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# Psychrotolerant spore-former growth characterization for the development of a dairy spoilage predictive model

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## ABSTRACT

Psychrotolerant spore-forming bacteria represent a major challenge regarding microbial spoilage of fluid milk. These organisms can survive most conventional pasteurization regimens and subsequently germinate and grow to spoilage levels during refrigerated storage. To improve predictions of fluid milk shelf life and assess different approaches to control psychrotolerant spore-forming bacteria in the fluid milk production and processing continuum, we developed a predictive model of spoilage of fluid milk due to germination and growth of psychrotolerant spore-forming bacteria. We characterized 14 psychrotolerant spore-formers, representing the most common *Bacillales* subtypes isolated from raw and pasteurized milk, for ability to germinate from spores and grow in skim milk broth at 6°C. Complete growth curves were obtained by determining total bacterial count and spore count every 24 h for 30 d. Based on growth curves at 6°C, probability distributions of initial spore counts in bulk tank raw milk, and subtype frequency in bulk tank raw milk, a Monte Carlo simulation model was created to predict spoilage patterns in high temperature, short time-pasteurized fluid milk. Monte Carlo simulations predicted that 66% of half-gallons (1,900 mL) of high temperature, short time fluid milk would reach a cell density greater than 20,000 cfu/mL after 21 d of storage at 6°C, consistent with current spoilage patterns observed in commercial products. Our model also predicted that an intervention that reduces initial spore loads by 2.2 $\mathrm{Log}_{10}$ most probable number/mL (e.g., microfiltration) can extend fluid milk shelf life by 4 d (end of shelf life was defined here as the first day when the mean total bacterial count exceeded 20,000 cfu/mL). This study not only provides a baseline understanding of the growth rates of psychrotolerant spore-formers in fluid milk, it also provides a stochastic model of spoilage by these organisms over the shelf life of fluid milk, which will ultimately allow for the assessment of different approaches to reduce fluid milk spoilage.

**Key words:** spore, fluid milk, psychrotolerant, Monte Carlo simulation

## INTRODUCTION

Microbial spoilage is an important component of food loss and can occur in products that have been heat-treated and are stored at refrigerated temperatures, such as fluid milk (Kantor et al., 1997; Buzby et al., 2014). Whereas microbial spoilage can occur due to postprocessing contamination, these problems can largely be addressed with improved sanitation strategies (Dogan and Boor, 2003; Martin et al., 2012). Gram-positive psychrotolerant endospore-forming bacteria (hereafter referred to as spore-formers) represent a more challenging problem to address in terms of microbial spoilage, as these organisms can survive many of the pasteurization heat treatments used to preserve foods and then germinate and grow during subsequent refrigerated storage (Huck et al., 2007; Ivy et al., 2012; Masiello et al., 2014). It is important to clarify that when we refer to spoilage in the current paper, we are referring to microbial spoilage of fluid milk, which we define as total bacterial counts exceeding 20,000 cfu/mL. This level is the legal limit set by the Pasteurized Milk Ordinance for grade A pasteurized fluid milk throughout shelf life (FDA, 2015). However, previous studies have suggested that total bacterial counts ≥1,000,000 cfu/mL are associated with sensory defects in pasteurized fluid milk detectable by consumers, suggesting that fluid milk that exceeds maximum permitted bacterial levels detailed in the Pasteurized Milk Ordinance would generally not be characterized as spoiled by consumers (Carey et al., 2005; Martin et al., 2012).

The genera *Bacillus* and *Paenibacillus* are the most common psychrotolerant spore-formers linked to spoilage of dairy products (Fromm and Boor, 2004; Durak et al., 2006; Huck et al., 2007). *Bacillus* spp. are typically isolated from fluid milk until 7 d of storage at 6°C, whereas *Paenibacillus* spp. have been isolated from fluid milk near the end of shelf life, from 17 d of

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storage at 6°C and beyond (Ranieri and Boor, 2009). Furthermore, previous characterization studies of bacterial isolates representing the genera *Bacillus* and *Paenibacillus* have shown that the majority of *Bacillus* spp., with the exception of *Bacillus weihenstephanensis*, are not able to grow during refrigerated storage of fluid milk whereas many *Paenibacillus* spp., as well as many *Viridibacillus* spp., are able to grow under such conditions (Ivy et al., 2012). Members of the genera *Bacillus*, *Paenibacillus*, and *Viridibacillus* are ubiquitous in nature and have been isolated throughout the dairy chain, including soil (Christiansson et al., 1999), silage (te Giffel et al., 2002), feed concentrate (Vaerewijck et al., 2001), bedding material (Magnusson et al., 2007), milking equipment (Bartoszewicz et al., 2008), and ultimately in raw and pasteurized milk (Huck et al., 2008). Additionally, members of these genera are capable of surviving harsh conditions, such as heat, desiccation, and sanitizers (Setlow, 2006; Checinska et al., 2015). Furthermore, isolates representing some species of the *Bacillales* order linked to fluid milk spoilage (e.g., *B. weihenstephanensis*, *Paenibacillus odorifer*, *Paenibacillus peoriae*, and *Viridibacillus arenosi*) have been shown to produce enzymes that cause off-flavors and curdling in the final product and that hence can degrade product quality (Ranieri et al., 2012; Trmčić et al., 2015). Consequently, the ability to reduce the presence or control the outgrowth of psychrotolerant spore-formers in the dairy system has the potential to considerably enhance the quality and prolong the shelf life of fluid milk.

Germination, the process where spores lose their dormancy and resistance properties, can be activated by sublethal heat treatments, such as those used in HTST pasteurization (Setlow, 2014; Moir and Cooper, 2016). Upon germination, spore-formers are then able to grow as vegetative cells and can grow to levels that ultimately spoil fluid milk. Previous studies suggest that currently more than 50% of fluid milk produced in New York reaches levels exceeding 20,000 cfu/mL over its shelf life because of the presence of psychrotolerant spore-formers when stored at 6°C (Ranieri and Boor, 2009). Whereas some studies have characterized psychrotolerant spore-formers for their ability to grow at refrigeration temperatures, a general lack of information exists on specific growth rates and parameters for psychrotolerant spore-formers (Ivy et al., 2012). Understanding specific growth parameters of psychrotolerant spore-formers is a first step to facilitate development and implementation of better control strategies to reduce psychrotolerant spore-former growth in fluid milk.

Many factors, including initial spore concentration in raw milk, spore-former frequency in raw milk, and their corresponding growth rates can influence the ultimate shelf life of fluid milk contaminated with psychrotolerant spore-formers. Monte Carlo simulations are a probabilistic modeling tool that can be used to account for the uncertainty and variability inherent in microbial dynamics (Nicolaï and Van Impe, 1996; Zwietering et al., 1996). By using probability distributions of data parameters in Monte Carlo simulations, more accurate predictions of shelf life are possible. Thus, the objectives of our study were to (1) understand the germination and growth characteristics of psychrotolerant spore-forming *Bacillus* and *Paenibacillus* spp. in fluid milk and (2) model contamination patterns and growth behavior of *Bacillus* and *Paenibacillus* spp. using Monte Carlo simulations to facilitate improved shelf life predictions of fluid milk.

## MATERIALS AND METHODS

### *Isolate Selection*

Isolates used for growth characterization were selected to represent a diversity of spore-forming *Bacillales* genera and species that have previously been associated with fluid milk spoilage and dairy-associated environments, focusing on isolates that have previously been reported to grow at 6°C. Specifically, 10 isolates were selected from a previously published standard dairy strain collection (Table 1; Trmčić et al., 2015). In addition to these 10 isolates, we also included (1) 1 isolate representing *Bacillus wiedmannii* (a newly described species that has been reported to grow at low temperatures) and (2) 3 isolates representing *Psychrobacillus* [as this genus was not included in the initial standard dairy strain collection, but has recently been reported from heat-treated raw milk (Kent et al., 2016)]. Isolate selection also considered the diversity of isolates within a given species. For example, allelic type (**AT**) 75 was not included, despite being the second most common *B. weihenstephanensis rpoB* AT, as this AT differs by only 1 SNP from AT 3; however, AT 513 was included, as this AT was not only included in the published standard dairy strain collection (Trmčić et al., 2015) but also differs from AT 3 by 4 SNP. Overall, the 14 isolates selected for in-depth growth characterization here represented the genera *Paenibacillus* (7 isolates), *Bacillus* (3 isolates), *Psychrobacillus* (3 isolates), and *Viridibacillus* (1 isolate). These 14 isolates were obtained from pasteurized fluid milk (10 isolates), and heat-treated raw milk samples (4 isolates) tested over their shelf life by using *Standard Methods for the Examination of Dairy Products* (Frank and Yousef, 2004). Specific isolate information can be found in the Food