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Spore test parameters matter: Mesophilic and thermophilic spore counts detected in raw milk and dairy powders differ significantly by test method

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ABSTRACT

United States dairy industry exports have steadily risen in importance over the last 10 yr, with dairy powders playing a particularly critical role. Currently, approximately half of US-produced nonfat dry milk and skim milk powder is exported. Reaching new and expanding existing export markets relies in part on the control of endospore-forming bacteria in dairy powders. This study reports baseline mesophilic and thermophilic spore counts and spore populations from 55 raw material samples (primarily raw milk) and 33 dairy powder samples from dairy powder processors across the United States. Samples were evaluated using various spore testing methodologies and included initial heat treatments of (1) 80°C for 12 min; (2) 100°C for 30 min; and (3) 106°C for 30 min. Results indicate that significant differences in both the level and population of spores were found for both raw milk and dairy powders with the various testing methods. Additionally, on average, spore counts were not found to increase significantly from the beginning to the end of dairy powder processing, most likely related to the absence of biofilm formation by processing plant-associated sporeformers (e.g., *Anoxybacillus* sp.) in the facilities sampled. Finally, in agreement with other studies, *Bacillus licheniformis* was found to be the most prevalent sporeformer in both raw materials and dairy powders, highlighting the importance of this organism in developing strategies for control and reduction of spore counts in dairy powders. Overall, this study emphasizes the need for standardization of spore enumeration methodologies in the dairy powder industry.

Key words: dairy powder, raw milk, spore test method, *Bacillus licheniformis*

INTRODUCTION

Aerobic endospore-forming bacteria of the *Bacillaceae* family have been recognized as major contributors to dairy product quality issues over the past 2 decades (Ralyea et al., 1998; Huck et al., 2007b; Ranieri and Boor, 2009). In spore form, these organisms are capable of surviving environmental stresses including low pH, high temperature, exposure to sanitizers, high pressure, and others (Logan and Devos, 2009). These qualities, combined with sporeformers' ubiquitous presence in natural environments (Carlin, 2011), have led to interest in controlling their entry into the dairy product continuum, on the farm (Vissers et al., 2006; Masiello et al., 2014; Miller et al., 2015a), in the transportation chain (Huck et al., 2008), and in the processing environment (Flint et al., 1997; Scott et al., 2007). In recent years, the presence of mesophilic and thermophilic spores in dairy powders has gained increasing attention, as specifications for these microorganisms in powders have become progressively more stringent (Watterson et al., 2014).

Mesophilic spores have been shown to be the most prevalent sporeformer found in bulk tank raw milk (Miller et al., 2015b). Organisms such as *Bacillus licheniformis* and *Bacillus pumilus* predominate in raw bulk tank milk (Ivy et al., 2012; Miller et al., 2015b) and appear to originate primarily from the dairy farm environment (te Giffel et al., 2002; Huck et al., 2008). In contrast, thermophilic spores are more prevalent in dairy powders (Watterson et al., 2014). Studies across the globe have consistently identified *Bacillus licheniformis*, *Anoxybacillus* sp., and *Geobacillus* sp. as the 3 primary sporeformers present in dairy powders (Ronimus et al., 2003; Rückert et al., 2004; Scott et al., 2007; Yuan et al., 2012). While *Anoxybacillus* sp. and *Geobacillus* sp. are considered obligate thermophiles (i.e., optimum growth temperatures of 50 to 62°C and 55 to 65°C, respectively; Pikuta, 2009; Logan et al., 2009) and are generally associated with the dairy processing environment (Flint et al., 1997; Scott et al., 2007), *Bacillus licheniformis* is capable of growing at mesophilic temperatures as well as thermophilic temperatures and

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is found throughout the dairy production and processing continuum (Ivy et al., 2012).

Strategies to reduce the prevalence and levels of spores in dairy powders include reducing their entry into raw milk (Masiello et al., 2014; Miller et al., 2015a) and controlling their presence and growth in processing environments (Flint et al., 1997; Scott et al., 2007). The success of these approaches is evaluated based on results of spore testing in the final product (i.e., reducing spore counts). Unfortunately, this seemingly straightforward process is more complicated than it would seem due to the lack of standardization in spore testing methodologies. Although a host of testing methodologies have been devised to enumerate spores in dairy products (Murphy et al., 1999; Hill, 2004; Scheldeman et al., 2005; ISO-IDF, 2009), there is little in the way of standardization when it comes to spore tests. Initial heat treatments to eliminate vegetative cells and select for spores range from 80°C to 125°C for 10 to 30 min; combined with incubation temperatures to select for mesophilic (i.e., 30–32°C) or thermophilic (i.e., 55°C) spores and various plating media, this leads to the potential for hundreds of unique spore test combinations. This makes national and global benchmarking and comparison nearly impossible. Additionally, although some spore tests are designed to target specific groups of sporeforming bacteria, in general, little is known regarding the effects of different spore treatments on the population of spores that will be detected.

To this end, the objective of this study was to utilize various commonly used spore enumeration methodologies to compare baseline mesophilic and thermophilic spore levels and populations in raw milk and dairy powders sourced from across the United States, and to test the specific hypotheses that (1) increasing spore counts throughout a processing run would indicate the presence of in-plant associated sporeforming bacteria (i.e., *Anoxybacillus*); and (2) spore testing parameters affect both the level and types of spores recovered from dairy powders. Results of this study will enable the US and global dairy industries to compare and reference spore levels in both raw milk and dairy powders, and define standard methods for enumeration of spores in dairy powder products, thereby allowing for targeted efforts to reduce spore levels in these products.

MATERIALS AND METHODS

Dairy Powder Processing Plants

Eleven dairy powder processing plants located either in the east (plants A, B, E, F, I, and K) or the west regions (C, D, G, H, J) of the United States participated in the survey study. Each of the 11 dairy plants manufactures one of the following finished powder products: whey protein concentrate (**WPC**; plant A), nonfat dry milk (NDM; plants B, C, D, E, F, and K), skim milk powder (plants G and H), and whole milk powder (plants I and J). All plants made milk powders from raw material (**RM**), primarily raw milk, except for plants A and E, which used cheese whey and condensed milk, respectively. The length of the production runs for each of the 11 dairy plants varied between 6.5 and 44 h.

Sample Collection

In total, 5 RM (representing RM used during the entire processing run) and 3 FP samples [representing the beginning (within 1 h of processing start-up), middle (within ±1 h of projected mid-point of processing run), and end (within 1 h of shut-down) of the processing run] were collected by plant personnel once from each of the 11 dairy plants over the 10-mo sampling period (July 2013 to April 2014). Detailed sampling instructions and checklists for sample collection, storage, and shipping were provided to plant personnel. Fluid samples and powder samples were aseptically collected in 10-oz. (296-mL) Capitol Plastics locking vials and 24-oz. (710-mL) Whirl-Pak bags, respectively, and were held at or below 6°C until tested within 24 h of arrival at the Milk Quality Improvement Program (MQIP) laboratory (Cornell University, Ithaca, NY).

Spore Treatment and Enumeration

Aerobic spores were enumerated using methods described previously (Watterson et al., 2014). Briefly, 11 g of each finished powder sample was rehydrated in 99 mL of PBS with magnesium chloride under aseptic conditions. Five spore tests were performed on 100 mL each of the RM and rehydrated finished powder samples, each test comprising a heat treatment to inactivate vegetative bacterial cells followed by spread-plating in duplicate on brain heart infusion (**BHI**) agar (Difco, BD, Sparks, MD) and incubation to recover viable spores. The methods used were (1) spore pasteurized mesophilic spore count (**SP-MSC**; 80°C for 12 min followed by incubation at 32°C for 48 h); (2) spore pasteurized thermophilic spore count (**SP-TSC**; 80°C for 12 min followed by incubation at 55°C for 48 h); (3) highly heat resistant mesophilic spore count (**HHR-MSC**; 100°C for 30 min followed by incubation at 32°C for 48 h); (4) highly heat resistant thermophilic spore count (**HHR-TSC**; 100°C for 30 min followed by incubation at 55°C for 48 h); and (5) specially thermo-