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

M. L. Ranieri and K. J. Boor[1]
Milk Quality Improvement Program, Department of Food Science, Cornell University, Ithaca, NY 14853

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 (Ralyea et al., 1998; Fromm and Boor, 2004; Durak et al., 2006; Huck et al., 2007a; Huck et al., 2008; M. Ranieri, unpublished data).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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[1]Corresponding author: kjb4@cornell.edu

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,