modeled (Dengremont and Membre, 1994; Lindberg and Borch, 1994). These studies were not conducted in milk nor did they utilize common milk bacteria.

The usefulness of any model for evaluating microbial growth is related to the accuracy and precision of the data upon which it is based. The variability in standard plating methods for microbial enumeration is inherently imprecise, subject to bias, and limited due to the number of data points that can be reasonably enumerated. Methods that produce a greater number of data points with a higher level of precision and accuracy will improve the statistical power of models. Conductivity measurement has the potential to deliver thousands of data points over short periods with minimal operator input.

Different microbial species may respond differently to CO_2 treatment, although few have attempted to characterize and compare growth kinetics to further define these differences. Our objective was to statistically compare the overall effect of a range of dissolved CO_2 concentrations on each growth parameter of representative native organisms in raw milk and to determine the effects on several specific organisms that frequently occur in raw milk. We used an abusive temperature in order to decrease the time required to reach stationary growth while acquiring a large amount of data under worst-case conditions. Temperatures commonly used to store milk (4.4 to 10°C) are known to increase the inhibitory effect of dissolved CO_2 (Law and Mabbitt, 1983). We used predictive models to precisely and accurately describe bacterial growth. Our desire to develop accurate models prompted the use of automated conductivity to gather a larger quantity of precise data. We used the Gompertz model in order to elucidate multiple growth parameters (lag-phase duration, exponential growth rate and maximum growth) that may be used to characterize and compare effects on individual microorganisms and mixed cultures.

MATERIAL AND METHODS

Milk Sample/Bacterial Isolate Sources

Raw milk samples were obtained from the Northeast Dairy Herd Improvement Association, Inc. (Ithaca, NY), a dairy analytical consulting laboratory. Milk was commingled from 236 farms from New York, Pennsylvania, and New Jersey and, thus, is likely representative of a wide range of milk flora. Ultra high temperature fat-free milk (Parmalat USA, Wallington, NJ) was obtained locally and stored at 6°C. Five percent (wt/vol) urea (Sigma-Aldrich Corp., St. Louis, MO) and arginine hydrochloride (Sigma-Aldrich Corp., St. Louis, MO) were filter sterilized in a Stericup vacuum filtration unit (SCGPU02RE, Millipore Corporation, Bedford, MA).

Isolates were selected for this study on the basis of their ability to grow in milk at 15°C, and either the potential to cause milk spoilage or produce illness if ingested. Microorganisms included *Pseudomonas fluorescens* R1-232 (Wiedmann et al., 2000), *Bacillus cereus* A1-029, and *Bacillus licheniformis* A1-030 (isolated from milk, Cornell University Milk Quality Improvement Program). *Listeria monocytogenes* R2-502 (isolated from chocolate milk) (Dalton et al., 1997), *Escherichia coli* DH5α (Promega Corporation, Madison, WI), and *Enterococcus faecalis* ATCC 19433 (American Type Culture Collection, Manassas, VA).

Analytical Tests

The pH was measured (Accumet pH Meter 925, Fisher Scientific, Springfield, NJ) at ambient room temperature. Carbon dioxide concentrations (% CO_2) were determined in triplicate as described elsewhere (Glass et al., 1999). A standard curve of dissolved CO_2 concentration (% CO_2) versus CO_2 concentration (ppm CO_2) was used for each milk product tested to determine concentration in milligrams per kilogram, which was then converted to mM (Glass et al, 1999). The data used to construct the standard curve typically produced a regression analysis with an R^2 value > 0.95.

Microbiological Maintenance and Quantification

SPC enumeration of all organisms studied was in accordance with Houghtby (1992) with the exception of the enumeration of *P. fluorescens*, *E. faecalis*, *L. monocytogenes*, and *E. coli*, where tryptic soy agar (**TSA**; Becton Dickinson and Co., Cockeysville, MD) was substituted for standard methods agar.

Pseudomonas fluorescens, *E. coli*, *L. monocytogenes*, or *E. faecalis* were streaked onto TSA slants in duplicate. The slants were incubated at 30°C for 24 h, stored at 3°C, and transferred weekly onto new stock slants. Single colonies were isolated from stock slants by streaking onto TSA plates and incubating at 32°C for 24 h. An isolated colony from the TSA plate was transferred to 9 ml of sterile Butterfield's phosphate buffer (US FDA, 1998), vortexed and a 100-μl aliquot spread on another TSA plate and incubated at 32°C for 24 h. One milliliter of phosphate buffer was added to the TSA

plate and a spreader used to suspend the bacteria. The suspension was removed by pipette, and added to 8 ml of sterile buffer. A 0.5 McFarland turbidity standard was used to create a known dilutions series for inoculation of the milk. The diluted standard was subsequently used to inoculate milks to 10^2–10^3 cfu/ml.

Bacillus cereus and *B. licheniformis* were maintained as a stock spore culture for up to 1 mo based on the methods of Mazas et al. (1995). Immediately before each experiment, 1 to 4 ml of *B. cereus* or *B. licheniformis* inoculum were heat shocked at 80°C under agitation for 12 min in a water bath to initiate germination and diluted with Butterfield's buffer at 6°C. Cell densities were estimated by McFarland equivalence turbidity standards (20410, Remel, Lenexa, KS), and verified by enumeration utilizing an Improved Neubauer Counting Chamber (Hausser, PA).

Raw Milk Sample Preparation and Analysis

Raw milk was stored at 6°C to allow the native bacterial counts to increase to $>10^3$ cfu/ml, then examined for native bacterial population and not further inoculated.

Twenty-four hours before each experiment, a sample of raw milk was enumerated for SPC. The raw milk was then diluted with UHT skim milk to bring SPC to approximately 10^3 cfu/ml. A sterile stainless steel sparger was inserted into 500 ml of milk and CO_2 (Airgas Mid-Atlantic, Inc. Elmira, NY) was bubbled through the milk to achieve added CO_2 levels of 0 to 61.4 m*M*. An aliquot was analyzed every 5 min until the desired CO_2 level was reached. The CO_2 level was then verified in triplicate and the pH taken. Urea and arginine hydrochloride (2 ml each) were added to 198 ml of the raw or UHT skim milk to amplify changes in conductivity as SPC increased (Suhren and Heeschen, 1987). The effect of CO_2 on conductivity was determined by adding known amounts of CO_2 to UHT milk and measuring conductivity. All conductivity measurements in this study were expressed as microsiemens (μS); electrical conductivity or specific conductance of solutions is typically measured in siemens, which is the reciprocal of the resistance in ohms (Eden and Eden, 1984).

Analysis of Amendment Effects

To determine whether added urea and arginine would influence the growth of bacteria, *P. fluorescens* was grown in UHT skim milk containing no amendments, 5 ml of 5% urea, 5 ml of 5% arginine-HCl, or 2.5 ml each of 5% urea/5% arginine-HCl. SPC were conducted in triplicate every 4 h and the results were fitted to the Gompertz equation.

Conductivity Test Apparatus and General Test Plan

In each growth experiment, amended, raw or inoculated milk was aseptically added to an autoclaved glass jar (220 ml, Ball Mason Jars, Alltrista Corp., Munci, IN), fitted with a metal lid. Autoclaved peristaltic pump tubing (6402-15 Norton Norprene Masterflex) was attached to the glass tubes protruding through the metal lid and the tubing placed into the Amicon peristaltic pump (LP-1, 115 V, 60 Hz), and connected to the flow through a conductivity probe (which had been treated with 10%, vol/vol, chlorine bleach for 10 min). The glass jar was placed into a 1.9-L cryogenic dewar (Alladin Industries, Nashville, TN) filled with water to just below the container lid. The refrigeration unit (model 1145, VWR Refrigerated Constant Temperature Circulator) recirculated 15 ± 0.5°C water through the copper coil placed within the dewar. Temperature was monitored with a calibrated mercury thermometer. The conductance probe was attached to an analog conductivity meter (model 19100-00, Cole Parmer Niles, IL), which was in turn connected to an Omega DAQ-802 (Omega Engineering, Inc., Stanford, CT) data acquisition hardware and software system. Data were recorded every 60 s by PC computer and automatically transferred to Microsoft Excel. The peristaltic pump circulated the milk through the probe at a flow rate of approximately 0.85 ml/min. A schematic of this experiment apparatus is illustrated in Figure 1.

SPC enumeration in triplicate was performed concurrently with conductance tests to determine how closely conductance followed microbial growth. *Pseudomonas fluorescens* was grown (10^3 cfu/ml initial concentration) in modified UHT skim milk at 15°C with agitation. Enumeration by SPC was performed as described above every 4 h. The plate count and conductance results were fitted to the Gompertz equation and compared.

Cultures were considered to be in stationary phase when the increase in conductance was < 10 μS over 6 h. When stationary phase was reached analytical tests (pH, infrared CO_2 analysis, and SPC) were performed.

Statistical Analysis and Gompertz Model Data Fit

Statistical analyses were performed using SigmaPlot 4.0 (San Rafael, CA). To compensate for differences in initial milk conductivity, raw data was normalized by subtracting the average of the first 10 values from all data. This adjusted data was then fit to the modified Gompertz equation (Buchanan, 1992), and growth curves were constructed. Each curve was derived from a single run consisting of 9600 to 24,000 data points, all of which were used to construct equations, but only every 10th data point was plotted in order to simplify the figures. In all cases, the Gompertz model fit the

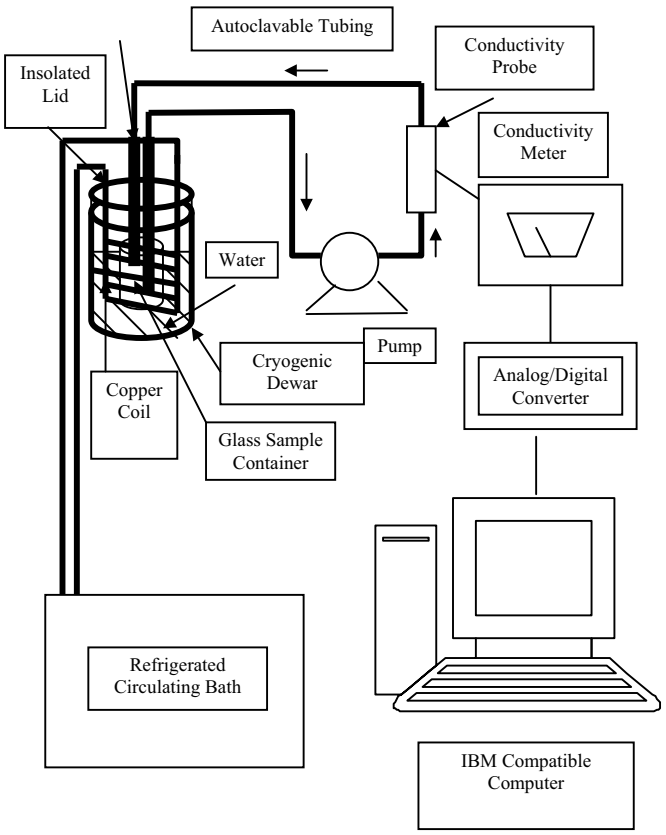

Figure 1. Conductivity experimental test apparatus.

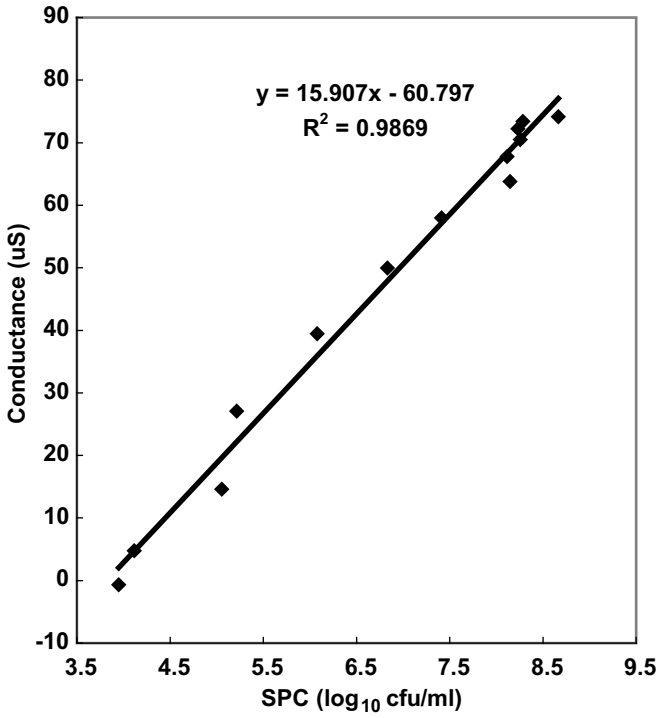

Figure 2. Relationship between *Pseudomonas fluorescens* counts ($\log_{10}$ cfu/ml) and conductivity (μS) in inoculated ultra high temperature skim milk amended with arginine hydrochloride and urea and incubated at 15°C. Conductance was normalized to reduce systematic variation.

data with an R^2 of >0.95, and in most cases R^2 was 0.99 or 1.00. The modified Gompertz equation utilized was: L(t)= A + C exp [–exp(–B(t–M))] where: L(t)= Log conductance μS at time t, t = time in hours, A = minimum conductance value, M = time (h) to reach maximum growth, C = amount of change in conductance, and B = relative growth rate at M (Buchanan, 1992).

ANOVA statistical analysis was conducted (Minitab, Release 9, State College, PA) for each organism and each of three Gompertz growth parameters (B, M, and C) to determine statistical differences ($P < 0.05$) between the different carbonation level treatments.

RESULTS AND DISCUSSION

Conductivity and the Gompertz Model to Describe Bacterial Growth Kinetics

Electrochemical methods measure the metabolic conversion of uncharged or weakly charged organic molecules (e.g., proteins, lipids, carbohydrates) to charged products, such as amino acids, lactate, and acetate and reflect metabolic activity. There is a high correlation between numbers of metabolically active bacteria and increases in conductivity (Eden and Eden, 1984). Conductivity provides more data than possible by standard plate counting and can be considered more precise because it does not have the methodological uncertainties of plating (McMeekin et al., 1993); electronic data collection additionally reduces bias. It is, however, an indirect method of determining numbers of organisms present.

To assess the relationship between conductivity and SPC, changes in *P. fluorescens* plate counts were compared to conductance. The change in conductivity was not significantly different ($P \leq 0.05$) from SPC and conductivity curves exhibited higher R^2 values (0.98), compared with 0.89 for SPC, and a lower standard deviation. There was a strong linear relationship between SPC and conductance (Figure 2).

The raw and UHT skim milk was amended with small amounts of urea and arginine-HCl as described by Suhren and Heeschen (1987), in order to increase the conductance signal. Comparison of SPC and conductivity curves showed that there was no effect of adding urea, arginine, or a combination of the two. The effect of dissolved CO_2 concentration on conductance was also evaluated. There was a small consistent increase in conductance with increasing CO_2 concentrations (y =

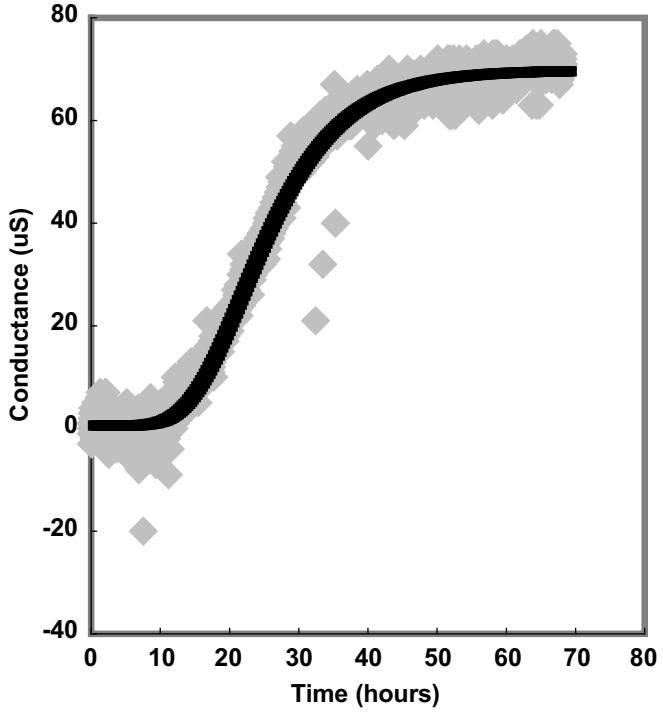

Figure 3. Change in conductance (μS) over time (hours) of *Pseudomonas fluorescens* inoculated urea/arginine amended ultra high temperature skim milk containing 11.2 m*M* CO_2 and incubated at 15°C. Conductance values (◆), data fitted to the Gompertz equation (–).

0.2353x + 0.7007; $R^2 = 0.95$). The addition of CO_2 increased conductance by 2 μS per 10 m*M*; however, the overall pattern of conductance signaling over time did not change.

The Gompertz model has been used in multiple studies to describe bacterial growth (Klemera and Doubal, 2000). Supported by a large number of data points (>4000 per analysis) obtained by conductance and the data acquisition software, the Gompertz model accurately described the empirical data (Figure 3). For example, Gompertz described the observed conductance data in raw milk with R^2 values of either 0.99 or 1.00 (Table 1). Several growth parameters derived from the Gompertz equation were compared at each CO_2 concentration tested (Table 1). ANOVA was used to determine whether there was a statistically significant effect of CO_2 concentration on these growth parameters.

CO_2 Fluctuations and General Effects of CO_2 on Conductivity and Bacterial Growth

In most cases, the CO_2 concentration at the end of each assay was lower than the initial concentration (Table 2). In two bacteria/CO_2 combinations, the CO_2 concentration increased beyond initial levels. Carbon dioxide is a byproduct of bacteria respiration and responsible for the increase in CO_2 in some samples. Samples with low increases in growth (i.e., slow increase in conductivity) did not show an increase in CO_2 concentration. The addition of CO_2 reduced the milk pH from an initial range of 5.90 to 6.80 to as low as 4.92, depending upon the total amount of CO_2 added and initial milk pH. The initial microbial counts were similar in all experiments, while final SPC decreased as CO_2 concentration increased (Table 2).

Changes in conductivity in raw milk and all individually tested microorganisms in UHT milk were significantly ($P \leq 0.05$) affected by the addition of CO_2 in a concentration-dependent manner. CO_2 influenced the lag phase, exponential phase, stationary phase, or a combination of these parameters. For raw milk, there was a significant increase in lag time, increase in conductance doubling time, and decrease in exponential growth (i.e., Gompertz parameter B) rate as the CO_2 concentration increased from 0.60 to 44.5 m*M* (Table 1 and Figure 4). The time to maximum change in conductance (i.e., Gompertz parameter M) increased from 26 h to 52.9 h with the addition of 44.5 m*M* CO_2 (Table 1), an effect most likely a result of lag phase extension. When taking into account the overall influence of increasing CO_2 in raw milk on the extension of the lag phase, reduction in exponential growth rate, and increase in time to maximum growth, the end result is more pronounced than if only one of these parameters were affected. To better understand these effects, and detect species-specific responses to CO_2, representative bacteria common to raw milk were selected for analysis as monocultures (gram-negative aerobic and facultative anaerobic rods, gram-positive sporogenic and asporogenic rods, gram-positive cocci).

Effects of CO_2 on Conductance and Growth Parameters of *Pseudomonas fluorescens*

The Gompertz equation closely described the observed changes in the *P. fluorescens* culture over time with R^2 of 0.99 (Table 1). Adding up to 46.3 m*M* CO_2 to the milk had a significant influence on all measured growth parameters. There was an overall increase in lag time and in the time to reach maximum growth (Table 1). The maximum change in conductance, which is comparable to the difference between initial and final cfu/ml values, decreased from 78.2 to 59.9 μS and then increased to 65.6 μS (Table 1). Conductance doubling time decreased from 2.7 to 2.3 h and then increased to 3.4 h. The log growth rate correspondingly increased from 0.112 μS/h to 0.130 μS/h, then declined to 0.088 μS/h. This change in growth characteristics between 0.4 and 27.1 m*M* and between 27.1 and 46.3 m*M* may

Table 1. Growth parameters calculated by fitting conductivity values to the modified Gompertz model L(t)= A + C exp [–exp(–B(t–M))] where: L(t)= Log count of bacteria at time t, t = time in hours, A = minimum conductance value, M = time (h) to reach maximum growth, C = amount of change in conductance, and B = relative growth rate at M.[1]

Sample	Initial [CO^2] (m*M*)	R^2	Exponential growth rate (B) (μS/h)	Time to maximum growth (M) (h)	Maximum change in conductance (C) (μS)	Conductance doubling time (h)	Lag time (h)
Raw milk	0.6	1.00	0.200[a]	26.0[a]	88.9[a]	1.8	20.0
	15.4	1.00	0.132[b]	33.0[b]	92.2[b]	2.3	25.4
	27.9	1.00	0.135[c]	40.2[c]	98.0[c]	2.2	32.8
	38.6	0.99	0.133[d]	44.3[d]	80.5[d]	2.3	37.7
	44.5	1.00	0.113[e]	52.9[e]	87.9[e]	2.7	44.1
Pseudomonas fluorescens	0.4	0.99	0.112[a]	11.7[a]	78.2[a]	2.7	3.3
	11.2	0.99	0.128[b]	21.1[b]	69.2[b]	2.4	13.3
	27.1	0.99	0.130[b]	22.7[c]	59.9[c]	2.3	15.0
	33.6	0.99	0.088[c]	27.3[d]	61.9[d]	3.4	16.0
	46.3	0.99	0.088[c]	37.5[e]	65.6[e]	3.4	26.1
Escherichia coli	0.5	0.99	0.064[a]	47.6[a]	56.0[a]	4.7	29.4
	49.4	0.97	0.055[b]	53.8[b]	22.0[b]	5.5	38.1
Listeria monocytogenes	0.5	0.98	0.136[a]	22.6[a]	118.0[a]	2.2	15.2
	48.9	0.99	0.100[b]	44.4[b]	71.5[b]	3.0	34.4
Enterococcus faecalis	0.5	0.99	0.055[a]	51.8[a]	69.0[a]	5.5	33.6
	51	0.98	0.076[b]	50.7[b]	40.7[b]	4.0	37.6
Bacillus cereus	0.5	1.00	0.128[a]	33.9[a]	79.3[a]	2.4	26.1
	47.1	0.99	0.105[b]	37.6[b]	77.9[b]	2.9	28.1
	61.4	0.99	0.057[c]	44.4[c]	74.8[c]	5.3	26.7
Bacillus licheniformis	0.5	0.99	0.057[a]	48.4[a]	51.4[a]	5.3	30.9
	49.4	0.96	0.057[a]	54.1[b]	31.2[b]	5.2	36.7

[1]For each organism parameter, different superscript letters denotes that parameters are statistically different from each other ($\alpha = 0.05$).

Table 2. Initial and final analytical results for raw and inoculated milk incubated in the presence of CO_2 at 15°C over several days

	Carbon dioxide concentration		pH		Mirobial counts	
Sample	Initial (m*M*)	Final (m*M*)	Initial	Final	Initial cfu/ml $\times 10^3$	Final cfu/ml $\times 10^9$
Raw milk	0.6	1.1	6.69	4.71	7.9	3.8
	15.4	6.8	6.46	4.70	3.3	3.7
	27.9	7.8	6.10	4.67	1.0	3.8
	38.6	13.5	6.20	4.83	3.7	3.7
	44.5	36.0	6.00	5.02	1.9	3.2
Pseudomonas fluorescens	0.4	0.3	6.80	6.10	4.7	3.2
	11.2	5.2	6.50	6.30	4.9	2.3
	27.1	9.3	6.23	5.80	4.8	1.7
	33.6	14.7	6.10	5.60	3.2	1.7
	46.3	43.1	5.92	5.27	1.2	1.7
Escherichia coli	0.5	0.4	5.90	5.79	3.9	8.3
	49.4	31.2	4.92	5.21	1.6	0.2
Listeria monocytogenes	0.5	5.7	6.40	5.64	3.3	3.2
	48.9	28.9	5.20	5.11	4.0	2.8
Enterococcus faecalis	0.5	0.4	6.77	4.93	2.3	3.7
	51.0	26.9	5.84	5.09	5.3	1.2
Bacillus cereus	0.5	0.4	6.79	5.80	1.6	3.0
	47.1	3.8	5.72	4.99	2.2	2.3
	61.4	36.9	4.92	5.31	2.0	1.2
Bacillus licheniformis	0.5	0.4	6.61	5.75	2.4	5.6
	49.4	32.3	5.86	4.84	2.2	2.4

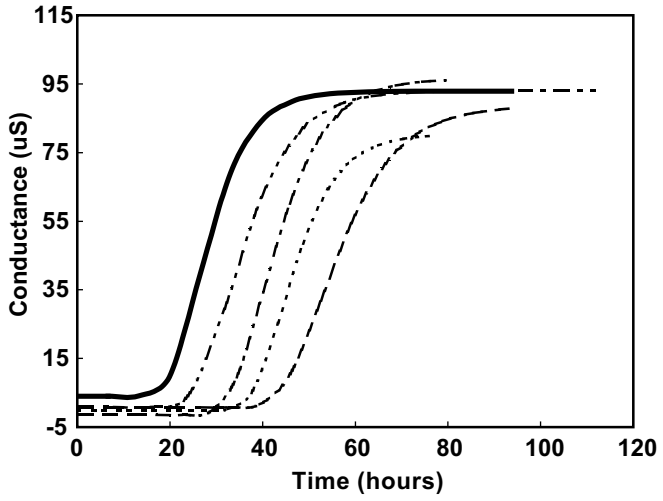

Figure 4. Changes in conductance (μS) over time (hours) of urea/arginine amended raw milk, held at 15°C and containing (——) 0.57 m*M*, (— · · —) 15.4 m*M*, (— · —) 27.9 mM, (· · · · · · · · ·) 38.6 m*M*, and (— — —) 44.5 m*M* carbon dioxide. Conductance values normalized and 1 of 6 data points plotted.

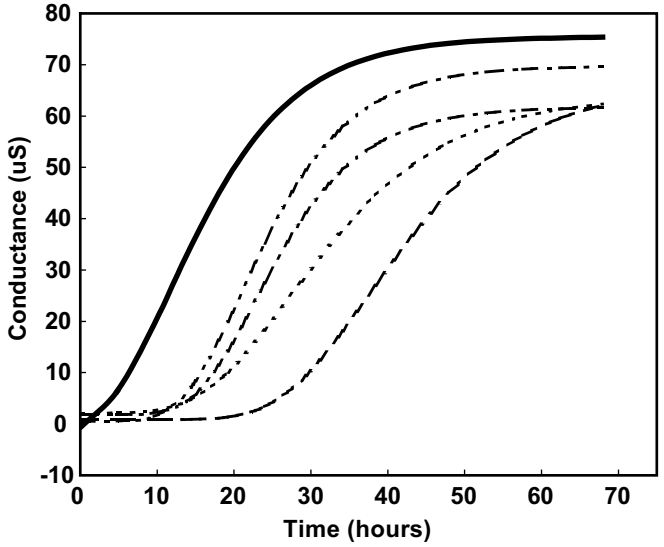

Figure 5. Changes in conductance (μS) over time (hours) of urea/arginine amended ultra high temperature milk inoculated with *Pseudomonas fluorescens*, held at 15°C and containing (——) 0.45 m*M*, (— · · —) 11.2 m*M*, (— · —) 27.1 m*M*, (· · · · · · · · ·) 33.6 m*M*, and (— — —) 46.3 m*M* carbon dioxide. Conductance values normalized and 1 of 6 data points plotted.

be significant; similar trends were observed in raw milk for log growth rate, maximum change in conductance, and conductance doubling time. This trend may be due to a species-specific effect of CO_2 particularly on *Pseudomonas* spp., which is a predominant spoilage organism in raw milk. The mechanism of effect of CO_2 on microorganisms is thought to be multifaceted and degree of influence species specific; thus, the response to increasing CO_2 levels at any one point cannot be assumed to be linear.

Others have observed a strong effect of dissolved CO_2 on the growth of *Pseudomonas* spp. Gill and Tan (1979) found an increase in lag phase of approximately four days for *P. fluorescens* at 8°C with 30 m*M* CO_2. However, others have found no effect of 30 m*M* CO_2 on growth rate of *P. fluorescens* (King and Mabbitt, 1982). We observed a small increase in the rate of growth up to 33.6 m*M* CO_2, with a sharp decrease in the growth rate at higher CO_2 concentrations (Figure 5). However, there was an overall inhibition of this microorganism due to the large increase in the lag phase due to CO_2.

Effects of Carbon Dioxide on Conductance and Growth Parameters of *Escherichia coli*

The changes in conductivity for *E. coli* growth fit the Gompertz model closely (R^2 = 0.99 and 0.97 for 0.5 m*M* and 49.4 m*M* CO_2, respectively) (Table 1). The exponential growth rate significantly decreased as the added CO_2 concentration increased from 0.5 m*M* to 49.4 m*M*. There was a significant increase in the time to reach maximum growth rate from 47.6 to 53.8 h, and an increase in the lag phase from 29.4 to 38.1 h (Table 1). The addition of CO_2 resulted in significantly smaller overall changes in conductance compared with milk without added CO_2 (Tables 1 and 2). Conductance doubling time increased from 4.7 to 5.5 h, corresponding to a significant decrease in the slope of the exponential growth rate phase. The lag phase also increased.

Inhibition of *E. coli* growth has been reported under 100% pCO_2, at 30°C in buffered TSA (Kimura et al., 1999). The lag phase increased and exponential growth rate decreased under CO_2, but specific growth statistics and dissolved CO_2 concentration were not given.

Effects of Carbon Dioxide on *Listeria monocytogenes*

The Gompertz model closely described growth characteristics of *L. monocytogenes* (R^2 = 0.98 and 0.99; Table 1) and growth was strongly affected by the added CO_2. There was a statistically significant increase in the time to maximum change in conductance (i.e., growth rate) when CO_2 levels of 0.5 and 48.9 m*M* were compared (Table 1). Carbon dioxide significantly decreased the exponential growth rate, increased the conductance doubling time and decreased the maximum change in conductance for CO_2 levels of 0.5 and 48.9 m*M* (Table 1).

Previous workers have reported that atmospheric CO_2 increases in lag phase of *L. monocytogenes* by 2 to 3 d at 8 to 10°C in BHI or phosphate buffer (Farber et al., 1996). Our observed lag-phase extension was not as pronounced, probably due to the elevated experimental temperatures compared with 8 to 10°C. Other studies have concluded that CO_2 did not affect *L. monocytogenes* growth (Nilsson et al., 1997; Karagul-Yuceer et al., 2001). For example, 1.27 volumes of CO_2 was ineffective when added to yogurt inoculated with *L. monocytogenes* and held at 4°C (Karagul-Yuceer et al., 2001).

Effects of Carbon Dioxide on Conductance and Growth Parameters of *Enterococcus faecalis*

The Gompertz equation fit the observed growth of *E. faecalis* (R^2 = 0.99 and 0.98 for 0.5 and 51.0 m*M* CO_2, respectively; Table 1). There was a significant decrease in the maximum conductance and a decrease in conductance doubling time from 5.5 h to 4 h for the CO_2 treated milk compared with the control. There was also significant increase in the lag phase as CO_2 levels increased (Table 1). However, there was also a statistically significant increase in the exponential growth rate as the CO_2 concentration was increased from 0.5 to 51.0 m*M* (Table 1). Maximum microbial counts and conductance at stationary phase (1.2×10^9 cfu/ml) with 51.0 m*M* CO_2 was statistically lower than the maximum microbial levels with 0.5 m*M* CO_2 (3.7×10^9 cfu/ml) (Table 2). However, the large decrease in maximum microbial counts resulted in a decrease in the time to reach maximum growth rate. The overall effect of CO_2 was, however, to decrease the growth of *E. faecalis*.

Effects of Carbon Dioxide on Conductance and Growth Parameters of *Bacillus spp.*

The conductance data for *B. cereus* and *B. licheniformis* both fit the Gompertz model as the CO_2 concentration increased from 0.5 to 61.4 m*M* (R^2 = 0.99 and 1.00, and 0.96 and 0.99 for each bacterium, respectively, Table 1). *Bacillus* spp. were weakly influenced by CO_2 (Figure 6). For *B. cereus*, statistically significant growth kinetic changes included a decrease in the maximum change in conductance, an increase in the lag time, an increase in the time to maximum growth and a decrease in the exponential growth rate (Table 1). The conductance doubling time was influenced by the exponential growth rate with values of 2.4 to 5.3 h.

Bacillus licheniformis growth kinetic responses to CO_2 mirrored that of *B. cereus* (Figure 6) in terms of lag phase, time to maximum growth and maximum change in conductance. There was insufficient evidence to demonstrate an effect by CO_2 on the exponential growth rate of *B. licheniformis* by CO_2.

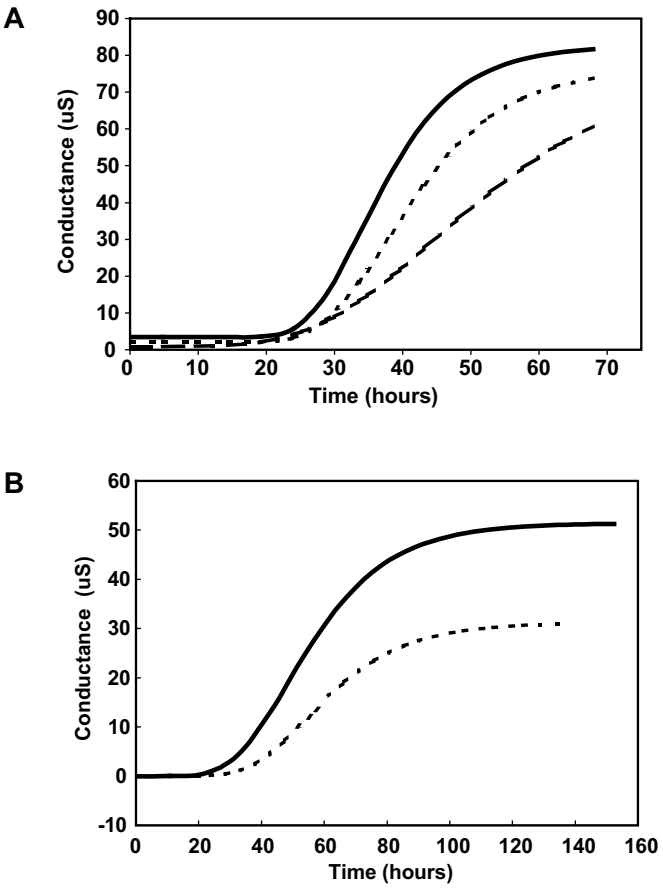

Figure 6. Changes in conductance (μS) over time (hours) of urea/arginine amended ultra high temperature milk at 15°C inoculated with: A. *Bacillus cereus* and added carbon dioxide to levels of (——) 0.48 m*M*, (·····) 47.1 m*M*, (— — —) 61.4 m*M* ; and B. *Bacillus licheniformis* and added carbon dioxide to levels of (——) 0.51 m*M* and (·····) 49.4 m*M*. Conductance values normalized and 1 of 6 data points plotted.

There are few available reports on the effect of CO_2 on *Bacillus* spp. In whole milk, there was no effect on spore germination and outgrowth during storage at 6.1°C with CO_2 concentrations of 11.9 m*M* (Werner and Hotchkiss, 2002). In buffered BHI, there was a slight decrease in the exponential growth rate and a slight increase in lag phase demonstrated for *B. circulans* (Devlieghere and Debevere, 2000), showing responses similar to that found in this study with *B. cereus* and *B. licheniformis*.

CONCLUSIONS

The addition of dissolved CO_2 to representative raw milk increased the lag phase and time to maximum growth by 100%, even at 15°C. This effect is likely to be much greater at lower milk storage temperatures. Dissolved CO_2 reduced the microbial growth on all

tested organisms, however, the overall inhibition was greater on gram-negative than gram-positive bacteria. Carbon dioxide influenced lag, log, and stationary phases, but not equally for all organisms. For gram-negative bacteria there was a significant increase in lag time and time to maximum growth, and a decrease in maximum change in conductance. Gram-positive bacteria had a statistically significant increase in lag time and decrease in maximum change in conductance. The effects of CO_2 on the exponential growth rate and conductance doubling time varied for gram-negative and gram-positive bacteria.

The combined effects of CO_2 and temperature could be an appropriate way to significantly decrease microbial growth in raw milk.

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