Research Note

Bacillus cereus in Refrigerated Milk Submitted to Different Heat Treatments

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ABSTRACT

The possibility of the survival, germination, and multiplication of Bacillus cereus in extended-shelf-life milk prompted research into the occurrence of the bacteria in refrigerated milk submitted to different heat treatments. Samples were submitted to ultrapasteurization (138°C for 2 s), “superpasteurization” (96°C for 13 s), and pasteurization (74°C for 15 s) and stored under refrigeration at 4 ± 2°C for up to 6 weeks. The milk was analyzed for its sensory quality and for the quantitative determination of mesophilic and psychrotrophic B. cereus in the vegetative form at incubation temperatures of 7 and 30°C, in addition to standard plate counts and psychrotrophic counts. In the three experimental trials, the psychrotrophic B. cereus counts were below the detection limit of the methodology (<10 CFU/ml) in all of the samples analyzed, independent of the heat treatment and storage period. The count of mesophilic B. cereus was restricted to samples of superpasteurized and pasteurized milk from a single trial, reaching 4.0 × 103 and 7.0 × 105 CFU/ml, respectively. Although the pasteurized milk had higher populations of mesophilic B. cereus after the second week of storage, flavor defects resulting in sensory rejection of the product did not appear before the fourth week of storage. The results of this research indicate that superpasteurization and ultrapasteurization are adequate for maintaining the product at refrigeration temperatures for 6 weeks. Pasteurized milk produced under clean conditions should have a shelf life limited to less than 2 weeks.

The current processing and filling techniques used for pasteurized milk have practically eliminated recontamination by the vegetative cells of microorganisms capable of developing at refrigeration temperatures, prolonging the shelf life of the product (11, 30). The microbes that most often limit the shelf life of milk are the psychrotrophic spore-forming bacteria, which can resist pasteurization and can germinate and multiply under refrigerated storage conditions (15). Bacillus cereus is one of the main spoilage organisms of pasteurized milk, resulting in defects commonly described as bitty cream and sweet coagulation (8, 9). Contaminated milk becomes a potential source of two types of foodborne disease, the emetic and diarrheal syndromes, caused by heat-stable emetic toxins and heat-sensitive enterotoxins, respectively, produced by this bacterium (14).

Heat resistance of the vegetative cells of B. cereus is relatively low; these cells are easily destroyed by conventional pasteurization methods: low temperature–long time and high temperature–short time (10, 28). B. cereus spores resist pasteurization and all other heat treatments below 100°C, which are commonly used in the production of extended-shelf-life milk. These sublethal treatments can increase the ability of the milk to support the germination of B. cereus spores either by the production of activation factors or the destruction of inhibitors (16, 17, 34).

The only treatments recognized as sufficiently effective for reducing populations of B. cereus spores to levels where there is practically no risk to public health are conventional in-package sterilization and continuous ultrahigh-temperature methods combined with aseptic filling, and ultrapasteurization (UP) (4). However, bacteria have been detected in UP milk stored at room temperature. This contamination could be the result of the presence of highly resistant strains (23, 26, 27), elevated populations in the raw milk, failures in the heat treatment, or recontamination.

The continued search for methods of prolonging the shelf life of milk and methods to inhibit the survival, germination, and multiplication of B. cereus prompted the present study, whose main objective was to evaluate the occurrence of the B. cereus in noninoculated (naturally contaminated) milk submitted to different heat treatments and stored under refrigeration.

MATERIALS AND METHODS

Raw milk. Refrigerated raw milk was obtained from 60 mechanically milked Dutch-Friesian and Gyr cows (Fazenda São Pedro do Imbirucu farm, Espírito Santo do Pinhal, São Paulo, Brazil) that were regularly fed corn silage and soy bran. The milk from the morning milking on the day of processing was cooled to 12°C with a plate heat exchanger, and this new milk was mixed with...
the milk from the previous afternoon, which was stored in an expansion tank at 4°C. The 200 liters of raw milk was placed into four 50-liter containers and transported under nonrefrigerated conditions from the rural property to the processing and filling pilot plant, a transport time of approximately 1 h. The milk was examined for the presence of antibiotic residues with the Delvotest kit.

Sanitization of the processing line and packages. The experimental production was carried out in a pilot plant developed for research with aseptic systems (24). Precautions were taken to prevent postprocessing contamination of the heat-treated product and packages. The processing line, including the tanks, plate heat exchanger, valves, and tubes, was sanitized with a 0.1% (wt/vol) peracetic acid solution at 45°C for 30 min. The line was rinsed with microfiltered water (three polypropylene filtration elements of pores sizes 5, 1, and 0.2 μm; Tech Filter Indústria e Comércio Ltda., Indaiatuba, Brazil) until a hydrogen peroxide residue below 0.5 mg/liter was obtained (32).

The 500-ml high-density polyethylene (HDPE) bottles, pigmented with titanium dioxide, and the HDPE screw caps with a low-density aluminum-polyethylene seal were sanitized by immersion in a 0.1% peracetic acid solution at room temperature for 10 min and then rinsed with microfiltered water.

Heat treatments and filling. Initially, the raw milk in the four containers was mixed and poured in equal proportions into the raw milk tank, which had no stirrer. The milk was then heat treated with a plate heat exchanger for the following treatments: UP at 138°C for 2 s (UP milk); “superpasteurization” at 96°C for 13 s (SP milk), and pasteurization at 74°C for 15 s (P milk). The treated milk was then cooled to approximately 10°C.

The milk from each heat treatment was placed in 500-ml HDPE bottles directly from the processing line tubes to prevent contamination from the filling machine. Filling was conducted manually with a previously sterilized funnel and applying aseptic techniques in an ISO class 8 clean room to prevent contamination from the environment. The bottles were closed with a screw cap and heat sealed by induction. The products were stored in a refrigerated incubator at 4 ± 2°C for up to 6 weeks.

The heat treatments were conducted in order of the most intense to the mildest. Between treatments, the respective holding tubes were exchanged and the processing line was depressurized, cleaned (2% sodium hydroxide at 65°C for 20 min), and returned to the production regime using microfiltered water. The holding tubes were previously wrapped in Kraft paper and sterilized at 121°C for 20 min in an autoclave. These procedures made it possible to carry out the three heat treatments with the same original batch of raw milk on the same day.

Microbiological and sensory analyses. Raw, UP, SP, and P milk samples were quantitatively analyzed for mesophilic and psychrotrophic B. cereus in the vegetative form according to the method of Bennett and Belay (3). The cultures were spread plated on mannitol–egg yolk–polymyxin agar and incubated at 30°C for 24 h for mesophilic strains and at 7°C for 10 days for psychrotrophic strains. Confirmatory tests were conducted when typical colonies developed.

The standard plate count (20) and psychrotrophic count (7) methods were also used on these samples. Packages were analyzed by the standard plate count procedure using the rinsing method (13). For the sensory evaluation of the products, the scorecard method of the American Dairy Science Association (5) was used as adapted by Aires (1). The sensory panel consisted of five judges that were trained and selected according to the methods of Shipe et al. (29).

Three samples from each of the three treatments per lot were selected at random and analyzed 1 day after production and then at successive 1-week intervals during the 6-week storage period or until the product reached the end of its bacteriological or sensory shelf life.

RESULTS AND DISCUSSION

Microbiological characterization of the raw milk and packages. Table 1 shows the results of the microbiological analyses of the raw milk used in the three experimental trials. The standard plate counts were high compared with those allowed by the U.S. (33) and the European Union (12) regulations. However, according to Brazilian legislation (6), the target for standard plate counts that was in effect until 1 July 2008 for refrigerated raw milk was a maximum of 1.0 × 10⁶ CFU/ml. The new standard allowed for some regions is in effect until 1 July 2012 and is 7.5 × 10⁵ CFU/ml. For the psychrotrophic count, only the raw milk of the first batch was considered to be satisfactory for producing milk of good quality and extended shelf life. Thomas and Thomfas (31) recommended a psychrotrophic count of up to 1.0 × 10⁴ CFU/ml for stored raw milk. Raw milk used in this study represents part of the milk produced in Brazil (2, 21, 22, 25).

The sanitized 500-ml HDPE bottles with screw caps and seals had standard plate counts below the detection limit of the methodology used, i.e., <2 CFU per package. This level is in compliance with the U.S. standards (33) for the bacterial counts of single-service containers used for packaging pasteurized milk and milk products, which fixes a maximum of 50 CFU per package in three of four samples analyzed.

<table>
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<tr>
<th>TABLE 1. Microbiological characterization of raw milk</th>
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<tr>
<td>Organism</td>
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<td>Standard aerobic bacteria</td>
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<td>Psychrotrophs</td>
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<td>Mesophilic B. cereus</td>
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<td>Psychrotrophic B. cereus</td>
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<th>TABLE 2. Mesophilic B. cereus counts in three samples of different bottles of pasteurized milk stored at 4 ± 2°C in experimental trial 2a</th>
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<tr>
<td>B. cereus count (CFU/ml)</td>
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<td>Time (days)</td>
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<td>7</td>
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<td>14</td>
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<td>21</td>
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a In trials 1 and 3, the mesophilic B. cereus counts in the three samples of pasteurized milk were <10 CFU/ml.
Microbiological and sensory characterization of the products. The psychrotrophic *B. cereus* counts for all the samples analyzed, independent of heat treatment, storage period, and experimental trial, were below the detection limits of the methodology used, i.e., <1 CFU/ml. These results also were obtained for mesophilic *B. cereus* counts in the UP milk for the three experimental trials and in the UP milk in trials 1 and 3.

The *B. cereus* was thus restricted to the mesophilic strains found exclusively in the samples of P milk and SP milk from trial 2. Table 2 shows the mesophilic *B. cereus* counts found in the P milk from trial 2. In the SP milk, colonies were found in only one of the three samples analyzed for weeks 2 and 6 of storage, at 30 and 40 CFU/ml, respectively. In the other samples collected during the 6 weeks of storage, no *B. cereus* was found, and the results were expressed as less than the limit of detection of 10 CFU/ml.

The occurrence of high levels of *B. cereus* in P milk after 1 week of storage agrees with previously published data. Conventional pasteurization treatments are incapable of causing substantial destruction of the spores (10, 28) and can make the product a more susceptible substrate for the germination of *B. cereus* spores (34). In the same way, the results for the UP milk corroborated the conclusions of Bergère and Cerf (4). These authors summarized data on the heat resistance of *B. cereus* and concluded that the ultrahigh-temperature heating methods were sufficient to reduce the spore population to levels at which public health risks were practically nonexistent. They also concluded that heat-resistant *B. cereus* spores were uncommon and should not cause concern when evaluating the efficacy of the ultrahigh-temperature treatment.

The low levels of *B. cereus* in the SP milk were unexpected because heating at 96°C for 13 s usually fails to reduce the population of *B. cereus* spores by even 1 log unit (4). Temperatures between 85 and 120°C are known spore germination inducers, and temperatures above 120°C with retention times of a few seconds are commonly used in the production of extended-shelf-life milk (19). The low levels of *B. cereus* verified in the SP milk could be attributed to the combined effect of the prevention of postprocessing contamination, transformation of the product into a medium less hospitable for spore germination, and storage conditions.

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at 4°C. Wilkinson and Davies (34) found that milk submitted to heat treatments at 80 to 95°C for 15 s became less favorable for the germination of *B. cereus* spores, despite the fact that such heat treatments caused spore activation and consequently germination in the product incubated at 30°C. In the present research, the inhibitory effect of the medium transformation combined with refrigerated storage supposedly counteracted activation. The counts of mesophilic *B. cereus*, verified exclusively in the plates incubated at 30°C, support this hypothesis.

In the P milk samples of trial 2, the counts of mesophilic *B. cereus* ranged between <10 and >10⁵ CFU/ml during storage, revealing differences between samples analyzed at a single time point (Table 2). The highest levels were reached in the weeks 2 and 3 of storage, even though the product was analyzed for up to 4 weeks. These findings may have resulted from initial contamination of the P milk at nonuniform levels during the production period or with *B. cereus* strains with different germination and multiplication abilities. Variations in the initial contamination levels could have been caused by the operational conditions applied in these experiments, particularly by the fact that the raw milk remained in the raw milk tank without stirring until processed and the fact that filling of the milk was done directly from the processing line tubes without storage in a tank before filling to make a homogenous lot. These conditions could have influenced distribution of the microorganism in the raw milk and consequently in the P milk samples.

Another situation that could have contributed to unequal contamination of the product is the phenomenon of splitting the bacterial aggregates at different moments during processing. Extreme differences in microbial counts also can occur in industrial products. Larsen and Jørgensen (18) analyzed 27 samples of P milk that had been collected from three dairies and stored at 7°C for 9 days. *B. cereus* counts in these samples ranged between <10 and 10⁵ CFU/ml. These results provide evidence that a single sample may not be representative of the entire batch, and thus samples for analysis should be randomly selected at different stages of production.

The standard plate counts and psychrotrophic counts in the samples from trial 2 are illustrated in Figures 1 and 2. The P milk from trial 2 supported the development of both mesophilic and psychrotrophic microorganisms. Mesophiles did not develop in the product in trials 1 and 3, but the growth curve of the psychrotrophs was similar to that of trial 2, suggesting that the mesophilic population in the P milk of trial 2 was composed predominantly of *B. cereus*. This relationship cannot be extended to the SP milk because the counts of mesophilic *B. cereus* were much lower than the standard plate counts.

Figure 3 shows the results of the sensory evaluation of the UP, SP, and P milk samples from the trial 2. The products from this trial had sensory profiles similar to those of the products from the other trials. No sensory defects were detected in the SP and P milk that could be associated with the development of *B. cereus*.

The UP heat treatment (138°C for 2 s) was sufficient to prevent the occurrence of both mesophilic and psychrotrophic *B. cereus* in milk stored for 6 weeks under refrigeration at 4°C. In the SP milk (96°C for 13 s), mesophilic *B. cereus* was found at levels presenting practically no public health risks. In the P milk (74°C for 15 s), the populations of mesophilic *B. cereus* reached above 10⁵ CFU/ml from week 2 of storage, despite not showing the development of psychrotrophic strains. However, the P milk had no sensory alterations until week 4 of storage. Thus, P milk produced under clean conditions should have its shelf life limited to less than 2 weeks or should be submitted to a heat treatment or storage temperature that prevents the survival, germination, and development of *B. cereus*.

REFERENCES


