High temperature, short time pasteurization temperatures inversely affect bacterial numbers during refrigerated storage of pasteurized fluid milk

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ABSTRACT

The grade A Pasteurized Milk Ordinance specifies minimum processing conditions of 72°C for at least 15 s for high temperature, short time (HTST) pasteurized milk products. Currently, many US milk-processing plants exceed these minimum requirements for fluid milk products. To test the effect of pasteurization temperatures on bacterial numbers in HTST pasteurized milk, 2% fat raw milk was heated to 60°C, homogenized, and treated for 25 s at 1 of 4 different temperatures (72.9, 77.2, 79.9, or 85.2°C) and then held at 6°C for 21 d. Aerobic plate counts were monitored in pasteurized milk samples at d 1, 7, 14, and 21 postprocessing. Bacterial numbers in milk processed at 72.9°C were lower than in milk processed at 85.2°C on each sampling day, indicating that HTST fluid milk-processing temperatures significantly affected bacterial numbers in fluid milk. To assess the microbial ecology of the different milk samples during refrigerated storage, a total of 490 psychrotolerant endospore-forming bacteria were identified using DNA sequence-based subtyping methods. Regardless of processing temperature, >85% of the isolates characterized at d 0, 1, and 7 postprocessing were of the genus *Bacillus*, whereas more than 92% of isolates characterized at d 14 and 21 postprocessing were of the genus *Paenibacillus*, indicating that the predominant genera present in HTST-processed milk shifted from *Bacillus* spp. to *Paenibacillus* spp. during refrigerated storage. In summary, 1) HTST processing temperatures affected bacterial numbers in refrigerated milk, with higher bacterial numbers in milk processed at higher temperatures; 2) no significant association was observed between genus isolated and pasteurization temperature, suggesting that the genera were not differentially affected by the different processing temperatures; and 3) although typically present at low numbers in raw milk, *Paenibacillus* spp. are capable of growing to numbers that can exceed Pasteurized Milk Ordinance limits in pasteurized, refrigerated milk.

Key words: milk, pasteurization, *Bacillus*, *Paenibacillus*

INTRODUCTION

In the United States, *Bacillus* spp. and *Paenibacillus* spp. have been identified as the biological barriers currently limiting HTST pasteurized fluid milk shelf life (Fromm and Boor, 2004; Durak et al., 2006; Huck et al., 2007b, 2008). *Bacillus* spp. and *Paenibacillus* spp. are capable of forming heat-resistant spores that can survive HTST pasteurization (Collins, 1981; Huck et al., 2007b), and some strains are able to germinate and grow at refrigeration temperatures, ultimately causing spoilage of processed products (Washam et al., 1977; Huck et al., 2008). Because *Bacillus* spp. and *Paenibacillus* spp. spores are ubiquitously present in nature, it is difficult to exclude them from the milk supply. Representatives from both genera have been isolated from dairy farms (Crielly et al., 1994; Sutherland and Murdoch, 1994; Lukasova et al., 2001; Huck et al., 2008), processing plants (Lin et al., 1998; Huck et al., 2007b), and pasteurized packaged products (Griffiths and Phillips, 1990; Lin et al., 1998; Douglas et al., 2000; Fromm and Boor, 2004; Durak et al., 2006; Huck et al., 2007b).

In recent years, both food safety concerns and the desire to extend fluid milk shelf life have prompted many dairy processors to increase HTST pasteurization temperatures above the minimum conditions specified by the Pasteurized Milk Ordinance (72°C for 15 s; Pasteurized Milk Ordinance, 2005; Gandy et al., 2008). Anecdotal reports from multiple milk processors of higher bacterial numbers and more rapid spoilage in fluid milk products after an increase in processing temperatures prompted this investigation of the effects of commonly applied HTST pasteurization temperatures on aerobic plate counts (APC) in refrigerated fluid milk products. To test the hypothesis that higher HTST processing temperatures result in higher bacterial numbers in fluid milk products during refrigerated storage, the objectives of this study were 1) to determine the effect of processing temperature on APC in milk samples that had been processed under a range of

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commonly applied HTST temperatures and held under refrigerated storage conditions (6°C); and 2) to monitor the microbial ecology of pasteurized products processed at each temperature during storage.

MATERIALS AND METHODS

Experimental Design

To measure the effects of HTST pasteurization temperatures on APC in pasteurized fluid milk, 4 independent batches of raw 2% fat homogenized milk were each processed for 25 s at one of the following temperatures: 72.9, 77.2, 79.9, or 85.2°C. A complete block design was used to allow all combinations of position in the processing order (first, second, third, or fourth) for each processing temperature (72.9, 77.2, 79.9, or 85.2°C) to ensure that each temperature held a different position in processing in each replicate.

Pasteurization of Raw Milk

Raw bovine milk from approximately 300 cows in Hartford, New York, was pooled, and representative samples were collected and transported to the Cornell University Food Processing and Development Laboratory (Ithaca, NY). The raw milk was cold-separated into cream and skim fractions with a DeLaval separator (Model 590; DeLaval, Poughkeepsie, NY), and then held refrigerated at 5°C for up to 72 h. On the day of processing, raw skim milk and cream were blended to make 2% fat milk. Approximately 5 gallons (18.93 L) of 2% fat raw milk was added to a jacketed steam kettle, heated to 60°C, and then homogenized with 2 passes through a Gaulin APV homogenizer (Model 200 E; Gaulin, Everett, MA). After homogenization, the milk was pasteurized at 72.9, 77.2, 79.9, or 85.2°C for 25 s as described by Ma and Barbano (2003). Milk samples (2 L) pasteurized at each temperature were collected aseptically and held on ice until all processing treatments were completed. Immediately postprocessing, 8 aliquots of approximately 200 mL were poured from each of the 2-L containers into sterile 250-mL screw-capped Pyrex bottles. The pasteurized milk samples were held at 6°C for up to 21 d.

Microbiological Testing of Milk Samples

To determine the microbiological quality of the raw milk before pasteurization, a sample was taken from the standardized 2% fat milk before heating and homogenization. Milk samples were assessed for total APC, coliform count, psychrotrophic bacteria count (**PBC**), and laboratory pasteurized count (**LPC**) according to the Standard Methods for the Examination of Dairy

Products (Frank and Yousef, 2004), except that the samples for APC, PBC, and LPC tests were spread-plated onto brain heart infusion agar (**BHI**; Difco, BD Diagnostics, Franklin Lakes, NJ). Preliminary incubation counts were performed on raw 2% fat milk samples according to the Standard Methods for the Examination of Dairy Products (Richardson, 1985), except that the raw 2% fat samples were spread-plated onto BHI agar and incubated at 32°C for 24 h. For the mesophilic spore count (**MSC**) and psychrotrophic spore count (**PSC**) tests, milk samples were heated at 80°C for 12 min, and then the milk was cooled rapidly, incubated overnight at 6°C, and spread-plated the following day on BHI agar, with subsequent incubation at 32°C for 24 h (for the MSC) or at 6°C for 10 d (for the PSC).

Two aliquots of milk that had been processed at each temperature were tested for APC at d 1, 7, 14, and 21 postpasteurization. Samples were serially diluted in PBS (Weber Scientific, Hamilton, NJ), spread-plated onto BHI agar, and incubated at 32°C for 24 h. On d 1, plating was performed by spreading 1 mL of milk sample over 5 plates to allow bacterial enumeration in samples with low bacterial counts.

Statistical Analyses

A mixed model was used to analyze the APC data (JMP Version 7.0; SAS Institute Inc., Cary, NC). For all analyses, log-transformed bacterial count data were used as a response. The model included temperature of processing and time of refrigerated storage of pasteurized milk as independent fixed variables. Replicate and temperature were random variables in the model. Temperature was a between-replicate effect and day was a within-replicate effect. Models were tested for an interaction between time and temperature. When an effect was significant, multiple comparisons were done with a Tukey correction. Distribution of bacterial subtypes was analyzed in JMP using a chi-square test for independence.

Bacterial Isolation

For each milk sample, bacterial colonies present on BHI that had been plated at d 0, 1, 7, 14, and 21 postprocessing were visually examined and a colony representative of each distinct morphology present on 1 plate that had been used for colony enumeration for each sample was chosen for isolation and later identification. Typically, 5 to 10 colonies per sample were selected and streaked for purity on BHI agar. Purified isolates were characterized for gram reaction using a 3-step gram stain kit (Becton, Dickinson and Co., Sparks, MD) and subsequently frozen at -80°C in 15% glycerol.

Table 1. Log-transformed bacterial numbers from 2% fat raw milk used in 4 independent replicates¹

Replicate	APC	MSC	LPC	PI	CC	PBC
1	4.98	2.88	1.95	4.90	1.63	3.18
2	3.94	1.36	1.45	5.66	2.04	3.36
3	3.92	0.85	1.00	2.19	1.18	4.18
4	3.91	1.59	1.08	>5.40 ²	1.08	>4.40 ²
Mean	4.19	1.67	1.37	4.25	1.48	3.57
SD	0.53	0.86	0.48	1.82	0.38	0.53

¹APC = aerobic plate count; MSC = mesophilic spore count; LPC = laboratory pasteurized count; PI = preliminary incubation count; CC = coliform count; PBC = psychrotrophic bacteria count. In all 4 replicates, psychrotrophic spore count analyses yielded no colonies, indicating the presence of less than 1 spore capable of growth at 6°C per milliliter of raw milk sample.

²Estimated counts were not included in mean or standard deviation calculations; bacteria from these samples were “too numerous to count.”

rpoB Sequencing

Species identification and subtyping of *Bacillus* spp. and *Paenibacillus* spp. isolates were performed by determining the DNA sequence of a 632-nucleotide fragment of the *rpoB* gene, as described previously by Huck et al. (2007a). This method was selected for bacterial identification and differentiation purposes because it allows for phylogenetic characterization of *Bacillus* and *Paenibacillus* isolates in addition to subtype identification, and because it is more economical than most banding pattern-based subtyping methods such as ribotyping or pulsed-field gel electrophoresis (Kabuki et al., 2004). The software DnaSP version 4.0 (Rozas and Rozas, 1999) was used for *rpoB* allele assignment, and different allelic types (AT) were assigned to gene sequences that differed from each other by 1 or more nucleotides. Isolates with different AT were considered to represent different molecular subtypes.

16S Ribosomal DNA Sequencing

Although the majority of isolates collected here could be characterized to genus and species through phylogenetic analyses by using the existing *rpoB* sequence database (Durak et al., 2006; Huck et al., 2007a,b, 2008), 16S ribosomal DNA (rDNA) sequencing 1) was used to confirm the genus or species identification, or both, of *rpoB* AT that had not been identified in any of our previous studies, and 2) was used for those isolates we could not identify by *rpoB* sequencing. Specifically, 1 isolate representing each newly identified *rpoB* allelic type was characterized by sequencing the 3' end of the 16S rDNA, as described previously by Huck et al. (2007a). Final partial 3' 16S rDNA sequences were used for similarity searches against the National Center for Biotechnology Information nucleotide sequence database, using the Blast Local Alignment Search Tool (McGinnis and Madden, 2004). Genus or species assignments, or both, for a specific 16S rDNA sequence

were based on the top matches returned by the search with the Blast Local Alignment Search Tool.

RESULTS

Microbiological Analysis of Raw Milk

To assess the microbiological quality of the raw milk before pasteurization, a sample of the 2% fat raw milk was collected immediately after the skim and cream portions had been blended, but before heat tempering and homogenization on each processing day. A complete listing of log-transformed bacterial counts for each replicate is presented in Table 1. Mean raw 2% fat milk APC from the 4 replicates was 4.19 log cfu/mL, and ranged from 3.91 (replicate 4) to 4.98 log cfu/mL (replicate 1). The mean MSC was 1.67 log cfu/mL, and ranged from 0.85 (replicate 3) to 2.88 log cfu/mL (replicate 1). On 2% fat raw milk samples, we observed a mean LPC of 1.37 log cfu/mL, a mean preliminary incubation count of 4.25 log cfu/mL, a mean coliform count of 1.48 log cfu/mL, a mean PBC of 3.57 log cfu/mL, and no growth (<1 spore/mL) for the PSC (Table 1).

Microbiological Analysis of Pasteurized Milk

Total aerobic bacterial numbers for the HTST pasteurized milk samples that had been stored at 6°C are shown in Figure 1. For d 1, 7, 14, and 21, the mean bacterial numbers for milk that had been processed at 72.9°C were lower than the mean numbers for the milk that had been processed at 85.2°C. In general, as pasteurization temperatures increased, mean aerobic bacterial plate counts also increased. At d 1 and 7 post-pasteurization, the mean APC for the milk processed at the 3 highest temperature treatments were similar and all were higher than the mean for the milk processed at 72.9°C.

Overall, results from statistical analyses of our data showed a significant effect of temperature ($P = 0.03$)

Table 2. Genus distribution of bacterial isolates from raw and heat-treated milk samples

Genus	Isolates from raw milk, ¹ no.	Isolates from pasteurized milk, no.	Total
Gram negative			
<i>Pseudomonas</i>	31	0	31
<i>Stenotrophomonas</i>	6	0	6
<i>Acinetobacter</i>	3	0	3
<i>Klebsiella</i>	2	0	2
<i>Pantoea</i>	2	0	2
<i>Comamonas</i>	1	0	1
Total	45	0	45
Gram positive			
<i>Bacillus</i> ²	29	229	258
<i>Paenibacillus</i> ³	2	230	232
<i>Enterococcus</i>	4	6	10
<i>Micrococcus</i>	0	7	7
<i>Staphylococcus</i>	1	5	6
<i>Streptococcus</i>	1	1	2
<i>Aerococcus</i>	1	0	1
<i>Brevibacillus</i>	0	1	1
<i>Corynebacterium</i>	0	1	1
<i>Macroccoccus</i>	1	0	1
Total	39	480	519
Total	84	480	564

¹Raw milk isolates were obtained from multiple tests performed on 2% raw milk, including aerobic plate counts, mesophilic spore counts, and laboratory pasteurized counts.

²Of 258 *Bacillus* isolates, 249 were identified by *rpoB* sequencing, and 9 isolates were identified by 16S ribosomal DNA (rDNA) sequencing.

³Of 232 *Paenibacillus* isolates, 229 were identified by *rpoB* sequencing, and 3 isolates were identified by 16S rDNA sequencing.

and day postpasteurization ($P < 0.001$) on bacterial numbers in pasteurized milk. The interaction between temperature and day was tested and found to be insignificant ($P = 0.82$). On d 1 postprocessing, mean APC ranged from 1.69 log cfu/mL for milk pasteurized at 72.9°C to 2.18 log cfu/mL for milk pasteurized at 79.9°C. Mean APC were slightly higher at d 7 postprocessing relative to the mean counts at d 1. At d 7, the mean APC for milk processed at 72.9°C was 1.74 log cfu/mL, whereas the mean APC for milk processed at 85.2°C was 2.30 log cfu/mL. Between d 7 and 14, the average increases in APC were 2.33, 2.66, 2.95, and 3.06 log cfu/mL for milk processed at 72.9, 77.2, 79.9, and 85.2°C, respectively. On d 21, the mean APC for milk processed at 72.9°C was 5.79 log cfu/mL (approximately 616,000 cfu/mL), whereas the mean APC for milk processed at 85.2°C was 7.10 log cfu/mL (approximately 12,590,000 cfu/mL). Mean APC for milk processed at 72.9°C was significantly lower ($P < 0.05$) than for milk processed at 85.2°C on each sampling day.

Bacterial Species Distribution as Determined by *rpoB* and 16S rDNA Subtyping Data

A total of 564 isolates were collected in the 4 replicates from both the raw and pasteurized product. Of those, 478 isolates were determined by *rpoB* sequenc-

ing to belong to genera characterized as gram-positive spore-forming bacteria (i.e., *Bacillus* and *Paenibacillus*). The remaining 86 isolates, which were not typable by the *rpoB* sequencing method, were identified by sequencing the 3' end of the 16S rRNA gene. Among these 86 isolates, 45 represented gram-negative bacteria and 41 represented gram-positive bacteria. Table 2 includes the genus distribution of the bacterial isolates identified (by 16S and *rpoB* sequencing) in this study. All gram-negative bacteria were isolated from raw milk, and the majority (31 isolates) were *Pseudomonas* spp. The other gram-negative genera identified included *Stenotrophomonas* (6 isolates), *Acinetobacter* (3 isolates), *Klebsiella* (2 isolates), *Pantoea* (2 isolates), and *Comamonas* (1 isolate). The most frequently isolated gram-positive bacteria included *Bacillus* (258 isolates) and *Paenibacillus* (232 isolates). Other gram-positive genera identified were *Enterococcus*, *Micrococcus*, *Staphylococcus*, *Streptococcus*, *Aerococcus*, *Brevibacillus*, *Corynebacterium*, and *Macroccoccus*.

Overall, 96 *rpoB* AT were differentiated among the 478 spore-forming bacterial isolates, indicating considerable genetic diversity among even the closely related microbes that were present in this milk-production and milk-processing system. An additional 9 *Bacillus* and 3

Table 3. Distribution of isolates obtained from pasteurized milk and characterized as *Bacillus* spp. or *Paenibacillus* spp.

Pasteurization temperature, °C	Day postpasteurization	Genus	
		<i>Bacillus</i> ¹	<i>Paenibacillus</i> ²
72.9	1	39	2
	7	21	7
	14	3	22
	21	0	23
	All days	63	54
77.2	1	31	4
	7	19	8
	14	3	27
	21	1	25
	All days	54	64
79.9	1	29	0
	7	20	6
	14	3	18
	21	0	31
	All days	52	55
85.2	1	32	1
	7	22	8
	14	2	24
	21	4	24
	All days	60	57
Total ³		229	230

¹Of 229 *Bacillus* isolates, 222 were identified by *rpoB* sequencing, and 7 isolates were identified by 16S ribosomal DNA (rDNA) sequencing.

²Of 230 *Paenibacillus* isolates, 227 were identified by *rpoB* sequencing, and 3 isolates were identified by 16S rDNA sequencing.

³Isolates obtained from raw milk or raw milk receiving heat treatments (laboratory pasteurized counts or mesophilic spore counts) are not included in these distributions.

Figure 1. Aerobic plate counts (APC) for 2% fat milk that had been pasteurized at 1 of 4 different temperatures (72.9, 77.2, 79.9, or 85.2°C) and held at 6°C for up to 21 d. Data represent mean APC for milk processed at each temperature from 4 independent replicates. Bars indicate mean $\pm$ 1 SD for each treatment.

Paenibacillus isolates that did not yield *rpoB* products were identified by 16S rDNA sequencing. Of the isolates obtained from pasteurized milk, 229 were identified as *Bacillus* and 230 as *Paenibacillus*. Table 3 shows the distribution of *Bacillus* and *Paenibacillus* isolates by pasteurization temperature and day postpasteurization. There was a significant association between genus identified and day postpasteurization ($P < 0.001$). As shown in Table 4, at d 0, 1, and 7, the number of *Bacillus* isolates collected was significantly higher than that of *Paenibacillus* ($P < 0.001$). Of 280 isolates characterized at d 0, 1, or 7 postpasteurization, 242 were characterized as *Bacillus*. The 38 remaining isolates were characterized as *Paenibacillus*. However, the number of *Paenibacillus* isolates collected was significantly higher than the number of *Bacillus* isolates at d 14 and 21 postpasteurization ($P < 0.001$). Of 210 isolates collected at d 14 or 21 postpasteurization, 194 were characterized as *Paenibacillus*. There was no significant association between genus isolated and pasteurization temperature ($P = 0.82$; chi-square analysis), suggesting that the genera were not differentially affected by the different processing temperatures.

Subtype Patterns Across Replicates and Temperature Treatments

One important outcome of this study was the further insight obtained regarding the diversity of microbes that pose a barrier to the extension of pasteurized milk shelf life. In the present study, only 8 of the 96 AT identified (4 *Bacillus* and 4 *Paenibacillus*) were isolated both from multiple replicates and more than 10 times (Table 5). The most frequently isolated *Bacillus* AT were AT1, AT6, AT20, and AT158. Allelic type 1 and AT6 were identified as *Bacillus licheniformis*, whereas AT20 and AT158 were identified as *Bacillus pumilus* and *Bacillus cereus*, respectively. The most commonly isolated AT of the genus *Paenibacillus* were AT13, AT15, AT25, and AT27. Research on *Paenibacillus* spp. is just emerging; thus, the majority of isolates that classify to this genus have not yet been speciated.

Certain *Bacillus* and *Paenibacillus* subtypes were found in samples collected from at least 3 replicates, indicating the persistence of these particular subtypes in the milk-production and milk-processing system. For example, AT1 (*B. licheniformis*) was isolated 4, 14, 6,

Table 4. Proportion of endospore-forming isolates collected from raw and heat-treated milk by day postpasteurization and by heat treatment (i.e., raw, laboratory pasteurization, mesophilic spore count, pasteurization)

Day postpasteurization (heat treatment)	Isolates characterized, no.	Isolates characterized, no. (%) ¹	
		<i>Bacillus</i> spp.	<i>Paenibacillus</i> spp.
0 (raw milk, none)	2	2 (100)	0 (0)
1 (laboratory pasteurization)	11	10 (91)	1 (9)
1 (mesophilic spore count)	18	17 (94)	1 (6)
1 (pasteurized) ²	138	131 (95)	7 (5)
7 (pasteurized) ²	111	82 (74)	29 (26)
14 (pasteurized) ²	102	11 (11)	91 (89)
21 (pasteurized) ²	108	5 (5)	103 (95)
Total	490	258 (53)	232 (47)

¹Percentages are calculated based on the number of isolates given in each row.

²Isolates were obtained from milk that had been pasteurized at all 4 experimental temperatures.

and 3 times in replicates 1, 2, 3, and 4, respectively. Furthermore, AT1 was isolated 5 times from raw milk, 16 times on d 1 postpasteurization, and 6 times on d 7 postpasteurization. Allelic type 1 was not obtained from any milk on d 14 or 21 postpasteurization. The most commonly isolated *Bacillus* allelic type was AT158 (*B. cereus*). Allelic type 158 was isolated 149 times and was found in all 4 replicates. All AT158 isolates were collected from pasteurized samples; 92 were isolated on d 1, 52 were isolated on d 7, 5 were isolated on d 14, and 0 were isolated on d 21. Allelic type 158 was isolated 49, 36, 29, and 35 times in replicates 1, 2, 3, and 4, respectively. When comparing the relationship between AT and pasteurization temperature that had been applied, no pattern was observed ($P = 0.85$; chi-square analysis; Table 6). The most common *Bacillus* isolate, AT158, was found 33, 36, 39, and 41 times in the 72.9, 77.2, 79.9, and 85.2°C temperature treatments, respectively. Allelic type 158 was the only *Bacillus* isolate found solely in pasteurized products. *Bacillus* AT1, AT6, and AT20 were isolated from raw milk samples receiving MSC or LPC heat treatments (from samples that had not been homogenized or pasteurized) 9, 2, and 4 times, respectively (Table 6). The presence of AT158 isolates in pasteurized samples and not in raw samples suggests the source of AT158 is likely the processing environment. This hypothesis is further supported by the observation that although AT158 was not found in raw milk, it was most frequently isolated from pasteurized milk at d 1 postprocessing and that it decreased in isolation frequency with increasing refrigerated storage time, suggesting that this strain is not particularly successful at reproducing under refrigerated storage conditions.

The common *Paenibacillus* subtypes identified in this study were AT13, AT15, AT25, and AT27 (Table 5). The most frequently isolated *Paenibacillus* subtype was AT27, which was isolated 26 times and was found in

all 4 replicates. Allelic type 27 was isolated 7, 5, 2, and 12 times in replicates 1, 2, 3, and 4, respectively. All AT27 isolates were collected from pasteurized samples at d 7, 14, or 21 postpasteurization; 4 were isolated on d 7, 13 were isolated on d 14, and 9 were isolated on d 21. When comparing the relationship between AT and pasteurization temperature that had been applied, no pattern was observed ($P = 0.85$; chi-square analysis; Table 6). The most common *Paenibacillus* isolate, AT27, was found 9, 6, 6, and 5 times in the 72.9, 77.2, 79.9, and 85.2°C temperature treatments, respectively. None of the common *Paenibacillus* isolates was obtained from the raw milk, MSC heat treatment, or LPC heat treatment. These results suggest that the *Paenibacillus* spp. that are present in high numbers at d 14 and 21 postprocessing are likely to be initially present in raw milk at numbers below the detection limit of 1 spore/mL. They also may be introduced from the processing environment.

DISCUSSION

Higher HTST Pasteurization Temperatures Result in Higher Numbers of Spore-Forming Spoilage Organisms During Refrigerated Storage

Psychrotolerant microorganisms present a considerable challenge to the dairy industry because fluid milk is stored at temperatures permissive of the growth of these organisms. Currently, *Pseudomonas* spp. represent the most predominant psychrotolerant gram-negative bacteria contributing to milk spoilage, but these microbes are heat sensitive and usually do not survive pasteurization (Cousin, 1982) unless present in raw milk at very high numbers (Griffiths and Phillips, 1984). Typically, such bacteria enter milk through postpasteurization contamination at the filling machine (Eneroth et al., 1998; Ralyea et al., 1998). In recent years, many com-

Table 5. Common¹ molecular subtypes of endospore-forming bacterial isolates obtained from raw and pasteurized milk samples

Genus, species	Allelic type	Total isolated, no.	Replicate (no. of isolates per replicate)	Sample day postpasteurization (no. of isolates) ²
<i>Bacillus</i> spp.				
<i>B. licheniformis</i>	1	27	1 (4) 2 (14) 3 (6) 4 (3)	0 (5) 1 (16) 7 (6) 14 (0) 21 (0)
<i>B. licheniformis</i>	6	13	1 (1) 2 (3) 3 (4) 4 (5)	0 (0) 1 (5) 7 (7) 14 (1) 21 (0)
<i>B. pumilus</i>	20	10	1 (3) 2 (2) 3 (4) 4 (1)	0 (1) 1 (8) 7 (1) 14 (0) 21 (0)
<i>B. cereus</i>	158	149	1 (49) 2 (36) 3 (29) 4 (35)	0 (0) 1 (92) 7 (52) 14 (5) 21 (0)
<i>Paenibacillus</i> spp.				
<i>Paenibacillus</i> spp.	13	13	1 (0) 2 (6) 3 (4) 4 (3)	0 (0) 1 (0) 7 (1) 14 (4) 21 (8)
<i>Paenibacillus</i> spp.	15	25	1 (11) 2 (8) 3 (3) 4 (3)	0 (0) 1 (0) 7 (2) 14 (11) 21 (12)
<i>Paenibacillus</i> spp.	25	16	1 (12) 2 (0) 3 (3) 4 (1)	0 (0) 1 (0) 7 (1) 14 (12) 21 (3)
<i>Paenibacillus</i> spp.	27	26	1 (7) 2 (5) 3 (2) 4 (12)	0 (0) 1 (0) 7 (4) 14 (13) 21 (9)

¹Allelic types represented by ≥ 10 isolates shown; allelic types with ≤ 10 isolates included 50 isolates classified as *Bacillus* and 149 isolates classified as *Paenibacillus*.

²Total numbers of isolates obtained on d 0, 1, 7, 14, and 21 were 13, 150, 107, 100, and 108, respectively.

mercial fluid milk processors have reduced postpasteurization contamination through implementation of improved filler and packaging technology to produce milk that does not result in consumer complaints at 15 to 25 d postpasteurization (Carey et al., 2005). When gram-negative postpasteurization contaminants are excluded from fluid milk-processing systems, the presence of psychrotolerant endospore-forming spoilage bacteria, particularly *Bacillus* and *Paenibacillus* spp., are the next biological barrier to extending the shelf life of HTST milk (Meer et al., 1991; Ralyea et al., 1998; Fromm and Boor, 2004; Durak et al., 2006; Huck et al., 2007a, 2008).

Heat treatment survival and subsequent outgrowth of psychrotolerant endospore-forming spoilage microbes are problematic for mildly heated refrigerated food products such as dairy products and pasteurized vegetable purees (Carlin et al., 2000; Guinebreteire et al., 2001). Specifically, in milk-processing systems, the spores of psychrotolerant endospore-forming spoilage bacteria can survive HTST pasteurization, germinate, and grow at refrigeration temperatures (Collins, 1981). Psychrotolerant spore-forming bacteria have been isolated from silage (te Giffel et al., 2002), pasture (Slaghuis et al., 1997), soil (Christiansson et al., 1999), and fecal material (Labots et al., 1965); hence, they

Table 6. Relationship between heat treatment and allelic type (AT) isolated

Heat treatment	<i>Bacillus</i> allelic type						<i>Paenibacillus</i> allelic type					
	AT1	AT6	AT20	AT158	Other AT ¹	Isolates without AT ²	AT13	AT15	AT25	AT27	Other AT ¹	Isolates without AT ³
Raw milk ⁴	9	2	4	0	12	2	0	0	0	0	2	0
72.9°C	7	6	1	33	14	2	2	4	6	9	31	2
77.2°C	5	0	2	36	9	2	6	9	5	6	38	0
79.9°C	1	4	3	39	5	0	1	5	1	6	42	0
85.2°C	5	1	0	41	10	3	4	7	4	5	36	1
Total	27	13	10	149	50	9	13	25	16	26	149	3

¹“Other AT” represent combined AT, each found fewer than 10 times.

²*Bacillus* were identified by 16S ribosomal DNA (rDNA) because they did not amplify with *rpoB* PCR; therefore, no AT are available for these isolates.

³Three *Paenibacillus* were identified by 16S rDNA because they did not amplify with *rpoB* PCR; therefore, no AT are available for these isolates.

⁴Raw milk isolates were obtained from different tests performed on 2% fat raw milk, including aerobic plate counts, mesophilic spore counts, and laboratory pasteurized counts.

are widely present in milk-production systems. Spores have also been found in raw milk (Shehata and Collins, 1971; Crielly et al., 1994; te Giffel et al., 2002; Huck et al., 2007a) and pasteurized milk (Griffiths and Phillips, 1990; Lin et al., 1998; Douglas et al., 2000; Fromm and Boor, 2004; Durak et al., 2006; Huck et al., 2007b).

Application of DNA sequence-based subtyping strategies has helped identify transmission pathways of the spore-forming *Bacillus* and *Paenibacillus* spp. from dairy farms, tank trucks, and plant storage silos to pasteurized milk (Huck et al., 2007a, 2008), indicating that initial contamination of raw milk with these microbes can occur at the farm. Clearly, however, the potential exists for contamination to occur at various points in the production continuum (Huck et al., 2008). To this point, *B. cereus* AT158 was isolated 149 times from 4 independent batches of pasteurized milk and across each of the 4 processing temperatures. Notably, we found AT158 only in processed milk, indicating that this microbe was most likely introduced into the milk in the processing environment. In support of this notion, further investigation of the microbial ecology of the processing environment used in this study revealed the presence of a diverse collection of endospore-forming bacteria, including an isolate with 99.8% sequence similarity with AT158 (M. L. Ranieri, unpublished data).

Ironically, the heat treatment used to destroy vegetative bacterial cells can also activate bacterial spores, actually enhancing spore germination and outgrowth (Meer et al., 1991). In the present study, HTST pasteurization temperatures were shown to inversely affect postprocessing APC of fluid milk products after storage at 6°C for 21 d. The lower APC for milk pasteurized at 72.9°C may be due to reduced activation of the spore population resident in the fluid milk relative to spore

activation achieved at the higher temperatures. Supporting this hypothesis, at d 1, the 79.9°C temperature treatment had the highest mean APC (2.18 log cfu/mL), consistent with a previous report of optimal spore germination after an 80°C heat treatment (Moran et al., 1990). At d 21 postpasteurization, however, the mean APC was highest for milk that had been treated at 85°C. These results suggest that thermosensitive factors intrinsic to the milk, to the spores, or both influence spore germination and outgrowth in fluid milk systems. Such factors could include differential availability to bacteria of milk-based nutrients as a function of heat treatment, possible destruction of indigenous antibacterial factors at higher processing temperatures, and possible pro- or antagonistic interactions between spore formers that are influenced by heat (McGuiggan et al., 2002). One example of a naturally occurring antimicrobial factor in milk is the lactoperoxidase system. Previous work has indicated that the lactoperoxidase system is sensitive to temperature, possibly contributing to the improved storage quality of milk pasteurized at 72°C for 15 s relative to milk pasteurized at 80°C for 15 s (Barrett et al., 1999).

***Paenibacillus* Is the Predominant Spoilage Organism Present at d 14 and 21 Postprocessing**

In the present study, the predominant organisms isolated at d 0 (raw samples), 1, and 7 were *Bacillus*, whereas the predominant microbes isolated at d 14 and 21 were *Paenibacillus*. None of the common *Bacillus* AT (i.e., AT1, AT6, AT20, or AT158) was isolated at d 21 postpasteurization, and none of the common *Paenibacillus* AT (i.e., AT13, AT15, AT25, or AT27) was isolated from raw or d-1 samples. These results

indicate that *Paenibacillus* spp. are generally present in raw milk at very low numbers or are entering milk through the processing environment.

The shift in the predominant population of endospore-forming spoilage organisms from *Bacillus* spp. to *Paenibacillus* spp. over the product shelf life in HTST pasteurized milk has been observed previously. Huck et al. (2008) found that 76.2% of isolates collected from pasteurized milk on d 12 of shelf life or beyond were *Paenibacillus* spp., whereas *Bacillus* spp. constituted 87.0% of environment and raw milk isolates. Additionally, Fromm and Boor (2004) found a greater proportion of *Paenibacillus* spp. than *Bacillus* spp. in commercial fluid milk samples at d 17 of shelf life. These results suggest that *Paenibacillus* spp. are better able to grow in milk at 6°C than the *Bacillus* spp. that constitute the predominant spore-former microflora in raw milk. In the present study, the relative percentage of *Bacillus* spp. isolated decreased from 95, 74, and 11% to 5% on d 1, 7, 14, and 21 postprocessing, respectively, whereas the percentage of *Paenibacillus* spp. isolated increased from 5, 26, and 89% to 95% on d 1, 7, 14, and 21, respectively. These trends were consistent for each temperature treatment applied in the study, indicating that the heat treatments within the range studied (72.9 to 85.2°C) did not preferentially affect a subpopulation of the endospore-forming bacteria commonly present in milk.

The fact that *Paenibacillus* was not isolated from raw milk yet was found as the predominant spoilage organism at 21 d postpasteurization across all 4 temperature treatments may explain why the microbiological tests currently used to assess raw milk quality do not effectively predict shelf-life performance of HTST pasteurized fluid milk (N. Woodcock, Cornell University, Ithaca, NY, personal communication). Because of the predominance of *Paenibacillus* spp. during product storage at refrigerated temperatures, targeting them for control or elimination appears to be an important goal for improving the quality and extending the shelf life of processed milk. Three of the 4 common *Paenibacillus* AT (AT13, AT15, and AT27) identified in the present study matched isolates previously reported by Huck et al. (2008) as isolated from multiple stages of the milk-processing continuum. Allelic type 15 and AT27 were identified in the dairy farm environment, tank trucks, plant storage silos, and pasteurized milk (Huck et al., 2008). Allelic type 13 was isolated from pasteurized milk and from the dairy farm environment. The fact that these common *Paenibacillus* isolates have been found throughout the processing continuum and that they are rarely isolated in pasteurized milk at d 1 postprocessing suggests that they are likely present in raw milk at low numbers. Representatives of these

common AT will be excellent candidates for future investigation of *Paenibacillus* spp. growth characteristics, of the response of *Paenibacillus* spp. to heat treatments, and, most important, of *Paenibacillus* spp. as targets for the development of sensitive and specific assays needed to enable detection of these microbes at low levels. Additionally, the collection and identification of more isolates across a broader sample of farms and processing plants will further improve our understanding of *Paenibacillus* diversity and the persistence of specific subtypes in the environment.

CONCLUSIONS

High temperature, short time pasteurization temperatures can affect APC present in processed milk during refrigerated storage. Specifically—and counterintuitively—higher bacterial numbers were found in milk that had been processed at higher temperatures. The predominant bacterial genera isolated from the milk samples did not differ by heat treatment, suggesting that the heat treatments within the range studied (72.9 to 85.2°C) did not preferentially affect a subpopulation of the endospore-forming bacteria present. We conclude that the endospore-forming psychrotolerant bacteria present in milk grow more effectively in pasteurized milk after a higher heat treatment. Additionally, *Paenibacillus* spp., which are likely present at <1 spore/mL of raw milk, are capable of growing to numbers that can exceed the Pasteurized Milk Ordinance limit of 20,000 cfu/mL for grade A pasteurized milk, illustrating the need for a comprehensive strategy to limit the entry of endospore-forming bacteria into milk systems. Ultimately, new processing methods may need to be used to physically remove psychrotolerant endospore-forming bacteria from raw milk. Microfiltration represents one possible processing tool for removal of bacterial spores, with the goal of extending milk shelf life (Elwell and Barbano, 2006). Continued efforts to improve the bacteriological quality of pasteurized milk will require an increased emphasis on limiting bacterial and spore contamination along the farm-to-table continuum.

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