Effect of Carbon Dioxide on the Growth of Bacillus cereus Spores in Milk During Storage

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ABSTRACT
The effects of the addition of 11.9 mM CO2 on the growth of Bacillus cereus spores inoculated into sterile homogenized whole milk at 101 and 106 spores/ml and stored at 6.1°C, was examined weekly for 35 d. Colony-forming units from CO2 treated inoculated milks decreased over 35 d at a rate similar to that of untreated inoculated milk, as defined by linear regression. Plate counts for treated and control milks inoculated at 101 cfu/ml were not significantly different on sampling d 0, 14, 21, and 28. Plate counts at d 7 were significantly different and counts at d 35 were at undetectable levels for both treated and control milks. Plate counts for milk inoculated at 106 cfu/ml were not significantly different on d 0, 28, and 35; they were significantly different on d 7, 14, and 21. There was no consistency as to whether the control or test milks were higher in counts on days when the differences were significant. Added CO2 reduced the pH of the milk from an average value of 6.61 to an average value of 6.31; however, this drop did not correlate with changes in any other parameter measured. These data suggest that moderate levels of CO2 do not enhance the outgrowth of B. cereus spores over long-term storage and do not increase the risk of foodborne illness due to the organism.

(Key words: Bacillus cereus spores, milk, carbon dioxide, shelf-life extension)

Abbreviation key: SPC = standard plate counts.

INTRODUCTION
Bacillus cereus, a facultative anaerobe, is commonly found in milk and milk-based products and can cause either emetic or diarrheal foodborne illness due to toxins. Concentrations of viable B. cereus in food ranging from 103 to 1010 cfu/ml have been implicated in foodborne disease outbreaks (Kramer and Gilbert, 1989; Andersson et al., 1995). Lower concentrations may cause illness in infants, who are more susceptible to enteric illnesses and to toxins than are children and adults (Rowan and Anderson, 1998). Bacillus cereus may also cause spoilage of aseptically packaged milk, canned milks, and nonaseptic packaged refrigerated milk. In the absence of competition and over the course of an extended storage period, low concentrations of B. cereus can cause defects such as coagulation and off-odors due to proteolysis and lipolysis (Meer et al., 1991; Andersson et al., 1995).

Spores of the organism are thermally resistant and can survive pasteurization and storage at refrigeration temperatures. Psychrotrophic strains of B. cereus isolated from milk have been shown to grow at temperatures as low as 1°C (Coghill and Juffs, 1979). Germination of spores can proceed over a wider range of temperatures than that required for outgrowth of a vegetative cell (Dufrenne et al., 1994; Knaysi, 1964). The activation of germination during storage may result from such factors as exposure to nutrients or nonnutrient chemical agents, reduction in pH, extended low temperatures, or sublethal heat treatment (Moir, 1992; Smoot and Pierson, 1982).

CO2 has been used effectively in extending the shelf life of a variety of cold-stored dairy products, including cottage cheese. Investigations have shown that CO2 is effective in lengthening the lag phase and slowing the growth phase of primarily gram-negative bacteria and other vegetative bacterial cells (Hendricks and Hotchkiss, 1997; King and Mabbitt, 1982; Roberts and Torrey, 1988). CO2 has been shown to inhibit, stimulate, or have little effect on germination and toxigenesis (Smoot and Pierson, 1982) of spore formers such as Clostridium botulinum, depending upon CO2 concentration, pH, temperature, media, and the presence of other bacterial species. However, the effect on Bacillus spp. spores is not well documented. The mode of action of CO2 as a preservative has been debated and may involve a complexity of interactions; mechanisms investigated have included displacement of oxygen, influence on pH, disruption of cell membranes, and metabolic interference (Daniels et al., 1985).

The objective of this study was to determine whether moderate levels of CO2 added to milk have any effect,
stimulatory or inhibitory, on the germination and subsequent outgrowth of either high or low concentrations of *B. cereus* spores during long-term storage at refrigeration temperatures. The results will be useful in further examinations of the effect of CO₂ in milk on spores and further investigations into the use of CO₂ as a bacteriostatic agent in milk and milk products.

**MATERIALS AND METHODS**

HTST homogenized whole milk was sterilized in a Vernitron model B3660 steam sterilizer (Vernitron Medical Products, Carlstadt, NJ) for 5 min at 121°C. Milk was sparged with CO₂ to a concentration of 11.9 mM, a concentration within the approximate flavor threshold upper limit for CO₂ in milk (Hotchkiss et al., 1999). The pH of the bulk treated milk was 6.12. Untreated milk had a CO₂ content of <1 mM and a pH of 6.61.

Four *B. cereus* isolates were used, three isolated from fluid milk samples obtained from the New York State Milk Quality Improvement Program Voluntary Shelf Life Program, Cornell University, and culture ATCC 14579. The three isolates were selected because their original source was milk; culture ATCC 14579 was selected because it was a type strain. All cultures used exhibited vegetative growth after 7 to 14 d of incubation at 6°C when streaked on tryptic soy agar, standard methods agar, and tryptic soy agar supplemented with 2% sheep blood; streak inoculum used for this growth check was from a 24-h tryptic soy agar culture incubated at 32°C. A pooled isolate inoculum of spores of 99% purity was prepared (Mazas et al., 1995) and stored at 6°C for 2 d before use; purity level was checked immediately prior to use. Milk was inoculated to final concentrations of 7 × 10⁴ and 5.8 × 10⁶ cfu/ml. Uninoculated milks were included in the study as controls.

Milk treatments were aliquotted into sterile 4-ounce Ball® glass jelly jars with banded dome lids, with approximately 1 cm of headspace. Tightly capped jars were stored at 6.1°C for 0, 7, 14, 21, 28, and 35 d; two jars per treatment were removed for testing at each
Table 1. Regression analysis (regression equation, slope and R-squared values) of standard plate count (log10 cfu/ml) over time (days) for sterilized whole homogenized milk untreated or treated with 11.9 mM CO2 and inoculated with 10^1 or 10^6 spores/ml Bacillus cereus.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>R-Squared</th>
<th>Regression equation</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>No CO2 10^1 spores/ml</td>
<td>87.10%</td>
<td>Log SPC = 1.872 – 0.0544286 Day</td>
<td>-0.0544286</td>
</tr>
<tr>
<td>No CO2 10^6 spores/ml</td>
<td>93.40%</td>
<td>Log SPC = 6.99905 – 0.0711837 Day</td>
<td>-0.0711837</td>
</tr>
<tr>
<td>CO2 Added 10^1 spores/ml</td>
<td>83.40%</td>
<td>Log SPC = 7.05762 – 0.0693878 Day</td>
<td>-0.0693878</td>
</tr>
<tr>
<td>CO2 Added 10^6 spores/ml</td>
<td>94.00%</td>
<td>Log SPC = 1.924 – 0.0495714 Day</td>
<td>-0.0495714</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

A plot of the mean SPC of all treatments during 35 d of storage shows a gradual decline in viable cells detected in milks inoculated at both levels (Figure 1). There was no significant difference in plate counts between the CO2-treated and untreated samples from the 10^1 cfu/ml inoculation level groups at d 0, 14, 21, and 28 (P > 0.05); plate counts for d-7 samples were significantly different. Day 35 results were not analyzed statistically as counts were <1 cfu/ml. The treated milks from the 10^6 cfu/ml inoculum level were not significantly different on sampling d 0, 28, or 35. On sampling d 7 and 21 treated milk plate counts were higher. On sampling d 14, the control milk counts were higher. Linear regression indicated that all four growth curves (added CO2 and not treated, inoculated with 10^1 or 10^6 cfu/ml spores) resulted in a high correlation/fit of data (all R^2 > 87%), with similar regression equations and negative slopes for all inoculated treatments (Table 1).

Table 2. Mean CO2 concentrations (mM) for sterilized whole homogenized milk untreated or treated with 11.9 mM CO2, inoculated with 10^1 or 10^6 spores of Bacillus cereus/ml or not inoculated, and stored at 6.1°C for up to 35 d.

<table>
<thead>
<tr>
<th>CO2 (mM)/Time (days)</th>
<th>0</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>No CO2 No Inoculum</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>No CO2 10^1 spores/ml</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>No CO2 10^6 spores/ml</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CO2 Added No Inoculum</td>
<td>12.6</td>
<td>12.6</td>
<td>12.8</td>
<td>13.0</td>
<td>12.7</td>
<td>12.7</td>
</tr>
<tr>
<td>CO2 Added 10^1 spores/ml</td>
<td>12.7</td>
<td>12.9</td>
<td>13.2</td>
<td>12.8</td>
<td>13.6</td>
<td>13.6</td>
</tr>
<tr>
<td>CO2 Added 10^6 spores/ml</td>
<td>12.4</td>
<td>12.8</td>
<td>12.5</td>
<td>12.8</td>
<td>13.1</td>
<td>13.1</td>
</tr>
</tbody>
</table>

milk during long-term storage at refrigeration temperatures. The numbers of viable organisms declined in all inoculated samples. No visual defects were detected in any of the milk samples. The addition of CO₂ reduced the pH of the milk samples to an average value of 6.31 from an average of 6.61; however, this drop did not correlate with changes in any other parameter measured. These data would suggest that moderate levels of CO₂ have no effect on spoilage and risk of food poisoning due to germinating spores of \textit{B. cereus} over long-term storage. Our previous work with \textit{Clostridium botulinum} inoculated milk made similar conclusions (Glass et al., 1999).

REFERENCES


