BACTERIAL ECOLOGY OF HIGH TEMPERATURE SHORT TIME PASTEURIZED FLUID MILK

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

Matthew Louis Ranieri

May 2009

ABSTRACT

The Grade "A" Pasteurized Milk Ordinance (PMO) 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% milkfat raw milk was heated to 60°C, homogenized, and treated for 25 s at one of four 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 post-processing. Bacterial numbers in milk processed at 72.9°C were lower ($P < 0.05$) than in milk processed at the other three temperatures on each sampling day, indicating that HTST fluid milk processing temperatures significantly affect 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 post-processing were of the genus *Bacillus*, while over 92% of isolates characterized at d 14 and 21 post-processing were of the genus *Paenibacillus*, indicating that the predominant genera present in HTST-processed milk shifts from *Bacillus* spp. to *Paenibacillus* spp. during refrigerated storage.

To determine the microbial ecology of pasteurized milk from different geographical regions within the United States, 2% fat pasteurized fluid milk samples were obtained from 18 dairy plants from five geographical areas representing the Northeast, Southeast, South, Midwest, and West. Of the 589 bacterial isolates identified using DNA sequence-based subtyping methods, 348 belonged to genera characterized as gram-positive endospore forming bacteria (i.e., *Bacillus* and

Paenibacillus). 244 of the 348 gram-positive endospore forming bacteria isolated in the present study classified into 46 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. >84% of the gram-positive sporeforming isolates characterized at d 1, 7, and 10 were of the genus *Bacillus*, while over 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. Gram-positive endospore forming bacteria with the same allelic types were obtained from milk samples originating from all 5 geographical regions in the US.

In summary, (i) HTST processing temperatures affected bacterial numbers in refrigerated milk, with higher bacterial numbers in milk processed at higher temperatures; (ii) no significant association was observed between genus isolated and pasteurization temperature, suggesting that the genera were not differentially affected by the different processing temperatures; (iii) although typically present at low numbers in raw milk, *Paenibacillus* spp. are capable of growing to numbers that limit HTST pasteurized refrigerated milk shelf-life; and (iv) gram-positive endospore forming bacteria with the same allelic types were obtained from milk samples originating from all 5 geographical regions in the US.

BIOGRAPHICAL SKETCH

Matthew L. Ranieri was born in Syracuse, NY and grew up nearby in Elbridge, NY. He later attended Jordan-Elbridge High School, where he was captain of the soccer, basketball, and golf teams. Matthew graduated from Cornell University in Ithaca, NY in 2006 with a Bachelor of Science degree in Food Science. After graduating, he returned to Cornell University for graduate research in Food Science. He was a member of the Chi Phi Fraternity, Institute of Food Technologists, and International Association for Food Protection. In 2007 Matthew placed third in the International Association for Food Protection Developing Scientist Competition.

To Mom, Dad, Elizabeth, Alison and Dr. Kathryn Boor for their continuous guidance and encouragement.

ACKNOWLEDGEMENTS

This project was supported by the New York State Milk Promotion Advisory Board through the New York State Department of Agriculture and Markets – New York State dairy farmers committed to production of high quality dairy products. Thank you to Dr. Kathryn Boor and Dr. Martin Wiedmann for their support, guidance and motivation throughout my studies at Cornell. Thank you to Dr. David Barbano for research assistance and helpful conversations, Esref Dogan, Robert Kaltaler, and Mark Newbold for their support in the pilot plant, and Nicole Woodcock, Nancy Carey, Shelley Laue, Robert Ralyea, Steven Murphy, Tanya Pierik, Pat Wood and Jason Huck for technical support. Thank you to the management and employees of the 18 processing plants involved in this research. Finally, thank you to my friends, family, and Brianne for their never-ending support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

APC – Aerobic Plate Count

AT – Allelic Type

BHI – Brain Heart Infusion Agar

BLAST – Blast Local Alignment Search Tool

CC – Coliform Count

HTST – High Temperature Short Time

LPC – Lab Pasteurized Count

MSC – Mesophilic Spore Count

MW – Midwest

NE – Northeast

NT – Nucleotide

NYS – New York State

PBC – Psychrotrophic Bacteria Count

PI – Pre-incubation

PMO – Pasteurized Milk Ordinance

PSC - Psychrotrophic Spore Count

S – South

SE – Southeast

SPC – Standard Plate Count

W – West

CHAPTER ONE

IMPROVING FLUID MILK QUALITY

In the past decade, the implementation of effective strategies for reducing or preventing the entry of spoilage bacteria in pasteurized milk in the processing plant has allowed dairy plants in the northeastern US to make significant strides in extending the length of time that fluid products taste fresh (Carey et al., 2005). A point of emphasis for dairy plant operators has been post-pasteurization contamination that can be present in a dairy processing plant, especially at fluid milk filler sites. Control of gram-negative, post-pasteurization contamination is essential for extending the shelf-life of fluid products beyond 14 days, however, the reduction of gram-negative spoilage bacteria has also led to the discovery of a new microbiological hurdle.

The presence of *Bacillus* and *Paenibacillus* spp. has been identified as a key biological barrier to extension of High Temperature Short Time (HTST) pasteurized fluid milk shelf-life beyond 2-3 weeks for milk stored at 6° C (Fromm and Boor, 2004; Durak et al., 2006; Huck et al., 2007b; Huck et al., 2008). *Bacillus* spp. and *Paenibacillus* spp. are capable of forming heat resistant spores that can survive High Temperature Short Time (HTST) pasteurization (Collins, 1981; Huck et al., 2007b) and some strains are able to grow at refrigeration temperatures following germination, ultimately causing spoilage of processed milk products (Washam et al., 1977; Huck et al., 2008). Representatives from both genera have been isolated from dairy farms, processing plants, and pasteurized packaged products (Huck et al., 2008) in New York State. The group of gram-positive sporeforming organisms represents a major barrier to the extension of product shelf lives because these microbes can survive HTST

pasteurization processes typically used for fluid milk, and they can grow in milk at refrigeration temperatures (~6°C).

The first study was initiated by anecdotal reports that higher bacterial numbers were found in fluid milk products following an increase in pasteurization temperatures at dairy processing plants. To test the hypothesis that higher HTST processing temperatures result in higher bacterial numbers in fluid milk products over refrigerated product shelf-life, the objectives of this study were: (i) to determine the effect of processing temperature on aerobic plate counts in milk samples that had been processed under a range of commonly applied HTST temperatures and held under refrigerated storage conditions (6^{o}C); and (ii) to monitor the microbial ecology of pasteurized products processed at each temperature during storage.

After conducting pasteurization trials with heat treatments ranging from 72.9 to 85.2°C, we found bacterial numbers in milk processed at 72.9°C were lower ($P < 0.05$) than in milk processed at the other three temperatures on each sampling day. These results indicate that HTST fluid milk processing temperatures significantly affect bacterial counts in HTST fluid milk. To determine the microbial ecology of the different milk samples during refrigerated storage, a total of 564 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 post-processing were of the genus *Bacillus*, while over 92% of isolates characterized at d 14 and 21 post-processing were of the genus *Paenibacillus*, indicating that the predominant genera present in HTST-processed milk shifts from *Bacillus* spp. to *Paenibacillus* spp. during refrigerated storage.

A second study was conducted to compare the microbial ecology of milk from 5 different geographical regions within the United States. We hypothesized that milk processed across the United States would harbor gram-positive sporeforming isolates

sharing the same AT as those found in NYS. Therefore, to identify and compare the spore-forming bacteria in milk processed across the country, an *rpoB* subtyping method (Durak et al., 2006; Huck et al., 2007a) was used to characterize bacterial isolates obtained from 2% milkfat pasteurized milk samples processed at 18 different fluid milk processing plants (representing 5 geographical regions: Northeast (NE), Southeast (SE), South (S), Midwest (MW), and West (W)) across the United States).

A total of 589 bacterial isolates were identified using DNA sequence-based subtyping methods, of which 348 were determined to belong to genera characterized as gram-positive endospore forming bacteria (i.e. *Bacillus* and *Paenibacillus*) by *rpoB* sequencing. Overall, 46 allelic types representing 244 of the 348 gram-positive endospore forming bacteria, shared identical allelic types to bacteria previously isolated in New York State. Furthermore, of the isolates identified by *rpoB* sequencing, >84% of isolates characterized at d 1, 7, and 10 were of the genus *Bacillus*, while over 92% of isolates characterized at d 17 of shelf life were of the genus *Paenibacillus*, indicating that the predominant genera present in HTST-processed milk lacking gram-negative contamination shifts from *Bacillus* spp. to *Paenibacillus* spp. during refrigerated storage.

Our data suggest that *Paenibacillus* spp. are the most common spore forming bacteria present at the end of HTST pasteurized milk shelf life. *Paenibacillus* spp. pose significant problem, as they are present in raw milk at very low numbers and are capable of growing to numbers which affect fluid milk quality. To further extend fluid milk product shelf lives it is necessary to implement strategies that prevent the initial entrance of sporeforming organisms into milk systems. Ultimately an improved shelf-life for fluid milk products may increase market opportunities for fluid milk products.

CHAPTER TWO

HIGH TEMPERATURE SHORT TIME (HTST) PASTEURIZATION TEMPERATURES INVERSELY AFFECT BACTERIAL NUMBER DURING REFRIGERATED STORAGE OF PASTEURIZED FLUID MILK[1]

INTRODUCTION

In the United States, *Bacillus* spp. and *Paenibacillus* spp. have been identified as the biological barriers currently limiting High Temperature Short Time (**HTST**) pasteurized fluid milk shelf-life (Fromm and Boor, 2004; Durak et al., 2006; Huck et al., 2007b; Huck et al., 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). As *Bacillus* 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 (**PMO**) (72°C for 15 sec) (PMO, 2005; Gandy et al., 2008). Anecdotal reports from multiple milk processors of higher bacterial numbers and more rapid

[1]Submitted for publication to the Journal of Dairy Science.

spoilage in fluid milk products following an increase in processing temperatures prompted this investigation of the effects of commonly applied HTST pasteurization temperatures on aerobic plate counts in refrigerated fluid milk products. To test the hypothesis that higher HTST processing temperatures result in higher bacterial numbers in fluid milk products over refrigerated product shelf-life, the objectives of this study were: (i) to determine the effect of processing temperature on aerobic plate counts in milk samples that had been processed under a range of commonly applied HTST temperatures and held under refrigerated storage conditions (6^{o}C); and (ii) 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 aerobic plate counts in pasteurized fluid milk, four 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 processing order (1st, 2nd, 3rd or 4th) 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, NY 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; Poughkeepsie, NY), 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 of 2% fat raw milk were added to a jacketed steam

kettle, heated to 60°C, and then homogenized with 2 passes through a Gaulin APV (Model 200 E; Everett, MA) homogenizer. 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 (2L) pasteurized at each temperature were collected aseptically and held on ice until all processing treatments were completed. Immediately post-processing, eight aliquots of ~200 mL were poured from each of the 2 L containers into sterile 250 mL screw cap 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 prior to pasteurization, a sample was taken from the standardized 2% fat milk before heating and homogenization. Milk samples were assessed for total aerobic plate count (**APC**), coliform count (**CC**), 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). Pre-incubation (**PI**) 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 minutes, then the milk was cooled rapidly and 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 post-pasteurization. Samples were serially diluted into PBS (Weber Scientific, Hamilton, NJ) and spread-plated onto BHI agar and incubated

at 32°C for 24 hours. On d 1, plating was performed by spreading 1mL 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 aerobic plate count data (JMP Version 7.0 SAS Institute Inc., Cary, NC, 1989-2007). 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*time were random variables in the model. 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 post-processing were visually examined and a colony representative of each distinct morphology present on one plate that had been used for colony enumeration for each sample was picked 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.

***rpoB* Sequencing.** Species identification and subtyping of *Bacillus* and *Paenibacillus* spp. isolates were performed by determining the DNA sequence of a 632 nucleotide (**nt)** fragment of the *rpoB* gene, as previously described by Huck et al. (2007a). This method was selected for bacterial identification and differentiation purposes as it allows for phylogenetic characterization of *Bacillus* and *Paenibacillus* isolates in addition to subtype identification, and as it is more economical than most banding pattern-based subtyping methods such as ribotyping or Pulsed Field Gel

Electrophoresis (Kabuki et al., 2004). 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 one or more nucleotides. Isolates with different allelic types were considered to represent different molecular subtypes.

16S rDNA Sequencing. While the majority of isolates collected here could be characterized to genus and species through phylogenetic analyses using the existing *rpoB* sequence database (Durak et al., 2006; Huck et al., 2007a,b; Huck et al., 2008), 16S rDNA sequencing was used to: (i) confirm the genus and/or species identification of *rpoB* allelic types that had not been identified in any of our previous studies; and (ii) for those isolates we could not identify by *rpoB* sequencing. Specifically, one isolate representing each newly identified *rpoB* allelic type was characterized by sequencing the 3' end of the 16S rDNA, as previously described 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 (**BLAST**) (McGinnis and Madden, 2004). Genus and/or species assignments for a specific 16S rDNA sequence were based on the top matches returned by the BLAST search.

RESULTS

Microbiological Analysis of Raw Milk. To assess the microbiological quality of the raw milk prior to pasteurization, a sample of the 2% fat raw milk was collected immediately after the skim and cream portions had been blended, but prior to heat tempering and homogenization on each processing day. A complete listing of log transformed bacterial counts for each replicate is presented in Table 2.1. Mean raw 2% fat milk APC from the four replicates was 4.19 log cfu/ml, and ranged from 3.91

log cfu/ml (Replicate 4) to 4.98 log cfu/ml (Replicate 1). The mean MSC was 1.67 log cfu/ml, and ranged from 0.85 log cfu/ml (Replicate 3) to 2.88 cfu/ml (Replicate 1). On 2% fat raw milk samples, we observed a mean LPC of 1.37 log cfu/ml, mean PI count of 4.25 log cfu/ml, mean CC of 1.48 log cfu/ml, mean PBC of 3.57 log cfu/ml and no growth (<1 spore per mL) for the PSC (Table 2.1).

Table 2.1. Log transformed bacterial numbers from 2% milkfat raw milk used in four independent replicates[1]

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[1]
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

[1]In all four replicates, PSC analyses yielded no colonies, indicating the presence of less than 1 spore capable of growth at 6°C per milliliter of raw milk sample
[2]Estimated counts were not included in mean or standard deviation calculations; results from these samples were "too numerous to count"

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 2.1. 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 three highest temperature treatments were similar and all were higher than the mean for the milk processed at 72.9°C.

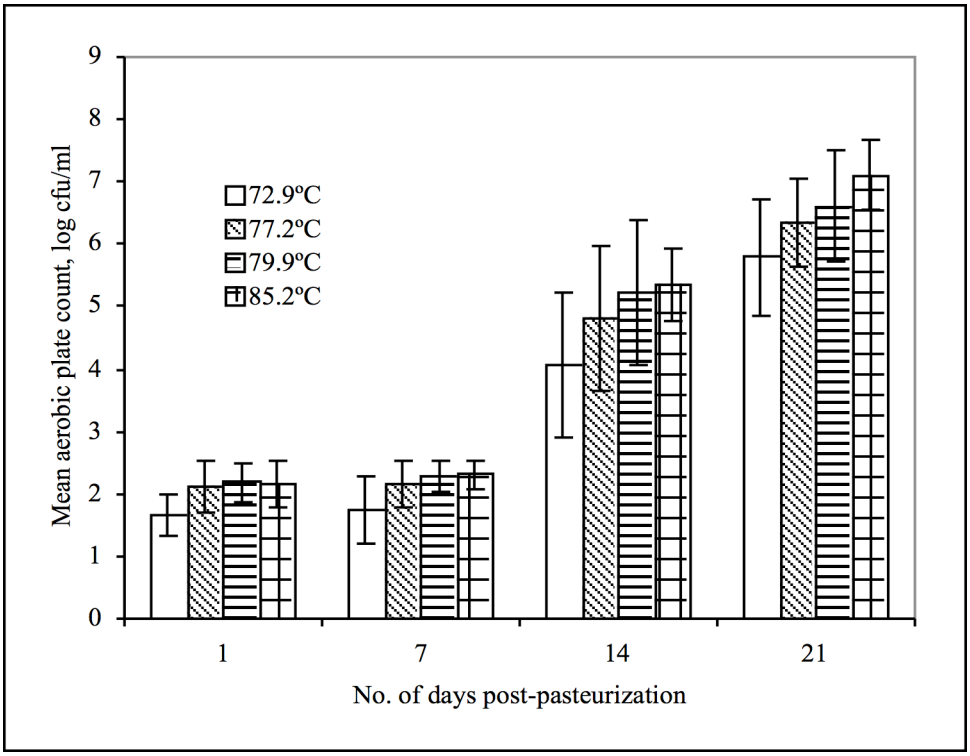


Figure 2.1. Aerobic plate counts (APC) for 2% fat milk that had been pasteurized at one of four different temperatures (72.9, 77.2, 79.9 or 85.2°C) and held at 6°C for up to 21 d. Data represent mean APC for milk processed at each temperature from four independents replicates. Bars indicate mean ± 1 SD for each treatment.

Overall, results from statistical analyses of our data showed a significant effect of temperature, day and replicate on bacterial numbers in pasteurized milk. On d 1 post-processing, 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 post-processing 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, while the mean APC for milk processed at 85.2°C was 2.30 log cfu/ml. Between d 7 and 14, the average increase in APC was 2.33 log cfu/ml, 2.66 log cfu/ml, 2.95 log cfu/ml, 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 (~616,000 cfu/mL), while the mean APC for milk processed at 85.2°C was 7.10 log cfu/ml (~12,590,000 cfu/mL). Mean APC for milk processed at 72.9°C was lower ($P < 0.05$) than for milk processed at the other three temperatures on each sampling day. In addition to a significant effect of temperature, both replicate and day were significant variables in the model ($P < 0.01$), which indicates associations between these variables and the mean bacterial numbers observed.

Bacterial Species Distribution as Determined by *rpoB* and 16S rDNA Subtyping Data. A total of 564 isolates were collected in the four replicates from both raw and pasteurized product. Of those, 478 isolates were determined by *rpoB* sequencing to belong to genera characterized as gram-positive spore-forming bacteria, i.e., *Bacillus* and *Paenibacillus*. The remaining 86 isolates, which were not typable using 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 2.2 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 *Macrococcus*.

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 processing system.

Table 2.2. Genus distribution of bacterial isolates from raw and heat-treated milk samples

Genus	No. of Isolates from Raw Milk[1]	No. of Isolates from Pasteurized Milk	Total
Gram-negative			
Pseudomonas	31	0	31
Stenotrophomonas	6	0	6
Acinetobacter	3	0	3
Klebsiella	2	0	2
Pantoea	2	0	2
Comamonas	1	0	1
Total	45	0	45
Gram-positive			
Bacillus[2]	29	229	258
Paenibacillus[3]	2	230	232
Enterococcus	4	6	10
Micrococcus	0	7	7
Staphylococcus	1	5	6
Streptococcus	1	1	2
Aerococcus	1	0	1
Brevibacillus	0	1	1
Corynebacterium	0	1	1
Macrococcus	1	0	1
Total	39	480	519
Total	84	480	564

[1]Raw milk isolates were obtained from multiple tests performed on 2% raw milk, including APC, MSC, and LPC
[2]249 of 258 *Bacillus* isolates were identified by *rpoB* sequencing, 9 isolates were identified by 16S rDNA sequencing
[3]229 of 232 *Paenibacillus* isolates were identified by *rpoB* sequencing, 3 isolates were identified by 16S rDNA sequencing

An additional 9 *Bacillus* and 3 *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 2.3 shows the

Table 2.3. Distribution of isolates obtained from pasteurized milk and characterized as *Bacillus* spp. or *Paenibacillus* spp.

Pasteurization temperature (°C)	Day post-pasteurization	Genus	
		Bacillus[2]	*Paenibacillus*[3]
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[1]		229	230

[1]Isolates obtained from raw milk or raw milk receiving heat treatments (LPC or MSC) are not included in these distributions
[2]222 of 229 *Bacillus* isolates were identified by *rpoB* sequencing, 7 isolates were identified by 16S rDNA sequencing
[3]227 of 230 *Paenibacillus* isolates were identified by *rpoB* sequencing, 3 isolates were identified by 16S rDNA sequencing

distribution of *Bacillus* and *Paenibacillus* isolates by pasteurization temperature and day post-pasteurization. There was a significant association between genus identified and day post-pasteurization ($P < 0.001$). As shown in Table 2.4, at d 0, 1, and 7, the

Table 2.4. Proportion of endospore-forming isolates collected from raw and heat-treated milk by day post-pasteurization and by heat treatment (i.e., raw, lab-pasteurization, mesophilic spore count, pasteurization)

Day post-pasteurization (heat-treatment)	Number of isolates characterized	Number of isolates characterized as:	
		Bacillus spp.	*Paenibacillus* spp.
0 (raw milk, none)	2	2 (100%)[2]	0 (0%)
1 (LP)	11	10 (91%)	1 (9%)
1 (MSC)	18	17 (94%)	1 (6%)
1 (pasteurized)[1]	138	131 (95%)	7 (5%)
7 (pasteurized)[1]	111	82 (74%)	29 (26%)
14 (pasteurized)[1]	102	11 (11%)	91 (89%)
21 (pasteurized)[1]	108	5 (5%)	103 (95%)
Total	490	258 (53%)	232 (47%)

[1]Isolates were obtained from milk that had been pasteurized at all four experimental temperatures
[2]Percentages are calculated based on the number of isolates given in each row

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 post-pasteurization, 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 post-pasteurization ($P < 0.001$). Of 210 isolates collected at d 14 or 21 post-pasteurization, 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 96 AT identified (4 *Bacillus* and 4 *Paenibacillus*) were isolated both from multiple replicates and more than 10 times (Table 2.5). The most frequently isolated *Bacillus* AT were 1, 6, 20 and 158. AT1 and 6 were identified as *Bacillus licheniformis,* while AT 20 and 158 were identified as *Bacillus pumilus* and *Bacillus cereus*, respectively. The most commonly isolated AT of the genus *Paenibacillus* were 13, 15, 25 and 27. 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 processing system. For example, AT1 (*Bacillus licheniformis*) was isolated 4, 14, 6, 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 and 6 times on d 7 post-pasteurization. AT1 was not obtained from any milk on d 14 or 21 post-pasteurization. The most commonly isolated *Bacillus* allelic type was AT158 (*Bacillus cereus*). AT158 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 on d 7, 5 on d 14 and 0 on d 21. AT158 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. AT158 was the only *Bacillus* isolate found solely in

Table 2.5. Common[1] molecular subtypes of endospore forming bacterial isolates obtained from raw and pasteurized milk samples

Genus, species	Allelic Type	Total No. Isolated	Replicate (no. of isolates per replicate)	Sample Day Post-Pasteurization (no. of isolates)[2]
Bacillus spp.				
licheniformis	1	27	1 (4) 2 (14) 3 (6) 4 (3)	0 (5) 1 (16) 7 (6) 14 (0) 21 (0)
licheniformis	6	13	1 (1) 2 (3) 3 (4) 4 (5)	0 (0) 1 (5) 7 (7) 14 (1) 21 (0)
pumilus	20	10	1 (3) 2 (2) 3 (4) 4 (1)	0 (1) 1 (8) 7 (1) 14 (0) 21 (0)
cereus	158	149	1 (49) 2 (36) 3 (29) 4 (35)	0 (0) 1 (92) 7 (52) 14 (5) 21 (0)
Paenibacillus spp.				
spp.	13	13	1 (0) 2 (6) 3 (4) 4 (3)	0 (0) 1 (0) 7 (1) 14 (4) 21 (8)

Table 2.5 (continued)

Genus, species	Allelic Type	Total No. Isolated	Replicate (no. of isolates per replicate)	Sample Day Post-Pasteurization (no. of isolates)[2]
Paenibacillus spp.				
spp.	15	25	1 (11) 2 (8) 3 (3) 4 (3)	0 (0) 1 (0) 7 (2) 14 (11) 21 (12)
spp.	25	16	1 (12) 2 (0) 3 (3) 4 (1)	0 (0) 1 (0) 7 (1) 14 (12) 21 (3)
spp.	27	26	1 (7) 2 (5) 3 (2) 4 (12)	0 (0) 1 (0) 7 (4) 14 (13) 21 (9)

[1] AT represented by ≥10 isolates shown; AT with ≤10 isolates included 50 isolates classified as *Bacillus* and 149 isolates classified as *Paenibacillus*
[2] Total numbers of isolates obtained on d 0, 1, 7, 14 and 21 were 13, 150, 107, 100 and 108, respectively

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 to be the processing environment. This hypothesis is further supported by the observation that while AT158 was not found in raw milk, it was most frequently isolated from pasteurized milk at d 1 post-processing and decreased in

Table 2.6. Relationship between heat treatment and AT isolated

Heat Treatment	*Bacillus* allelic type						*Paenibacillus* allelic type					
	AT1	AT6	AT20	AT158	Other AT[2]	Isolates Without AT[3]	AT13	AT15	AT25	AT27	Other AT[2]	Isolates Without AT[4]
Raw Milk[1]	9	2	4	0	12	2	0	0	0	0	2	0
72.9	7	6	1	33	14	2	2	4	6	9	31	2
77.2	5	0	2	36	9	2	6	9	5	6	38	0
79.9	1	4	3	39	5	0	1	5	1	6	42	0
85.2	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]Raw milk isolates were obtained from different tests performed on 2% fat raw milk, including APC, MSC, and LPC

[2] "Other AT" represent combined AT, each found fewer than 10 times

[3]9 *Bacillus* were identified by 16S rDNA because they did not amplify with *rpoB* PCR, therefore, no AT are available for these isolates

[4]3 *Paenibacillus* were identified by 16SrDNA because they did not amplify with *rpoB* PCR, therefore, no AT are available for these isolates

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 2.5). The most frequently isolated *Paenibacillus* subtype was AT27, which was isolated 26 times and was found in all 4 replicates. AT27 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 post-pasteurization; 4 were isolated on d 7, 13 on d 14, and 9 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 2.6). 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 were 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 post-processing 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 as fluid milk is stored at temperatures permissive for 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 et al., 1984). Typically, such bacteria enter milk through post-pasteurization contamination at the filling machine (Eneroth et al., 1998; Raylea et al., 1998). In recent years, many commercial fluid milk processors have reduced post-pasteurization contamination through implementation of improved filler and packaging technology to produce milk that does not result in consumer complaint at 15 to 25 d post-pasteurization (Carey et al., 2005). When gram-negative post-pasteurization 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; Huck et al., 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., 1999; Guinebretiere 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 (Slaguis et al., 1997), soil (Christiansson et al., 1999), and fecal material (Labots et al., 1965), hence they 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 sporeforming *Bacillus* and *Paenibacillus* spp. from dairy farms, tank trucks, and plant storage silos to pasteurized milk (Huck et al., 2007a; Huck et al., 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, *Bacillus cereus* AT158 was isolated 149 times from four independent batches of pasteurized milk and across each of the four 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. 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 post-processing aerobic plate counts of fluid milk products following 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 following an 80°C heat treatment (Moran et al., 1990). At d 21 post-pasteurization, 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 sporeformers 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 keeping quality of milk pasteurized at 72°C for 15 s relative to milk pasteurized at 80°C for 15 s (Barrett et al., 1998).

***Paenibacillus* is the Predominant Spoilage Organism Present at Days 14 and 21 Post-Processing.** 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., AT 1, 6, 20 or 158) was isolated at d 21 post-pasteurization, and none of the common *Paenibacillus* AT (i.e., AT 13, 15, 25 or 27) 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 product shelf-life in HTST pasteurized milk has been previously observed. Huck et al. (2008) found 76.2% of isolates collected from pasteurized milk on d 12 of shelf life or beyond were *Paenibacillus* spp., while *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 comprise the predominant sporeformer microflora in raw milk. In the present study, the relative percentage of *Bacillus* spp. isolated decreased from 95%, 74%, 11%, to 5% on d 1, 7, 14 and 21 post-processing, respectively, while the percentage of *Paenibacillus* spp. isolated increased from 5%, 26%, 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 sub-population 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 post-pasteurization across all four temperature treatments may explain why 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). Due to their predominance during product storage at refrigerated temperatures, targeting *Paenibacillus* spp. 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 four common *Paenibacillus* allelic types (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. AT15 and 27 were identified in the dairy farm environment, tank trucks, plant storage silos, and pasteurized milk (Huck et al., 2008). AT13 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 post-processing 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, response to heat treatments

and most importantly, as targets for 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

HTST pasteurization temperatures can affect aerobic plate counts present in processed milk during refrigerated storage. Specifically -- and counter-intuitively -- 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) do not preferentially affect a sub-population of the endospore forming bacteria present. We conclude that the endospore-forming psychrotolerant bacteria present in milk grow more effectively after a higher heat treatment. Additionally, *Paenibacillus* spp., which are likely present at <1 spore per mL of raw milk, are capable of growing to numbers that limit HTST pasteurized milk shelf-life, 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 employed to physically remove psychrotolerant endospore forming bacteria from raw milk. Micro-filtration 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.

CHAPTER THREE

BACTERIAL ECOLOGY OF HIGH TEMPERATURE SHORT TIME (HTST) PASTEURIZED MILK PROCESSED IN FIVE DIFFERENT GEOGRAPHIC REGIONS IN THE UNITED STATES[1]

INTRODUCTION

Bacterial spoilage currently limits the shelf-life of conventionally processed high-temperature short-time (**HTST**) pasteurized fluid milk to the 2-3 weeks typical of US products (Fromm and Boor, 2004). Previous research on milk and dairy environment samples in New York State has demonstrated that when post-pasteurization 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 sporeforming 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 to over

[1]Submitted for publication to the Journal of Dairy Science.

200 unique allelic types (**AT**), illustrating the rich diversity of sporeforming 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% milkfat pasteurized milk samples processed at 18 different 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**)). NE plants included one from New York State and three from Pennsylvania. The SE was represented by one plant each from Florida, Georgia and Tennessee. Three plants from Texas represented the S. Two plants from Michigan, one plant from Wisconsin and one plant from Minnesota represented the MW. Two plants from California, one plant from New Mexico, and one plant from Idaho represented the W.

MATERIALS AND METHODS

Pasteurized 2% milkfat fluid milk samples in half-gallon containers were shipped to the Milk Quality Improvement Laboratory (Ithaca, NY) in coolers packed with ice. Upon receipt, one half-gallon container was selected as a temperature control, 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. Following temperature verification, two half-gallon containers from a given plant were inverted 25 times and wiped with ethanol. Approximately 250 mL from each of the two half-gallon containers was poured into each of five separate sterile 500 mL Pyrex glass containers. The Pyrex glass containers were then inverted 25 times and stored at

6°C. Microbiological testing was performed on one of the five containers at each d 1, 7, 10, 14 and 17 post-pasteurization. Aerobic plate counts were determined in duplicate by spread plating appropriate dilutions made with PBS (Weber Scientific, Hamilton, NJ) on Brain Heart Infusion agar (Difco, BD Diagnostics, Franklin Lakes, NJ) and all plates were incubated at 32°C for 24 h prior to enumeration.

For each milk sample, bacterial colonies present on BHI that had been plated at d 1, 7, 10, 14, and 17 post-pasteurization were visually examined. A single colony representing each distinct colony morphology present on one of the two 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, with increasing time post-pasteurization (e.g., at d 14 and 17 post-pasteurization), bacterial colonies on the plates appeared to be more homogeneous, suggesting predominance of specific types of bacteria following extended refrigerated storage. 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.

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 previously described by Huck et al. (2007a). This method was selected as it allows for phylogenetic characterization of isolates in addition to subtype identification. BioEdit (Hall, 1999) was used for *rpoB* allele assignment. A unique 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, 1989-2007), using a chi-square test for independence; APC means were compared using Student's t-tests.

As the *rpoB* subtyping method is an emerging technique 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; Huck et al., 2008; M. Ranieri, unpublished data). Therefore, 16S rDNA sequencing was used to confirm the genus and/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, (i) one isolate representing each newly identified *rpoB* allelic type; or (ii) 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 previously described 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 (**BLAST**) (McGinnis and Madden 2004). Genus and/or species assignments for a specific 16S rDNA sequences were based on the top matches returned by the BLAST search.

RESULTS

A total of 589 isolates were collected from the pasteurized 2% fat milk obtained from the 18 plants. Of these, 348 isolates were determined by *rpoB* sequencing to belong to genera characterized as gram-positive spore-forming bacteria, i.e., *Bacillus* and *Paenibacillus*. The remaining 241 isolates, which were not typable using the *rpoB* sequencing method, were identified by sequencing the 3' end of the 16S rRNA gene. Among these 241 isolates, 68 represented gram-positive bacteria and 173 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 3.1 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* (238 isolates), which was isolated 36, 42, 61, 48, and 51 times in the MW, NE, S, SE and W, respectively.

Table 3.1. Bacterial isolates obtained from 2% fat HTST-pasteurized milk, categorized by geographic origin of the milk sample

Bacterial Isolate	MW	NE	S	SE	W	No. Isolates	% of Isolates
Gram-Positive							
Bacillus	36	42	61	48	51	238	40
Paenibacillus	23	28	10	35	30	126	21
Staphylococcus	7	4	4	1	3	19	3
Leuconostoc	2		2	3	11	18	3
Enterococcus	5					5	1
Streptococcus		4				4	1
Brevibacillus				1		1	<1
Corynebacterium	1					1	<1
Lactococcus			1			1	<1
Microbacterium			1			1	<1
Micrococcus			1			1	<1
Oceanobacillus			1			1	<1
Gram-Negative							
Pseudomonas	53	38	2	8	19	120	20
Acinetobacter	1	9	2		2	14	2
Yersinia			11			11	2
Enterobacter	4	1			3	8	1
Enterobacteriaceae	8					8	1
Shewanella	1		3			4	1
Aeromonas			3			3	<1
Flavobacterium	2					2	<1
Pantoea		1			1	2	<1
Sphingobacterium						1	<1
Total	143	127	102	96	121	589	100

Paenibacillus was the second most frequently isolated gram-positive genus (126 isolates), and was isolated 23, 28, 10, 35, and 30 times in the MW, NE, S, SE and W,

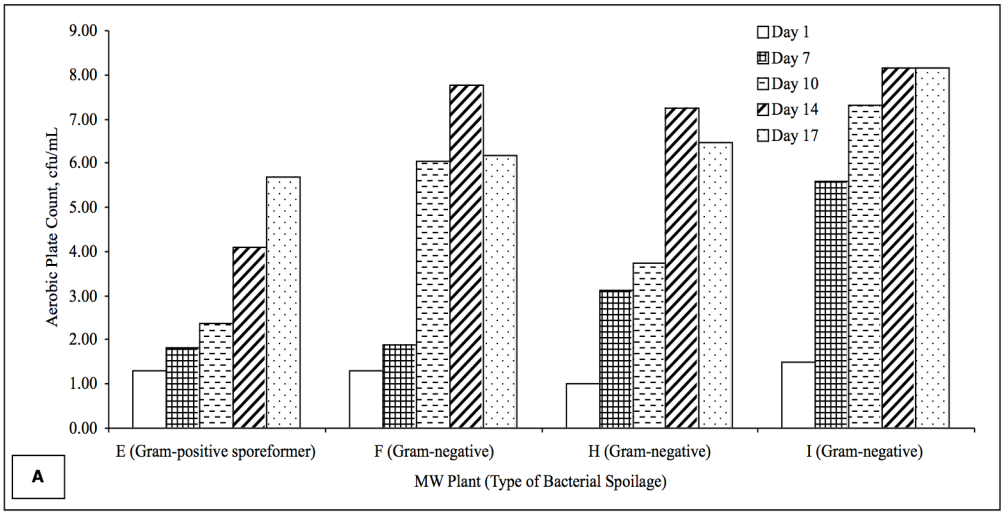
respectively. Other gram-positive bacteria identified were: *Staphylococcus*, *Leuconostoc*, *Enterococcus*, *Streptococcus*, *Brevibacillus*, *Corynebacterium*, *Lactococcus*, *Microbacterium*, *Micrococcus*, and *Oceanobacillus*.

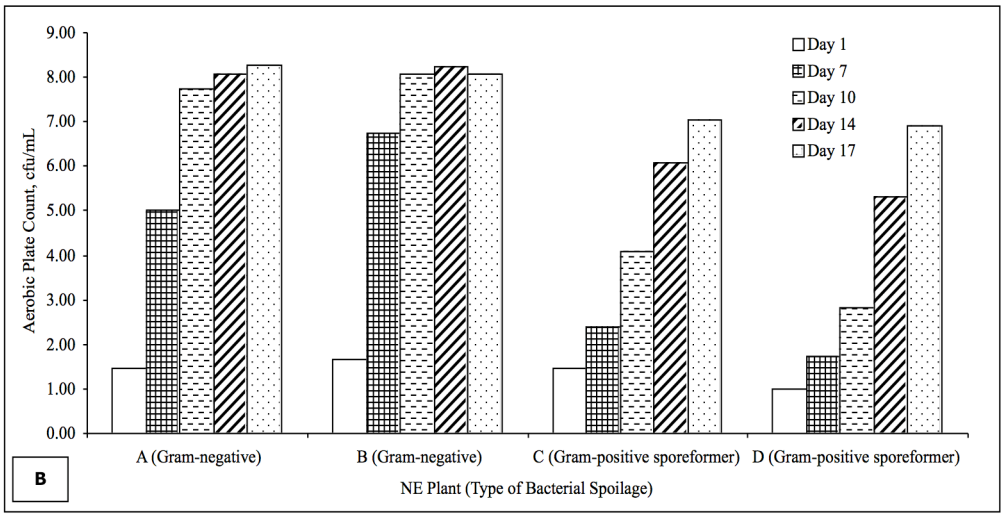
The most frequently isolated gram-negative genus was *Pseudomonas* (120 isolates), which was isolated 53, 38, 2, 8, and 19 times in the MW, NE, S, SE, and W, respectively. In total, milk samples from 7 plants (~39% of the plants sampled) contributed 114 of the 120 (95%) of the *Pseudomonas* obtained in this study. Only ~20% (74 of 364) 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*.

Figure 3.1 and Table 3.2 present the aerobic plate counts from samples from each plant on each day of testing. All 589 bacteria isolated during this study are accounted for in this Table 3.2, 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. "Gram-negative spoilage" was defined as identification of *Pseudomonas* spp. in milk samples from the same plant on at least two of the five testing days. Plants F, H, and I (MW), A and B (NE), G (SE), and L (W) were characterized as having gram-negative spoilage. To illustrate the rapid APC development in milk spoiled by gram-negative growth, on day 10 post-pasteurization, Plant F milk had an APC of 6.04 log cfu/mL, with 8 *Pseudomonas* spp. isolated from testing plates. On day 14 post-pasteurization, Plant F had an APC of 7.78 log cfu/mL, with *Pseudomonas* spp. isolated 9 times. Figure 3.1 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 due to the presence of gram-negative bacteria (e.g., *Pseudomonas* spp.; upper curve) vs. gram-positive sporeformers (e.g., *Paenibacillus* and *Bacillus*; lower curve). While at day 1 post-pasteurization, the APC were not significantly different for those bearing predominantly gram-positive vs. gram-negative spoilers (1.43 vs. 1.58 log cfu/mL for gram-positive sporeformers and *Pseudomonas*, respectively), the mean APC for those with *Pseudomonas* spoilage was significantly higher than those with gram-positive spoilage at days 7, 10, and 14 ($P<0.05$). By day 7, the APC of milk samples with *Pseudomonas* spoilage was a 2.5 log cfu/mL higher than in milk with gram-positive bacteria. At days 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 demonstrate the likelihood that rapid reproduction of gram-negative bacteria can mask the presence of gram-positive endospore-forming bacteria in milk. Our data illustrate that gram-positive sporeformers typically grow more slowly in refrigerated pasteurized milk than gram-negative microbes, and thus gram-positive organisms such as *Bacillus* and *Paenibacillus* spp., while likely to be present simultaneously with the gram-negative organisms, generally are found at lower numbers, particularly early in product shelf-life.

Figure 3.1. APC for days 1, 7, 10, 14 and 17 post-pasteurization 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 sporeformer or gram-negative). Log cfu/mL is indicated on the Y-axes. Panel F shows the mean APC from milk samples obtained from the seven plants characterized as having gram-negative spoilage patterns (upper curve) vs. APC from samples obtained from the eleven plants characterized as having gram-positive spoilage patterns. APC were significantly greater for the gram-negative samples than for the gram-positive samples at d 7, 10, and 14 post-pasteurization ($P<0.05$).


Aerobic Plate Count, cfu/mL
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Day 1
Day 7
Day 10
Day 14
Day 17
E (Gram-positive sporeformer)
F (Gram-negative)
H (Gram-negative)
I (Gram-negative)
MW Plant (Type of Bacterial Spoilage)
A


Aerobic Plate Count, cfu/mL
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Day 1
Day 7
Day 10
Day 14
Day 17
A (Gram-negative)
B (Gram-negative)
C (Gram-positive sporeformer)
D (Gram-positive sporeformer)
NE Plant (Type of Bacterial Spoilage)
B

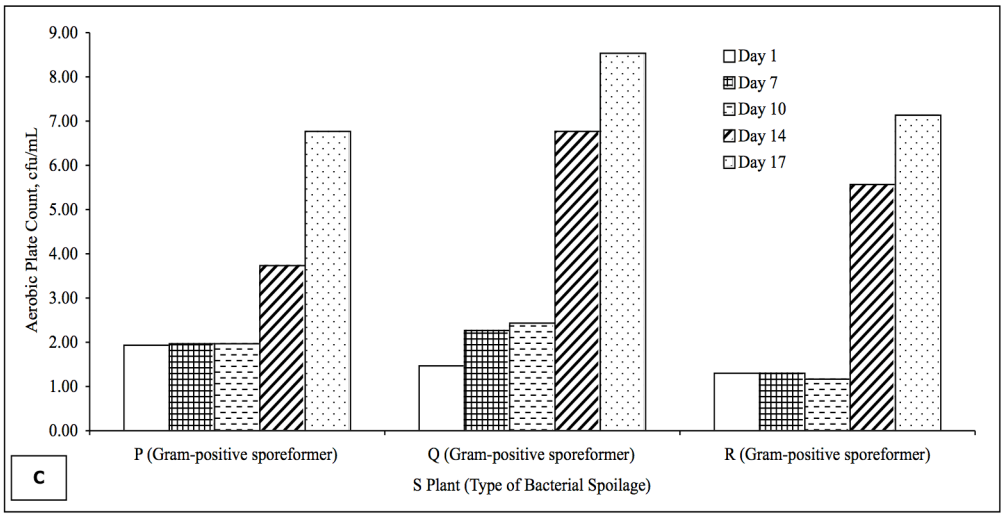
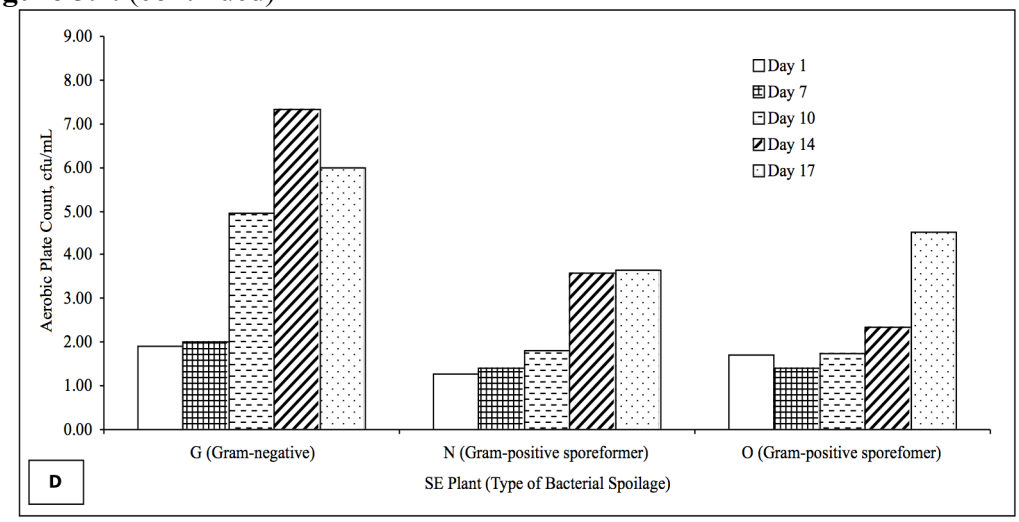
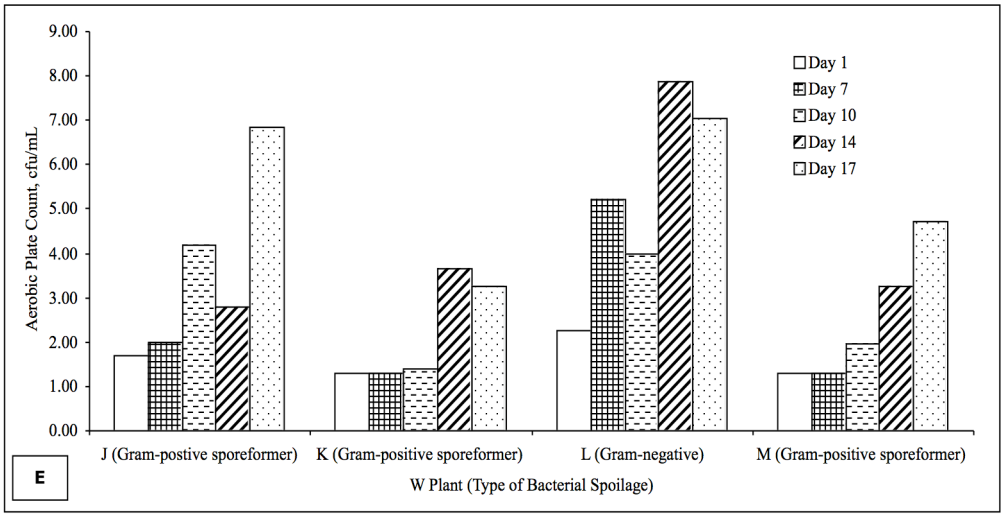

Aerobic Plate Count, cfu/mL
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Day 1
Day 7
Day 10
Day 14
Day 17
P (Gram-positive sporeformer)
Q (Gram-positive sporeformer)
R (Gram-positive sporeformer)
S Plant (Type of Bacterial Spoilage)
C

Figure 3.1. (continued)





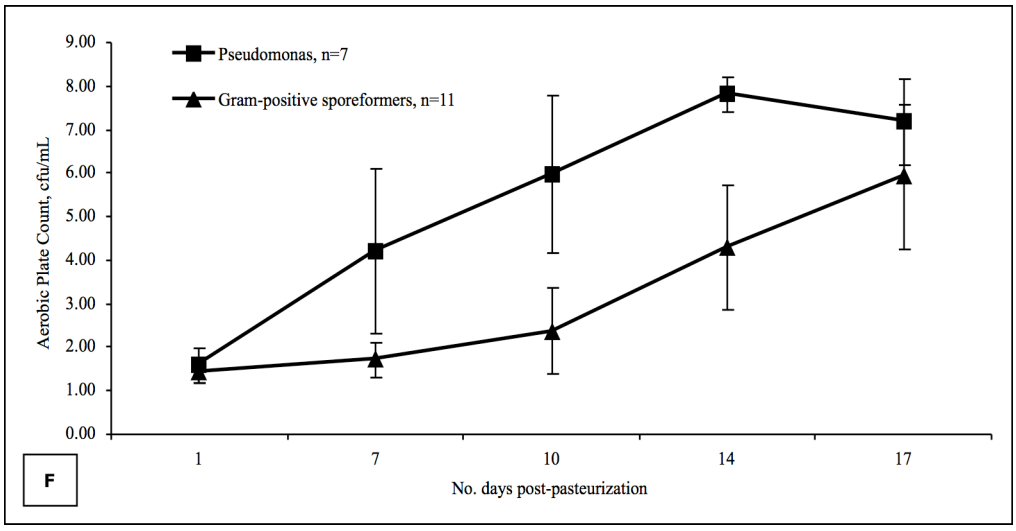


Table 3.2. Aerobic plate counts and isolation frequency of different bacterial genera obtained from milk samples originating from 18 fluid milk processing plants in the U.S.

Region	Plant	Aerobic Plate Count, log cfu/mL [No. isolates, genera[1]]				
		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[2]	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, 1Ec, 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]
	I	1.51 [9 B]	5.60 [**7 Ps**, 2 St]	7.32 [**8 Ps**]	8.15 [**6 Ps**]	8.15 [**8 Ps**]
NE	**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 P, **1 Ps**, 2 St]	6.76 [1 P, **4 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]
	D	1.00 [2 B, 1 P, 1 St]	1.74 [6 B]	2.83 [5 B, 2 P]	5.32 [8 B, 2 P]	6.92 [2 B, 2 P]
S	P	1.94 [8 B, 1 O, 1 P, 2 St]	1.95 [8 P]	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]
	R	1.30 [5 B, 2 St]	1.30 [4 B]	1.18 [2 B]	5.57 [2 Le]	7.15 [8 P]
SE	**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]
	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]
W	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 Sc]	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]

[1]Genera abbreviated as: 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*, Ps=*Pseudomonas*, S=*Shewanella*, and Sp=*Sphingobacterium*

[2]Bold font indicates plants with products characterized as spoiling due to 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 two of five plating days

Overall, 120 *rpoB* AT were differentiated among the 348 spore-forming bacterial isolates characterized by *rpoB* sequencing. Of the 120 AT identified, 46 AT, representing 244 of the 348 (~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 46 AT that had been previously obtained from NYS sites, 74 new AT, representing 104 isolates, were isolated for the first time in the present study. Figure 3.2 presents, by geographic region, the isolates obtained most frequently (n≥8 times) in this study, as well as an indication of the

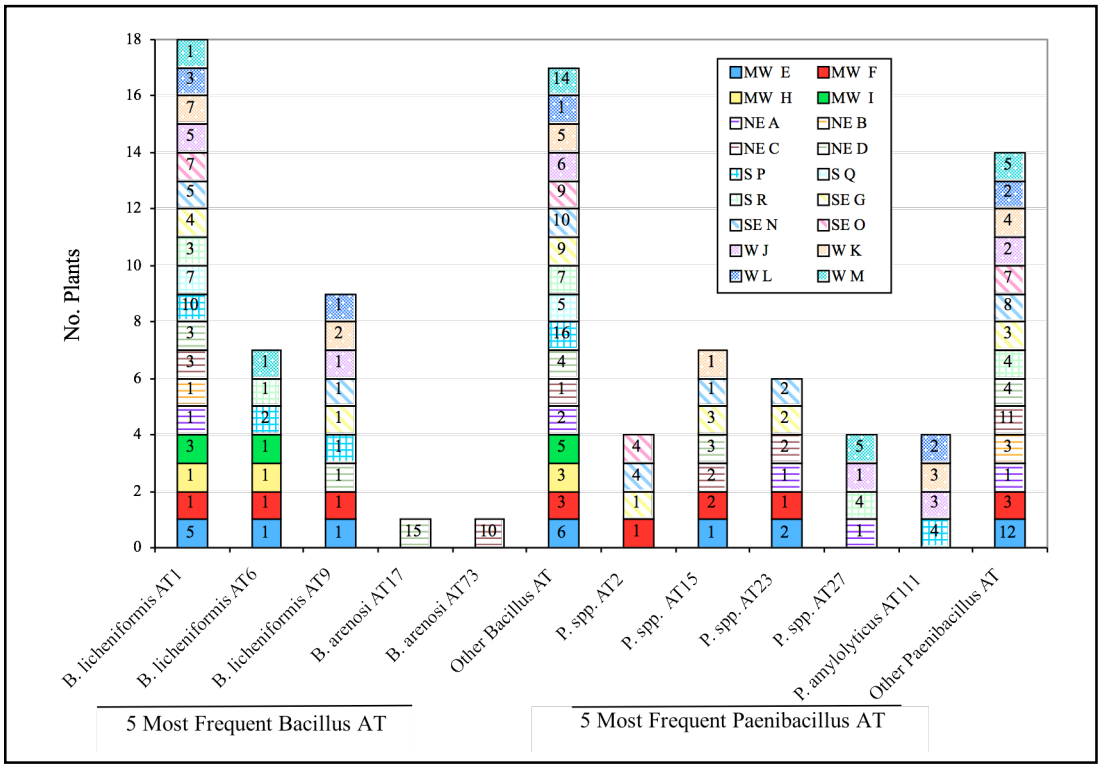


Figure 3.2. Distribution of the 10 most frequently isolated *Bacillus* and *Paenibacillus* AT obtained from 18 processing plants located within 5 different geographical regions across the US. Each region (W, SE, S, MW, NE) is coded by pattern and plant (A-R) is coded by color.

distribution of all other *rpoB* AT. The five 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 17 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. While both *Bacillus* and *Paenibacillus* were isolated from milk from all geographical regions *Paenibacillus* was more frequently isolated at the end of shelf-life. Figure 3.3 shows the total number of *Bacillus* and *Paenibacillus* isolated at each post-pasteurization test day. There was a significant association between genus identified and test day ($P<0.001$) at days 1, 7, 10 and 17. At days 1, 7 and 10, the number of *Bacillus* isolates collected was significantly higher than that of *Paenibacillus* ($P<0.001$). Of 248 gram-positive spore-forming isolates characterized at d 1, 7 or 10, 211 of those isolates were characterized as *Bacillus*. The 37 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 ($P=.33$). 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.

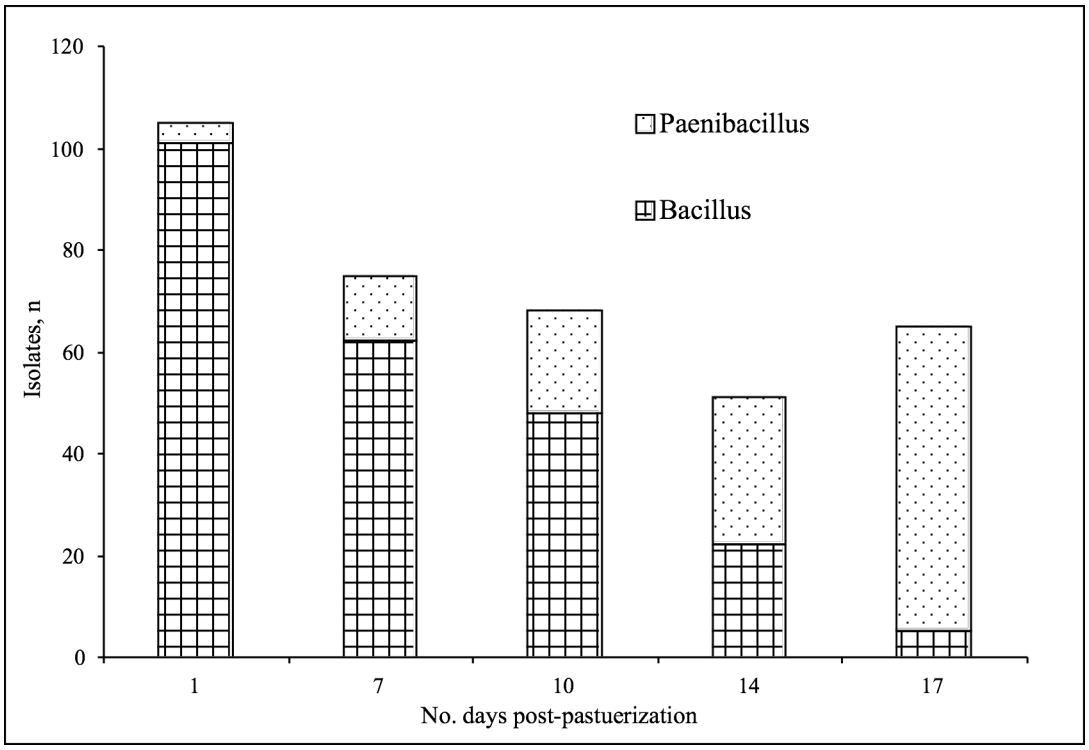


Figure 3.3. Number of isolates characterized as *Bacillus* (n=222) or *Paenibacillus* (n=125) obtained from each day tested.

CONCLUSIONS

In the present study, we found that endospore forming bacteria with AT identical to those of isolates previously found in New York State are present in milk processed throughout the US. Furthermore, AT1, 15, 23, and 27 represent AT that have been previously isolated throughout the milk processing continuum, from farm to finished product. 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, 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., while *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 96%, 83%, 71%, 43%, to 8% on d 1, 7, 10, 14, and 17 post-pasteurization, respectively, while the percentage of *Paenibacillus* spp. isolated increased from 4%, 17%, 29%, 57%, to 92% on d 1, 7, 10, 14, and 17, respectively. The low rate of isolation of *Paenibacillus* spp. at d 1 post-pasteurization 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. 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; 16 new *Paenibacillus* AT were identified in this study. Due to 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. 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.

CHAPTER 5

CONCLUSIONS

The presence of psychrotolerant spore forming bacteria (e.g., *Bacillus* and *Paenibacillus* spp.) remains the key hurdle in extending product shelf-lives beyond 2-3 weeks (Fromm and Boor, 2004; Durak et al., 2006). The pasteurization temperature study determined that the heat-treatment used for HTST pasteurization can affect aerobic plate counts present in processed milk during refrigerated storage. Specifically, and counter-intuitively, 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) do not preferentially affect a sub-population of the endospore forming bacteria present. We conclude that the endospore-forming psychrotolerant bacteria present in milk grow more effectively after a higher heat treatment. Additionally, low numbers of *Paenibacillus* spp. present in raw milk are capable of growing to numbers that limit HTST pasteurized milk shelf-life, illustrating the need for a comprehensive strategy to limit the entry of endospore-forming bacteria into milk systems.

The survey of milk across the US demonstrated that endospore forming bacteria are common in milk processed throughout the United States, and have identical AT to isolates previously found in New York State. Furthermore, AT1, 15, 23, and 27 represent AT common throughout the geographical regions tested within the United States and to AT previously isolated throughout the milk processing continuum, from farm to finished product in NYS. 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, identical allelic types were found in several geographic locations and environments indicates the ubiquitous nature of these spore-forming organisms. Representatives of these common AT will be excellent candidates for future investigation of sporeformers, especially the *Paenibacillus* spp. (AT15, 23, and 27) that are frequently isolated at the end of product shelf life. These AT make excellent candidates for growth characteristics, response to heat treatments and most importantly, as targets for development of sensitive 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. Ultimately, the dairy industry needs to identify and implement an appropriate, cost-effective strategy to limit the number of psychrotrophic spores that enter final products. This will likely require a combination of improved farm practices, milk handling strategies, sanitation, and possibly the application of processing equipment such as microfiltration. However, continued research is necessary to verify which bacteria are capable of causing spoilage and how they enter milk systems.

REFERENCES

Barrett, N.E., A.S., Grandison. and M.J. Lewis. 1998. Contribution of the lactoperoxidase system to the keeping quality of pasteurized milk. J. Dairy Research. 66:73-80.

Boor, K.J. and Murphy, S.C. 2002. Microbiology of market milks. Pages 91-122 in Dairy Microbiology handbook: the microbiology of milk and milk products. Robinson, R.K. 3rd ed. Wiley-Interscience, New York.

Carlin, F., M. H. Guinebretiere, C. Choma, R. Pasqualini, A. Braconnier, and C. Nguyen. 2000. Spore-forming bacteria in commercial cooked, pasteurised and chilled vegetable purees. Food Microbiol. 17:153-165.

Carey, N. R., S. C. Murphy, R. N. Zadoks, and K. J. Boor. 2005. Shelf lives of pasteurized fluid milk products in New York State: A ten-year study. Food Prot. Trends 25:102-113.

Christiansson, A., J. Bertilsson, and B. Svensson. 1999. *Bacillus cereus* spores in raw milk: factors affecting the contamination of milk during the grazing period. J. Dairy Sci. 82:305-314.

Collins, E. B. 1981. Heat resistant psychrotrophic organisms. J. Dairy Sci. 64:157-160.

Crielly, E. M., N. A. Logan, and A. Anderton. 1994. Studies on the *Bacillus* flora of milk and milk products. J. Appl. Bacteriol. 77:256-263.

Cousin, M. A. 1982. Presence and activity of psychrotrophic microorganisms in milk and dairy products: a review. J. Food Prot. 45:172-207.

Douglas, S. A., M. J. Gray, A. D. Crandall, and K. J. Boor. 2000. Characterization of chocolate milk spoilage patterns. J. Food Prot. 63:516-521.

Durak, M. Z., H. I. Fromm, J. R. Huck, R. N. Zadoks, and K. J. Boor. 2006. Development of molecular typing methods for *Bacillus* spp. and *Paenibacillus* spp. isolated from fluid milk products. J. Food Sci. 71:50-56.

Elwell, M. W. and D. M. Barbano. 2006. Use of microfiltration to improve fluid milk quality. J. Dairy Sci. 89:E20-E30.

Eneroth, S., A. Christiansson, J. Brendehaug, and G. Molin. 1998. Critical contamination sites in the production line of pasteurised milk, with reference to the psychrotrophic spoilage flora. Int. Dairy J. 8:829-834.

Frank, J.F., and A.E. Yousef. 2004. Tests for groups of microorganisms. Pages 227-248 in Standard Methods for the Examination of Dairy Products. 17th ed. M. Wehr, ed. Am. Public Health Assoc., Washington, DC.

Fromm, H. I. and K. J. Boor. 2004. Characterization of pasteurized fluid milk shelf-life attributes. J. Food Sci. 69:207-214.

Gandy, A. L., M. W. Schilling, P. C. Coggins, C. H. White, Y. Yoon, and V. V. Kamadia. 2008. The effect of pasteurization temperature on consumer acceptability, sensory characteristics, volatile compound composition, and shelf-life of fluid milk. J. Dairy Sci. 91:1769-1777.

Griffiths, M. W. and J. D. Phillips. 1984. Effect of aeration on extracellular enzyme synthesis by psychrotrophs growing in milk during refrigerated storage. J. Food Prot. 47:697-702.

Griffiths, M. W. and J. D. Phillips. 1990. Incidence, source and some properties of psychrotrophic *Bacillus* spp. found in raw and pasteurized milk. J. Soc. Dairy Technol. 43:62-66.

Guinebretiere, M. H., O. Berge, P. Normand, C. Morris, F. Carlin, and C. Nguyen. 2001. Identification of bacteria in pasteurized zucchini purees stored at different temperatures and comparison with those found in other pasteurized vegetable purees. Appl. Environ. Microbiol. 67:4520-4530.

Hall, T. A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95–98.

Huck, J. R., B. H. Hammond, S. C. Murphy, N. H. Woodcock, and K. J. Boor. 2007a. Tracking spore-forming bacterial contaminants in fluid milk processing systems. J. Dairy Sci. 90:4872-4883.

Huck, J. R., N. H. Woodcock, R. D. Ralyea, and K. J. Boor. 2007b. Molecular subtyping and characterization of psychrotolerant endospore-forming bacteria in two New York State fluid milk processing systems. J. Food Prot. 70:2354-2364.

Huck, J. R., M. Sonnen, and K. J. Boor. 2008. Tracking heat-resistant, cold-thriving fluid milk spoilage bacteria from farm to packaged product. J. Dairy Sci. 91:1218-1228.

Kabuki, D. Y., A. Y. Kuaye, M. Wiedmann, and K. J. Boor. 2004. Molecular subtyping and tracking of *Listeria monocytogenes* in Latin-style fresh-cheese processing plants. J. Dairy Sci. 87:2803-2812.

Labots, H., G. Hup, and T. E. Galesfoot. 1965. *Bacillus cereus* in raw and pasteurized milk. III. The contamination of raw milk with *Bacillus cereus* spores during its production. Neth. Milk Dairy J. 19:191-221.

Lin, S., H. Schraft, J. A. Odumeru, and M. W. Griffiths. 1998. Identification of contamination sources of *Bacillus cereus* in pasteurized milk. Int. J. Food Microbiol. 43:159-171.

Lukasova, J., J. Vyhnalkova, and Z. Pacova. 2001. *Bacillus* species in raw milk and in the farm environment. Milchwissenschaft 56:609-611.

Ma, Y. and D. M. Barbano. 2003. Milk pH as a function of CO_2 concentration, temperature, and pressure in a heat exchanger. J. Dairy Sci. 86:3822-3830.

McGinnis, S. and T. L. Madden. 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Res. 32:W20-W25.

McGuiggan, J. T. M., D. R. McCleery, A. Hannan, and A. Gilmour. 2002. Aerobic spore-forming bacteria in bulk raw milk: factors influencing the numbers of psychrotrophic, mesophilic and thermophilic *Bacillus* spores. Int. J. Dairy Technol. 55:100-107.

Meer, R. R., J. Baker, F. W. Bodyfelt, and M. W. Griffiths. 1991. Psychrotrophic *Bacillus* spp. in fluid milk products: a review. J. Food Prot. 54:969-979.

Moran, L., M. T. Rowe, and J. A. Hagan. 1990. The effect of various heat activation treatments on fast, intermediate and slow germinating spores of *Bacillus* spp. Lett. in Appl. Microbiol. 10:43-46.

PMO. 2005 Revision. Pasteurized Milk Ordinance. Section 1. EE. The Food and Drug Administration, US Department of Health and Human Services, Washington, DC.

Ralyea, R. D., M. Wiedmann, and K. J. Boor. 1998. Bacterial tracking in a dairy production system using phenotypic and ribotyping methods. J. Food Prot. 61:1336-1340.

Richardson, G. K. 1985. Preliminary incubaction for raw milk. Pages 146-147. 15th ed. Am. Public Health Assoc., Washington, DC.

Rozas, J. and R. Rozas. 1999. DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics 15:174-175.

Shehata, T. E., Collins, E. B. 1971. Isolation and identification of psychrotrophic strains of *Bacillus* from milk. Appl. Environ. Microbiol. 21:466-473.

Slaghuis, B. A., M. C. t. Giffel, R. R. Beumer, and G. Andre. 1997. Effect of pasturing on the incidence of *Bacillus cereus* spores in raw milk. Int. Dairy J. 7:201-205.

Sutherland, A. D. and R. Murdoch. 1994. Seasonal occurrence of psychrotrophic *Bacillus* species in raw milk, and studies on the interactions with mesophilic *Bacillus* spp. Int. J. Food Microbiol. 21:279-292.

te Giffel, M. C., A. Wagendorp, A. Herrewegh, and F. Driehuis. 2002. Bacterial spores in silage and raw milk. Antonie Van Leeuwenhoek 81:625-630.

Washam, C. J., Olson, H.C., Vedamuthu, E.R. 1977. Heat-resistant psychrotrophic bacteria isolated from pasteurized milk. J. Food Prot. 40:101-108.