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Research Note

Effect of Heat Treatment of Milk on Activation of *Bacillus* Spores

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ABSTRACT

The quality and shelf life of fluid milk products are dependent on the amount and type of microorganisms present following pasteurization. This study evaluated the effects of different pasteurization processes on the microbial populations in fluid milk. The objective was to determine whether certain pasteurization processes lead to an increase in the amount of bacteria present in pasteurized milk by activating *Bacillus* spores. Samples of raw milk were collected on the day of arrival at the dairy plant. The samples were pasteurized at 63°C for 30 min (low temperature, long time), 72°C for 15 s (high temperature, short time), 76°C for 15 s, and 82°C for 30 min. The pasteurized samples were then stored at 6 and 10°C for 14 days. The samples were analyzed for standard plate count and *Bacillus* count immediately after pasteurization and after 14 days of storage. Pasteurization of milk at 72 and 76°C significantly ($P < 0.05$) increased the amount of *Bacillus* spore activation over that of 63°C. There was no detection of *Bacillus* in initial samples pasteurized at 82°C for 30 min, but *Bacillus* was present in samples after storage for 14 days, indicating that injury and recovery time preceded growth. The majority of isolates were characterized as *Bacillus mycoides* and not *Bacillus cereus*, suggesting that this organism might be more a cause of sweet curdling of fluid milk than previously reported.

Pasteurization is a process designed to eliminate disease-causing organisms, significantly reduce total bacterial load, and destroy lipase and other natural enzymes in milk products *(15)*. The Code of Federal Regulations *(3)*, Title 21, Section 131.3b, defines pasteurized dairy products as using properly operated equipment to heat every particle of the product to a specified temperature and held at or above that temperature for a specified time. The pasteurization of milk at 63°C for 30 min is known as batch pasteurization, or low temperature, long time pasteurization *(17)*. High temperature, short time pasteurization is when milk is pasteurized at 72°C for 15 s. Unless pasteurization temperatures are carried out above the minimums, thermoduric bacteria are capable of survival and growth in pasteurized milk products *(10)*.

One problem that is found to cause a reduction in the shelf life of fluid milk is the presence and growth of *Bacillus* spp. *(9, 13)*. *Bacillus* is a gram-positive spore-forming microorganism. Specifically, the spores of *Bacillus cereus* and *Bacillus mycoides* are known to be extremely heat stable and are capable of surviving pasteurization temperatures. The high pasteurization temperatures destroy vegetative cells but activate the spores, which leads to germination and growth *(12)*. These microorganisms are known to cause a defect in fluid milk called sweet curdling. The defect is first manifested by the formation of a sweet (nonacid) curd on the bottom of the milk container that has a bitter off-flavor *(5)*. The formation of the sweet curd is due to an extracellular enzyme produced by *Bacillus* spp. that causes the casein to precipitate *(8)*.

Over the years, the high temperature, short time pasteurization process has been increased from 72 to 76°C to ensure the elimination of non–spore-forming pathogens such as *Listeria monocytogenes (1, 2)*. The objective of this study was to determine whether this increase in heat treatment of fluid milk has any effect on *Bacillus* spore activation. The information provided in this study should help determine whether one pasteurization process is better for providing a product with a longer shelf life.

MATERIAL AND METHODS

Collection and storage of raw milk. Raw milk was collected on the day it was delivered to the Babcock Hall Dairy Plant (Madison, Wis.). Approximately 4.5 liters of raw milk were collected in a sterile container with a lid and stored at approximately 4°C until picked up for testing. The raw milk was picked up within 3 h of collection. Samples were collected on five different receiving days.

Pasteurization of raw milk. The raw milk was pasteurized under four different conditions: 63°C for 30 min, 72°C for 15 s, 76°C for 15 s, and 82°C for 30 min. Pasteurization at 82°C for 30 min was performed to duplicate yogurt production and to evaluate the effect of high pasteurization temperatures on spore activation. For pasteurization at 63°C, nine sterile 50-ml polypropylene conical tubes were filled with raw milk, placed in a 63°C water bath, held for 30 min when the samples reached 63°C, and immediately cooled in an ice bath. One tube was equipped with a thermometer

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to determine when the samples reached 63°C. Pasteurization at 72 and 76°C was accomplished with the use of a small plate heat exchanger (AGC Engineering PRO013, Portland, Oreg.). A pump (Cole-Parmer Instrument Co. Masterflex 7015, Chicago, Ill.) was used to pump the milk through the plate heat exchanger at a total time in the heat exchanger of 15 s. The pasteurized milk was collected in sterile 50-ml polypropylene conical tubes and immediately cooled in an ice bath. Pasteurization at 82°C was done by heating 500 ml of raw milk in a beaker on a stirring hot plate and holding for 30 min at 82°C. The pasteurized milk was then poured into sterile 50-ml conical polypropylene tubes and cooled in an ice bath.

Handling and storage of pasteurized samples. After the pasteurized samples had been cooled, they were divided and placed in storage cabinets at two different temperatures, 6 and 10°C, to represent normal and abuse conditions, respectively, for 14 days. Approximately 120 ml of each sample was stored at each temperature.

Microbiological analysis. Raw milk samples for each of the sampling times were evaluated for standard plate count (SPC), aerobic spore count, and *Bacillus* spp. count. SPC and *Bacillus* spp. count were also conducted on the pasteurized milk samples on days 0 and 14. The SPC was determined by preparing serial dilutions with Butterfield's phosphate buffer (Nelson-Jameson, Marshfield, Wis.) and plated on aerobic plate count petrifilm (3M, St. Paul, Minn.) with incubation at 32°C for 48 h. The aerobic bacterial spore count was conducted by heating raw milk to 80°C and holding for 12 min, immediately cooling in an ice bath, and plating in triplicate on aerobic plate count petrifilm. As per section 8.9 of the *Standard Methods for the Examination of Dairy Products (11)*, the samples were incubated at 7°C for 10 days to determine the psychrotrophic spore count and at 32°C for 48 h to determine the mesophilic spore count. Mesophilic spore count was determined by *Bacillus* spp. having a tendency to grow very slowly or not at all at 7°C, depending on the strain *(6).* The *Bacillus* spp. count was determined according to chapter 14 of the *Bacteriological Analytical Manual* (BAM) *(16).* The samples were diluted and blended with Butterfield's phosphate-buffered dilution water prepared in the laboratory in a sterile blender. The appropriate serial dilutions were made and plated in duplicate on mannitol–egg yolk–polymyxin agar (Difco, Becton Dickinson, Sparks, Md.) plates. Numbers (CFU per milliliter) were determined following the guidelines presented in BAM. Isolates were further tested with Gram staining and presence or absence of rhizoid growth on nutrient agar in an attempt to further classify the isolates as *B. cereus*, specifically.

Statistical analysis. The results were analyzed by Minitab statistical software with a one-way analysis of variance *(14).* All trials were replicated five times. Reported mean values represent duplicate determinations of duplicate samples at each treatment for the five replicates. Fisher's pairwise comparisons were used to provide confidence intervals for all pairwise differences between level means. The level of significance was $P < 0.05$.

RESULTS AND DISCUSSION

Raw milk analysis. The SPC for the raw milk samples for the five trials ranged from 3.19 to 4.42 log CFU/ml, with a mean SPC of 4.02 log CFU/ml. The mesophilic aerobic spore count ranged from 3 to 48 CFU/ml, with an average of 17 CFU/ml. The *Bacillus* presumptive count ranged from <10 to 50 CFU/ml, with a mean of 10 CFU/ml. This indicated that there were spore-forming organisms present in the raw milk that had the potential to survive pasteurization and cause problems in finished products. In all five trials, psychrotrophic aerobic spore-forming organisms were undetected.

TABLE 1. *Microbial counts of* Bacillus *spp. in milk following various pasteurization treatments*

Pasteurization treatment	*Bacillus* spp. (log CFU/ml)[a]		
	0 days	14 days at 6°C	14 days at 10°C
63°C for 30 min	<1 B	1.44	6.08
72°C for 15 s	1.88 A	1.41	6.07
76°C for 15 s	1.90 A	1.47	6.28
82°C for 30 min	<1 B	1.06	6.32

[a] Mean *Bacillus* spp. count in raw milk before pasteurization was 1.0 log CFU/ml. Means ($n = 5$) in the same column with a different letter are significantly different ($P < 0.05$).

Spore activation. Table 1 shows the effect of heat treatment on the amount of *Bacillus* spore activation. There was a significant increase in *Bacillus* spp. count with the 72 and 76°C samples. These data suggest that pasteurization temperatures between 72 and 76°C caused a greater increase in the number of *Bacillus* spores that are activated compared with lower-temperature pasteurization processes. These results agree with previous reports that heat treatment between 65 and 75°C was optimal for *Bacillus* spore activation *(4, 13).* There was no significant difference in activation of *Bacillus* spores between pasteurization temperatures of 72 and 76°C. The *Bacillus* spp. count of the milk samples after incubation for 14 days at 6 and 10°C showed that there was no significant difference in the *Bacillus* spp. counts of the samples 14 days after heat treatment. For all trials, there were no *Bacillus* spp. spores detected in the samples immediately following heat treatment at 82°C for 30 min. However, 14 days after pasteurization at 82°C, on average there were 1.06 and 6.32 log CFU/ml at 6 and 10°C, respectively. The high heat damages the *Bacillus* spores and slows the recovery and detection immediately following the heat treatment. With incubation, the spores are able to recover and grow in the pasteurized product. In an experiment by McGuiggan et al. *(12)*, it was shown that maximum recovery of *Bacillus* spores was obtained after incubating the sample for 15 days.

The *Bacillus* isolates were further classified by Gram stain and rhizoid growth capability on nutrient agar as described in BAM *(16). B. mycoides* exhibits rhizoid growth on nutrient agar, whereas *B. cereus* does not. Generally, the majority of the microorganisms isolated were *B. mycoides* and not *B. cereus.* For the samples stored at 6°C, all the samples had over 50% of the isolates characterized as *B. mycoides.* All the milk samples stored at 10°C had at least 70% of the isolates identified as *B. mycoides.* A fermented or fruity aroma was noticed in the samples incubated at 10°C, which is a sensory defect associated with sweet curdling *(5). B. mycoides* is closely related to *B. cereus (12)* and might possibly be more responsible for the sweet cur-

TABLE 2. *Standard plate count (SPC) of milk after various pasteurization treatments*

Pasteurization treatment	SPC (log CFU/ml)[a]		
	0 days	14 days at 6°C	14 days at 10°C
63°C for 30 min	1.71 B	3.22 AB	7.00
72°C for 15 s	3.55 A	4.32 A	6.95
76°C for 15 s	3.24 A	2.45 B	6.50
82°C for 30 min	<1.4 C	<1.4 C	6.47

[a] Mean SPC of raw milk before pasteurization was 4.02 log CFU/ml. Means ($n = 5$) in the same column with a different letter are significantly different ($P < 0.05$).

dling problem in fluid milk than *B. cereus* itself. The greater prevalence of *B. mycoides* in the pasteurized milk samples could indicate that *B. mycoides* is more easily activated than *B. cereus*, especially at temperatures between 72 and 76°C, or that the raw milk might have contained a higher level of *B. mycoides* spores than *B. cereus* spores.

The results of the SPC on the heat-treated milk samples are summarized in Table 2. There was a significant difference in the SPC between samples pasteurized under different conditions both initially and after 14 days of storage at 6°C. In the initial samples, pasteurization at 72°C for 15 s and 76°C for 15 s yielded samples with higher SPCs than samples pasteurized under other conditions. After storage for 14 days at 6°C, the highest SPCs were observed in the samples pasteurized at 63°C for 30 min and 72°C for 15 s. After the lower temperature pasteurization, the psychrotrophic spore-forming bacteria flourished. At 10°C, the mesophilic spore formers outgrew the psychrotrophic thermodurics. Increasing the pasteurization temperature from 72 to 88°C has been reported to significantly decrease the SPCs *(7)*. In this experiment, the SPCs in the initial samples increased as the pasteurization temperature increased from 63 to 76°C and then significantly decreased at 82°C. This observation can be explained by activation of *Bacillus* spp. spores at 72 and 76°C, as seen in Table 1, and caused the increase in the SPCs.

From the results of this study, we conclude that there was a difference in the amount of *Bacillus* spp. spores that were activated at different pasteurization conditions. Pasteurization processes between the temperatures of 72 and 76°C caused more spores to be activated than pasteurization treatments at other temperatures. The majority of the *Bacillus* spp. present were *B. mycoides*, suggesting that they might be more easily heat activated than *B. cereus* and could be a greater cause of sweet curdling in fluid milk, or they might have been present in the raw milk at a higher level. This study also indicated that proper refrigeration temperatures are important to extend the shelf life of milk products because traditional pasteurization will activate *Bacillus* spores and allow for growth at abusive temperatures.

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