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Isolation and genetic identification of spore-formers associated with concentrated-milk processing in Nebraska

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ABSTRACT

Spore-forming bacteria are heat-resistant microorganisms capable of surviving and germinating in milk after pasteurization. They have been reported to affect the quality of dairy products by the production of enzymes (lipolytic and proteolytic) under low-temperature conditions in fluid milk, and have become a limiting factor for milk powder in reaching some selective markets. The objective of this research was to isolate and identify the population of spore-forming bacteria (psychrotrophic and thermophilic strains) associated with concentrated milk processing in Nebraska. During 2 seasons, in-process milk samples from a commercial plant (raw, pasteurized, and concentrated) were collected and heat-treated (80°C/12 min) to recover only spore-formers. Samples were spread-plated using standard methods agar and incubated at 32°C to enumerate mesophilic spore counts. Heat-treated samples were also stored at 7°C and 55°C to recover spore-formers that had the ability to grow under those temperature conditions. Isolates obtained from incubation or storage conditions were identified using molecular techniques (16S or *rpoB* sequencing). Based on the identification of the isolates and their relatedness, strains found in raw, pasteurized, and concentrated milk were determined to be similar. *Paenibacillus* spp. were associated with both raw and concentrated milk. Due to their known ability to cause spoilage under refrigeration, this shows the potential risk associated with the transferring of these problematic organisms into other dairy products. Other *Bacillus* species found in concentrated milk included *Bacillus clausii*, *Bacillus subtilis*, *Lysinibacillus* sp., *Bacillus safensis*, *Bacillus licheniformis*, *Bacillus sonorensis*, and *Brevibacillus* sp., with the last 3 organisms being capable of growing at thermophilic temperatures. These strains can also be translocated to other dairy products, such as milk powder, representing a quality problem. The results of this research highlight the importance of understanding spore-formers associated with the processing of condensed milk, which then may allow for specific interventions to be applied to control these microorganisms in this processing chain. To our knowledge, this is the first study evaluating spore-formers associated with concentrated milk in the United States.

Key words: spore-forming bacteria, thermophilic, mesophilic, processing

INTRODUCTION

Spore-forming bacteria are often present in dairy products, ranging from raw milk to packaged products, due to their ability to survive different heat treatments including the pasteurization process (Huck et al., 2008; Ranieri et al., 2009). These bacterial communities are widely distributed in dairy farm environments and are easily introduced into raw milk and at subsequent points during handling and processing (te Giffel et al., 2002; Magnusson et al., 2007; Watterson et al., 2014). More importantly, the presence of certain spore-forming strains may produce quality defects in different dairy products. For the fluid milk industry, psychrotrophic spore-formers are an issue due to the reduction of shelf-life during refrigerated storage caused by the ability of these strains to produce different enzymes (lipolytic and proteolytic) at low temperatures (Meer et al., 1991). Among those spore-former species are *Paenibacillus* spp., *Viridibacillus* spp., and *Bacillus weihenstephanensis* (Fromm and Boor, 2004; Ivy et al., 2012; Estrada, 2014).

For the milk powder industry, high levels of spore-formers (>500 spores/mL) in the final product limits their potential markets, leading to loss of opportunities when quality standards of more profitable markets (e.g., global and infant formula markets) cannot be met (Bienvenue, 2014). Some spore-former strains can germinate and grow under warm (45–60°C) conditions; therefore, these thermophilic strains are of special interest to this industry due their ability to grow and

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establish themselves on equipment surfaces during processing (Burgess et al., 2010; Watterson et al., 2014).

Commercial sweetened condensed milk typically contains high sugar levels, which are usually added during processing, followed by a final canning step. Due to the addition of these multiple hurdles, such as high osmotic pressure and a killing step, the final product is rendered safe and shelf-stable. It is believed that the main spore-former bacteria surviving this heat treatment are thermophilic strains (i.e., *Bacillus licheniformis*, *Bacillus coagulans*, *Bacillus macerans*, *Bacillus subtilis*, and *Geobacillus stearothermophilus*). Therefore, their growth is limited when product is stored at room temperature conditions (<43°C), especially when osmotic pressure is an additional hurdle (Karaman and Alvarez, 2014).

When concentrated or evaporated milk is used as an ingredient, it may not require a heating step (i.e., canning) or the addition of sugars. However, this product is still susceptible to bacterial spoilage and refrigeration is required to maintain its integrity until further use (Karaman and Alvarez, 2014). This intermediate product is used to produce a multitude of other dairy products, including spray-dried milk powder, yogurt, pudding, and cheeses. Therefore, concentrated milk may serve as a vehicle for spore-formers, leading to potential contamination of consumer products. The quality and the safety of those foods could be at risk if strains with the ability to affect their quality or safety (i.e., *Bacillus cereus* or toxigenic strains) are present. Due to these multiple issues caused by spore-formers in different dairy sectors, the dairy industry is interested in understanding this bacterial population.

To date, spore-forming bacteria associated with concentrated milk produced in the United States has not been well described. It is not clear if the main entry point of spore-formers is the raw milk or perhaps processing equipment due to the conditions encountered during the manufacturing of condensed milk. Therefore, the objective of our research was to isolate and genetically identify spore-forming bacteria (psychrotrophic and thermophilic strains) associated with a concentrated milk processing facility in Nebraska.

MATERIALS AND METHODS

Collection and Analysis of Samples

Samples of raw milk were collected during spring (April) and fall (October) 2014 from a concentrated skim milk processing facility in Nebraska. In each season, samples were collected from tankers (PR, Cl, and DF; samples were given random letters to maintain confidentiality) representing different farms that deliver at the processing facility. From each tanker samples were collected in duplicate and raw milk was transferred from trucks to silos using a QMI (QualiTru Sampling System, Oakdale, MN) aseptic sampling system with a 250-mL sterile bag. One sample was also collected from each raw milk silo (bulk milk cooling tank numbers 4 and 5; **BM4** and **BM5**), and 1 additional sample was collected right before the pasteurizer (Figure 1). Similarly, pasteurized milk samples (collected after pasteurization and before the evaporation process, at the evaporator) and concentrated skim milk samples (collected at holding tanks) were obtained in duplicate during each season using the QMI aseptic system (250-mL bags). Samples from evaporator and final holding tanks were collected in the middle of a processing run, which usually is no longer than 5 h. A diagram of sampling points at the processing plant and the number of samples collected in each season per sampling point are represented in Figure 1. All collected samples were stored under refrigeration at the plant until they were transported to the laboratory. During transport they were maintained on ice and upon arrival they were processed. The whole process of sample collection and transport never exceeded 24 h.

The selected processing plant produces mostly class II and III concentrated milk, which is an ingredient to produce yogurt, pudding, cottage, and other cheeses. The commercial plant uses a pasteurization protocol of 75°C (167°F) per 20 s. The evaporation process runs at 61.1°C (~142°F) for 20 min and, finally, concentrated milk is stored at 1.6°C (~35°F) for future use. Process fluctuations were within 1°C.

For all collected samples, microbial quality was determined, including total plate count (**TPC**), coliforms (**Col**), *Escherichia coli* (**EC**), yeast count (**YC**), molds counts (**MC**), and mesophilic spore counts (**MSC**). The detection limit was equal to 1 cfu/mL for all microbial methods previously mentioned. These analyses were performed with the intent to describe the microbial quality of the milk entering into the processing facility and the in-process product. Figure 2 shows the steps performed for microbial analysis in raw, pasteurized, and concentrated milk samples.

For detection of spore-formers, milk samples (250 mL collected at processing plant) were homogenized in the laboratory by thoroughly mixing the contents of the bag, and aliquots (150 mL) were transferred into sterile 250-mL screw-capped bottles. Vegetative cells were killed by heating samples at 80°C for 12 min, using a temperature-controlled water bath (Model 148007, Bockel Industries, Philadelphia, PA). This method has been widely used for the isolation of spore-formers by other research groups (Ivy et al., 2012; Estrada, 2014).