Short communication: Bacterial ecology of high-temperature, short-time pasteurized milk processed in the United States

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ABSTRACT

To determine the microbial ecology of pasteurized milk within the United States, 2% fat pasteurized fluid milk samples were obtained from 18 dairy plants from 5 geographical areas representing the Northeast, Southeast, South, Midwest, and West. Of the 589 bacterial isolates identified using DNA sequence-based subtyping methods, 346 belonged to genera characterized as gram-positive endospore-forming bacteria (i.e., *Bacillus* and *Paenibacillus*). Of the 346 gram-positive endospore-forming bacteria isolated in the present study, 240 were classified into 45 allelic types identical to those previously identified from samples obtained in New York State, indicating the widespread presence of these microbes in fluid milk production and processing systems in the United States. More than 84% of the gram-positive spore-forming isolates characterized at d 1, 7, and 10 were of the genus *Bacillus*, whereas more than 92% of isolates characterized at d 17 of shelf life were of the genus *Paenibacillus*, indicating that the predominant gram-positive spoilage genera shifts from *Bacillus* spp. to *Paenibacillus* spp. during refrigerated storage.

Key words: fluid milk spoilage, *Bacillus*, *Paenibacillus*, bacterial subtyping

In general, in the United States, bacterial spoilage limits the shelf-life of conventionally processed HTST pasteurized fluid milk to approximately 2 to 3 wk (Simon and Hansen, 2001; Hayes et al., 2002; Fromm and Boor, 2004; Gandy et al., 2008; He et al., 2009). Previous research on milk and dairy environment samples in New York State has demonstrated that when postpasteurization bacterial contaminants are excluded from fluid milk processing systems, the biological barrier to extension of HTST fluid milk shelf-life is the presence of psychrotolerant endospore-forming spoilage bacteria, particularly *Bacillus* and *Paenibacillus* spp. (Ralyea et al., 1998; Fromm and Boor, 2004; Durak et al., 2006;

Huck et al., 2007a; Huck et al., 2008; M. Ranieri, unpublished data). *Bacillus* and *Paenibacillus* spp. can survive pasteurization; some strains are capable of subsequent germination and growth under refrigeration conditions in pasteurized milk (Meer et al., 1991; Boor and Murphy, 2002; Huck et al., 2007b).

To enable identification and transmission tracking of gram-positive bacteria contributing to the spoilage of HTST milk, an *rpoB* subtyping method was developed and applied to psychrotolerant endospore-forming bacteria isolated from milk production and processing systems (Durak et al., 2006; Huck et al., 2007a). To date, over 1,100 gram-positive spore-forming isolates have been collected from New York State (NYS) farms, dairy processing environments, and raw and pasteurized milk samples. Based on *rpoB* subtyping analysis, these isolates have been classified into over 200 unique allelic types (AT), illustrating the rich diversity of spore-forming microbes present in fluid milk production and processing systems in NYS (Huck et al., 2008; M. Ranieri, unpublished data).

We hypothesized that the strains of *Bacillus* and *Paenibacillus* spp. that have been identified in pasteurized fluid milk to date are not unique to NYS. Therefore, to identify and compare the gram-positive spore-forming bacteria in milk processed across the country, the *rpoB* subtyping method was used to characterize bacterial isolates obtained from 2% milk fat pasteurized milk samples processed at 18 fluid milk processing plants representing 5 geographical regions across the United States [i.e., the Northeast (NE), Southeast (SE), South (S), Midwest (MW), and West (W)]. Northeast plants included 1 from New York State and 3 from Pennsylvania. The SE was represented by 1 plant each from Florida, Georgia, and Tennessee. Three plants from Texas represented the S. Two plants from Michigan, 1 plant from Wisconsin and 1 plant from Minnesota represented the MW. Two plants from California, 1 plant from New Mexico, and 1 plant from Idaho represented the W.

On the day they were pasteurized, 2% milk fat fluid milk samples in 4 half-gallon containers were shipped in coolers packed with ice by overnight courier to the Milk Quality Improvement Laboratory (Ithaca, NY).

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Upon receipt, 3 half-gallon containers from each cooler were moved to a refrigerator at 6°C. One half-gallon container, which was selected as a temperature control, was inverted 25 times and the temperature of the contents was measured with a thermometer probe to verify that the milk arrived at the laboratory at or below 6°C. Two half-gallon containers representing each plant were removed from the refrigerator, inverted 25 times, and wiped with ethanol. Approximately 250 mL from each of the 2 half-gallon containers was poured into 5 separate sterile 500-mL Pyrex glass containers. The Pyrex glass containers were then inverted 25 times and stored at 6°C. Microbiological sampling was performed on one of the 5 containers at each test day on d 1, 7, 10, 14, and 17 postpasteurization. Aerobic plate counts were determined in duplicate by spread plating appropriate dilutions made with PBS (Weber Scientific, Hamilton, NJ) on brain heart infusion (BHI) agar (Difco, BD Diagnostics, Franklin Lakes, NJ) and all plates were incubated at 32°C for 24 h before enumeration.

For each milk sample, bacterial colonies present on BHI that had been plated at d 1, 7, 10, 14, and 17 postpasteurization were visually examined. On each day of testing, a single colony representing each distinct colony morphology present on 1 of the 2 plates that had been used for enumeration for each sample was picked for isolation and identification. Typically, 5 to 10 colonies per sample were selected and streaked for purity on BHI agar. In general, the appearance of the bacterial colonies on the plates became more homogeneous with increasing time postpasteurization (e.g., at d 14 and 17 postpasteurization), suggesting predominance of specific types of bacteria following extended refrigerated storage. Purified isolates (i.e., those that produce only one colony morphology when re-streaked onto rich medium) 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.

Species identification and subtyping of *Bacillus* and *Paenibacillus* spp. were performed by determining the DNA sequence for a 632-nucleotide fragment of the *rpoB* gene for each isolate, as described previously by Huck et al. (2007a). This method was selected because it allows for phylogenetic characterization of isolates in addition to subtype identification. BioEdit (Hall, 1999) was used for *rpoB* allele assignment. A unique allelic type (AT) was assigned to a gene sequence that differed from any previously obtained sequence by one or more nucleotides. Isolates with different AT were considered to represent different subtypes. Distribution of bacterial subtypes was analyzed in JMP (Version 7.0, SAS Institute Inc., Cary, NC), using a chi-squared test

for independence; aerobic plate count (APC) means were compared using Student's *t*-tests.

As the *rpoB* subtyping method was developed to enable specific differentiation of *Bacillus* and *Paenibacillus* spp., not all isolates collected here could be characterized to genus and species through phylogenetic analyses with previous *rpoB* sequence data (Durak et al., 2006; Huck et al., 2007a,b, 2008; M. Ranieri, unpublished data). Therefore, 16S rDNA sequencing was used to confirm the genus or species identification of *rpoB* allelic types that had not been identified in any of our previous studies, as well as to identify gram-negative or *rpoB* PCR negative gram-positive bacteria. Specifically, 1) one isolate representing each newly identified *rpoB* allelic type; or 2) each isolate that could not be identified by sequencing a portion of the *rpoB* gene was characterized by sequencing the 3' end of the gene encoding 16S rRNA, 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 and species assignments for a specific 16S rDNA sequence were based on the top matches returned by the search.

A total of 589 isolates was collected over 5 sampling days per sample from the pasteurized 2% fat milk obtained from the 18 plants. Of these, 346 isolates were determined by *rpoB* sequencing to belong to genera characterized as gram-positive spore-forming bacteria; that is, *Bacillus* and *Paenibacillus*. The remaining 243 isolates, which were not typable using the *rpoB* sequencing method, were identified by sequencing the 3' end of the 16S rRNA gene. Among these 243 isolates, 68 represented gram-positive bacteria and 175 represented gram-negative bacteria. Overall, 21 different bacterial genera were identified by 16S rDNA sequencing, indicating a considerable diversity of bacteria present in milk. Table 1 includes the genus distribution of bacterial isolates identified (by both 16S rDNA and *rpoB* sequencing) in this study. The most frequently isolated gram-positive genus was *Bacillus* (240 isolates), which was isolated 36, 44, 61, 48, and 51 times in the MW, NE, S, SE, and W, respectively. *Paenibacillus* was the second most frequently isolated gram-positive genus (122 isolates), and was isolated 23, 24, 10, 35, and 30 times in the MW, NE, S, SE, and W, respectively. Other gram-positive bacteria identified were *Staphylococcus*, *Leuconostoc*, *Enterococcus*, *Streptococcus*, *Brevibacillus*, *Corynebacterium*, *Lactococcus*, *Microbacterium*, *Micrococcus*, and *Oceanobacillus*.

The most frequently isolated gram-negative genus was *Pseudomonas* (122 isolates), which was isolated 53,

Table 1. Bacterial isolates obtained from 2% milk fat high-temperature, short-time pasteurized milk, categorized by geographic origin of the milk sample

Bacterial isolate	Region ¹					Isolates, n	Isolates, %
	MW	NE	S	SE	W		
Gram-positive							
<i>Bacillus</i>	36	44	61	48	51	240	40
<i>Paenibacillus</i>	23	24	10	35	30	122	21
<i>Staphylococcus</i>	7	4	4	1	3	19	3
<i>Leuconostoc</i>	2		2	3	11	18	3
<i>Enterococcus</i>	5					5	1
<i>Streptococcus</i>		4				4	1
<i>Brevibacillus</i>				1		1	<1
<i>Corynebacterium</i>	1					1	<1
<i>Lactococcus</i>			1			1	<1
<i>Microbacterium</i>			1			1	<1
<i>Micrococcus</i>			1			1	<1
<i>Oceanobacillus</i>			1			1	<1
Gram-negative							
<i>Pseudomonas</i>	53	40	2	8	19	122	20
<i>Acinetobacter</i>	1	9	2		2	14	2
<i>Yersinia</i>			11			11	2
<i>Enterobacter</i>	4	1			3	8	1
<i>Enterobacteriaceae</i>	8					8	1
<i>Shewanella</i>	1		3			4	1
<i>Aeromonas</i>			3			3	<1
<i>Flavobacterium</i>	2					2	<1
<i>Pantoea</i>		1			1	2	<1
<i>Sphingobacterium</i>						1	<1
Total	143	127	102	96	121	589	100

¹MW = Midwest; NE = Northeast; S = South; SE = Southeast; W = West.

40, 2, 8, and 19 times in the MW, NE, S, SE, and W, respectively. *Pseudomonas* spp. are killed by pasteurization, thus their presence in these commercial milk samples strongly suggests that the milk products were contaminated following pasteurization. In total, milk samples from 7 plants (~39% of the plants sampled) contributed 116 of the 122 (~95%) of the *Pseudomonas* obtained in this study. Only ~20% (72 of 362) of all gram-positive spore-forming isolates obtained in this study were collected from these same 7 plants. Other gram-negative bacteria identified were *Acinetobacter*, *Yersinia*, *Enterobacter*, *Enterobacteriaceae*, *Shewanella*, *Aeromonas*, *Flavobacterium*, *Pantoea*, and *Sphingobacterium*.

Table 2 presents the APC from samples from each plant on each day of testing. All 589 bacteria isolated during this study are accounted for in this table, next to the respective plate count obtained on each sample day. The rapid growth of gram-negative bacteria caused rapid APC increases in milk samples from 7 of 18 fluid milk processing plants in this study; these plants are highlighted in bold font (Table 2). "Gram-negative spoilage" was defined as identification of *Pseudomonas* spp. and other gram-negative microbes as the predominant organisms in milk samples from the same plant on at least 2 of the 5 testing days. Plants F, H, and I (MW), A and B (NE), G (SE), and L (W) were charac-

terized as having gram-negative spoilage. To illustrate, on d 10 postpasteurization, plant F, characterized as having gram-negative spoilage, had an APC of 6.04 log cfu/mL, with 8 *Pseudomonas* spp. isolated from testing plates. On d 14 postpasteurization, plant F had an APC of 7.78 log cfu/mL, with *Pseudomonas* spp. isolated 9 times. Figure 1 compares APC over shelf life for products from all 18 plants grouped by geographical region and characterized by type of bacterial spoilage. In the lower right panel (F), the mean APC at each day tested were plotted for the samples that spoiled because of the presence of gram-negative bacteria (e.g., *Pseudomonas* spp.; upper curve) versus gram-positive spore formers (e.g., *Paenibacillus* and *Bacillus*; lower curve). Whereas at d 1 postpasteurization, the APC were not significantly different for those bearing predominantly gram-positive versus gram-negative spoilers (1.43 vs. 1.58 log cfu/mL for gram-positive spore formers and *Pseudomonas*, respectively), the mean APC for those with *Pseudomonas* spoilage was significantly higher than those with gram-positive spoilage at d 7, 10, and 14 ($P < 0.05$). By d 7, the APC of milk samples with *Pseudomonas* spoilage was 2.5 log cfu/mL higher than in milk with gram-positive bacteria. At d 10 and 14, the *Pseudomonas* mean APC was 3.62 and 3.52 log cfu/mL higher. These data illustrate the rapid growth of *Pseudomonas* spp. in milk stored at 6°C and

Table 2. Aerobic plate counts and isolation frequency of different bacterial genera obtained from milk samples originating from 18 fluid milk processing plants in the United States

Region ¹	Plant	Aerobic plate count, log cfu/mL [No. isolates, genus ²]				
		Day 1	Day 7	Day 10	Day 14	Day 17
MW	E	1.30 [4 B, 1 C, 3 St]	1.81 [6 B, 1 P]	2.38 [3 B, 2 P]	4.08 [1 B, 7 P]	5.70 [1 B, 5 P]
	F ³	1.30 [4 B]	1.88 [2 B, 2 P]	6.04 [8 Ps]	7.78 [9 Ps]	6.18 [5 P, 2 St]
	H	1.00 [6 B]	3.11 [1 A, 3 Eb, 2 Ec, 2 Ps]	3.73 [2 E, 1 Ec, 1 F, 2 Le, 1 P, 1 S]	7.26 [1 E, 3 Eb, 5 Ps]	6.48 [1 E, 2 Eb, 2 Ec, 1 F]
NE	I	1.51 [9 B]	5.60 [7 Ps , 2 St]	7.32 [8 Ps]	8.15 [6 Ps]	8.15 [8 Ps]
	A	1.45 [4 B]	5.00 [5 B, 2 P]	7.74 [2 A, 5 Ps]	8.08 [8 Ps , 2 Sc]	8.28 [2 A, 1 E, 1 P, 1 Pa, 1 Ps , 2 Sc]
	B	1.65 [1 B, 2 Ps , 2 St]	6.76 [5 Ps]	8.08 [7 Ps]	8.23 [8 Ps]	8.08 [4 Ps]
	C	1.46 [2 B]	2.40 [4 B, 1 P, 1 St]	4.08 [2 B, 3 P]	6.08 [4 B, 6 P]	7.04 [2 B, 5 P]
S	D	1.00 [3 B, 1 St]	1.74 [6 B]	2.83 [5 B, 2 P]	5.32 [8 B, 2 P]	6.92 [2 B, 2 P]
	P	1.94 [8 B, 1 Mi, 1 O, 2 St]	1.95 [8 B]	1.95 [15 B]	3.73 [2 B, 1 M]	6.76 [2 P]
	Q	1.46 [10 B]	2.28 [1 A, 3 B, 2 Y]	2.45 [1 A, 4 B, 1 L, 2 Ps, 3 S]	6.76 [5 Y]	8.52 [3 Ae, 4 Y]
SE	R	1.30 [5 B, 2 St]	1.30 [4 B]	1.18 [2 B]	5.57 [2 Le]	7.15 [8 P]
	G	1.91 [7 B]	2.00 [7 B, 3 P]	4.94 [2 Le, 2 P, 3 Ps]	7.32 [1 P, 5 Ps]	6.00 [1 Le, 3 P, 1 St]
	N	1.26 [10 B]	1.40 [3 B, 1 P]	1.81 [2 B, 3 P]	3.59 [2 B, 4 P]	3.64 [7 P]
W	O	1.70 [5 B]	1.40 [5 B]	1.74 [4 B, 1 P]	2.34 [3 B, 1 Br, 3 P]	4.53 [7 P]
	J	1.69 [7 B, 1 P]	2.00 [6 B, 1 P]	4.18 [7 Le]	2.78 [1 B, 4 P]	6.85 [4 Le]
	K	1.30 [6 B]	1.30 [4 B, 1 P]	1.40 [5 B, 1 P]	3.64 [4 Ps]	3.26 [6 P]
	L	2.26 [5 B, 2 St]	5.20 [10 Ps , 1 Sp]	4.00 [3 E, 2 P]	7.87 [5 Ps]	7.04 [2 A, 4 P]
	M	1.28 [6 B]	1.30 [4 B]	1.95 [6 B, 3 P]	3.26 [1 B, 2 P, 1 Pa, 1 St]	4.72 [5 P]

¹MW = Midwest; NE = Northeast; S = South; SE = Southeast; W = West.

²Genera: A = *Acinetobacter*, Ae = *Aeromonas*, B = *Bacillus*, Br = *Brevibacillus*, C = *Corynebacterium*, E = *Enterobacter*, Eb = *Enterobacteriaceae*, Ec = *Enterococcus*, F = *Flavobacterium*, L = *Lactococcus*, Le = *Leuconostoc*, M = *Microbacterium*, Mi = *Micrococcus*, O = *Oceanobacillus*, P = *Paenibacillus*, Pa = *Pantoea*, Ps = *Pseudomonas*, S = *Shewanella*, Sc = *Streptococcus*, Sp = *Sphingobacterium*, St = *Staphylococcus*, and Y = *Yersinia*.

³Bold font indicates plants with products characterized as spoiling because of the presence of gram-negative organisms (e.g., *Pseudomonas* spp.). For each of the seven plants highlighted, *Pseudomonas* spp. were isolated from samples on at least 2 of 5 plating days.

Figure 1. Aerobic plate count (APC) for d 1, 7, 10, 14, and 17 postpasteurization for fluid milk samples obtained from each of 18 processing plants, grouped by geographic region (A through E), including a description of type of spoilage (i.e., gram-positive spore former or gram-negative). Log cfu/mL is indicated on the y-axes. Panel F shows the mean APC from milk samples obtained from the 7 plants characterized as having gram-negative spoilage patterns (upper curve) versus APC from samples obtained from the 11 plants characterized as having gram-positive spoilage patterns. Aerobic plate counts were significantly greater for the gram-negative samples than for the gram-positive samples at d 7, 10, and 14 postpasteurization ($P < 0.05$).

demonstrate the likelihood that rapid reproduction of gram-negative bacteria can mask the presence of gram-positive endospore-forming bacteria in milk (i.e., the milk dilutions required to enumerate the gram-negative bacteria would obscure the simultaneous presence of the gram-positive bacteria). Our data illustrate that gram-positive spore formers typically grow more slowly in refrigerated pasteurized milk than gram-negative microbes, and thus gram-positive organisms such as *Bacillus* and *Paenibacillus* spp., although likely to be present simultaneously with the gram-negative organisms, are generally found in lower numbers, particularly early in product shelf-life.

Overall, 120 *rpoB* AT were differentiated among the 346 spore-forming bacterial isolates characterized by *rpoB* sequencing. Of the 120 AT identified, 45 AT, representing 240 of the 346 (~70%) isolates obtained in this study, had previously been isolated from multiple NYS sample locations, including dairy farms, raw milk tank trucks, raw milk storage silos, and pasteurized milk samples (Huck et al., 2008; M. Ranieri, unpublished data). In addition to the 45 AT that had been previously obtained from NYS sites, 75 new AT, representing 106 isolates, were isolated for the first time in the present study. Figure 2 presents, by geographic region, the isolates obtained most frequently ($n \geq 8$

Figure 2. Distribution of the 10 most frequently isolated *Bacillus* and *Paenibacillus* allelic types obtained from 18 processing plants located within 5 different geographical regions across the United States. Each region [West (W), Southeast (SE), South (S), Midwest (MW), Northeast (NE)] is coded by pattern, and plant (A to R) is coded by color.

times) in this study, and illustrates the distribution of all other *rpoB* AT. The 5 most frequently isolated *Bacillus* AT were *Bacillus licheniformis* AT1, AT6, and AT9 and *Bacillus arenosi* AT17 and AT73. *Bacillus licheniformis* AT1 was found in processed milk from all regions and in all 18 processing plant samples. A total of 109 “other” *Bacillus* AT, representing those AT collected fewer than 8 times each, were collected from 18 plants. The most frequently isolated *Paenibacillus* AT were *Paenibacillus* spp. AT2, AT15, AT23, AT27, and *Paenibacillus amylolyticus* AT111. The most frequently isolated *Paenibacillus* subtype was AT15, which was isolated a total of 13 times across 7 plants. Although both *Bacillus* and *Paenibacillus* were isolated from milk from all geographical regions, *Paenibacillus* was more frequently isolated at the end of shelf life (i.e., beyond 14 d of storage at 6°C).

Figure 3 shows the total number of *Bacillus* and *Paenibacillus* isolated at each postpasteurization test day. There was a significant association between genus identified and test day ($P < 0.001$) at d 1, 7, 10, and 17. At d 1, 7, and 10, the number of *Bacillus* isolates collected was significantly higher than that of *Paenibacillus* ($P < 0.001$). Of 246 gram-positive spore-forming

isolates characterized at d 1, 7, or 10, 213 of those isolates were characterized as *Bacillus*. The 33 remaining isolates were characterized as *Paenibacillus*. Conversely, at d 17, the number of *Paenibacillus* isolates collected was significantly greater than the number of *Bacillus* isolates ($P < 0.001$). There was no significant difference in the proportion of *Bacillus*/*Paenibacillus* isolates obtained on d 14 ($P = 0.26$). This day appears to be at or near a transition point where *Paenibacillus* surpasses *Bacillus* as the predominant gram-positive spore-former present in pasteurized milk.

In the present study, we found that endospore-forming bacteria with identical AT to isolates previously found in NYS are present in milk processed throughout the United States. Furthermore, AT1, 15, 23, and 27 represent AT that have been previously isolated throughout the milk processing continuum, from farm to finished product, suggesting that milk can be contaminated with endospore-forming bacteria on the farm, as well as at various points throughout the processing continuum. Specifically, AT1, 15, 23 and 27 were isolated from either 3 or 4 of the following locations: dairy farm, raw milk tank truck, raw milk storage silos, and pasteurized milk (Huck et al., 2008). Overall, the fact that multiple,

Figure 3. Number of isolates characterized as *Bacillus* (n = 222) or *Paenibacillus* (n = 125) obtained from each day tested.

identical allelic types were found in several geographic locations and environments indicates the ubiquitous nature of these spore-forming organisms.

In addition to finding common AT in milk from several geographic regions, we observed a shift in the predominant population of endospore-forming spoilage organisms from *Bacillus* spp. to *Paenibacillus* spp. over product shelf life. Huck et al. (2008) found that 76.2% of isolates collected on d 12 of shelf life or beyond were *Paenibacillus* spp., whereas *Bacillus* spp. constituted 87% of isolates obtained from environment and raw milk samples. In this study, the relative percentage of *Bacillus* spp. isolated decreased from 99, 84, 71, 43, to 8% on d 1, 7, 10, 14, and 17 postpasteurization, respectively, whereas the percentage of *Paenibacillus* spp. isolated increased from 1, 16, 29, 57, to 92% on d 1, 7, 10, 14, and 17, respectively. Because of their predominance during product storage at refrigerated temperatures, targeting *Paenibacillus* spp. for control or elimination may prove to be a good strategy for improving the quality and extending the shelf life of processed milk. Additionally, the collection and identification of isolates across a broader sample of farms and processing plants will further improve our understanding of *Paenibacillus* ecology and the persistence of specific subtypes in the environment.

Sixteen new *Paenibacillus* AT were identified in this study. The low rate of isolation of *Paenibacillus* spp. at d 1 postpasteurization suggests that these organisms are generally present in low numbers in raw milk, but that they are capable of growing to numbers that limit HTST pasteurized milk shelf-life. In summary, our results illustrate the need for a comprehensive strategy to limit the entry of endospore-forming bacteria into milk systems to improve product shelf life.

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