The Pennsylvania State University

The Graduate School

Department of Food Science

DEVELOPMENT OF A SEQUENCE-BASED SUBTYPING METHOD FOR *BACILLUS CEREUS* DAIRY ISOLATES

A Dissertation in

Food Science

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2008

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ABSTRACT

Recent research has suggested Gram-positive spore-forming microorganisms including *Bacillus cereus* are the predominant microorganisms in pasteurized milk during refrigerated storage. The presence of *B. cereus* in pasteurized milk is of concern to the dairy industry because this microorganism can influence the quality and safety of the product. During growth in milk, *B. cereus* can produce proteinases and lipases which cause quality defects in milk and limit shelf life. In addition, the presence of *B. cereus* in pasteurized milk may pose a health hazard to the consumer because *B. cereus* is a human pathogen and has been shown to cause emetic and diarrheal illnesses.

Numerous international studies have been conducted to determine the incidence and level of *B. cereus* in pasteurized milk, but recent research in the U.S. is limited. The objectives of the first portion of this work were to evaluate the microbiological quality of commercial milk from four manufacturers and to determine the level and incidence of *B. cereus*. Microbiological analysis was conducted on whole, 2% milk fat, 1% milk fat, and skim milks stored at 4 and 7°C and included aerobic plate counts, mesophilic and psychrotrophic spore counts, and *B. cereus* counts. Analysis was conducted on the sell-by date (day 0), 12 days before the sell-by date (-12), 7 days before the sell-by date (-7), and 7 days after the sell-by date (+7).

On the sell-by-date, milks stored at 4 and 7˚C, yielded aerobic plate counts ranging from ≤1.0 to 8.5 $\log_{10}$ CFU/mL and ≤1.0 to 8.6 $\log_{10}$ CFU/mL,

respectively. Seven (25%) milk samples stored at 4˚C had aerobic plate counts ≥4.3 $\log_{10}$ CFU/mL (20,000 CFU/mL), the limit outlined in the Pasteurized Milk Ordinance, while 23 (82%) milk samples stored at 7˚C exceeded the limit. Mesophilic and psychrotrophic spore counts of milks stored at 4 and 7˚C were low and ranged from ≤1.0 to 2.2 $\log_{10}$ CFU/mL and ≤1.0 to 1.7 $\log_{10}$ CFU/mL, respectively. The growth of *B. cereus* during refrigerated storage appeared to be influenced by storage temperature. On the sell-by date, *B. cereus* was detected in 2 of 28 (7%) cartons stored at 4˚C, and counts of milks ranged from ≤1.0 to 4.2 $\log_{10}$ CFU/mL. However, *B. cereus* was detected in 16 (57%) milk samples at 7°C, and counts ranged from ≤1.0 to 8.9 $\log_{10}$ CFU/mL. On the sell-by date, 9 (32%) of the milk samples stored at 7°C yielded *B. cereus* counts exceeding the minimum infectious dose for the diarrheal illness.

The data from the first portion of the research suggested further work needed to be conducted to improve the microbiological quality of pasteurized milk. Such work would include tracking studies to identify contamination sites in milk production and processing.

The second objective of this work was to develop a sequence-based subtyping method for *B. cereus* dairy isolates. Currently, the RAPD-PCR method developed by Nilsson et al. (1998) is the subtyping method used most extensively for tracking *B. cereus* in milk production and in the processing environment. This method is well-suited for large-scale typing studies because it is highly discriminatory and is relatively simple and inexpensive compared to

other subtyping methods; however, this method has several disadvantages associated with fragment-based methods including difficulties in standardization and interpretation of the results and low portability of the data.

The focus of the second portion of this work was to develop a two- or three-gene MLST scheme for tracking *B. cereus* dairy isolates using housekeeping and virulence gene sequences. *Bacillus cereus* possesses a number of virulence genes that could potentially be included in a MLST scheme, but in order for these genes to be included in a MLST scheme, the genes would need to be widely distributed among *B. cereus* isolates. The incidence of nine virulence genes was evaluated among 13 *B. cereus* reference strains and milk isolates, and two virulence genes, *entFM* and *nheC* were detected in all of the strains.

The next part of the work involved comparison of the number of allelic types obtained with housekeeping gene sequences in the MLST scheme of Helgason et al. (2004) with sequences of virulence genes, *entFM* and *nheC.* It was expected that the virulence gene sequences would have a greater number of polymorphic sites and yield a greater number of allelic types than the housekeeping genes.

The number of allelic types obtained with sequences of housekeeping genes in the MLST scheme of Helgason et al. (2004) ranged from four (*adk*, *recF,* and *sucC*) to seven (*ftsA* and *glpT*). The percentage of polymorphic sites among these genes ranged from 1.7% (*ftsA*) to 7.3% (*glpT*). Virulence genes *entFM* and *nheC* yielded seven and six allelic types, respectively, and these

genes exhibited a higher percentage of polymorphic sites (10.2 and 12.1%) than the housekeeping genes.

Finally, various combinations of housekeeping and virulence gene (*entFM* and *nheC*) sequences were evaluated in the development of a two- or three-gene MLST scheme for *B. cereus*. The number of sequence types obtained with the two-gene MLST schemes ranged from five (*ccpA* and *pyrE*, *adk* or *recF* and *sucC*) to eight (any housekeeping gene and *nheC,* or *entFM* and *nheC*). The addition of a third gene to the MLST scheme did not increase the number of sequence types. The seven-gene MLST scheme described by Helgason et al. (2004) yielded seven sequence types, and the combination of the seven housekeeping genes and two virulence genes yielded eight sequence types.

The two-gene MLST schemes could be used for large-scale tracking studies to identify sources of *B. cereus* contamination. Such tracking studies could lead to a reduction in the level and incidence of *B. cereus* in pasteurized milk and result in improvements in the quality and shelf life of the product.

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ACKNOWLEDGEMENTS

I would like to thank my thesis advisors Dr. Doores and Dr. Roberts for their encouragement and guidance during my thesis research. I would particularly like to thank Dr. Doores for the freedom she gave me to explore new research topics when my initial project did not turn out as planned. I would like to thank Dr. Roberts for joining my committee and serving as a co-advisor when I began working with molecular subtyping methods. I greatly appreciate him allowing me to work in his lab and the encouragement he provided as I began research with subtyping methods.

I would like to acknowledge the contributions of my committee members Dr. DebRoy, Dr. Dudley, and Dr. Knabel. I am grateful for their willingness to help with my research and many valuable suggestions

I would like to thank Dr. K. J. Boor at Cornell University and Dr. L. Beuchat at the University of Georgia for generously providing *B. cereus* strains that were used in this work.

I would like to express my appreciation to Dr. Floros for his support throughout this project.

I would also like to thank my lab mates past and present and other graduate students in the Department of Food Science for their assistance, friendship and support. I am especially grateful to my most recent lab mates Neetu Motwani and Denise Gardner for all that they did to make working in 420 an enjoyable and memorable experience.

I would to acknowledge Dr. Elizabeth Panko Briczinski and Elisabeth Roberts for helping me learn the molecular subtyping methods used in my research.

I would like to thank Dr. Yi Chen for the time he spent helping me learn the software used for editing sequences, and answering my many questions about sequencing.

I would like to express my appreciation to Tom Palchak and the Creamery staff for their helpfulness and assistance with my thesis research.

I am especially grateful to my husband, Phil Miller and his family for their love and support during my graduate work.

Chapter 1

Statement of the Problem

In the beverage market, pasteurized milk is facing increased competition as many consumers are opting for more shelf stable and convenient products. In order to develop a more a competitive position within the beverage market, the dairy industry has sought to improve distribution of milk beyond supermarkets. Distribution of pasteurized milk could be expanded by shipping the product to wider geographical areas or through new sales outlets such as vending machines. However, expanded distribution through these new venues would require extension of the product's shelf-life.

Bacillus cereus is recognized as a foodborne pathogen and is a spoilage microorganism that has been associated with the development of quality defects which limit the shelf life of pasteurized milk. Recent research has suggested Gram-positive spore-forming microorganisms such as *B. cereus* are the predominant microorganisms in pasteurized milk during refrigerated storage. However, further research is necessary to assess the level and incidence of *B. cereus* contamination because recent research in the U.S. is limited.

Bacillus cereus is particularly challenging to control because contamination of milk may occur at numerous points in production and processing. Contamination may occur through spores in raw milk or at the processing facility. Because of the wide range of potential sources, there is

interest in identifying critical contamination sites for these microorganisms so that appropriate intervention strategies can be developed.

Such tracking studies require the use of a reliable subtyping method. The randomly amplified polymorphic-PCR method developed by Nilsson et al. (1998) has been used most extensively to track *B. cereus* dairy isolates. This method is well-suited for large-scale tracking studies because it is relatively simple and easy to perform compared to other subtyping methods. However, this method also has significant drawbacks associated with fragment-based methods including difficulties in standardization and interpretation of the results as well as low portability of the data. Currently, there is a need for a sequence-based subtyping method that can be used for large-scale tracking studies.

The objectives of the present research were:

- To conduct microbiological analysis of pasteurized milk and enumerate *B. cereus.*
- To develop a sequence-based molecular subtyping method for *B. cereus* dairy isolates.

Chapter 2

Literature review

2.1 Introduction

Bacillus cereus is a Gram-positive, aerobic to facultatively anaerobic, large (1.0-1.2 µM by 3.0-5.0 µm) rod-shaped bacterium *(74, 77)*. The term "bacillus" in Latin denotes a small rod, and "cereus" means waxen or wax-like *(74)*. On common agar media, *B. cereus* forms large (3-8 mm diameter) colonies that are flat with a dull or frosted glass appearance and irregular borders *(74, 77)*. *Bacillus cereus* is capable of forming endospores, and in the sporulated state, cells are resistant to a variety of adverse conditions including heat, radiation, and chemicals *(71)*.

2.1.1 *Bacillus cereus* group

Bacillus cereus is a member of the *Bacillus cereus* group, which is composed of six closely related species including *B. cereus sensu stricto*, *B. anthracis, B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, and *B. weihenstephanensis (77)*. Three members of the *B. cereus* group–*B. anthracis, B. cereus,* and *B. thuringiensis*–are recognized pathogens. These three species

exhibit a high level of genetic similarity and are differentiated primarily by genes carried on plasmids.

2.1.1.1 *Bacillus anthracis,* and *Bacillus thuringiensis*

Bacillus anthracis can cause infections in humans and animals and is classified by the Centers for Disease Control and Prevention as a Category A bioterrorism agent *(12, 41, 58)*. *Bacillus anthracis* differs from *B. cereus* in that it carries two virulence plasmids pOX1 and pOX2 *(91)*. The virulence genes on these plasmids encode for capsule production, edema factor, lethal factor, and protective antigen.

Bacillus thuringiensis is an insect pathogen and is used extensively as a biopesticide *(68). Bacillus thuringiensis* produces Cry proteins, which when ingested by insects can cause lysis of mid-gut epithelial cells, septicemia, and death. The genes for Cry proteins are located on plasmids, and production of the proteins occurs during sporulation. Cry proteins form large crystal aggregates that account for up to 25% of the dry weight of the spore and are visible by microscopic examination after staining *(9, 68)*. The presence of Cry protein crystals is the only phenotypic means to differentiate *B. thuringiensis* from *B. cereus.*

Currently, *B. anthracis, B. cereus,* and *B. thuringiensis* are classified as distinct species; however, there has been considerable debate as to whether the

present taxonomy should be revised. Ash et al. *(7)* reported the 16S rDNA sequence (1,446 bp) of *B. anthracis* Sterne was identical to that of *B. cereus* NCTC 11143. Ash et al. *(6)* also reported only two nucleotide differences in the 23S rDNA sequences of *B. anthracis* and *B. cereus.* Harrell et al. *(31)* sequenced a 143-144 bp fragment of the intergenic spacer region (ISR) between genes for 16S and 23S rDNA in two *B. anthracis* strains (Ames and Vollum) and *B. cereus* ATCC 14579^T. They found only one nucleotide difference, a deletion, between the sequences of *B. anthracis* strains and *B. cereus*. One nucleotide difference was also detected in the 121-bp ISR between the housekeeping genes *gyrA* and *gyrB.* Helgason et al. *(32)* recommended the three species should be classified as a single species based on the high level of similarity (92.2-99.6%) observed in pairwise comparison of sequences from nine housekeeping genes. Ko et al*. (42)* studied the genetic relationship of 52 *B. anthracis, B. cereus,* and *B. thuringiensis* isolates by sequencing seven genes (*rpoB, gyrB, pycA, mdh, mbl, mutS,* and *plcR*) and reported *B. cereus* and *B. thuringiensis* strains were not separated into distinct clusters based on the sequences of these genes. For five of the seven genes, *B. anthracis* sequences yielded a distinct cluster. However, for *pycA,* one *B. cereus* strain had a sequence identical to that of *B. anthracis,* and for *mdh,* two *B. cereus* strains and two *B. thuringiensis* strains yielded sequences identical to that of *B. anthracis.* Priest et al. *(67)* studied the phylogenetic relationship of 11 *B. anthracis,* 38 *B. cereus,* and 53 *B. thuringiensis* isolates using sequences of seven housekeeping genes, and isolates were separated into eight distinct lineages. It is noteworthy that *B. cereus* isolates

were present in seven of the eight lineages and were absent only from the cluster of *B. anthracis* strains.

In addition, several researchers have reported isolates with characteristics that do not allow for unequivocal classification of strains as *B. anthracis* or *B. cereus*. Hoffmaster et al. *(35)* reported a *B. cereus* isolate recovered from a patient with life-threatening pneumonia had phenotypic characteristics similar to *B. cereus* and yet carried a plasmid (pBCOX1) that had 99.6% similarity to the pOX1 plasmid of *B. anthracis*. The isolate was also found to produce a capsule like *B. anthracis*.

Marston et al. *(55)* reported 23 *B. anthracis* strains from the culture collection at the Centers for Disease Control and Prevention yielded anomalous results when screened with standard microbiological and PCR methods. It was found that six of the isolates yielded amplicons with primers for *B. anthracis* chromosomal and plasmid markers. However these isolates were found to be gamma phage-resistant like *B. cereus*. Seventeen of the isolates did not yield the expected PCR reaction products for *B. anthracis* but were found to be gamma phage susceptible.

Differentiation of *B. anthracis, B. cereus*, and *B. thuringiensis* may be hindered by horizontal gene transfer among these species. Van der Auwera et al. *(89)* demonstrated plasmid exchange between an emetic strain of *B. cereus* and *B. thuringiensis.* Rasko et al. *(69)* compared the chromosomes of *B. anthracis* Ames, *B. cereus* ATCC 10987 (cheese isolate), and *B. cereus* 14579^{T} and found *B. cereus* ATCC 10987 possesses a plasmid that resembles pOX1 of

B. anthracis. They reported 65% of proteins coded by genes on the *B. cereus* ATCC10987 plasmid were homologous to proteins coded by genes on pOX1, and 50% of the genes were in a syntenic location. However, the plasmid of *B. cereus* ATCC 10987 did not carry genes for lethal factor, protective antigen, or edema factor, which are characteristic of *B. anthracis.*

2.1.1.2 *Bacillus mycoides, Bacillus pseudomycoides*, and *Bacillus weihenstephanensis*

Bacillus cereus can be differentiated from the remaining members of the *B. cereus* group by colony morphology and growth temperature. *Bacillus mycoides* and *B. pseudomycoides* exhibit rhizoidal growth on agar plates while *B. cereus* does not *(59, 60, 74)*. *Bacillus weihenstephanensis* was proposed to differentiate psychrotrophic and mesophilic strains of *B. cereus*. Lechner et al. *(46)* classified strains as *B. weihenstephanensis* on the basis of signature sequences for genes encoding for cold shock protein A and 16S rDNA and by growth at 7℃ but not 43℃. These criteria have been contested by Stenfors and Granum *(78)*, who provided evidence for intermediates between the two species.

2.1.2 Reservoirs

Bacillus cereus is commonly associated with soil, and until recently, it was widely believed that *B. cereus* exists in soil only in the sporulated state. However, Vilain et al. *(90)* recently presented evidence the microorganism is capable of a complete saprophytic life cycle–growth, germination, and sporulation–in soil. The prevalence of *B. cereus* and *B. weihenstephanensis* in soil is reportedly influenced by climate, and von Stetten et al. *(92)* evaluated 1,060 *B. cereus* group isolates from soil samples collected from alpine, temperate, and tropical climates. They reported only mesophilic *B. cereus* spores were isolated from soil in the tropical climate, but both *B. cereus* and *B. weihenstephanensis* were present in temperate climate soil. *Bacillus weihenstephanensis,* however, was found to predominate in alpine soil samples.

Bacillus cereus has been isolated from several different types of soil. Hendriksen et al. *(34)* isolated *B. cereus*-like colonies from Danish sandy loam and reported counts of 1.8×10^5 CFU/g. One hundred ninety-two isolates were selected for identification at the species level, and from these isolates, it was found 93.7% were *B. weihenstephanensis* and 6.3% were *B. cereus.* Altayar and Sutherland *(4)* determined the level and incidence of *B. cereus* in soil collected from parks, woodlands, and fields and reported the highest incidence and level of *B. cereus* occurred in samples collected from fields growing arable crops. *Bacillus cereus* was detected in 20 (100%) soil samples collected from such fields with a mean count of 2.5×10^4 CFU/mL. *Bacillus cereus* was also detected

in 15 (75%) samples collected from fields grazed by livestock with a mean count of 1.3 $\times 10^3$ CFU/mL. The prevalence of *B. cereus* in soil samples obtained from woodlands was lower as the microorganism was detected in only 5 (25%) of the samples. Christiansson et al. *(13)* collected soil samples from a dairy environment and found 72 (100%) samples contained *B. cereus* spores with counts ranging from 3.0×10^2 to 1.4×10^5 CFU/ g. Te Giffel et al. *(86)* enumerated *B. cereus* in soil, grass, bedding, feedstuff, and feces samples collected from six farms in the Netherlands and found the highest counts for *B. cereus* vegetative cells (10^3-10^7 CFU/g) and spores (10^2-10^5 CFU/mL) occurred in soil. It was also noted that among the environmental samples, soil yielded the highest percentage (66%) of psychrotrophic isolates.

2.2 *Bacillus cereus* in milk

2.2.1 Spoilage microorganism

As a soil bacterium, *B. cereus* is a common contaminant of raw agricultural commodities, and it is frequently associated with raw milk *(44, 49, 81, 84)*. *Bacillus cereus* spores may be transferred to the udders of cows through soil and grass as the animals are pasteured *(28, 56)*. Contamination of raw milk then can occur during the milking process. Christiansson et al. *(13)* suggested soil is the primary source of *B. cereus* contamination in raw milk and reported

significant correlations between dirtiness of the teats and access alley and contamination of raw milk with spores. Slaghuis et al. *(72)* investigated the effect of housing on the prevalence of *B. cereus* spores in raw milk from seven farms in the Netherlands. *Bacillus cereus* spores were detected in 23% of bulk tank milk samples collected from farms at which the cows were pasteured whereas *B. cereus* was found in only 4% of bulk tank milk samples from farms at which the cows were housed throughout the year. McKinnon and Pettipher *(56)* reported a seasonal effect in contamination of raw milk with sporeformers. They examined spore contamination in milk from three farms in which the herds were pastured during the summer months and from one farm at which the cows were housed indoors throughout the year. During the summer months, psychrotrophic spore counts in grass and soil ranged from 2.35 to 5.46 $\log_{10}$ CFU/g, and a 10 to 24% increase in the proportion of psychrotrophic sporeformers to the total spore count was observed in milk from farms at which the cows were pasteured.

Contamination of raw milk with *B. cereus* spores can lead to the development of quality defects in pasteurized milk. Spores in raw milk are sufficiently heat-resistant to survive high-temperature short-time (HTST) pasteurization (72°C), and decimal reduction times well exceeding the 15 sec heat treatment have been published. For example, Novak et al. *(63)* reported $D_{72°C}$=246 min for *B. cereus* ATCC 9818 spores in skim milk. Instead of destroying spores, the heat treatment can trigger spores to germinate, and temperatures between 65 and 75°C have been reported as optimal for heat-activation of *B. cereus* spores *(70)*. After pasteurization, psychrotrophic strains

are capable of replicating and reaching high levels in milk during refrigerated storage.

During growth in milk, *B. cereus* can produce lipases and proteinases that degrade milk components. The term “bitty cream” is used to describe quality defects caused by the activity of lipases, produced by *B. cereus,* leading to aggregation of fat globules and development of rancid and fruity off-flavors *(57)*. “Sweet curdling” is the term used to describe quality defects caused by proteinases produced by *B. cereus*. The action of proteinases may lead to “sweet curdling” gelation of the milk in the absence of a reduction in pH and development of bitter off-flavors *(57)*. In milk that is sweet curdled, the consumer may notice the formation of a curd on the bottom of the carton *(65)*.

2.2.2 Foodborne pathogen

The presence of *B. cereus* in pasteurized milk is also a concern because this microorganism is recognized as a foodborne pathogen and is capable of causing two types of foodborne illness–diarrheal and emetic syndromes.

The diarrheal syndrome is a toxicoinfection that occurs when ingested cells or spores produce enterotoxin *(77)*. Symptoms typically begin 8 to 16 hours after the consumption of the contaminated food and include abdominal pain and diarrhea *(70)*. Cases of the diarrheal syndrome may be misdiagnosed as *Clostridium perfringens* food poisoning because the symptoms are similar. The

diarrheal syndrome is often associated with consumption of cooked meats, and the infectious dose has been estimated to range from 5 x 10^4 to 10^{11} cells *(19, 51, 70, 73)*

Bacillus cereus has been shown to produce five enterotoxins: hemolytic enterotoxin (Hbl), non-hemolytic enterotoxin (Nhe), enterotoxin FM (EntFM), enterotoxin T (BceT), and cytotoxin K *(*CytK, *Table 2-1, 70)* Three of these toxins, CytK, Hbl, and Nhe, have been associated with foodborne illnesses, and it is currently thought that each of the toxins can act independently to cause symptoms associated with the diarrheal illness. Most research has focused on Hbl and Nhe because these toxins were associated with the diarrheal syndrome earlier than the other toxins. Hbl and Nhe are both three component toxins, and all components are required for biological activity *(50, 70)*. Currently commercial tests are only available for detection of Hbl and Nhe (Table 2-1). The BCET-RPLA (Oxoid) is used for detection of the L_2 component of Hbl, whereas the *Bacillus cereus* Diarrhoeal Enterotoxin Visual Immunoassay (Tecra) is specific for NheA.

Table 2-1: Toxins associated with diarrheal and emetic illnesses caused by *B. cereus (23, 28, 70).*

Toxin	Type	Size (kDa)	Genes	Commercial tests available for detection
Diarrheal illness				
Hemolytic enterotoxin (Hbl)	Protein, three components[1]			
	B	38	*hblA*	
	L_2	43	*hblC*	BCET-RPLA (Oxoid)
	L_1	39	*hblD*	
Non-hemolytic enterotoxin (Nhe)	Protein, three components			
	NheA	41	*nheA*	*Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay (Tecra)
	NheB	40	*nheB*	
	NheC	37	*nheC*	
Enterotoxin T (BceT)	Protein, one component	41	*bceT*	
Enterotoxin FM (EntFM)	Protein, one component	45	*entFM*	
Cytotoxin K (CytK)	Protein, one component	34	*cytK*	
Emetic illness				
Emetic toxin (cereulide)	Cyclic peptide synthesized by a non-ribosmal peptide synthetase	1.2	*ces*[2]	

[1] A fourth gene, *hblB*, is present in the operon, but the protein has not yet been isolated.

[2] Gene encoding cereulide peptide synthetase.

The emetic syndrome is an intoxication that occurs when an individual ingests food containing the emetic toxin, cereulide (Table 2-1). Cereulide is a small cyclic dodecadepsipeptide that is synthesized by a nonribosomal peptide synthetase *(22)*. Cereulide is acid, heat, and trypsin resistant, and therefore is not degraded in the food by thermal processing or in the gastrointestinal tract *(22)*. The symptoms associated with the emetic illness typically commence 1 to 5 hours after consumption of food containing emetic toxin and include nausea, vomiting, and abdominal cramping. Cases of the emetic syndrome may be misdiagnosed as the symptoms mimic those of *Staphylococcus aureus* food poisoning *(70)*. The emetic syndrome has been associated with consumption of starch-based foods, and a majority of the cases have been linked to rice dishes. It is estimated that 10^5-10^8 CFU/g are required to produce illness, and the amount of emetic toxin in foods implicated in outbreaks has been reported to range from 0.01 to 1.28 µg/g body weight *(1, 28)*.

2.2.2.1 Association of milk with foodborne illness

In 1997, a risk assessment study of *B. cereus* in pasteurized milk was conducted by Notermans et al. *(62)* in the Netherlands. Information pertaining to milk consumption, storage time and temperature was collected from 273 households, and *B. cereus* was enumerated in pasteurized milk collected from six processing plants. Notermans et al. *(62)* estimated between 10^9 and 10^{10}

100 mL portions of milk were consumed in the Netherlands that year, and of these, 7 and 4% were estimated to contain >10^5 and >10^6 *B. cereus*/mL, respectively. That finding was significant because these portions contained *B. cereus* counts exceeding the minimum estimated infectious dose for the diarrheal and emetic syndromes. Notermans et al. *(62)* also reported spoilage of the milk was not likely to prevent individuals from consuming these portions as quality defects were not observed until *B. cereus* counts exceeded 10^7 CFU/mL.

Langeveld et al.*(43)* evaluated the effect of consuming pasteurized milk with *B. cereus* to obtain information about the allowable level of *B. cereus* for that product. Naturally contaminated milk was stored for 3 to 14 days at 7.5°C, and 34 healthy human subjects consumed milk samples over the course of three weeks. Of the 259 milk samples consumed, only 18 cases of gastrointestinal illness were reported (Table 2-2). The greatest number of complaints was observed among individuals who ingested >10^8 *B. cereus* cells, and complaints included abdominal pain, diarrhea, and nausea. Langeveld et al. *(43)* reported pasteurized milk containing up to 10^7 *B.* cereus/mL did not have off-flavors and concluded healthy adults are unlikely to become ill from consuming pasteurized milk containing less than 10^5 *B. cereus/* mL.

Table 2-2: Number of milk samples consumed and complaints by the number of *B. cereus* cells ingested *(43)*.

No. *B. cereus* cells ingested	No. of consumptions	No. complaints
$<10^6$	132	5 (3.8)[a]
10^6-10^7	32	2 (6.3)
10^7-10^8	26	2 (7.7)
$>10^8$	69	9 (13.0)

[a] Percentage of consumptions that yielded complaints

Numerous researchers have speculated as to why *B. cereus* foodborne illnesses have not been associated with consumption of pasteurized milk. Granum *(28)* has suggested the number of foodborne illnesses attributed to pasteurized milk may be greatly underestimated because of the mildness and duration of the illness, misdiagnosis of the causative agent, or quality defects caused by *B. cereus* prevent consumers from ingesting highly contaminated milk. Stadhouders et al. *(76)* has suggested the ability of *B. cereus* to cause the diarrheal illness is related to the state (vegetative or sporulated) of the cell. In pasteurized milk, *B. cereus* cells are predominantly in the vegetative state because HTST pasteurization leads to heat activation of the spores, and *B. cereus* is not capable of sporulation in pasteurized milk *(76)*. In the vegetative state, *B. cereus* cells are susceptible to the acidic conditions in the gastrointestinal tract; however, in the sporulated state, cells are capable of surviving. This hypothesis is supported by the work of several other researchers. Clavel et al. *(14)* evaluated survival of *B. cereus* vegetative cells and spores inoculated at 10^6-10^7 CFU/mL in acid media simulating the human stomach.

Bacillus cereus spores survived in the acidic conditions and exhibited less than a 1.5 $\log_{10}$ CFU/mL reduction in simulated gastric medium with pH values between 1.0 and 5.2. In contrast, the vegetative cells were highly suspectible to the acidic conditions, and 3.0 $\log_{10}$ CFU/mL reductions were observed within 2 h. Duc et al. *(20)* and Wilcks et al. *(95)* provided evidence for survival and persistance of *B. cereus* spores in the gastrointestintal tract. Duc et al. *(20)* reported three *B. cereus* strains were detected in the feces of mice 18 days after administration of 10^9 spores. Wilcks et al. *(95)* studied survival of *B. cereus* spores and vegetative cells in the gut of human-flora-associated rats and found *B. cereus* was only detected in the intestinal contents of rats which had ingested spores.

Typical storage conditions of pasteurized milk do not appear to be conducive for production of the emetic toxin, cereulide. Finlay et al. *(25)* evaluated the ability of seven emetic toxin-producing *B. cereus* strains to grow in skim milk medium between 10 and 50°C and found 12 and 46°C to be the minimum and maximum growth temperatures, respectively. Agata et al. *(1)* quantified the amount of cereulide in various foods inoculated with 10^3 *B. cereus*/g and incubated at 30°C for 24 h (Table 2-3). Cereulide was not detected in milk incubated statically; however with shaking, the level of cereulide produced was greater than levels in starch-based foods, which have been associated with the emetic illness. It should be noted such conditions, however, would not typically be encountered in the processing or storage of pasteurized milk.

Table 2-3: Quantification of cereulide, the emetic toxin, produced by *B. cereus* in various foods during incubation at 30℃ for 24 h *(1)*.

Food	Viable count (CFU/g)	Toxin titer (ng/g)
Boiled rice	3.4	320
Fried rice	3.2	320
Spaghetti	6.6	160
Tempura	2.7	160
Bread	5.2	20
Milk (stationary)	7.7	<5
Milk (shaking)	4.3	640

2.2.3 Level and incidence of *B. cereus* in milk

A number of studies have been conducted to determine the level of *B. cereus* contamination in pasteurized milk. Interestingly, most studies have been conducted outside the U.S.

Coghill and Juffs *(16)* examined the incidence of psychrotrophic spore-forming microorganisms in pasteurized milk and cream from ten dairy processing facilities in Queensland, Australia. Psychrotrophic sporefomers were detected in 31% of the 167 samples, and of the 23 representative isolates selected for identification, 15 were *B. cereus*. Coghill *(15)* also determined the incidence and characterized thermoduric psychrotrophs in raw and pasteurized milk, ice cream, and butter obtained from dairies in South East Queensland. Two-hundred psychrotrophic isolates were obtained from the milk and milk products, and of these isolates, 14 (7.0%) were reported to be resistant to a 15 sec heat treatment at 73℃. Of those 14, 5 were classified as *Bacillus* spp., but classification was

not conducted to the species level. Ahmed et al. *(3)* determined the level and incidence of *B. cereus* in 400 samples of milk and milk products collected from retail outlets in Madison, Wisconsin over a five-month period. *Bacillus cereus* was detected in 9 (9%) of raw milk samples, 35 (35%) of pasteurized milk samples, 7 (14%) of Cheddar cheese samples, and 48 (48%) ice cream, but was not isolated from any of 50 yogurt samples. Counts in the samples ranged from <10-3,800 CFU per g or mL. Wong et al. *(96)* determined the incidence of *B. cereus* in 293 dairy products purchased from local markets in Tapei, Taiwan. *Bacillus cereus* was detected in 27% of the milk powder samples, 17% of ice cream samples, 17% of fermented milks, 52% of ice cream samples, and 35% of soft serve ice cream samples. The microorganism was also detected in 2% of pasteurized milk and fruit-flavored reconstituted milk samples, but the number of samples evaluated was not provided. Griffiths and Phillips *(29)* analyzed retail packs of freshly pasteurized milk in Scotland and found 68.8% of 80 samples contained psychrotrophic spores of *Bacillus* spp. with counts ranging from less than 0.5 to 170 spores/L. Te Giffel et al. *(85)* collected 334 pasteurized milk samples from household refrigerators in the Netherlands, and *B. cereus* was isolated from 133 (40%) of the samples. Larsen and Jorgensen *(44)* examined 458 samples of Danish pasteurized full-fat milk (3.5%), low-fat milk (1.5%) and double cream (48%) stored for 8 days at 7˚C and found *B. cereus* was present in 257 (56%) of the samples. Although there was no significant difference in the percentage of *B. cereus* positive samples for each of the products, significantly

higher mean counts were observed in the double cream (3.2 x 10^6 CFU/mL) compared to the full-fat (6 x 10^2 CFU/mL) and low-fat (5 x 10^2 CFU/mL) milks.

In another study, Larsen and Jorgensen *(45)* studied changes in the aerobic plate count and *B. cereus* and *Bacillus* spp. counts throughout the shelf life of pasteurized milk. Although the population of *Bacillus* spp. remained constant (~1.5 $\log_{10}$ CFU/mL) during 8 days of storage at 7˚C, aerobic plate counts and *B. cereus* counts increased exponentially (Figure 2-1). *Bacillus cereus* counts increased from <0.1 $\log_{10}$ CFU/mL on day 0 to ~ 2.5 $\log_{10}$ CFU/mL on day 8. In addition, the population of *B. cereus* positive cartons also increased with time with *B. cereus* being isolated from 2 of 27 samples on day 0, and 24 of the 27 samples at the end of the incubation period (Figure 2-2).

In the U.S., Fromm and Boor *(26)* conducted a survey to identify the predominant spoilage microorganisms in pasteurized milk during refrigerated storage at 6˚C. Raw milk samples were collected from three commercial processing facilities in New York State, pasteurized (30 min at 63˚C), and then analyzed on days 0, 7, 14 and 17. In this study, Gram-positive sporeformers accounted for 87% of the 197 isolates recovered. In their study, the predominant genera isolated were *Paenibacillus* spp. (39%) and *Bacillus* spp. (32%). Interestingly, the most common species was *B. cereus*, accounting for 18 of the 63 isolates. *Bacillus cereus* was identified using API 50 CH strips and CHB medium and by partial 16S rDNA sequences.

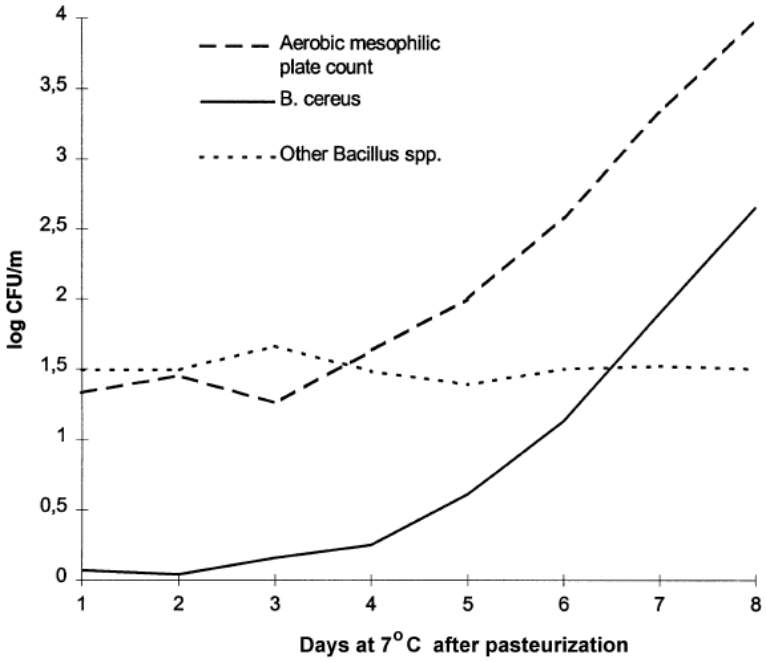

Figure 2-1: Mean counts of *Bacillus cereus*, aerobic mesophilic plate count (APC) and other *Bacillus* spp. in pasteurized milk stored at 7˚C after pasteurization *(45)*.

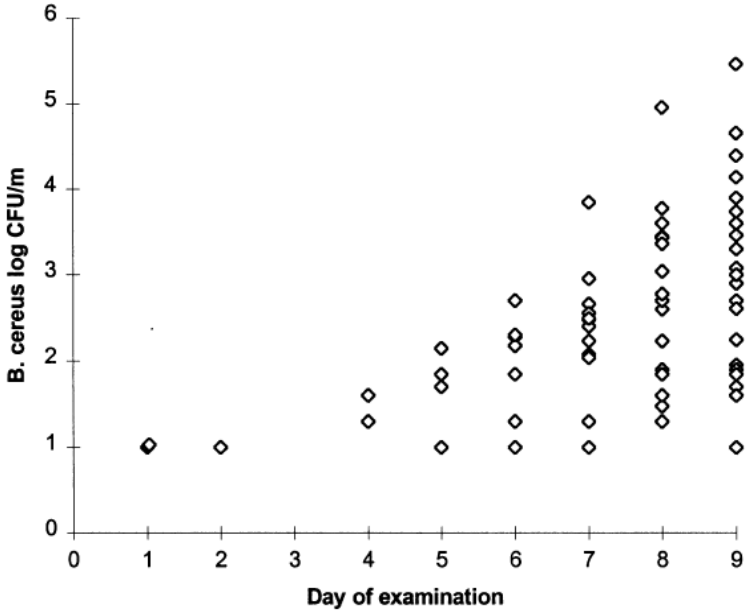

Figure 2-2: Scatterplot of the count of *Bacillus cereus*/mL in cartons with more than 10 *B. cereus*/mL versus day after production of pasteurized milk. Each point indicates the level of *B. cereus*/mL in one carton *(45)*.

Zhou et al. *(98)* collected 54 full-fat samples of milk from supermarkets in China and determined the incidence of *B. cereus, B. mycoides*, and *B. thuringiensis* in milks representing 4 brands. *Bacillus cereus* was isolated from 15 (71%) of 21 milks collected in the spring and 11 (33%) of 33 milks collected in the autumn. *Bacillus thuringiensis* and *B. mycoides* were detected in only 6 (11%) and 1 (2%) of the 54 samples, respectively.

Bartoszewicz et al. *(8)* determined the level of *B. cereus* group spores in 44 milk samples collected from a dairy farm and two dairy processing facilities in Poland. *Bacillus cereus* group spores were detected in 19 of 20 milk samples

collected from the dairy farm, and counts ranged from 32 spores/mL in the autumn to 200 spores/mL in the spring. *Bacillus cereus sensu stricto* and *B. weihenstephanensis* were the predominant species isolated from farm raw milk and accounted for 68 to 88% of the *B. cereus* group isolates.

2.2.4 Identification of contamination sources in dairy processing

Bacillus cereus is particularly challenging to control because contamination of milk may occur at numerous points in production and processing. Contamination may occur through spores in raw milk or post-pasteurization contamination. Several studies have been conducted to identify sources of *B. cereus* in pasteurized milk.

Te Giffel et al. *(84)* determined the incidence of *B. cereus* in raw and pasteurized milk from two dairy processing facilities in the Netherlands, and isolates were characterized according to growth at 7°C in milk and carbohydrate metabolism using API 50 CHB strips. In Dairy Plant I, the incidence of *B. cereus* in milk increased from 9 (35%) raw milk samples in the bulk tank to 20 (61%) samples collected from the outlet of the pasteurizer. *Bacillus cereus* was detected in 22 (71%) of the final packages from Plant I. At Plant II, which manufactured powdered milk, 7 (35%) raw milk samples contained *B. cereus* and 12 (60%) the milk powder samples contained the microorganism. Te Giffel et al. *(84)* reported more than 20 different carbohydrate utilization patterns were

observed among isolates from raw and pasteurized milk at the plants, and certain biotypes were only associated with isolates in milk after pasteurization. Te Giffel et al. *(84)* suggested *B. cereus* cells may adhere to and colonize equipment and processing lines, and thus serve as continuous source of contamination. In a subsequent study, Te Giffel et al. *(87)* studied adherence of *B. cereus* spores in a heat exchanger. *Bacillus cereus* spores were inoculated in milk at 1.6×10^3 CFU/mL and a 60-min processing run at 80°C was conducted. After the processing run, the unit was disassembled, and the tubes were swabbed. Counts from the tubes were reported to range from <1.0 to 2.6×10^3 CFU/cm^2.

Lin et al. *(49)* identified sources of *B. cereus* in pasteurized milk using fatty acid profiles. The study included 122 environmental samples and 232 milk samples collected along processing lines of two facilities, and the fatty acid profiles of 546 isolates were determined. The isolates were divided into 18 subgroups, and of these, 11 were found to contain isolates from both raw and pasteurized milk. Isolates in the 11 subgroups accounted for 94.8% of those analyzed. Five subgroups were composed only of isolates from pasteurized milk and accounted for 3.7% of the isolates analyzed. Lin et al. *(49)* noted that environmental sources of the post-pasteurization contamination were not identified for all the subgroups. Based on these findings, Lin et al. *(49)* concluded spores in raw milk were a major source of *B. cereus* in the pasteurized product and suggested further research should be conducted to identify sources of post-pasteurization contamination, particularly from sources in processing lines.

Svensson et al. *(82)* conducted a study to identify contamination sites and endemic strains in a dairy processing facility. A one-day in-depth study was conducted to identify sources of the microorganism during processing. Samples were collected from farm bulk tanks, the silo at the dairy processing facility, the balance tank, after the pasteurizer, the product tank, before the filling machine, and consumer packages, and *B. cereus* isolates were subtyped using randomly amplified polymorphic DNA (RAPD)-PCR. Raw milk samples were collected from the bulk tanks of 29 farms supplying milk to the dairy processing facility, and 36 *B. cereus* isolates from these samples yielded 29 RAPD-PCR profiles. Fifty-six *B. cereus* isolates were obtained from 7 raw milk samples collected at the dairy processing facility, and these isolates yielded 6 RAPD-PCR profiles. Only one isolate from farm bulk tank milk and milk in the silo at the dairy processing facility were found to have identical RAPD-PCR profiles. Twenty-one samples of pasteurized milk were collected from the dairy processing facility, and the 54 isolates from these samples yielded 19 RAPD-PCR patterns. Svensson et al. *(82)* found 17 of the 24 RAPD-PCR profiles from isolates in milk samples at the dairy processing facility were only associated with pasteurized milk. Based on this finding, Svensson et al. *(82)* suggested contamination of the milk occurred at the dairy processing facility, and a long-term study was then conducted to identify endemic *B. cereus* strains.

The long-term study involved collection of pasteurized milk samples from the product tank and consumer packages over the course of two years. The RAPD-PCR profiles obtained from isolates during the long-term study were

compared with isolates from the one-day in-depth study. Identical patterns were observed among isolates from pasteurized milk in the long-term study and unpasteurized milk at the dairy during the one-day in-depth study. Because of this finding, Svensson et al. *(82)* hypothesized contamination of milk occurred in the silo at the dairy processing facility.

Svensson et al. *(81)* studied *B. cereus* contamination in raw milk collected from the silos of eight dairy processing facilities in Sweden over the course of a year and reported evidence for the existence of in-house flora of *B. cereus.* They reported isolation of mesophilic *B. cereus* strains with identical RAPD-PCR profiles from all of the dairies on several sampling ocassions. Svensson et al. *(81)* hypothesized these strains may be able to persist in silos because they are better able to adhere to the surface in the raw milk tanks or are sufficiently heat resistant to survive cleaning procedures.

Svensson et al. *(83)* reported involvement of a pasteurizer in the contamination of milk. They observed a significant increase in *B. cereus* counts in milk collected after the pasteurizer during the first and second hours of a processing run, with counts of <10 and 8,500 *B. cereus* CFU/mL, respectively. In addition, from hour two onwards, more than 70% of the *B. cereus* isolates after the pasteurizer yielded identical RAPD-PCR profiles. Svensson et al. *(83)* suggested the increase in *B. cereus* counts between hours one and two of the processing run, may have been due to a biofilm that was present in the pasteurizer. It was thought the pasteurizer may not have been adequately cleaned between processing runs and a biofilm was able to form.

Eneroth et al. *(24)* reported contamination of pasteurized milk with *B. cereus* occurred during the filling operation. They evaluated milk samples immediately after pasteurization, from the product tank, immediately before filling, and from consumer packages, and found milk samples collected after the filling operation yielded isolates with more RAPD-PCR profiles than samples taken before the filling machine. One RAPD-PCR type was obtained from numerous samples collected throughout the processing day, and it was suggested these data indicated the presence of a stationary contamination source in the vicinity of the filling machine.

2.2.5 Summary

The results of the previously described studies provide evidence that contamination of milk with *B. cereus* is occurring in dairy processing facilities. It is noteworthy that all of these studies have been conducted outside the U.S. Currently there is limited information on the incidence and level of *B. cereus* in pasteurized milk in the U.S. Such information is important given the economic and public health significance of this microorganism.

These studies also highlight the need for additional work to improve our understanding of contamination in routes in dairy processing facilities and to characterize *B. cereus* strains that are able to persist in the processing environment. In order for such research to be conducted, a reliable subtyping method is needed for tracking isolates and identification of persistant strains.

2.3 Molecular methods for tracking *B. cereus*

Subtyping methods can be evaluated on the basis of several criteria including typeablity, discriminatory power, and reproducibility *(79, 93)*. The term "typeability" describes the proportion of isolates that can be classified using a given method, and ideally should be 100% *(79)*. Discriminatory power quantifies the probability that two unrelated strains will be classified as different subtypes using a given method and can be determined using Simpson's Index of Discrimination *(40)*. Ideally, a subtyping method should yield a value of 1. Reproducibility refers to the ability of a subtyping method to yield the same type with repeated testing of given strain *(79)*. Additional factors to consider in the evaluation and selection of a subtyping method are automation and ease of use, cost, and time required to obtain results *(93)*.

The RAPD-PCR method developed by Nilsson et al. *(61)* is the subtyping method that has been used most extensively for tracking *B. cereus* contamination in milk production and the processing environment *(5, 11, 13, 24, 52, 81, 82)*. RAPD-PCR is a nucleic acid pattern-based method that involves the use of arbitrary primers, approximately 9 to 10 bp in length for the PCR reaction *(64)*. If the primers anneal to the chromosome within several kilobases of each other and in the proper orientation, amplification of the region may occur. The resulting PCR products are then separated by agarose gel electrophoresis, and the banding patterns compared. It is expected that the number and size of bands will vary between strains, reflecting genetic differences.

RAPD-PCR is particularly attractive for large-scale typing studies aimed at identifying contamination sources on the farm and in the processing facility because it is highly discriminatory and the procedure is relatively simple and inexpensive compared to other molecular subtyping methods *(61)*. In addition, no prior knowledge of DNA sequences is necessary to use this method. This method, however, also has significant drawbacks including difficulties in standardization and interpretation of the results and low portability of the data *(18, 27, 64, 66)*.

The PCR reaction and subsequent banding patterns can be influenced by numerous factors including PCR reagents (template, primers, $MgCl_2$ concentration), PCR conditions, and variation between thermocyclers *(66)*. The banding patterns can also vary depending on the electrophoresis conditions and the staining and destaining protocol. This factor is particularly important in visualization of faint bands. Another issue with RAPD-PCR is the ambiguous nature of data interpretation. Results may vary depending on interpretation of faint bands and the number of bands used to differentiate strains *(66)*.

Durak et al. *(21)* recently developed a sequence-based subtyping method for *Bacillus* spp. and *Paenibacillus* spp. dairy isolates. The method involves sequencing a 632-bp fragment of *rpoB,* which encodes for the beta-subunit of RNA polymerase. This method has been used by Huck et al. *(37-39)* to study contamination of raw and processed milk with *Bacillus* spp. and *Paenibacillus* spp. However, a disadvantage of this method is that a single primer set is used for PCR amplification, and thus, not all isolates may yield amplification products.

Huck et al. *(39)* has suggested to overcome this problem, *rpoB* sequences could be used with additional gene sequences as part of a multigene sequencing strategy (e.g. MLST).

2.3.1 Multilocus sequence typing

An alternative method that could be used for subtyping *B. cereus* is multilocus locus sequence typing (MLST). Since the time MLST was first proposed by Maiden et al. *(54)*, this subtyping method has been widely applied for evolutionary and population studies of bacteria, and several MLST schemes have been developed for members of the *Bacillus cereus* group *(10, 33, 67, 75, 88)*. MLST involves sequencing and comparison of 400 to 600 bp fragments of six to ten housekeeping genes *(53)*. Housekeeping genes were selected for this analysis because these genes are ubquitous in a population, and mutations accumulate slowly over time and are under netural selection. The advantages of this method are that it yields data that is unambiguous, highly reproducible and highly portable.

Despite the advantages of MLST, this method may not be feasible for large-scale tracking studies because of the cost of sequencing six to ten housekeeping genes. Another option could be to use a MLST scheme that is composed of housekeeping and hypervariable genes. Hypervariable genes typically accumulate mutations at a faster rate than housekeeping genes and

may provide greater discriminatory power. Thus, fewer genes may be necessary to differentiate isolates *(17)*. The use of hypervariable genes in MLST schemes is particularly useful in local epidemiology as would be encountered in investigating an outbreak or tracking strains in a processing facility *(17, 97)*.

For pathogenic microorganisms such as *B. cereus*, hypervariable genes may include those which are associated with virulence *(17)*. Several other researchers have used virulence genes in MLST schemes for other foodborne pathogens *(2, 47, 48)*. For example, Zhang et al. *(97)* developed what has been termed a "multi-virulence-locus sequence typing scheme" for *Listeria monocytogenes.* That typing scheme was found to have greater discriminatory power than *EcoR*I-ribotyping, (D.I.=0.921) and pulsed-field gel electrophoresis (0.970). In addition, Zhang et al. *(97)* reported the virulence genes used in the typing scheme possessed a greater number of nucleotide polymorphisms than fragments of nine housekeeping genes.

Sukhnanand et al. *(80)* developed a three-gene MLST scheme for *Salmonella enterica* that consisted of two housekeeping genes and one virulence gene. In the development of the MLST scheme, they first compared the ability of seven genes–five housekeeping genes and two virulence genes– to differentiate 25 *Salmonella enterica* isolates (Table 2-4). The most discriminatory gene was *manB*, a housekeeping gene, which yielded 7 allelic types, and the two virulence genes, *fimA* and *spaN*, yielded 5 and 4 allelic types, respectively. Sukhnanand et al. *(80)* then developed a three-gene MLST scheme based on sequences of two three housekeeping genes, *mdh* and *manB* and one virulence gene, *fimB*.

The seven- and three- gene MLST schemes were compared for differentiating 25 *Salmonella enterica* isolates, and the three-gene scheme yielded the same number of sequence types as the scheme consisting of five housekeeping genes and two virulence genes.

Table 2-4: Summary of allelic types obtained from housekeeping and virulence gene sequences of 25 strains of *Salmonella enterica* (*80*).

Gene	Protein	Type of Gene	Length (bp)	Allelic Types	% Polymorphic Sites
aceK	Isocitrate dehydrogenase kinase/phosphatase	Housekeeping	1,598	4	1.75
icdA	Isocitrate dehydrogenase	Housekeeping	1,251	4	1.60
manB	Phosphomannomutase	Housekeeping	813	7	2.83
mdh	Malate dehydrogenase	Housekeeping	643	5	2.02
panB	Ketopantoate hydroxymethyltransferase	Housekeeping	792	4	4.92
fimA	Fimbrial gene A	Virulence	558	5	2.51
spaN	Surface presentation of antigens	Virulence	1,010	4	1.78

2.3.2 Development of a molecular subtyping method using housekeeping and virulence gene sequences

Bacillus cereus is known to possess a number of virulence genes that could potentially be used with housekeeping genes that are part of an existing MLST scheme. However, one issue with using hypervariable genes is that unlike housekeeping genes, these genes may not be ubiquitous in a population. Several virulence genes including enterotoxin FM (*entFM*), hemolytic enterotoxin (*hblA, hblB, hblC,* and *hblD*), non-hemolytic enterotoxin (*nheA, nheB,* and *nheC*)

and sphingomyelinase (*sph*) have been reported to be widely distributed among *B. cereus* strains and could be used as part of a MLST scheme *(30, 36, 94)*.

2.4 Objectives

Based on this, research will be undertaken to evaluate the microbiological quality of pasteurized milk. The study will include enumeration of *B. cereus* in pasteurized milk during refrigerated storage at 4 and 7°C. In addition, a MLST scheme will be developed for *B. cereus* using housekeeping and virulence gene sequences.

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Chapter 3

Microbiological Evaluation of Pasteurized Milks

3.1 Introduction

The presence of *B. cereus* in pasteurized milk is of concern to the dairy industry because this microorganism can influence the quality and safety of the product. *Bacillus cereus* can form endospores which are sufficiently heat-resistant to survive high-temperature short-time pasteurization (72°C for 15 sec) and capable of germination in milk during refrigerated storage *(12, 18)*. During growth, vegetative cells can produce proteinases and lipases which degrade milk components, leading to quality defects such as sweet curdling and bitty cream *(16)*. "Sweet curdling" is the term used to describe quality defects caused by proteinases produced by *B. cereus*. The action of proteinases may lead to gelation of the milk and development of bitter off flavors. In milk that is sweet curdled, the consumer may notice the formation of a curd on the bottom of the carton. The term "bitty cream" is used to describe quality defects caused by the activity of lipases. This quality defect is characterized by aggregation of fat globules and development of rancid and fruit off-flavors.

In addition to causing quality defects, the presence of *B. cereus* in pasteurized milk may also pose a health hazard for the consumer because *B. cereus* is a human pathogen and has been shown to cause emetic and diarrheal

illnesses *(23, 25)*. *Bacillus cereus* foodborne outbreaks have not been associated with pasteurized milk; however, some strains isolated from pasteurized milk have been shown to possess virulence genes and exhibit cytotoxicity *(26, 27)*. It has been suggested the discrepancy between the presence of *B. cereus* in milk and outbreaks may be due to underreporting, which is influenced by the mildness and duration of the illness or misdiagnosis of the causative agent *(9)*. The development of quality defects associated with high numbers of *B. cereus* may also prevent consumers from ingesting highly contaminated milk.

Numerous international studies have been conducted to determine the incidence and level of *B. cereus* in pasteurized milk, but recent research in the U.S. is limited. Te Giffel et al. *(29)* collected 334 pasteurized milk samples from household refrigerators in the Netherlands, and *B. cereus* was isolated from 133 (40%) of the samples. Larsen and Jorgensen *(14)* examined 458 samples of Danish pasteurized full-fat milk (3.5%), low-fat milk (1.5%) and double cream (48%) stored for 8 days at 7˚C and found *B. cereus* was present in 257 (56%) of the samples. Mean counts in double cream, full-fat, and low-fat milk were 3.2 x 10^6, 6 x 10^2, and 5 x 10^2 CFU/mL, respectively. Zhou et al. *(31)* collected 54 full-fat samples of milk from supermarkets in China and determined the incidence of *B. cereus* in milks representing 4 brands. *Bacillus cereus* was isolated from 15 (71%) of 21 milks collected in the spring and 11 (33%) of 33 milks collected in the autumn.

In the U.S., Fromm and Boor *(8)* conducted a study to identify the predominant microorganisms in pasteurized milk during 14 days of refrigerated storage. Pasteurized milk for the study was collected from three dairy processing facilities in New York State during 2002 and 2003 and stored at 6°C. Gram-positive sporeformers were found to account for 87% of the 197 isolates from the milk, and the predominant genera isolated were *Paenibacillus* spp. (39%) and *Bacillus* spp. (32%). Interestingly, the most common species of *Bacillus* isolated was *B. cereus,* accounting for 18 of 63 isolates. Based on these findings, Fromm and Boor *(8)* suggested Gram-positive sporeformers have considerable spoilage potential and may present the next hurdle in shelf-life extension of pasteurized milk.

The objectives of the present study were to evaluate the microbiological quality of commercial milk and to determine the level and incidence of *B. cereus*.

3.2 Materials and Methods

3.2.1 Collection and storage of the samples

Cartons (946 mL) of commercially pasteurized whole, 2%, 1%, and skim milk with the same sell-by date were purchased from grocery stores located in Centre County, PA, and stored at 4 and 7˚C (Table 3-1). Milks were collected from four manufacturers and 224 samples were analyzed.

Table 3-1: Summary of milk samples

Manufacturer	Type of milk[1]							
	Whole milk		2% milk		1% milk		Skim milk	
	4˚C	7˚C	4˚C	7˚C	4˚C	7˚C	4˚C	7˚C
1	2[2]	2	2	2	0	0	2	2
2	2	2	2	2	2	2	2	2
3	2	2	1	1	2	2	1	1
4	2	2	2	2	2	2	2	2
n=	8	8	7	7	6	6	7	7

[1]At least 12 different samples of each product were obtained. A fresh (unopened) package was used for each analytical time point.
[2]Indicates samples from two different sell-by dates collected at different times. Samples stored at 4 and 7˚C were from the same sell-by date. Samples were collected based on availability in the market.

3.2.2 Microbiological analysis of pasteurized milk

Since the date of manufacture for each of the products was not known, microbiological analysis of each product was conducted on the sell-by date (day 0), 12 days before the sell-by date (-12), 7 days before the sell-by date (-7), and 7 days after the sell-by date (+7).

Aerobic plate counts were determined by spread plating serial dilutions of milk prepared in phosphate buffer *(4)* on duplicate plates of Standard Methods Agar (SMA; Becton Dickinson and Co., (BD) Sparks, MD) followed by incubation of the inoculated plates for 48 h at 32˚C *(13)*.

Aerobic spore counts were determined by heating 5 mL of pasteurized milk for 12 min at 80˚C in 5 mL Wheaton gold band glass ampules (Wheaton, Millville, NJ). The heated milk was cooled on ice for 5 min, and then serial dilutions were spread plated on SMA supplemented with 0.1% soluble starch *(7)*. Mesophilic spore counts were determined by incubating duplicate plates for 48 h

at 32˚C. Psychrotrophic spore counts were determined by incubating duplicate plates for 10 d at 7˚C.

Bacillus cereus counts were determined by preparing duplicate spread plates of mannitol-egg yolk-polymyxin (MYP) agar (BD) followed by incubation of the inoculated plates for 48 h at 32˚C *(1)*. Colonies that appeared pink-red and were surrounded by a zone of precipitate on MYP agar were recorded as presumptive *B. cereus*.

Isolates from MYP agar were confirmed as members of the *B. cereus* group using the protocol described by Hansen et al. *(11, Table 3-2)*. Briefly, stock cultures were streaked for isolation on nutrient agar plates (BD) and incubated for 24 h at 32°C. A sterile toothpick was used to transfer cells from a single colony to the well of a PCR plate (Eppendorf, Germany), and the reaction mixture (25 µL) consisting of GoTaq Green Master Mix (Promega Corp., Madison, WI) and 0.5 µM of each primer was added. PCR conditions consisted of an initial denaturation for 10 min at 95°C, 45 s at 63°C, and 45 s at 72°C, followed by a final extension of 7 min at 72°C.

Isolates confirmed as members of the *B. cereus* group were then confirmed as *B. cereus* using the multiplex PCR protocol described by Choo et al. *(3, Table 3-2).* Briefly, stock cultures were streaked on nutrient agar plates and incubated for 24 h at 32°C. A sterile toothpick was used to transfer cells from a single colony to the well of a PCR plate, and the reaction mixture (25 µL)

Table 3-2: Primers used for confirmation of isolates as members of the *B. cereus* group and as *B. cereus.*

Gene amplified	Primer name	Sequence (5'—›3')	Product size	References
B. cereus group 16S rDNA (16S rDNA)	F[1]: S-S-Bc-200a-S-18	TCGAAATTGAAAGGCGGC	288 bp	*(3)*
	R: S-S-Bc-470-a-A-18	GGTGCCAGCTTATTCAAC		
DNA gyrase (*gyrB*)	F: BCFW1	GTTTCTGGTGGTTTACATGG	374 bp	*(11)*
	R: BCrevenew	TTTTGAGCGATTTAAATGC		
Crystal encoding gene (*cry*)	F: K3	GCTGTGACACGAAGGATATAGCCAC	1.6-1.7 kbp	*(11)*
	R: K5	AGGACCAGGATTTACAGGAGG		

[1]F: Forward primer, R: Reverse primer

consisting of GoTaq Green Master mix, 0.6 µM of BCFWI and BCrevenew primers, and 1.0 µM of K3 and K5 primers was added. PCR conditions consisted of an initial denaturation of 10 min at 95°C, 25 cycles of 1 min at 94°C, 2 min at 55°C, and 1.5 min at 72°C, followed by a final extension of 7 min at 72°C.

After amplification, 10 µL of PCR reaction mixture was added to 1.0% agarose gel and electrophoresed at 115 volts for approximately 1.5 h in 0.5x TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). Gels were stained with ethidium bromide, and bands were visualized with a UV transilluminator (302 nm). Results were recorded with an AlphaImager™ 3300 Gel Documentation System (Alpha Innotech Corp., San Leandro, CA).

Isolates confirmed as members of the *B. cereus* group using molecular methods were also confirmed as *B. cereus* group members by evaluating anaerobic utilization of glucose, nitrate reduction, production of acetylmethylcarbinol from glucose (VP test), tyrosine decomposition, and lysozyme resistance as described by Rhodehamel and Harmon *(21).*

3.3 Results and Discussion

The objectives of the study were to assess the microbiological quality of commercial pasteurized milk from four manufacturers and to determine the level and incidence of *B. cereus*. Storage temperatures of 4 and 7˚C were selected based on the recommendations made by the Dairy Practices Council (DPC) and the Pasteurized Milk Ordinance (PMO). The DPC recommends milk be stored at

4.4˚C or less and suggests that storage at 1.1 to 3.8˚C is most desirable for maximizing shelf-life *(22)*. The PMO allows milk to be stored at temperatures up to 7˚C *(30)*.

3.3.1 Aerobic plate counts

On day -12, aerobic plate counts of milk samples were low; milks stored at 4 and 7˚C, yielded counts ranging from ≤1.0 to 2.4 $\log_{10}$ CFU/mL and ≤1.0 to 2.0 $\log_{10}$ CFU/mL, respectively (Tables 3-3 & 3-4)**.** By day 0, the sell-by-date, milks stored at 4 and 7˚C, yielded aerobic plate counts ranging from ≤1.0 to 8.5 $\log_{10}$ CFU/mL and ≤1.0 to 8.6 $\log_{10}$ CFU/mL, respectively. On day 0, seven (25%) milk samples stored at 4˚C had aerobic plate counts equal to or greater than 4.3 $\log_{10}$ CFU/mL (20,000 CFU/mL), the limit outlined in the PMO. It is noteworthy that six of those seven cartons were processed by manufacturer 4; thus, 75% of the milk samples obtained from manufacturer 4 and incubated at 4°C had counts above the PMO limit. On day 0, 23 (82%) milk samples stored at 7˚C had aerobic plate counts equal to or greater than 4.3 $\log_{10}$ CFU/mL. All milk samples processed by manufacturers 2, 3, and 4 had counts exceeding 4.3 $\log_{10}$ CFU/mL when stored at 7˚C.

Table 3-3**:** Aerobic plate counts and presumptive *B. cereus* counts of pasteurized milk samples held at 4°C for up to 7 days past the sell-by date. Data are the range observed in samples including whole, 2% milk fat, 1% milk fat, and skim milk.

Manufacturer	No. samples	Range of microbial counts (log_{10} CFU/mL)							
		Day relative to the sell-by date							
		-12		-7		0		+7	
		APC[1]	BCC[2]	APC	BCC	APC	BCC	APC	BCC
1	6	ND-2.0	ND	ND-1.2	ND	ND-1.5	ND	ND-2.6	ND
2	8	ND-2.4	ND	1.5-2.0	ND	1.7-3.6	ND	2.7-7.5	ND
3	6	ND-1.7	ND	ND-3.3	ND	1.6-8.4	ND	3.4-8.9	ND-2.6
4	8	1.2-2.2	ND	ND-2.3	ND	2.1-8.5	ND-4.2	4.1-8.5	ND-3.2

[1]APC: Aerobic plate counts were determined on Standard Methods Agar incubated for 48 h at 32˚C.
[2]BCC: Presumptive *Bacillus cereus* counts were determined on MYP Agar incubated 48 h at 32˚C.
[3] ND: Not detected;≤1.0 log_{10} CFU/mL. The lowest dilution was 10^{-1}.

Table 3-4: Aerobic plate counts and presumptive *B. cereus* counts of pasteurized milk samples held at 7°C for up to 7 days past the sell-by date. Data are the range observed in samples including whole, 2% milk fat, 1% milk fat, and skim milk.

Manufacturer	No. samples	Range of microbial counts (log_{10} CFU/mL)							
		Day relative to the sell-by date							
		-12		-7		0		+7	
		APC[1]	BCC[2]	APC	BCC	APC	BCC	APC	BCC
1	6	ND	ND	ND-1.5	ND	ND-6.9	ND-6.7	ND-7.7	ND-4.2
2	8	1.2-1.9	ND	1.7-7.3	ND-2.3	4.5-7.4	ND-6.9	5.7-8.3	ND-7.3
3	8	1.2-1.6	ND	2.1-7.3	ND-6.9	6.9-8.6	ND-8.9	6.7-8.9	5.7-8.9
4	6	ND-2.0	ND	2.0-6.6	ND-2.5	4.3-8.6	ND-7.2	7.1-8.9	ND-8.1

[1]APC: Aerobic plate counts were determined on Standard Methods Agar incubated for 48 h at 32°C.
[2]BCC: Presumptive *Bacillus cereus* counts were determined on MYP Agar incubated 48 h at 32°C.
[3] ND: Not detected;≤1.0 log_{10} CFU/mL. The lowest dilution was 10^{-1}.

The findings of the present study highlight the need to improve the microbiological quality of pasteurized milk. The high aerobic plate counts observed in the milk samples from manufacturer 4 could have been the result of contamination of the milk during production or processing or temperature abuse during transport and retail. The initial counts of milk samples from manufacturer 4 were low, suggesting the issue is contamination of the milk with microorganisms which are capable of replication at low temperatures. Because these samples were collected from grocery stores, information pertaining to the processing facility and potential contamination sources was not available. A tracking study could be conducted in the processing facility of manufacturer 4 to identify sources of the psychrotrophic microorganisms. These microorganisms could be spore-formers which are present in the raw milk or post-pasteurization contaminants. The filling operation has been identified as a critical contamination site because psychrotrophic microorganisms in aerosols or condensation may enter the milk during this operation *(5, 20)*

Carey et al. *(2)* also reported a high percentage of pasteurized milk samples with aerobic plate counts exceeding the limit defined in the PMO. In that study, pasteurized milk samples were collected from 23 dairy processing facilties in New York State over a period of 10 years, and aerobic plate counts of the milks were determined during storage at 6.1°C for 14 days. On the initial day of testing (1 or 2 days after processing), the yearly percentage of samples with counts below 20,000 CFU/mL ranged from 99 to 100%, and none of the samples had counts exceeding 1.0×10^6 CFU/mL. After 7 days of storage, the

percentage of milks with counts below 20,000 CFU/mL ranged from 25 to 50%, and 19 to 36% of the samples yielded counts greater than 1.0×10^6 CFU/mL. By day 14, 12 to 32% of the milks had counts below 20,000 CFU/mL and 53 to 70% yielded counts greater than 1.0×10^6 CFU/mL. These findings along with those of the present study, suggest further work is needed to improve microbiological quality of pasteurized milk.

3.3.2 Mesophilic and psychrotrophic spore counts

Mesophilic and psychrotrophic spore counts of milks stored at 4 and 7˚C were low and ranged from ≤1.0 to 2.2 $\log_{10}$ CFU/mL and ≤1.0 to 1.7 $\log_{10}$ CFU/mL, respectively (Table 3-5). Low spore counts in raw and pasteurized milk have been reported by other researchers. Hanson et al. *(12)* determined mesophilic and psychrotrophic spore counts of raw milk produced in the U.S. Mesophilic spore counts ranged from 0.5 to 1.7 $\log_{10}$ spores/mL with an average of 1.2 $\log_{10}$ spores/mL. Psychrotrophic spores were not detected in any of the milk samples examined in the study. In 1990, Griffiths and Phillips *(10)* reported psychrotrophic spore counts in pasteurized milk ranged from <0.5 to 170 (2.2 $\log_{10}$) spores/L with an average of 17 (1.2 $\log_{10}$) spores/L.

Mesophilic and psychrotrophic spore counts were compared between milks of varying fat contents because previous research has suggested the association of spores with milk fat *(19)*. Mesophilic spores were detected in 47

Table 3-5: Mesophilic and psychrotrophic spore counts of pasteurized milk samples held at 4 and 7˚C for up to 7 days past the sell-by date. Data are the range observed in samples from 4 manufacturers.

Fat level[1]	No. samples	Spore counts (log_{10} CFU/mL)							
		Day relative to the sell-by date							
		-12		-7		0		+7	
		MS[2]	PS[3]	MS	PS	MS	PS	MS	PS
Whole	16	ND[4]-1.8	ND	ND-1.8	ND-1.2	ND-2.18	ND	ND-1.7	ND
2% milk fat	14	ND-1.7	ND	ND-1.6	ND	ND-1.74	ND-1.7	ND-1.6	ND
1% milk fat	12	ND-2.0	ND	ND-2.2	ND	ND-1.81	ND	ND-1.7	ND
Skim milk	14	ND-1.7	ND	ND-1.6	ND-1.2	ND-1.60	ND	ND-1.6	ND

[1]Combined data for milks stored at 4 and 7˚C.
[2] MS: Mesophilic spore counts were determined by plating heat-treated milk (12 min at 80˚C) on Standard Methods Agar supplemented with 0.1% soluble starch. Inoculated plates were incubated for 48 h at 32˚C.
[3] PS:Psychrotrophic spore counts were determined by plating heat-treated milk (12 min at 80˚C) on Standard Methods Agar supplemented with 0.1% soluble starch. Inoculated plates were incubated for 10 d at 7˚C.
[4] ND: Not detected; the lowest dilution was 10^{-1}.

(73%) of whole milk samples, 43 (77%) of 2% milk samples, 44 (92%) of 1% milk samples, and 44 (79%) of skim milk samples, and psychrotrophic spores were detected in 2 (3%) of whole milk samples, 2 (4%) of 2% milk samples, 2 (4%) of 1% milk samples, and 4 (7%) of skim milk samples. Psychrotrophic spores were detected in milk samples from manufacturers 1, 3, and 4.

The difference in the incidence of mesophilic and psychrotrophic spores in the pasteurized milk could be related to contamination of the milk at the dairy processing facility. Svensson et al. *(28)* conducted a survey of milk from silos of eight dairy processing facilities in Sweden and reported contamination of milk silos with mesophilic *B. cereus* was widespread. Mesophilic *B. cereus* strains with identical RAPD-PCR profiles were isolated from all of the dairies on several sampling ocassions. Svensson et al. *(28)* hypothesized these strains may be able to persist in silos because they are better able to adhere to the surface in the raw milk tanks or are sufficiently heat resistant to survive cleaning procedures.

The difference in the incidence of mesophilic and psychrotrophic spores in milk may also be influenced by the percentage of germination following pasteurization. Stadhouders et al. *(24)* reported what they termed “fast-” and “slow- germinating” spores. “Fast-germinating” spores exhibit extensive (97-99%) germination within 24 h when stored at 20°C whereas “slow-germinating” spores do not germinate within 24 h and require a more intensive heat treatment for activation. Thus, it may be that a higher percentage of psychrotrophic spores germinate in response to the pasteurization heat treatment. Griffiths and Phillips

(10) compared psychrotrophic spore counts in raw and pasteurized milk and reported >95% of the spores germinated after pasteurization. Griffiths and Phillips *(10)* also noted that after heat shock, psychrotrophic sporeformers exhibited a more extensive germination during storage at 5°C than mesophilic sporeformers.

3.3.3 *Bacillus cereus* counts

On day -12, *B. cereus* counts in milk samples stored at 4 and 7°C were ≤1.0 $\log_{10}$ CFU/mL (Tables 3-3 & 3-4). The growth of *B. cereus* during refrigerated storage appeared to be influenced by storage temperature. On day 0, the sell-by date, *B. cereus* was detected in 2 of 28 (7%) cartons stored at 4˚C, and counts of milks ranged from ≤1.0 to 4.2 $\log_{10}$ CFU/mL. However, *B. cereus* was detected in 16 (57%) of milk samples at 7°C, and counts ranged from ≤1.0 to 8.9 $\log_{10}$ CFU/mL. Milk processed by manufacturer 4 had the highest incidence of *B. cereus* as six (75%) of the samples tested positive for the microorganism on the sell-by date. *Bacillus cereus* was detected in 4 milk samples from manufacturers 2 and 3.

When Larsen and Jorgensen *(15)* studied growth of *B. cereus* in pasteurized milk stored for 9 days at 7˚C, they found on day 1, *B. cereus* was only detected in 2 (7.4%) of 27 milk samples, and counts in these 2 samples were low (≤ 1.0 $\log_{10}$ CFU/mL). By day 9, *B. cereus* was detected in 24 (89%) of 27 milk samples, and counts ranged from approximately 1.0 to 6.0 $\log_{10}$ CFU/mL.

Te Giffel et al*. (29)* studied the incidence of *B. cereus* in 334 pasteurized milk samples from household refrigerators in the Netherlands. For 258 (77%) milk samples, *B. cereus* counts were below the detection limit (<0.7 CFU/mL), but 5% yielded *B. cereus* counts >3.7 $\log_{10}$ CFU/mL. Te Giffel et al. *(29)* noted the influence of storage temperature on the number of samples testing positive for *B. cereus*. They reported one day after the expiration date, 67% of milks stored above 7˚C tested positive for *B. cereus* while only 25% of milks at temperatures below 7˚C tested positive for *B. cereus*.

A culture collection consisting of 58 presumptive *B. cereus* group isolates from MYP agar was screened using primers specific for *B. cereus* group 16S rDNA. Isolates confirmed as members of the *B. cereus* group were then screened using primers specific for the DNA gyrase sequence shared by *B. cereus* and *B. thuringiensis* and the crystal encoding gene of *B. thuringiensis.* Isolates were classified as *B. cereus* if PCR yielded only a 374 bp product (Figure 3-1).

Of the culture collection screened using molecular methods, 54 (93%) of the 58 presumptive *B. cereus* isolates were confirmed as members of the *B. cereus* group. These isolates were also confirmed as *B. cereus* group members using the biochemical tests described by Rhodehamel and Harmon (21). Of these 54, 17 (32%) were further confirmed as *B. cereus*.

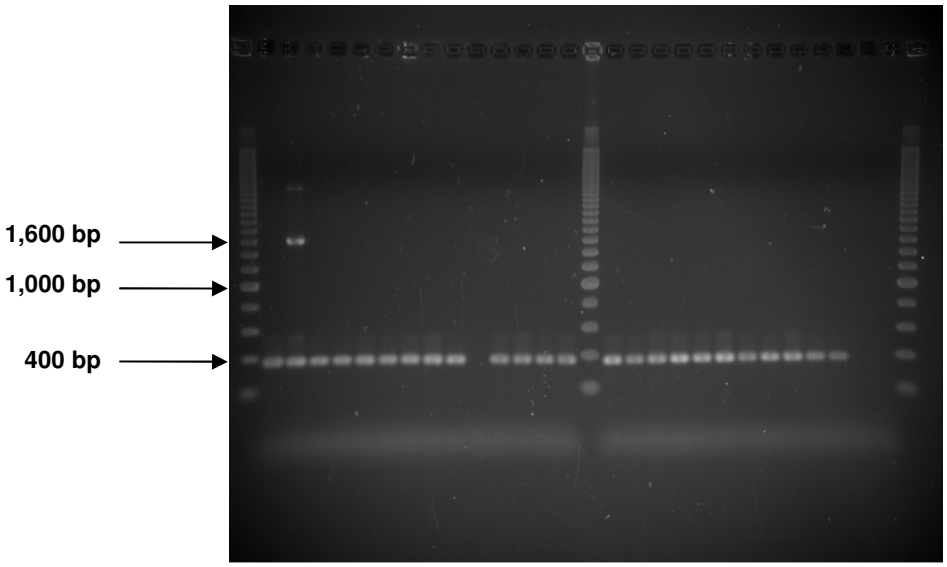

Figure 3-1: Confirmation of presumptive *B.* cereus dairy isolates using the multiplex PCR protocol described by Choo et al. *(3).* Presumptive *Bacillus cereus* isolates were recovered from milk using MYP agar and yielded pink colonies surrounded by a zone of precipitate. Using the multiplex PCR protocol, *B. thuringiensis* is expected to yield two PCR products: 374 bp (DNA gyrase) and 1.6-1.7 kbp (Crystal encoding gene). *Bacillus cereus* is expected to yield one band (374 bp). 1, 15 & 30 = 200 bp molecular weight markers; 2 = *Bacillus cereus* ATCC 14579^{T} (reference strain); 3 = *Bacillus thuringiensis* ATCC 33679 (reference strain); 4 to 12 = *B. cereus* (known isolates); 13-14, 16-28 = *B. cereus* milk isolates.

Because *B. cereus* is a human pathogen and has been associated with foodborne illness, contamination of milk with this microorganism could potentially present a public health hazard. *Bacillus cereus* can cause two types of foodborne illness—an emetic and a diarrheal syndrome. The emetic syndrome occurs when an individual consumes food containing cereulide, a toxin produced by *B. cereus* during growth. The emetic syndrome is not likely to be associated with the consumption of pasteurized milk under typical storage conditions

because 12°C has been reported as the minimum temperature for cereulide production by *B. cereus (6).*

The diarrheal syndrome is a toxicoinfection that occurs when an individual consumes contaminated food, and the microorganism subsequently proliferates in the gastrointestinal tract *(25)*. This type of syndrome is of concern with pasteurized milk because *B. cereus* has been shown to be a common contaminant and capable of reaching high levels during refrigerated storage. The minimum infectious dose for the diarrheal syndrome has been estimated to be 5.0 $\log_{10}$ CFU/mL, and in the present study 9 (32%) the milk samples yielded *B. cereus* counts to exceeding that number on the sell-by date.

In 1997, Notermans et al. *(17)* conducted a risk assessment study of *B. cereus* in pasteurized milk. The study involved collection of information from 273 households in the Netherlands on milk consumption, storage time, and temperature. In addition, *B. cereus* was enumerated in pasteurized milk from six processing facilities. Based on this information, Notermans estimated that 7% of the milk consumed in the Netherlands contains counts exceeding 5.0 $\log_{10}$ CFU/mL.

3.4 Conclusions

The findings of this study indicate further work is needed to improve the microbiological quality of pasteurized milk. On the sell-by date, a high percentage of pasteurized milks were found to contain microbial counts

exceeding the bacterial limit set in the PMO. In addition, approximately one-third of the milk samples at held at 7°C yielded *B. cereus* counts exceeding the minimum estimated infectious dose for the diarrheal syndrome. Although *B. cereus* is most widely recognized as a spoilage microorganism in milk, the results obtained in this study suggest, *B. cereus* could also present a health hazard to the consumer. MYP agar appears to be a reliable medium for the isolation of *B. cereus* group isolates as a high percentage of presumptive isolates were confirmed as members of the *B. cereus* group using biochemical and molecular methods.

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Chapter 4

Development of a sequence-based molecular subtyping method for *Bacillus cereus* dairy isolates

4.1 Introduction

Bacillus cereus is a Gram-positive spore-former that is recognized as a foodborne pathogen and has been associated with the development of quality defects in pasteurized milk *(9, 27, 34, 36)*. Low levels of *B. cereus* are commonly present in milk after pasteurization, and the microorganism is capable replication during refrigerated storage *(23, 30)*. During growth in milk, *B. cereus* can produce lipases and proteinases that degrade milk components leading to the development of quality defects, which limit the shelf life of the product.

Currently, there is interest in tracking *B. cereus* to identify sources of contamination in dairy processing facilities to allow for further study of endemic strains. The subtyping method that has been used most extensively for this purpose is randomly amplified polymorphic DNA (RAPD)-PCR *(7, 12, 25, 29, 39, 40)*. This fragment-based subtyping method is well-suited for large-scale tracking studies because it is highly discriminatory and relatively inexpensive and simple to perform compared to other subtyping methods. However, issues with reproducibility and low portability of the data are major drawbacks to using this

method *(31, 32)*. One alternative method for subtyping *B. cereus* dairy isolates is multilocus locus sequence typing (MLST). Several MLST schemes have been developed for the *Bacillus cereus* group, and Ehling-Schulz et al. *(11)* used the scheme of Helgason et al. *(16)* to study emetic-toxin producing *B. cereus* isolates *(4*, *16, 33, 35, 41)*. The advantages of MLST are that this subtyping method yields unambiguous, sequence-based data, which is highly portable. However, the cost associated with sequencing seven housekeeping genes per isolate may prohibit the use of this method for large-scale tracking studies.

A more cost-effective subtyping method for tracking *B. cereus* in dairy processing could be a two- or three-gene MLST scheme which utilizes a combination of housekeeping and virulence gene sequences. Such a subtyping method has not yet been developed for *B. cereus,* but methods are available for other pathogens. For example, Sukhnanand et al. *(38)* developed a three-gene MLST scheme for *Salmonella enterica* that consisted to two housekeeping genes and one virulence gene. The advantage of including virulence gene sequences in a MLST scheme is these genes may be more variable and, thus have greater discriminatory power than housekeeping genes *(1, 8, 43)*. Sukhnanand et al. *(38)* found their three-gene scheme yielded the same number of sequence types as a seven-gene scheme consisting of five housekeeping genes and two virulence genes.

Bacillus cereus possesses a number of virulence genes that could potentially be included in a MLST scheme, but in order for these genes to be included in a MLST scheme, they would need to be widely distributed among *B.*

cereus isolates. Numerous studies have indicated that *B. cereus* isolates differ in virulence gene carriage; however, several genes including those encoding for hemolytic enterotoxin (Hbl), non-hemolytic enterotoxin (Nhe), enterotoxin FM (EntFM), and sphingomyelinase (Sph) appear to be widely distributed *(14, 17, 37, 42)*.

The objectives of the present study were to i) determine the prevalence of genes enconding for the hemolytic enterotoxin (*hblA, hblB, hblC, hblD*), the non-hemolytic enterotoxin (*nheA, nheB, nheC*), enterotoxin FM (*entFM*), and sphingomyelinase (*sph*) among *B. cereus* isolates ii) to compare the number of allelic types obtained with housekeeping gene sequences in the MLST scheme of Helgason et al. *(16)* with selected virulence gene sequences, and iii) develop a two- or three-gene MLST scheme for *B. cereus* using housekeeping and virulence gene sequences.

4.2 Materials and Methods

4.2.1 *Bacillus cereus* isolates

The culture collection used for this study consisted of nine *B. cereus* reference strains and four *B. cereus* strains isolated from pasteurized milk from a single processing facility (Table 4-1). All strains exhibited pink-red colonies with precipitate on mannitol-egg yolk-polymyxin agar and were confirmed as *B.*

cereus using the multiplex PCR method described by Choo et al and referenced in Chapter 3 of this work *(6)*.

Table 4-1: *Bacillus cereus* strains used in this work

Isolate	Source	Provider	References
ATCC 14579[1]	Unknown	ATCC	*(15, 20, 28)*
ATCC 9818	Unknown	ATCC	*(21)*
038-2	Infant formula	R. Bennett	*(21)*
3802A/84	Milk	L. Beuchat	*(21)*
3812/84	Milk	L. Beuchat	*(21)*
F4512A/87	Milk	L. Beuchat	*(21)*
F4616/90	Milk	L. Beuchat	*(21)*
TJL-14	Sweet and sour pork	L. Beuchat	*(21)*
F4810/72	Cooked rice	L. Beuchat	*(15, 21)*
37	Pasteurized milk	D.Miller	
94	Pasteurized milk	D. Miller	
373	Pasteurized milk	D. Miller	
375	Pasteurized milk	D. Miller	

4.2.2 Prevalence of virulence genes among the isolates

Because numerous studies have suggested virulence gene carriage may vary among *B. cereus* isolates, the culture collection was screened for virulence genes including those encoding for the hemolytic enterotoxin (*hblA, hblB, hblC, hblD*), non-hemolytic enterotoxin (*nheA, nheB, nheC*), enterotoxin FM (*entFM*),

and sphingomyelinase (*sph*). Stock cultures were streaked on nutrient agar plates (BD, Becton, Dickinson and Co., Sparks, MD) and incubated for 24 h at 32℃. A sterile toothpick was used to transfer cells from a single colony to the wells of a PCR reaction plate. The PCR reaction mixture (25 µL) for all genes except *hblC* consisted of GoTaq Green Master Mix (Promega Corp., Madison, WI) and 0.5 µM of each primer (Table 4-2). For *hblC,* the PCR reaction mixture (25 µL) consisted of GoTaq Green Master mix and 1.0 µM of each primer. The PCR conditions for *hblA, hblC, hblD*, *nheA, nheB,* and *nheC* consisted of an initial denaturation of 10 min at 95˚C, 35 cycles of 1 min at 94˚C, 1 min at the annealing temperature, and 2 min at 72˚C, followed by a final extension of 5 min at 72˚C. For *hblB*, PCR conditions consisted of an initial denaturation of 10 min at 95˚C, 10 cycles of 10 s at 94˚C, 30 s at 58 ˚C, 2 min at 68˚C. This was followed by 20 cycles of 10 s at 94˚C, 30 s at 58 ˚C, 2 min (plus 20 s each cycle) at 68˚C, and a final extension of 7 min at 68˚C. For *entFM* and *sph*, the PCR conditions consisted of an initial denaturation of 10 min at 95˚C, 30 cycles of 30 s at 94˚C, 30 s at the annealing temperature, 30 s at 72 ˚C followed by final extension of 5 min at 72˚C.

After amplification, 10 µL of PCR reaction mixture was added to a 1.0% agarose gel and run at 115 volts for approximately 1.5 h with 0.5x TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). Gels were stained with ethidium bromide, and bands were visualized with a UV transilluminator (302 nm). Results were recorded with the AlphaImager™ 3300 Gel Documentation System (Alpha Innotech Corp., San Leandro, CA). Isolates were considered to

Table 4-2: Primer sets used for amplification of virulence genes in *B. cereus* reference strains and milk isolates.

Gene amplified	Primer name	Sequence (5'—›3')	Annealing temperature (˚C)	Product size	References
entFM	entFM-F	ATGAAAAAAGTAATTTGCAGG	52	1,269	*(3)*
	entFM-R	TTAGTATGCTTTTGTGTAACC			
hblA	ha-F[1]	AAGCAATGGAATACAATGGG	56	1,154	*(14)*
	ha-R	AGAATCTAAATCATGCCACTGC			
hblB	ha- F	AAGCAATGGAATACAATGGG	58	2,684	*(14)*
	hb-R	AATATGTCCCAGTACACCCG			
hblC	hc-F	GATAC(T,C)AATGTGGCAACTGC	58	740	*(14)*
	hc-R	TTGAGACTGCTCG(T,C)TAGTTG			
hblD	hd-F	ACCGGTAACACTATTCATGC	58	829	*(14)*
	hd- R	GAGTCCATATGCTTAGATGC			
nheA	na-F	GTTAGGATCACAATCACCGC	56	755	*(14)*
	na-R	ACGAATGTAATTTGAGTCGC			
nheB	nb-F	TTTAGTAGTGGATCTGTACGC	54	743	*(14)*
	nb-R	TTAATGTTCGTTAATCCTGC			
nheC	nc-F	TGGATTCCAAGATGTAACG	54	683	*(14)*
	nc-R	ATTACGACTTCTGCTTGTGC			
sph	sph-F	CGTGCCGATTTAATTGGGGC	58	558	*(17)*
	sph-R	CAATGTTTTAAACATGGATGCG			

[1]F: forward primer, R: reverse primer

be negative for the gene if the appropriate size band was not observed after two independent attempts to detect by PCR.

4.2.3 Comparison of housekeeping and virulence gene sequences

4.2.3.1 PCR amplification of housekeeping genes

DNA was isolated according to the method described by Choo et al. *(6)*. Briefly, a loop of each stock culture was transferred to 10 mL of trypticase soy broth (BD) and incubated for 24 h at 32˚C. A 2.0 mL aliquot of prepared culture was transferred to a sterile eppendorf tube and centrifuged at 14,000 rpm for 2 min to pellet the cells. The supernatant was discarded, and cells were resuspended in 1 mL sterile water. The resuspended cells were centrifuged at 14,000 rpm for 2 min, and the supernatant was discarded. The washed cells were resuspended in 0.2 mL of sterile water, and a loopful of Witcarb activated carbon (Witco Chemical Corp., NY) was added. Cells were held for 24 h at -20˚C and then heated in boiling water for 10 min. After heating, the Eppendorf tubes were placed in an ice water bath for 1 min. Cell debris was removed by centrifugation at 14,000 rpm for 5 min. The extracted DNA was transferred to a sterile eppendorf tube and stored at -20˚C. DNA concentrations were determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer by measuring absorbance at 260 and 280 nm (Nano-drop Technologies, Inc., Wilmington, DE).

Fragments of seven housekeeping genes including *adk* (encoding adenylate kinase), *ccpA* (catabolite control protein A), *ftsA* (cell division protein), *glpT* (glycerol-3-phosphate permease), *pyrE* (orotate phosphoriobosyltransferase), *recF* (DNA replication and repair protein), and *sucC* (succinyl coenzyme A synthetase, beta subunit) were amplified using primers and conditions described by Helgason et al. *(16, Table 4-3)*. The location of these genes on the 5.4 Mbp chromosome of *B. cereus* ATCC 14579^T from the origin or replication (*oriC*) as follows: *recF,* 3.3 kb; *adk* 137.0 kb; *glpT*, 651.8 kb; *sucC,* 3,813.2 kb; *pyrE,* 3,861.6 kb; *ftsA*, 3,886.8 kb; and *ccpA,* 4611.7 kb.

The reaction mixture (50 µL) consisted of Go Taq Green Master Mix with 0.8 mM each deoxynucleoside triphosphate, 0.4 µM of each primer, 2.4 mM $MgCl_2$, and 50 ng DNA. PCR conditions consisted of an initial denaturation of 10 min at 95˚C, 40 cycles of 1 min at 94˚C, 1 min at the annealing temperature, and 1 min at 72˚C, followed by a final extension of 7 min at 72˚C.

4.2.3.2 PCR amplification of virulence genes

A sterile toothpick was used to transfer cells from a single colony on a nutrient agar plate (BD) to the wells of a PCR reaction plate. The PCR reaction mixture (25 µL) consisted of GoTaq Green Master Mix (Promega Corp., Madison, WI) and 0.5 µM of each primer (Table 4-3). PCR conditions for *entFM* consisted of an initial denaturation of 10 min at 95˚C, 35 cycles of 1 min at 94˚C, 1 min at

Table 4-3: Primers for amplification and sequencing of housekeeping gene fragments as described in the MLST scheme Helgason et al. *(16)* and virulence genes *nheC* and *entFM*.

Genes	Primer name	Primer sequence (5'—›3')	Annealing temperature (˚C)	Fragment length (bp)
adk	adk F[1]	CAGCTATGAAGGCTGAAACTG	57	450
	adk R	CTAAGCCTCCGATGAGAACA		
ccpA	ccpA F	GTTTAGGATACCGCCCAAATG	59	418
	ccpA R	TGTAACTTCTTCGCGCTTCC		
ftsA	ftsA F	TCTTGACATCGGTACATCCA	57	401
	ftsA R	GCCTGTAATAAGTGTACCTTCCA		
glpT	glpT F	TGCGGCTGGATGAGTGA	52	330
	glpT R	AAGTAAGAGCAAGGAAGA		
pyrE	pyrE F	TCGCATCGCATTTATTAGAA	57	404
	pyrE R	CCTGCTTCAAGCTCGTATG		
recF	recF F	GCGATGGCGAAATCTCATAG	59	470
	recF R	CAAATCCATTGATTCTGATACATC		
sucC	sucC F	GGCGGAACAGAAATTGAAGA	59	504
	sucC R	TCACACTTCATAATGCCACCA		
entFM1	entFM1 F	TTTTAAGAGGATAGTAGGGAAAGGAA	54	703
	entFM1 R	CAACATAAGAACCACCAGTTTGA		
entFM2[2]	entFM2 F	CGAAACACAACAACCAACTACAA	54	739
	entFM2 R	GGAAAGCCTTTTCACTATACGA		
nheC	nc F	TGGATTCCAAGATGTAACG	54	683
	NC- R	ATTACGACTTCTGCTTGTGC		

[1] F: forward primer, R:reverse primer
[2] Two primers sets were used for amplification and sequencing of the entire gene.

54˚C, and 1 min at 72˚C, followed by a final extension of 7 min at 72˚C. PCR conditions for *nheC* were conducted as previously described.

4.2.3.3 Preparation of PCR reaction products for sequencing

After amplification, the size of the amplicons was determined using agarose gel electrophoresis, and then the PCR reaction products were purified as described by Huck *(18)*. Briefly, 10 µL of PCR reaction mixture was transferred to a PCR reaction tube and 0.5 µL of 10 U/ µL exonuclease in storage buffer (20 mM Tris-HCl [pH 7.5], 5 mM 2-mecaptoethanol, 0.5 mM EDTA, 50% glycerol, USB Corp., Cleveland, OH) and 0.5 µL of 1 U/ µL shrimp alkaline phosphatase in storage buffer (25 mM Tris-HCl [pH 7.6], 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 50% glycerol, USB) were added. The tubes were heated for 45 min at 37˚C to degrade the primers and unincorporated dNTPs and then for 15 min at 80˚C to inactivate the enzymes. Bidirectional sequencing with PCR primers was performed at the Pennsylvania State University Nucleic Acid Facility with an ABI PRISM® 3700 DNA Analyser (Applied Biosystems, Foster City, CA). Four sequences were obtained (two forward and two reverse) for each gene fragment.

4.2.3.4 Analysis of housekeeping and virulence gene sequences

Housekeeping and virulence gene sequences were aligned using MEGA *(*v. 4, *22)*. The *entFM* contigs were assembled using Proseq (v.2), and open reading frames were determined using Genemark.hmm *(13, 24)*. Phylogenetic trees were constructed for individual genes using the neighbor-joining method with 1,000 boostrap replicates (MEGA v. 4). For the MLST schemes, neighbor-joining trees based on allelic profiles were constructed with Tree drawing (www.PubMLST.org). The number of polymorphic sites was determined using ProSeq (v. 2). Simpson's Index of Diversity was calculated as described by Hunter and Gaston *(19)*.

4.3 Results

4.3.1 Prevalence of virulence genes among the *B. cereus* isolates

The incidence of the nine virulence genes was evaluated among 13 *B. cereus* isolates, and two virulence genes, *entFM* and *nheC* were detected in all of the strains (Table 4-4). Of the remaining genes, *nheB* and *sph* were the most prevalent and were detected in 12 (92%) and 11 (85%) of the isolates, respectively. *nheA* was detected in 10 (77%) of the isolates. PCR reaction products for *hblA, hblB, hblC,* and *hblD* were only obtained with 3 (23%), 4 (31%), 3 (23%), and 3 (23%) of the isolates, respectively. Virulence genes,

Table 4-4: Distribution of virulence genes among *B. cereus* reference strains and milk isolates.

Isolate	*nheA*	*nheB*	*nheC*	*hblA*	*hblB*	*hblC*	*hblD*	*entFM*	*sph*	Virulence gene profile[1]
ATCC 14579	+[2]	+	+	+	+	+	+	+	-	1
ATCC 9818	+	+	+	-	-	-	-	+	+	2
F4512A/87	+	+	+	-	-	-	-	+	+	2
F4616/90	+	+	+	-	-	-	-	+	+	2
038-2	+	+	+	-	-	-	-	+	+	2
3802A/84	+	+	+	-	-	-	-	+	+	2
3812/84	+	+	+	-	-	-	-	+	+	2
TJL-14	+	+	+	-	-	-	-	+	+	2
F4810/72	+	+	+	-	-	-	-	+	+	2
37	-	+	+	+	+	-	+	+	+	3
94	-	+	+	-	-	+	-	+	+	4
373	-	+	+	-	+	-	-	+	+	5
375	+	-	+	+	+	+	+	+	-	6
Positives	**10**	**12**	**13**	**3**	**4**	**3**	**3**	**13**	**11**	

[1] The virulence gene profile was based on the presence of absence of the nine virulence genes as determined by PCR.
[2] "+" indicates a positive result. "-"indicates a negative result. An isolate was considered negative for the gene after two independent attempts to detect by PCR.

entFM and *nheC*, were selected for further analysis in the development of the two-or three-gene MLST scheme.

Six virulence gene profiles were observed among the isolates (Table 4-4). The most common profile was detected among eight (62%) of the isolates and was positive for *nheA, nheB, nheC, entFM,* and *sph*¸ and negative for *hblA, hblB, hblC,* and *hblD.* Although isolates 37, 94, 373, and 375 were isolated from a single processing facility, these isolates were considered to be unique strains because of differences in the virulence gene profiles.

4.3.2 Comparison of housekeeping and virulence gene sequences

The number of allelic types obtained with sequences of housekeeping genes in the MLST scheme of Helgason et al. *(16)* ranged from four (*adk*, *recF,* and *sucC*) to seven (*ftsA* and *glpT*). The percentage of polymorphic sites among these genes ranged from 1.7% (*ftsA*) to 7.3% (*glpT*, Table 4-5 & 4-6). Virulence genes *entFM* and *nheC* yielded seven and six allelic types, respectively, and these genes exhibited a higher percentage of polymorphic sites (10.2 and 12.1%) than the housekeeping genes (Table 4-5 & 4-6).

Table 4-5**:** Genetic diversity of housekeeping gene and virulence sequences from 13 *B. cereus* strains. Housekeeping gene fragments were described in the MLST scheme Helgason et al. *(16)*.

Locus	Type of gene	Fragment length (bp)	No. alleles [c]	No.polymorphic sites
adk	Housekeeping	434 (648)[a]	4	16 (3.7)[b]
ccpA	Housekeeping	381 (996)	5	15 (3.9)
ftsA	Housekeeping	416 (1,299)	7	7 (1.7)
glpT	Housekeeping	342 (1,347)	7	25 (7.3)
pyrE	Housekeeping	423 (630)	6	13 (3.1)
recF	Housekeeping	474 (1,125)	4	32 (6.8)
sucC	Housekeeping	520 (1,158)	4	29 (5.6)
entFM	Virulence	1,332 (1,293)[b]	7	136 (10.2)
nheC	Virulence	586 (1,080) [b]	6	71 (12.1)

[a] The number in parentheses represents the total length of the gene.
[b] Size of the gene in *B. cereus* ATCC 14579^T.
[c] Each allelic type represents at least one nucleotide difference in the sequences.

Table 4-6: Allelic types and sequence types of the *B. cereus* isolates. Housekeeping gene fragments were described in the MLST scheme of Helgason et al. *(16)*.

Strain	Allelic types									Sequence type
	Housekeeping genes							Virulence genes		
	adk	*ccpA*	*ftsA*	*glpT*	*pyrE*	*recF*	*sucC*	*entFM*	*nheC*	
ATCC14579	1	1	1	1	1	1	1	1	1	1
ATCC9818	2	2	2	2	2	2	2	2	2	2
038-2	3	3	3	3	3	3	3	3	3	3
3802A/84	4	2	4	4	2	4	2	4	4	4
3812/84	4	2	4	4	2	4	2	4	4	4
F4512A/87	4	2	4	4	2	4	2	4	4	4
F4616/90	4	2	4	4	2	4	2	4	4	4
TJL-14	2	4	5	5	4	2	4	5	4	5
F4810/72	4	2	4	4	2	4	2	4	3	6
37	3	3	6	6	5	3	3	6	5	7
94	3	3	6	6	5	3	3	6	5	7
373	3	3	6	6	5	3	3	6	5	7
375	1	5	7	7	6	1	1	7	6	8

4.3.3 Development of a two- or three-gene MLST scheme

Various combinations of housekeeping genes from the MLST scheme and two virulence genes (*entFM* and *nheC*) were evaluated in the development of a two- or three-gene MLST scheme for *B. cereus*. Because the same distribution of allelic types was observed with the sequences of two housekeeping genes, *adk* and *recF*, *recF* was not included in the evaluation.

The number of sequence types obtained with the two-gene MLST schemes ranged from five (*ccpA* and *pyrE*, *adk* or *recF* and *sucC*) to eight (housekeeping genes and *nheC, entFM* and *nheC*, Table 4-7). The two-gene MLST schemes yielding eight sequence types all had a Discrimination Index of 0.88. The addition of a third gene to the MLST scheme did not increase the number of sequence types (Table 4-8). The seven-gene MLST scheme described by Helgason et al. *(16)* yielded seven sequence types, and the combination of the seven housekeeping genes and two virulence genes yielded eight sequence types (Table 4-8).

Table 4-7**:** The number of sequence types obtained with two-gene MLST schemes consisting housekeeping and virulence gene sequences.

Genes	No. sequence types
adk, ccpA	6
adk, ftsA	7
adK, glpT	7
adk, pyrE	7
adk, sucC	5
adk, entFM	7
adk, nheC	8
ccpA, ftsA	7
ccpA, glpT	7
ccpA, pyrE	6
ccpA, sucC	5
ccpA, entFM	7
ccpA, nheC	8
ftsA, glpT	7
ftsA, pyrE	7
ftsA, sucC	7
ftsA entFM	7
ftsA, nheC	8
glpT, pyrE	7
glpT, sucC	7
glpT, entFM	7
glpT, nheC	8
pyrE, sucC	6
pyrE, entFM	7
pyrE, nheC	8
sucC, entFM	7
sucC, nheC	8
entFM, nheC	8

Table 4-8: The number of sequence types and Discrimination Index obtained with three gene MLST schemes consisting of housekeeping and virulence gene sequences.

MLSTschemes	No. sequence	Discrimination Index[1]
ftsA, nheC, adk,	8	0.88
ftsA, nheC, ccpA	8	0.88
ftsA, nheC, glpT	8	0.88
ftsA, nheC, pyrE	8	0.88
ftsA, nheC sucC	8	0.88
ftsA, nheC entFM	8	0.88
glpT, nheC, entFM	8	0.88
adk, ccpA, ftsA, glpT, pyrE, recF, sucC	7	0.64
adk, ccpA, ftsA, glpT, pyrE, recF, sucC, entFM,	8	0.88

[1] The Discrimination Index was calculated according to the method of Hunter and Gaston (19).

4.4 Discussion

The objective of the present study was to develop a two- or three-gene MLST scheme consisting of housekeeping and virulence genes that could be applied for subtyping *B. cereus* dairy isolates. The first part of the project involved screening isolates to determine, which, if any, of the virulence genes were widely distributed and thus would be useful as part of a MLST scheme. Two of the *B. cereus* references strains, ATCC 14579^T and F4810/72, had previously been screened for virulence genes by Hansen et al*. (15)*, and the findings of this study are in agreement with that work.

Two virulence genes, *entFM* and *nheC*, were detected in all of the isolates. This finding is in agreement with the work of several other researchers. Guinebretiere et al. *(14)* screened 88 *B. cereus* strains, and genes encoding for Nhe (*nheA, nheB,* and *nheC*) were detected in 36 (97%) of 37 food isolates and 49 (96%) of 51 isolates associated with diarrheal illness. Andersen Borge et al. *(2)* screened 11 *B. cereus* strains isolated from milk and meat products for genes encoding Nhe, and all strains were found to carry the genes. Wijnands et al. *(42)* conducted a study to determine the pathogenic potential of *B. cereus* isolates from retail foods in the Netherlands. The study included 796 *B. cereus* isolates from 128 food samples. At least one gene encoding for Nhe was detected in 95% of the isolates examined. Ngamwongsatit et al. *(28)* screened 411 *B. cereus* strains including 121 food isolates and 290 soil isolates, for the presence of genes encoding Nhe (*nheA, nheB, nheC*) and enterotoxin FM (*entFM*) and found all strains possessed these genes. Cardazzo et al. *(5)* used the MLST scheme of Priest et al. *(33)* to study the phylogeny of 47 *B. cereus* food isolates, and isolates were screened for ten virulence genes including *nheA, nheB, nheC,* and *entFM.* Genes encoding for NheA, NheB, NheC, and EntFM were detected in 47, 47, 44, and 46 of the 47 isolates, respectively.

One of the limitations of the present study is that a single primer set was used for detection of each of the virulence genes. Thus, it is possible that other genes were present in all of the isolates, but were not detected due to mutations in the primer binding sites. It may be *entFM* and *nheC* are not more prevalent than the other virulence genes, but were detected in all of the isolates because

these genes are less variable than other virulence genes. Ehling-Schulz et al. *(10)* observed a high level of sequence polymorphisms in enterotoxin gene sequences from 49 *B. cereus* strains and suggested that false-negative results observed in PCR protocols for the detection of the hemolytic enterotoxin (Hbl) and the non-hemolytic enterotoxin (Nhe) might be due to a high level of sequence polymorphisms in these genes. Several other researchers have reported negative results for virulence genes using PCR but have obtained positive results using other methods such as BCET-RPLA (HblC, Oxoid)*, Bacillus* enterotoxin VIA (NheA, Tecra) or Southern blot analysis for detection of virulence genes *(14, 15, 26)*.

In the present study, the number of allelic types obtained with housekeeping and virulence gene sequences was compared because previous studies have suggested that virulence gene sequences may have greater discriminatory power than housekeeping gene sequences *(8, 43)*. In this study, two housekeeping genes, *ftsA* and *glpT*, and one virulence gene, *entFM*, were found to yield the highest number of allelic types. The two virulence genes did have a higher percentage of polymorphic sites than all of the housekeeping genes, but the number of polymorphic sites did not correspond with the number of allelic types. It was observed that the two housekeeping genes *ftsA* and *glpT,* which yielded the highest number of allelic types, also had the lowest and highest percentage of polymorphic sites (1.7 and 7.3%, respectively).

In the study by Helgason et al. *(16)*, 77 *B. cereus* group isolates were examined with the MLST scheme, and the genes with the greatest number of

allelic types (40) were *glpT* and *pyrE.* These two genes also had the highest percentage of variable nucleotide sites (26.1 and 23.4%, respectively). In both the study by Helgason *(16)*, and the present work, approximately 50% of the isolates were differentiated with the sequences of *glpT*. Notably, *ftsA* sequences in the study by Helgason *(16)* yielded the lowest number of allelic types (25) and had the lowest percentage of variable sites (9.2%).

In this work, various two- and three-gene MLST schemes were evaluated for subtyping *B. cereus*. Two-gene schemes consisting of any one of the housekeeping genes and the virulence gene *nheC* yielded the greatest number of sequence types. The same number of sequences types could also be obtained by the combination of sequences from the two virulence genes *entFM* and *nheC.* The addition of a third gene to the scheme did not result in an increase in the number of sequence types or the Discrimination Index. The two gene schemes were compared to the seven-gene MLST scheme described by Helgason et al. *(16, Table* 4-9*)*, and the two-gene schemes all yielded one more allelic type. The advantages of the two-gene MLST scheme are that it provides a viable alternative to RAPD-PCR, which is a fragment-based method, and it is more economical than using a “traditional” MLST scheme with seven housekeeping genes. It should be noted that the present research was conducted with a limited number of strains. It would be beneficial to compare the number of sequence types obtained with these two-gene schemes with a larger (>100 strains) culture collection.

4.5 Conclusions

In this study, a two-gene MLST scheme was developed for subtyping *B. cereus* dairy isolates. This sequence-based method could be used for large-scale tracking studies to identify sources of *B. cereus* contamination. Such tracking studies could lead to a reduction the level and incidence of *B. cereus* in pasteurized milk and result in improvements in the quality and shelf life of the product.

4.6 References

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Chapter 5

Summary and Future Research

5.1 Summary

Currently, there is interest in the identification of sources of microbial contamination in pasteurized milk so that the quality and shelf life of the product can be improved. Recent research has suggested Gram-positive spore-forming microorganisms such as *B. cereus* present the next hurdle in shelf-life extension of pasteurized milk. *Bacillus cereus* is foodborne pathogen and is also recognized as a spoilage microorganism that causes quality defects in pasteurized milk.

The objective of the first portion of this work was to conduct microbiological analysis and enumerate *B. cereus* in pasteurized milk obtained from four manufacturers because there are no recent studies on the level of *B. cereus* in milk processed in the U.S. It was found that on the sell-by date, 82% of milk samples held at 7°C had microbial counts exceeding 20,000 CFU/ mL, the limit outlined by the Pasteurized Milk Ordinance. In addition, 32% of the milks held at 7°C on the sell-by date, contained *B. cereus* counts exceeding the minimum infectious dose for the diarrheal illness. The findings of this study indicate that further research was necessary to improve the microbiological

quality of pasteurized milk. Such research would involve tracking studies to identify sources of contamination in milk processing.

The objective of the second portion of this work was to develop a sequence-based subtyping method for tracking *B. cereus* dairy isolates. The goal was to develop a two-or three-gene multi-locus sequence typing (MLST) scheme using a combination of housekeeping and virulence gene sequences. Thirteen *B. cereus* dairy isolates were screened for nine virulence genes, and two genes*, entFM* and *nheC*, were detected in all of the isolates. The number of polymorphic sites and allelic types obtained with sequences of these two genes were compared with the seven housekeeping genes in the MLST scheme of Helgason et al. (2004). Various combinations of housekeeping and virulence gene sequences were subsequently evaluated in the development of a two- or three-gene MLST scheme. MLST schemes were compared on the basis of the number of sequence types and the Discrimination Index. It was found that the combination of any housekeeping gene and *nheC* or the combination of the *entFM* and *nheC* yielded the greatest number of sequence types (8) and the highest Discrimination Index. The addition of a third gene to the scheme did not increase number of sequence types or Discrimination Index.

5.2 Future Research

Recommendations for future research include further evaluation of virulence genes for incorporation in the MLST scheme. Such work could involve screening isolates with different primer sets specific for virulence genes which were not detected in all strains in the culture collection. ELISA or blotting could also be used to determine if the genes are present but not detected by PCR. The sequences of entire virulence genes could be compared to identify regions with high variability.

Future research should also be conducted to evaluate the two-gene MLST schemes with a larger collection of *B. cereus* strains. Such a study should involve subtyping strains from a number of different farms and processing facilities, as well as clinical isolates. One of the two-gene schemes could then be used for tracking studies in dairy processing facilities. It is anticipated that a two-gene MLST scheme will allow for the identification of critical contamination sites within the processing facilities and endemic strains.

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