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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 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

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at 6°C, whereas *Paenibacillus* spp. have been isolated from fluid milk near the end of shelf life, from 17 d of 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 Ex-*

amination of Dairy Products (Frank and Yousef, 2004). Specific isolate information can be found in the Food Microbe Tracker Database (www.foodmicrobetracker.com; Vangay et al., 2013).

Sporulation

The isolates were streaked from frozen culture onto brain heart infusion (**BHI**) agar (Becton, Dickinson and Co., Sparks, MD) and incubated for 24 h at optimum temperatures (21, 32, or 37°C) as determined by Trmčić et al. (2015). Following incubation, an isolated colony was selected for each isolate and used to inoculate a tube containing 5 mL of BHI broth (Becton, Dickinson and Co.). Each tube was then incubated at optimum temperatures (21, 32, or 37°C) for 72 h. Following incubation, 100 μL of the inoculated BHI broth was spread plated in duplicate on sporulating media, AK Agar #2 (Becton, Dickinson and Co.). The plates were then incubated aerobically for 120 h at optimum temperatures (21, 32, or 37°C). Following incubation, sporulation was confirmed via microscopy with an endospore stain. Briefly, smears of the isolates were prepared on microscope slides and heat fixed. Each slide was then flooded with a 7.5% malachite green oxalate solution (J.T. Baker, Phillipsburg, NJ) for 20 min, rinsed gently with water, and then blotted and air-dried. Bacteria were visualized under 1,000× total magnification and spores were identified as unstained structures within the cell. For isolates with no spores confirmed, AK Agar #2 plates were placed in the incubator for an additional 2 wk. If spores were visualized from a given culture, spore suspensions were made as detailed in Gaillard et al. (1998). Briefly, spores were harvested by scraping the surface of the agar with PBS (Weber Scientific, Hamilton, NJ), and subsequently washed 3 times by centrifugation at 11,710 × *g* for 15 min. Following washing, 5 mL of distilled water and 5 mL of 96% ethanol were added to the spore pellet for overnight incubation at 4°C in a tube rotator (Dynal Inc., New Hyde Park, NY) to eliminate vegetative bacteria. The final suspension (approximately 10^6 spores/mL) was kept at 4°C.

Germination, Growth, and Enumeration of Spore Suspensions in Skim Milk Broth

Germination and growth for psychrotolerant spore suspensions was assessed in sterile skim milk broth (**SMB**; Becton, Dickinson and Co.) at 6°C. Spore suspensions were diluted in PBS to approximately 40,000 cfu/mL and then heated at 80°C for 12 min to stimulate germination (Ranieri et al., 2009). Aliquots of 40 mL of SMB precooled to 6°C were inoculated with heat-activated spore suspensions to achieve an initial population of approximately 1,000 cfu/mL, followed by incubation at 6°C. To monitor germination and growth, spore counts and vegetative cell counts were determined every day during the germination and lag phase and then every 2 d during the exponential and stationary phase until 3 time points were taken during the stationary phase. For each count determination, two 1-mL samples of inoculated SMB were placed in separate glass tubes. One tube was heated at 80°C for 12 min to allow for determination of spore numbers; the unheated tube was used for determination of vegetative cell counts. Serial dilutions with PBS were performed for each tube, followed by spiral plating in duplicate on BHI using an Autoplate 5000 (Advanced Instruments Inc., Norwood, MA). The BHI plates were incubated at each isolate's optimum growth temperature for 48 h. Following incubation, colonies were counted with the

Table 1. Genus and species identification, isolation source, and *rpoB* allelic type (AT) for the 14 study isolates

Genus	Species	FSL ID[1]	Source	*rpoB* AT
Bacillus	*weihenstephanensis*	H7–0687	Pasteurized fluid milk	3
	weihenstephanensis	J3–0123	Pasteurized fluid milk	513
	wiedmannii	W8–0169	Spore-count raw milk[3]	61
Paenibacillus	*amylolyticus* s.l.[2]	J3–0122	Pasteurized fluid milk	23
	glucanolyticus	R5–0808	Pasteurized fluid milk	159
	odorifer	H8–0237	Pasteurized fluid milk	15
	peoriae	A5–0030	Pasteurized fluid milk	179
	peoriae	J3–0120	Pasteurized fluid milk	340
	spp.	R7–0277	Pasteurized fluid milk	45
	xylanilyticus, pabuli	H8–0287	Pasteurized fluid milk	100
Psychrobacillus	cf. *psychrotolerans*	K6–2836	Spore-count raw milk[3]	564
	spp.	K6–2591	Spore-count raw milk[3]	147
	spp.	K6–1853	Spore-count raw milk[3]	321
Viridibacillus	*arenosi*	R5–0213	Pasteurized fluid milk	17

[1]FSL ID = Cornell University Food Safety Lab isolate designation.

[2]s.l. = sensu lato.

[3]Spore-count raw milk refers to raw milk heated at 80°C for 12 min and subsequently tested for spore-formers.

Table 2. Growth parameters of psychrotolerant spore-formers in skim milk broth[1]

Genus	Species	*rpoB* allelic type	Lag (d) at		μ_{max} (Log_{10} cfu/mL per day) at		N_{max} (Log_{10} cfu/mL)
			4°C[2]	6°C	4°C[2]	6°C	
Bacillus	*weihenstephanensis*	3	10.5	6.6	0.7	1.1	5.8
Bacillus	*weihenstephanensis*	513	9.1	5.7	0.5	0.7	6.4
Bacillus	*wiedmannii*	61	21.3	13.4	1.0	1.5	6.4
Paenibacillus	*amylolyticus* s.l.[3]	23	46.2	29.0	NA[4]	NA	NA
Paenibacillus	*glucanolyticus*	159	46.2	29.0	NA	NA	NA
Paenibacillus	*odorifer*	15	3.1	1.9	0.4	0.6	6.5
Paenibacillus	*xylanilyticus, pabuli*	100	16.6	10.4	0.6	1.0	6.5
Paenibacillus	spp.	45	29.0	18.2	0.6	1.0	7.6
Paenibacillus	*peoriae*	179	7.9	5.0	0.5	0.8	7.5
Paenibacillus	*peoriae*	340	5.6	3.5	0.5	0.8	7.4
Viridibacillus	*arenosi*	17	4.7	3.0	0.8	1.3	7.4

[1]μ_{max} = maximum growth rate; N_{max} = maximum cell density. The values for these parameters represent Buchanan growth model-fitted data.
[2]Lag and μ_{max} at 4°C represent Buchanan growth-model fitted data transformed to 4°C using Ratkowsky's square-root model.
[3]s.l. = sensu lato.
[4]Not applicable. These isolates failed to germinate and grow in skim milk broth.

QCount Automated Colony Counter (Advanced Instruments Inc.).

Growth Model

Cell density measurements were fitted to a 3-phase linear model as described by Buchanan et al. (1997) using the nlsmicrobio package 0.0–1(Baty and Delignette-Muller, 2017) in R v 3.3.2 (R Core Team, 2013). Based on this model, 4 growth parameters, including lag phase (days), maximum growth rate ($\boldsymbol{\mu_{max}}$; Log_{10} cfu/mL per day), initial cell density ($\boldsymbol{N_0}$; Log_{10} cfu/mL), and maximum cell density ($\boldsymbol{N_{max}}$; Log_{10} cfu/mL), were calculated for each isolate (Table 2).

To characterize the growth parameters as a function of temperature, the square root model for μ_{max} was used (Ratkowsky et al., 1983). According to this model, μ_{max} and lag time (Lt) are expressed as

$$\sqrt{\mu_{max}} = a(T - T_0), \quad [1]$$

and

$$\sqrt{\frac{1}{Lt}} = a(T - T_0), \quad [2]$$

where μ_{max} is the exponential growth rate (Log_{10} cfu/mL per day), T is the growth temperature (degrees C), T_0 is the extrapolated minimum notational growth temperature (degrees C), a is the slope parameter for psychrotolerant spore-formers in fluid milk, and Lt is the lag time (days). For this model, the value for T_0 was estimated as −3.62°C based on growth curves of *Paenibacillus odorifer* (*rpoB* AT 15) obtained at 4, 7, and 32°C in BHI broth (N. H. Martin, unpublished data). To estimate growth parameters for fluid milk stored at 4°C, μ_{max} and Lt experimentally measured at 6°C were transformed to 4°C, using the approach reported in Pradhan et al. (2009). Briefly, to obtain the μ_{max} at 4°C, a ratio of equation 1 was arranged, as shown in equation 3. Likewise, Lt at 6°C was converted to an equivalent Lt at 4°C by rearranging equation 2 as a ratio, as shown in equation 4:

$$\frac{\mu_{max4}}{\mu_{max6}} = \left[\frac{a(T_4 + 3.62)}{a(T_6 + 3.62)}\right]^2 = \left[\frac{7.62}{T_6 + 3.62}\right]^2, \quad [3]$$

and

$$\frac{Lt_4}{Lt_6} = \left[\frac{a(T_6 + 3.62)}{a(T_4 + 3.62)}\right]^2 = \left[\frac{T_6 + 3.62}{7.62}\right]^2, \quad [4]$$

where μ_{max4} and Lt_4 are kinetic parameters at 4°C, μ_{max6} and Lt_6 are kinetic parameters at the experimental temperature 6°C. These converted parameters (Table 2) were used in the simulation model to predict growth of psychrotolerant spore-formers in fluid milk that was stored at 4°C.

Predictive Model Development

Model Assumptions. In our model, bulk tank raw milk from the farm was assumed to be the only source of psychrotolerant spore-formers in the system. Previous studies have demonstrated that psychrotolerant spore-formers are ubiquitous in the dairy farm environment and enter the milk supply through bulk tank raw milk

Table 3. Variables used in Monte Carlo simulation of the shelf life of pasteurized milk

Description	Units	Variable name	Description and detail
Initial microbial population	Log_{10} most probable number/mL	N_0	Modeled as lognormal (−0.72, 0.99) distribution
Psychrotolerant spore-former *rpoB* allelic type frequency	Percent	F	Frequency table based on data reported by Masiello et al. (2014)
Maximum growth rate	Log_{10} cfu/mL per d	μ_{max}	Based on experimental data reported here; model described by Buchanan et al. (1997): $\mu_{max} = \frac{N_t - N_0}{t - t_{lag}}$
Lag	d	t_{lag}	Based on experimental data reported here; model described by Buchanan et al. (1997). For $t \leq t_{lag}$: $N_t = N_0$
Maximum microbial population	Log_{10} cfu/mL	N_{max}	Based on experimental data reported here; model described by Buchanan et al. (1997). For $t \geq t_{max}$: $N_t = N_{max}$

(Christiansson et al., 1999; te Giffel et al., 2002; Masiello et al., 2014). When studying the growth of various psychrotolerant spore-formers in milk, we assumed each simulated half-gallon of milk (1,900 mL) was only contaminated with 1 subtype. This is a simplifying assumption common in predictive microbiology (Malakar et al., 2003). Concentrations of psychrotolerant spore-formers over shelf life were determined assuming a constant storage temperature of 6°C. This assumption was made to compare simulated results to real-life milk sampled over shelf life at 6°C through Cornell University's Milk Quality Improvement Program Voluntary Shelf-Life Program (**VSL**; Martin et al., 2012).

Model Parameters. Five parameters were included in the Monte Carlo simulation model developed here (Table 3), including (1) initial farm bulk tank raw milk psychrotolerant spore-former concentration ($\mathbf{N_0}$), (2) farm bulk tank raw milk psychrotolerant spore-former *rpoB* AT frequency (**F**), (3) maximum growth rate by subtype (μ_{max}), (4) lag phase by subtype ($\mathbf{t_{lag}}$), and (5) maximum microbial population by subtype ($\mathbf{N_{max}}$). Raw most probable number (**MPN**) data from Masiello et al. (2014) was obtained to describe the lognormal distribution of N_0 in bulk tank raw milk at the farm. A frequency table of the psychrotolerant spore-formers obtained from 99 farms across New York State was retrieved from Masiello et al. (2014) to estimate frequency (Table 4). Growth characteristics (μ_{max}, t_{lag}, and N_{max}) were determined as described in the previous section (Table 2). To assign growth characteristics to psychrotolerant spore-former *rpoB* AT isolated at the bulk tank farm level, but for which no growth parameters were available, an *rpoB* region maximum-likelihood phylogenetic tree was constructed with sequences for the isolates characterized in the current study as well as sequences representing all *rpoB* AT found among the psychrotolerant spore-former isolates obtained by Masiello et al. (2014). This tree was constructed using the rapid maximum-likelihood algorithm RAxML (Stamatakis, 2006) with rapid bootstrapping and 100 bootstrap replicates (Supplemental Figure S1; https://doi.org/10.3168/jds.2018-14501). Pairwise distances between each *rpoB* sequence in the phylogenetic tree were computed using the package ape v. 4.1 (Popescu et al., 2012) in R v. 3.3.2 (R Core Team, 2013). When sampling from the frequency table, if an *rpoB* AT was selected with no growth parameters available, growth parameters were selected from the closest pairwise distance *rpoB* AT with available growth parameters. For example, for *rpoB* AT 75, which is the second most common *B. weihenstephanensis rpoB* AT found among fluid milk isolates based on data reported by Masiello et al. (2014), *rpoB* AT 3 growth data were used, as this AT shows the closest pairwise distance to *rpoB* AT 75 (1 SNP pairwise distance).

Model Simulations. The simulation model was programmed in R v 3.3.2 (R Core Team, 2013). Monte Carlo simulations comprised 100,000 iterations. The simulation model predicted the concentration of psychrotolerant spore-formers in a half-gallon of milk (1,900 mL) stored at 6°C from 14 to 24 d of shelf-life based on initial spore-former concentration in the farm bulk tank raw milk, frequency of psychrotolerant spore-formers in farm bulk tank raw milk, and growth characteristics of psychrotolerant spore-formers as inputs. Each iteration resulted in the prediction of a value for the initial concentration of psychrotolerant spore-formers in a half-gallon of milk stored at 6°C. This value was then traced over the milk's shelf life, from 14 to 24 d of storage at 6°C using Buchanan growth model parameters.

Sensitivity Analysis. Best- and worst-case scenario analyses were used to determine quantitatively the most important aspects affecting psychrotolerant spore-former concentrations (Zwietering and Van Gerwen, 2000); best-case scenarios were generally defined as changes that would reduce finished product spoilage

Table 4. Numbers and prevalence of psychrotolerant spore-former *rpoB* allelic types (AT) obtained from spore-pasteurized bulk tank milk samples collected from 99 New York State farms over 1 yr[1]

Genus	Species	rpoB AT	Total no. of isolates out of 159 isolates across 99 bulk tank milk samples	% of isolates[2]
Bacillus	*megaterium*	151	3	2
	weihenstephanensis	3	22	14
		75	18	11
		90	1	<1
		97	1	<1
		132	2	1
		342	1	<1
	wiedmannii	61	3	2
Lysinibacillus	spp.	299	1	<1
		303	3	2
Paenibacillus	*amylolyticus*	111	1	<1
	amylolyticus s.l.[3]	23	2	1
		28	2	1
		29	3	2
		83	1	<1
		184	1	<1
		189	1	<1
		274	1	<1
		345	1	<1
	borealis	41	1	<1
	cf. *cookii*[4]	138	4	3
		332	1	<1
	graminis	39	3	2
		87	4	3
		163	1	<1
		335	1	<1
		336	1	<1
		339	1	<1
		349	1	<1
	lactis	139	1	<1
	macerans	343	2	1
	odorifer	2	3	2
		7	1	<1
		13	1	<1
		15	15	9
		16	1	<1
		18	1	<1
		19	1	<1
		21	5	3
		27	1	<1
		35	2	1
		36	1	<1
		40	2	1
		346	1	<1
		348	1	<1
	cf. *pabuli*[3]	338	1	<1
	cf. *peoriae*[3]	157	2	1
		170	3	2
		179	10	6
		199	4	3
		239	2	1
		334	1	<1
		340	2	1
	spp.	50	3	2
		74	2	1
		77	1	<1
		168	1	<1
Viridibacillus	*arvi/arenosi*	17	2	1

[1]Data were calculated based on data reported by Masiello et al. (2014).

[2]Total number of isolates with specific *rpoB* AT/159 isolates characterized in the Masiello et al. (2014) study.

[3]s.l. = sensu lato; in the broad sense.

[4]cf. = short for the Latin *confer* ("compare with"); signifies AT that resemble the given named species, but where identification represents considerable uncertainty.

(e.g., reduced initial spore levels), whereas worst-case scenarios were defined as those that increased fluid milk spoilage. The effects of 5 major aspects of psychrotolerant spore-former contamination and growth parameters contributing to spoilage of fluid milk were evaluated using best- and worst-case scenario analysis. The 5 aspects of psychrotolerant spore-former growth and contamination considered were (1) the initial farm-level bulk tank milk contamination concentration, N_0, Buchanan psychrotolerant spore-former growth parameters including (2) t_{lag} and (3) μ_{max}, and the frequency of the 2 most prevalent psychrotolerant spore-former subtypes, (4) *rpoB* AT 15 and (5) *rpoB* AT 3, in the farm-level bulk tank raw milk. For the initial farm-level bulk tank milk contamination concentration, the worst-case scenario was calculated as a 1-log (low) and 2-log (high) increase of the mean of N_0; likewise, the best-case scenario was calculated as a 1-log (low) and 2-log (high) decrease of the mean of N_0. For t_{lag}, the worst-case scenario was calculated by decreasing lag phase by 20 (low) and 40% (high); similarly, the best-case scenario was calculated by increasing t_{lag} by 20 (low) and 40% (high). For μ_{max}, the worst-case scenario was calculated by increasing μ_{max} 20 (low) and 40% (high); the best-case scenario was calculated by decreasing μ_{max} 20 (low) and 40% (high). The frequency of *rpoB* AT 15 and 3 (initially at 30.8 and 30.2%, respectively) were independently decreased to 10 (a high level change) and 20% (a low level change) for worst-case scenario calculations and were independently increased to 40 (low) and 50% (high) for best-case scenario calculations (for AT frequencies, higher frequencies were classified as best-case scenarios, as these 2 AT represented subtypes with relatively low μ_{max} values, representing slower growth). Best- and worst-case scenarios were calculated as the difference between the percent of half-gallons of milk that contained greater than 4.3 Log_{10} cfu/mL (20,000 cfu/mL) at 21 d of 100,000 simulated half-gallon psychrotolerant spore-former concentrations for each aspect independently and the baseline model, where all 5 aspects were set to their original values. Ultimately, these scenarios helped to identify aspects of psychrotolerant spore-former growth that affect the prediction of half-gallons that are above the legal limit according to the Pasteurized Milk Ordinance (>20,000 cfu/mL; FDA, 2015).

What-If Analysis. Two what-if scenarios were used to evaluate the effect of control strategies that might be employed to reduce fluid milk spoilage by psychrotolerant spore-formers. The control strategies considered were (1) lower refrigeration temperature during shelf life storage and (2) spore removal technologies (e.g., microfiltration) applied to raw milk. Previous studies have documented that lower storage temperatures of fluid milk results in a longer shelf life (i.e., time to reach >20,000 cfu/mL; Elwell and Barbano, 2006). To evaluate the effect of refrigeration temperature during shelf life storage, the growth curve parameters experimentally collected at 6°C were adjusted using Ratkowsky's square-root model to 4°C (Table 2; see Growth Model section for details of calculation). These adjusted growth curve parameters were used in the simulation model.

To evaluate the effect of microfiltration applied to raw milk, a 2.2-log reduction for the mean N_0 was used. This value was used because Doll et al. (2017) described a 2.2-log reduction on average of psychrotolerant spore-formers after microfiltration of milk in Germany.

Model Validation. Simulated model counts at 14 d were compared with actual counts on 14 d obtained experimentally from commercial market milk that was sampled across New York State from October 2016 to June 2017 in the Cornell VSL program. Through the VSL program, commercially packaged pasteurized fluid milk samples are collected twice a year from New York State dairy plants and evaluated for total gram-negative bacterial counts and SPC, determined over shelf life at 6°C (Martin et al., 2012). Samples that tested positive for gram-negative bacteria, based on plating on crystal-violet tetrazolium agar (Becton, Dickinson and Co.), were classified as showing evidence of gram-negative bacteria postpasteurization contamination and were excluded from the data set used for model validation. Simulated model counts <1 Log_{10} cfu/mL were excluded in analysis to account for the limit of detection for actual count data. The distribution of observed SPC counts from the VSL program (30 total samples) was compared with the distribution of the simulation model's predicted counts using the Kolmogorov-Smirnov test in R v 3.3.2 (R Core Team, 2013), with the null hypothesis that samples are drawn from the same distribution and the alternative hypothesis that samples are drawn from different distributions (Wilcox, 2005). Additionally, empirical cumulative probability distributions and boxplots were constructed for the simulated counts and the observed SPC counts from the VSL program to compare the distributions.

RESULTS

Germination and Growth of Psychrotolerant Spore Suspensions

Spore suspensions were successfully prepared for 11 of 14 isolates. Three *Psychrobacillus* isolates (*rpoB* AT 147, 321, and 564) failed to sporulate after 3 wk of

incubation at 32°C. Spore suspensions of *Paenibacillus amylolyticus* s.l. (*rpoB* AT 23) and *Paenibacillus glucanolyticus* (*rpoB* AT 159) failed to germinate and grow in skim milk broth at 6°C over 29 d. As the spore counts for these isolates were constant for 29 d, we concluded that heating at 80°C for 12 min did not kill these isolates; rather, these isolates remained as spores and failed to germinate and grow. However, both of these isolates were included in the model as remaining in lag phase for the entire 24 d of simulated shelf life.

The remaining spore suspensions germinated and grew in skim milk broth at 6°C, and their growth parameters are described in Table 2. Briefly, for these isolates, lag phases at 6°C ranged from 1.9 to 18.2 d, maximum growth rate at 6°C ranged from 0.6 to 1.5 Log_{10} cfu/mL per day, and maximum cell density ranged from 5.8 to 7.6 Log_{10} cfu/mL.

Initial Psychrotolerant Spore-Former Populations in Bulk Tank Milk

The MPN data for psychrotolerant spore-former levels in raw milk bulk tanks were available for 56 farms included in a previous study (Masiello et al., 2014); these data were used to determine the distribution of psychrotolerant spore-former populations at the bulk tank level and to fit a log-normal distribution, which was used as an input for our model. As MPN assay results for samples with all negative or all positive MPN tubes yield an upper or lower boundary, respectively, but not a numerical Log_{10} MPN per milliliter value, we regarded our observations as censored and fit the distribution using the "fitdistrplus" package in R (Delignette-Muller and Dutang, 2015). If all tubes in the MPN assay were negative, the data were left censored and regarded as an observation of <-2 Log_{10} MPN/mL. If all the tubes in the assay were positive, the data were right censored and regarded as an observation of >1.38 Log_{10} MPN/mL. All other cases were taken to be an observation of the MPN estimate calculated from the configuration of positive tubes. The fitted distribution had a mean of -0.72 Log_{10} MPN/mL and a standard deviation of 0.99 Log_{10} MPN/mL (Figure 1). This corresponds roughly to 1 spore per 5 mL of bulk tank milk. Our observation of low levels of psychrotolerant spore-formers in bulk tank milk is in agreement with previous studies (Mayr et al., 1999; McGuiggan et al., 2002; Doll et al., 2017).

Distribution of Simulated Concentrations of Psychrotolerant Spore-Formers over Shelf Life

At a storage temperature of 6°C, over half (56%) of simulated half-gallons of fluid milk reached >20,000 cfu/mL (4.3 Log_{10} cfu/mL) of psychrotolerant spore-formers by 20 d of storage, and 83% of simulated half-gallons of fluid milk reached >20,000 cfu/mL by 24 d (Figure 2). The mean concentration of psychrotolerant spore-formers per half-gallon of simulated fluid milk at 14 d was 2.21 ± 1.64 Log_{10} cfu/mL, and this concentration increased to a mean of 5.28 ± 1.44 Log_{10} cfu/mL by 24 d of storage at 6°C. At 21 and 24 d, the second most frequent *rpoB* AT (AT 3) reached its N_{max} at 5.8 Log_{10} cfu/mL, resulting in the prominent bars in the histogram panels C and D of Figure 2.

Sensitivity Analysis

Best- and worst-case scenario analyses revealed that μ_{max}, followed by initial farm-level bulk tank milk contamination concentration, N_0, have the largest effect on the model output (Figure 3). Decreasing μ_{max} by 40% resulted in a reduced mean concentration of 2.47 ± 1.49 Log_{10} cfu/mL on 21 d compared with the base concentration level of 4.54 ± 1.71 Log_{10} cfu/mL on 21 d. Reducing the initial contamination population by 2 Log_{10} MPN/mL resulted in a mean concentration of 3.08 ± 1.83 Log_{10} cfu/mL on d 21 of simulated storage as compared with a base concentration (4.54 ± 1.71 Log_{10} cfu/mL). The lag phase parameter had a moderate effect on the model output, increasing lag phase by 40% reduced the mean concentration to 3.79 ± 2.00 Log_{10} cfu/mL on 21 d. The frequencies of *rpoB* AT 3 and 15 had very little effect on the model output. Increasing *rpoB* AT 15 to 50% (from an estimate of

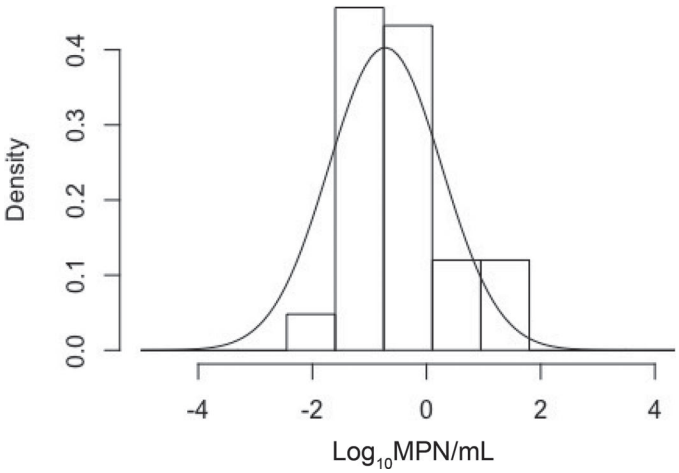

Figure 1. Initial bulk tank raw milk psychrotolerant spore-former populations based on data reported by Masiello et al. (2014) and the simulated log normal distribution of initial bulk tank raw milk psychrotolerant spore-former populations with a mean of -0.72 Log_{10} most probable number (MPN)/mL and a standard deviation of 0.99 Log_{10} MPN/mL.

30.8%) resulted in a mean concentration of 4.66 ± 1.53 Log_{10} cfu/mL on 21 d; similarly, increasing *rpoB* AT 3 to 50% (from an estimate of 30.2%) resulted in a mean concentration of 4.63 ± 1.60 Log_{10} cfu/mL on 21 d.

Model Validation

To assess the simplifying assumptions made in the development of this model, it is important to evalu-

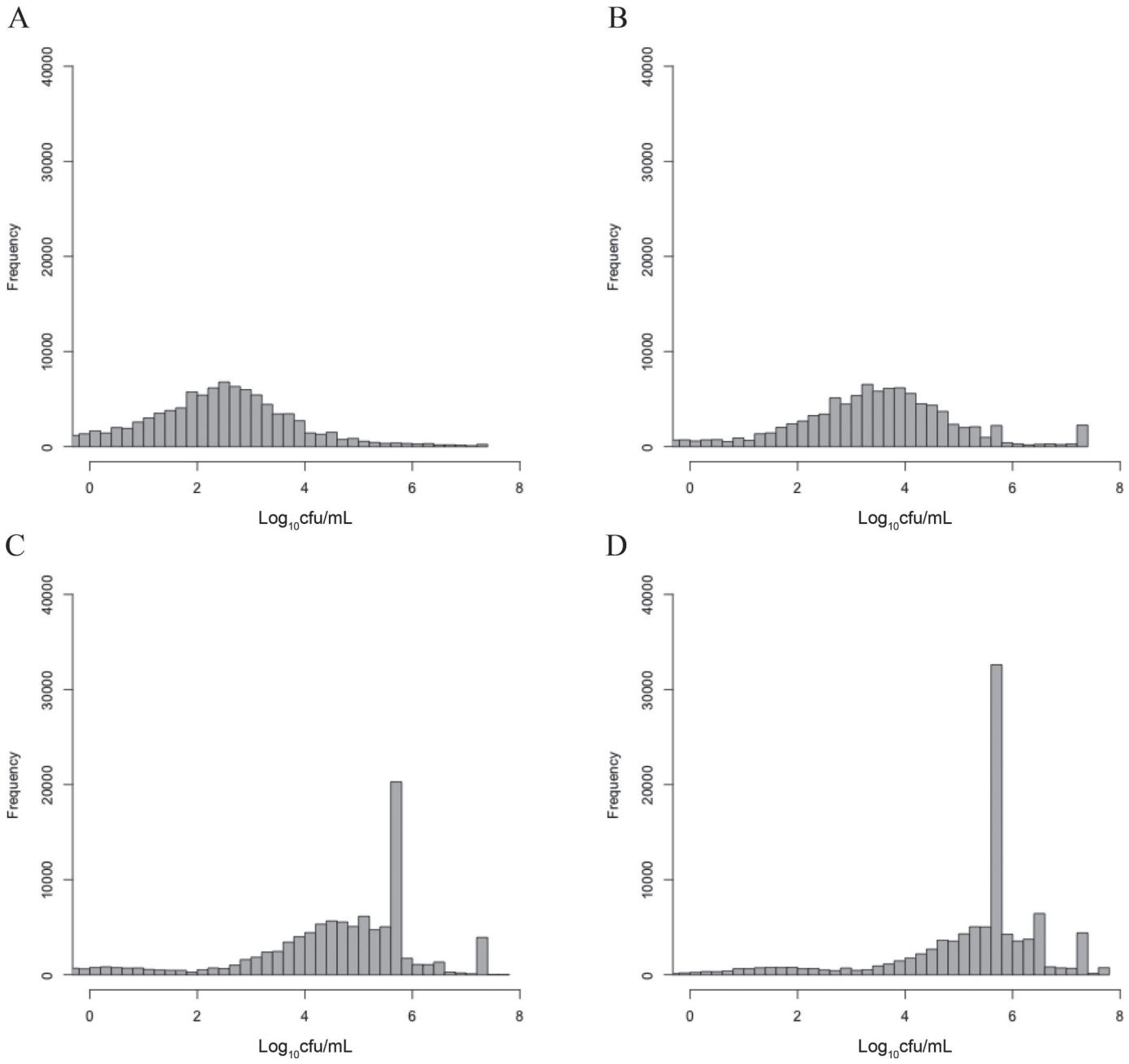

Figure 2. Histograms of the simulated concentration of psychrotolerant spore-formers in fluid milk per half-gallon over shelf-life when stored at 6°C for (A) 14, (B) 17, (C) 21, and (D) 24 d. Monte Carlo simulations comprised 100,000 iterations and were based on 5 model parameters: (1) initial farm bulk tank raw milk psychrotolerant spore-former concentration, (2) farm bulk tank raw milk psychrotolerant spore-former *rpoB* allelic type (AT) frequency, (3) maximum growth rate by subtype, (4) lag phase by subtype, and (5) maximum microbial population by subtype. For the 21 and 24 d histograms, the prominent bar at 5.8 Log_{10} cfu/mL (around 24,000 and 39,000 out of 100,000 iterations, respectively, for 21 and 24 d) can be explained by the fact that the second most frequent *rpoB* AT (AT 3) has a maximum microbial population (N_{max}) of 5.8 Log_{10} cfu/mL. A similar prominent bar is not found for the most frequent AT (AT 15), as this AT has a higher N_{max} and a slower maximum growth rate (μ_{max}) than AT 3, hence a prominent bar at 6.5 Log_{10} cfu/mL is only visible at times past 24 d (data not shown here).

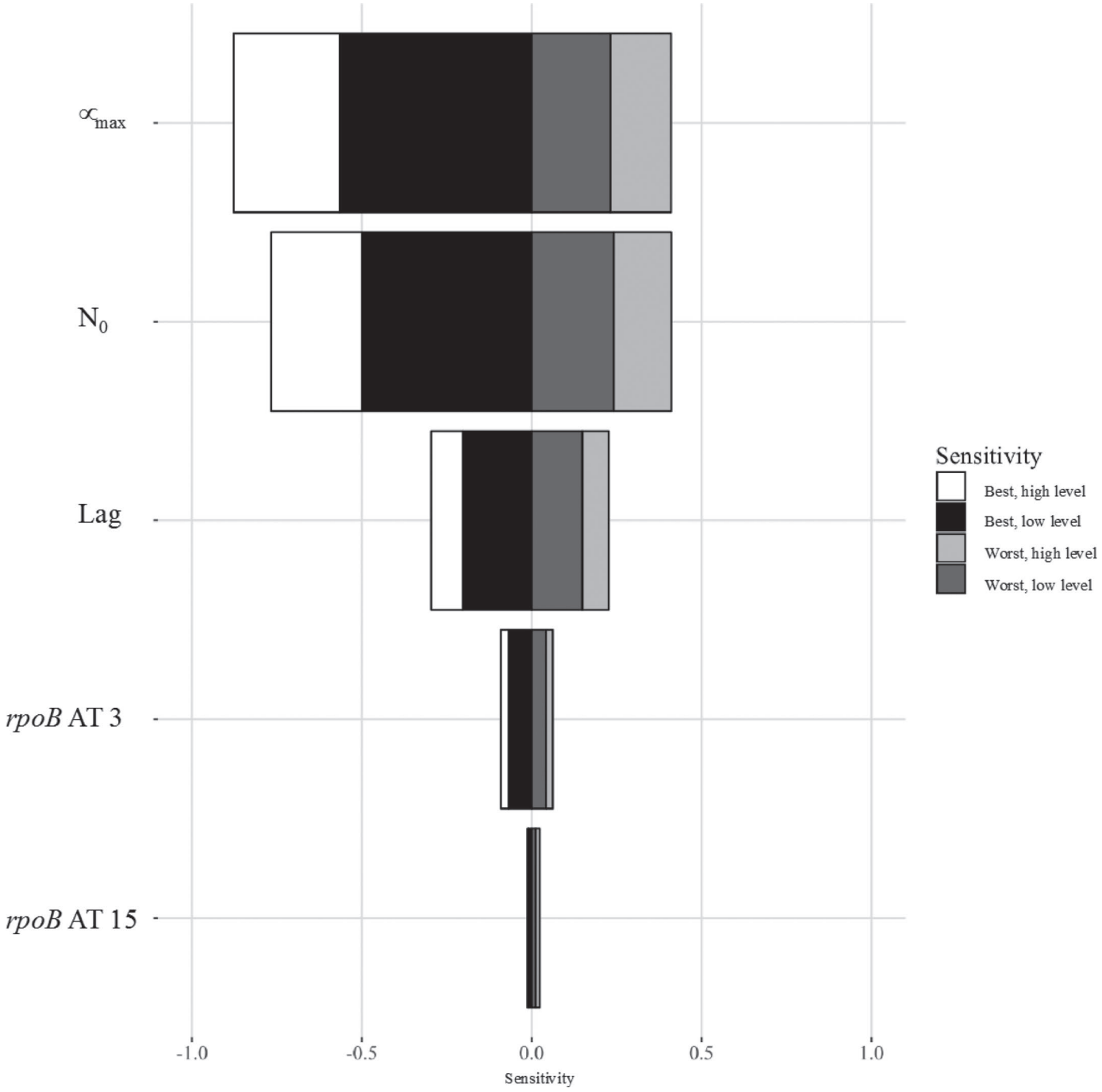

Figure 3. Sensitivity analyses assessing the effects of best-case (white and black) and worst-case (light gray) scenarios on the percent of half-gallons of fluid milk that exceed 4.3 Log_{10} cfu/mL on d 21 of refrigerated storage at 6°C for different aspects of psychrotolerant spore-former growth. μ_{max} represents the maximum growth rate, N_0 represents the farm bulk tank raw milk psychrotolerant spore-former concentration, lag represents the time (d) where the growth rate equals 0, and *rpoB* AT 3 and 15 represent the respective frequencies of these allelic types (AT). Best- and worst-case scenarios were calculated as the difference between the percent of half-gallons of milk that contained greater than 4.3 Log_{10} cfu/mL at 21 d for each input value and the baseline model. Worst-case scenarios were calculated as 1-log (low) and 2-log (high) increase of the mean of N_0, 20 (low) and 40% (high) decrease of lag phase, 20 (low) and 40% (high) increase of μ_{max}, and decreasing the frequencies of *rpoB* AT 15 and 3 independently to 10 (a high level change) and 20% (a low level change). Best-case scenarios were calculated similarly, with a 1-log (low) and 2-log (high) decrease of the mean of N_0, 20 (low) and 40% (high) increase of lag phase, 20 (low) and 40% (high) decrease of μ_{max}, and increasing the frequencies of *rpoB* AT 15 and 3 independently to 40 (low) and 50% (high).

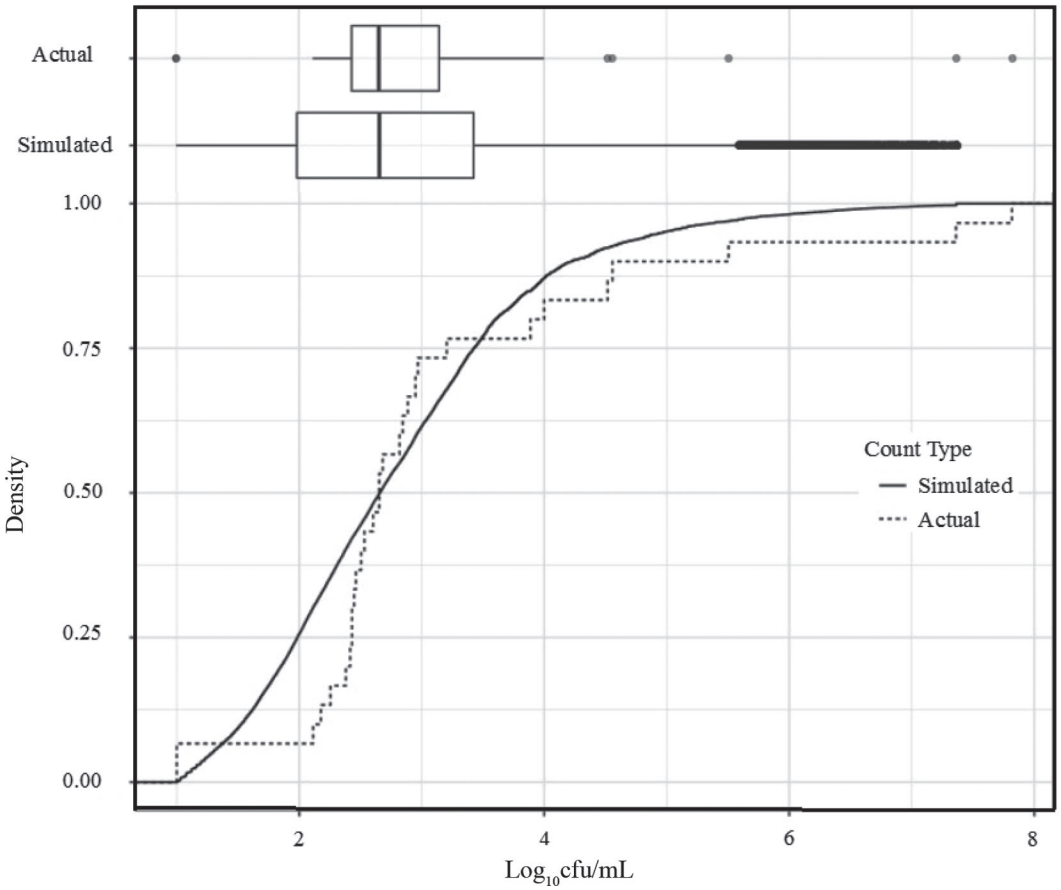

Figure 4. Empirical cumulative probability distributions and corresponding boxplots of the simulated concentration of psychrotolerant spore-formers (Log_{10} cfu/mL) in fluid milk per half-gallon at 14 d of storage at 6°C (solid line) and actual concentrations of presumptive psychrotolerant spore-formers in fluid milk per half-gallon at 14 d of storage at 6°C, based on 30 commercial fluid milk samples tested as part of Cornell University's Milk Quality Improvement Program Voluntary Shelf Life Program (dashed line). For each boxplot, the box extends from the first to the third quartile. The upper whisker extends from the upper end of the box to the largest value no further than 1.5× the interquartile range. The lower whisker extends similarly, to the smallest value no further than 1.5× the interquartile range. Data beyond the end of the whiskers are plotted individually as dots. The median is the line in the box.

ate the model simulation results in context of real-life fluid milk systems. This was done by comparing the distribution of counts in commercial samples spoiled by psychrotolerant spore-formers to the distribution of the simulated counts (Figure 4). The Kolmogorov-Smirnov test statistic of 0.21 ($P = 0.16$) failed to reject the null hypothesis that the distributions of simulated and actual counts are different. Moreover, the centers of the distributions were very close [means of 2.78 $\log_{10}$ cfu/mL (simulated) and 3.14 Log_{10} cfu/mL (actual) on 14 d], though the actual counts had higher spread (1.53 vs. 1.10 Log_{10} cfu/mL for simulated).

What-If Analyses

The predicted effects of refrigeration temperature and microfiltration on fluid milk spoilage by psychrotolerant spore-formers are shown in Table 5. Lowering the refrigeration temperature by 2°C during storage had a dramatic effect on the percent of samples spoiled [9 vs. 66% simulated samples microbiologically spoiled (>4.3 Log_{10} cfu/mL or 20,000 cfu/mL) on 21 d]. Additionally, the mean concentration of psychrotolerant spore-formers on d 21 of simulated storage at 4°C was 2.37 ± 1.52 Log_{10} cfu/mL, compared with an original mean concentration of 4.54 ± 1.71 Log_{10} cfu/mL for storage at 6°C for 21 d (Figure 5). Moreover, the shelf life of simulated fluid milk half-gallons, defined by the time (days) for the mean total bacterial count to exceed 4.3 Log_{10} cfu/mL, was extended by 9 d from an original estimated shelf life of 21 d for storage at 6°C to an estimated shelf life of 30 d for storage at 4°C.

Microfiltration of raw milk (implemented by reducing the mean initial psychrotolerant spore-former population by 2.2 Log_{10} MPN/mL) was estimated to lower the mean concentration of psychrotolerant spore-formers

Table 5. Summary of what-if scenario analysis outcomes

What-if condition	Storage temperature (°C)	Concentration at 21 d (Log_{10} cfu/mL)		Percent of half-gallon containers that exceed 4.3 Log_{10} cfu/mL at d 21
		Mean	SD	
Initial condition	6	4.54	1.71	66
Lower refrigeration temperature	4	2.37	1.52	9
Lower initial raw milk contamination levels by 2.2 Log_{10} most probable number/mL via microfiltration	6	3.03	1.83	13

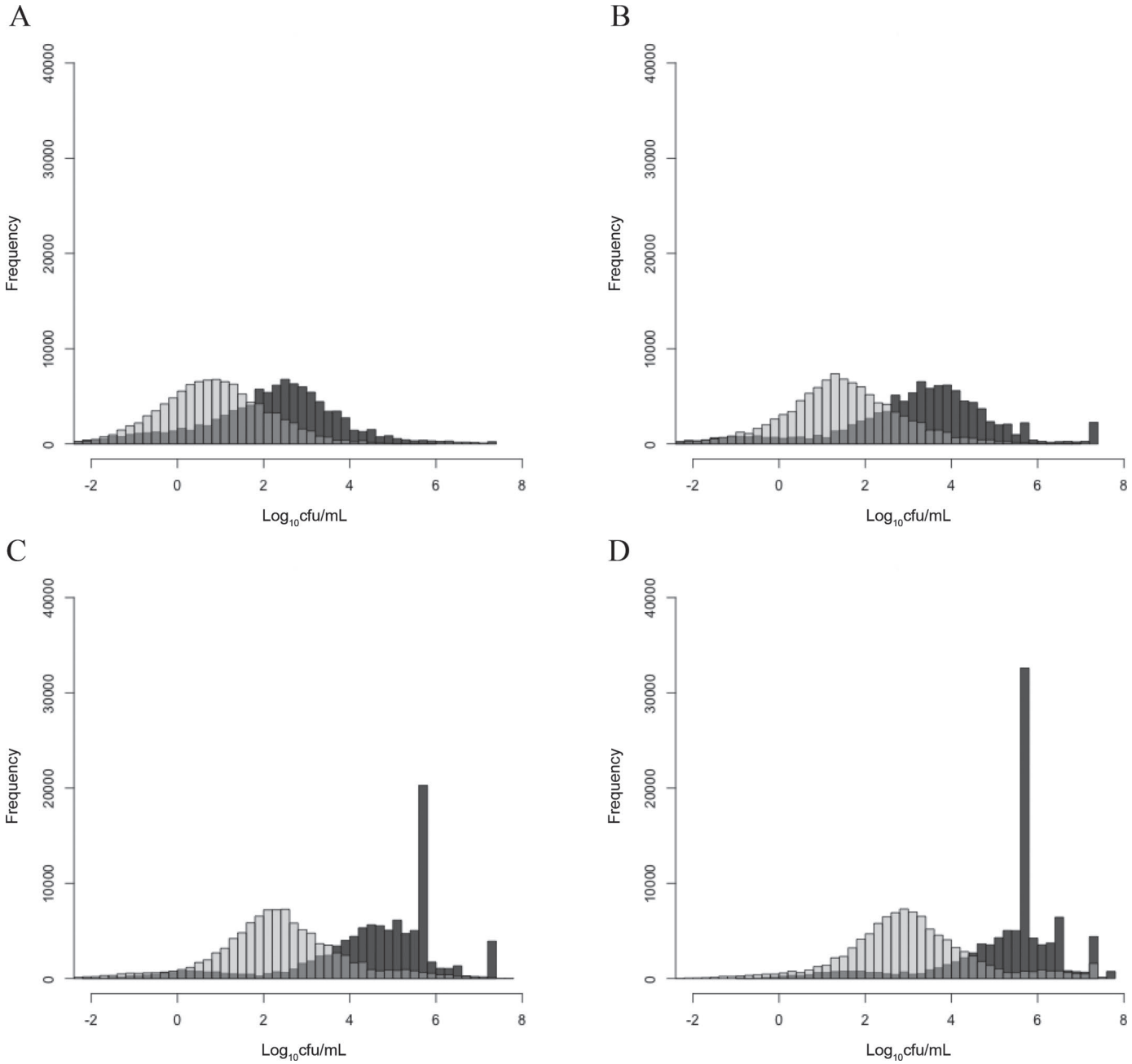

Figure 5. Histograms of the simulated concentration of psychrotolerant spore-formers (Log_{10} cfu/mL) in fluid milk per half-gallon assuming storage of milk at 4°C (light gray) compared with storage of milk at 6°C (dark gray) over shelf-life at (A) 14, (B) 17, (C) 21, and (D) 24 d.

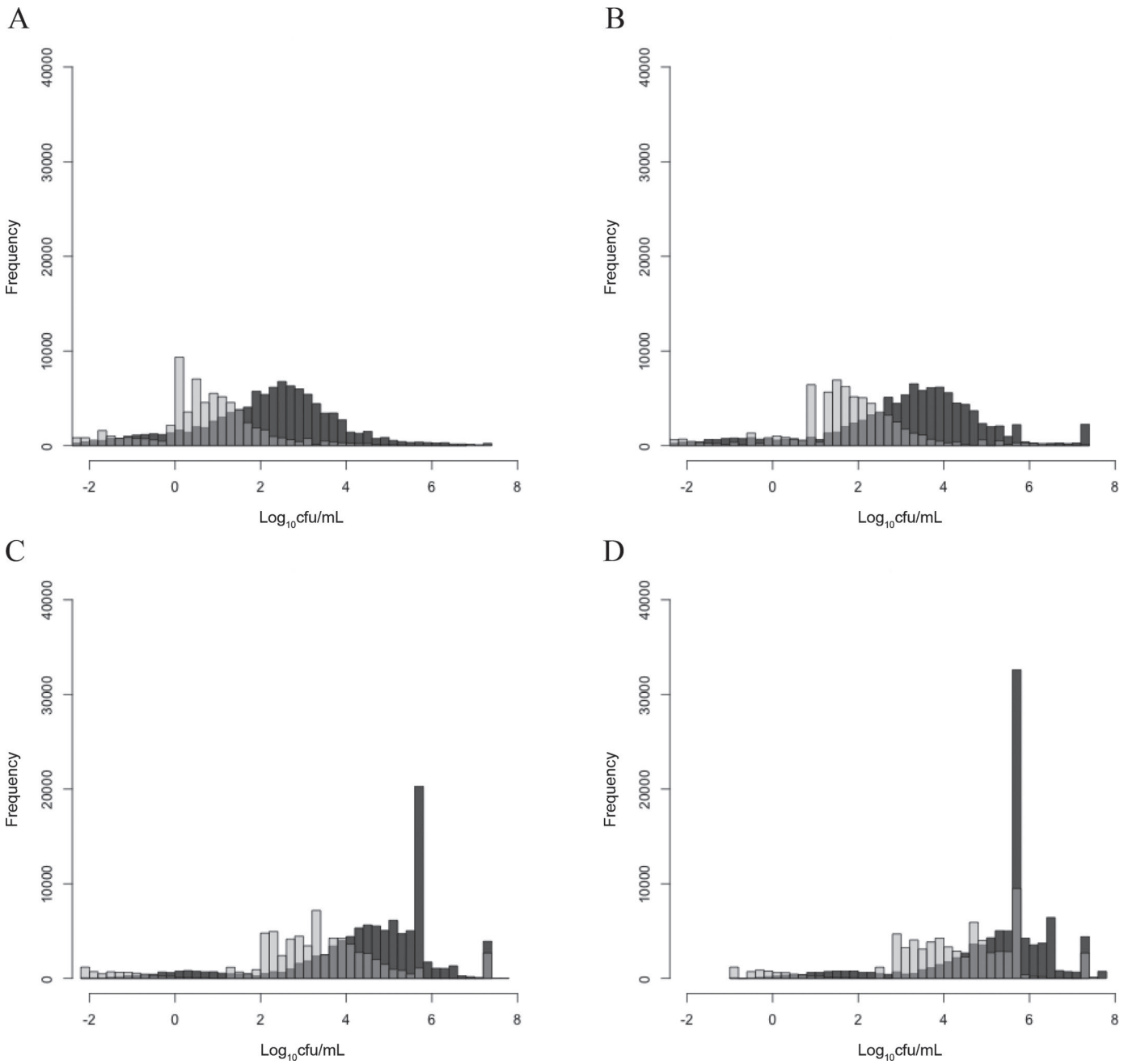

Figure 6. Histogram of the simulated concentration of psychrotolerant spore-formers (Log_{10} cfu/mL) in fluid milk per half-gallon assuming microfiltration of raw milk (yielding a 2.2 Log_{10} most probable number/mL reduction of spore numbers; light gray) compared with no treatment of raw milk (dark gray) over shelf-life at (A) 14, (B) 17, (C) 21, and (D) 24 d.

in simulated half-gallons to 3.03 ± 1.83 Log_{10} cfu/mL at 21 d, corresponding to only 13% of simulated half-gallons spoiled (>4.3 Log_{10} cfu/mL) on 21 d (Figure 6). This extended the shelf life of the simulated fluid milk half-gallons [defined as the time (days) until the mean total bacterial count to exceeds 4.3 Log_{10} cfu/mL] 4 d from an original estimated shelf life of 21 d to a new estimated shelf life of 25 d.

DISCUSSION

Our study characterized psychrotolerant spore-former growth patterns and subsequently developed a predictive model to estimate the concentration of psychrotolerant spore-formers in fluid milk over its shelf life. The predictive model was then used to determine which model parameters contributed most to model outcomes,

and how different management decisions can affect the concentration of psychrotolerant spore-formers in fluid milk over its shelf life. Importantly, our study provides a foundation for the development of improved stochastic models that can be used to predict fluid milk shelf life and assess shelf life extension strategies.

Psychrotolerant Spore-Formers Differ in Ability to Sporulate and Germinate

Among the 14 spore-former isolates tested, all 3 isolates that belong to the genus *Psychrobacillus* (*rpoB* AT 564, 147, and 321) failed to sporulate under the laboratory conditions used (i.e., growth on AK#2 agar over 3 wk). This is consistent with several previous reports that achieving successful sporulation of wild-type spore-former isolates can be challenging (Duncan and Strong, 1968; Cazemier et al., 2001; Nguyen Thi Minh et al., 2011). *Bergey's Manual of Systematic Bacteriology* also specifically indicates that sporulation is infrequently observed for the genus *Psychrobacillus* (Logan and De Vos, 2009). Whereas *Psychrobacillus* isolates were included in our isolate set to capture the diversity of psychrotolerant spore-former genera associated with raw milk, *Psychrobacillus* spp. tended to be infrequently isolated from heat-treated raw milk, and specifically fluid milk. For example, no *Psychrobacillus* isolates were found among 444 *Bacillales* isolates obtained from a large cross-sectional study of bulk tank milk samples collected from New York State farms (Masiello et al., 2014). In addition, no *Psychrobacillus* spp. were isolated from 336 isolates obtained from commercial fluid milk samples; however, *Psychrobacillus* spp. were isolated from the dairy farm environment in the same study, representing 9 of 33 isolates from soil, manure, and bedding pack samples (Huck et al., 2008). The 3 isolates included in our study were obtained from raw milk collected in dairy powder plants or collected on a dairy farm. In dairy powder plants, *Psychrobacillus* spp. only represented 2 of 209 isolates from raw milk samples (Kent et al., 2016). Due to the overall infrequent occurrence of *Psychrobacillus* in raw milk and pasteurized fluid milk products, the inability to obtain spore preparations for isolates representing this genus will not have a major effect on the model outcome. However, future experiments could be conducted to test different conditions for their ability to induce sporulation of *Psychrobacillus*. For example, Hoxey et al. (1985) showed that some spore-formers only sporulated on specific media, and Garcia et al. (2010) indicated that sporulation rates may be affected by temperature.

Among the 11 spore-formers for which spore preparations were successfully obtained, 2 *Paenibacillus* isolates [representing *P. amylolyticus* s.l., and *P. glucanolyticus* (*rpoB* AT 23 and 159, respectively)] did not germinate and grow in skim milk broth over 29 d under the conditions used here. Of note, however, these isolates were selected for their ability to grow at cold temperatures and have previously demonstrated vegetative growth at 6°C (Ivy et al., 2012; Trmčić et al., 2015). Although we did not observe germination and growth for these isolates, isolates with these *rpoB* AT are rarely found in raw milk; *rpoB* AT 23 was only isolated once during a cross-sectional study of the frequency of psychrotolerant spore-formers in spore-pasteurized bulk tank milk at the farm level and *rpoB* AT 159 was never isolated during the same study (Masiello et al., 2014; Table 4). This suggests that these *rpoB* AT may exist as vegetative cells in the farm environment rather than as spores; vegetative cells would not survive pasteurization and hence would not be expected to spoil fluid milk. Hence, data for these isolates will likely have a minimal effect on our model findings. Lack of germination in these isolates could be due to several factors, including (1) sporulation conditions that yielded spores with reduced ability to germinate, (2) heat activation step conditions that did not facilitate germination, and (3) environmental conditions after heat activation that did not facilitate germination. Sporulation conditions have previously been shown to affect the ability of spore-formers to germinate (Raso et al., 1998a,b; Black et al., 2005; Nguyen Thi Minh et al., 2011). For example, a study in the United States investigated how sporulation temperature (20, 30, and 37°C) influenced the initiation of germination of *Bacillus cereus* spores, and found that *B. cereus* sporulated at 20°C exhibited the lowest rate of germination compared with *B. cereus* sporulated at 30 or 37°C (Raso et al., 1998b). Conditions for the heat activation step have also been shown to affect whether spore-formers germinate or not (Vary and Halvorson, 1965; Levinson and Hyatt, 1970; Ghosh et al., 2009). Ghosh et al. (2009) reported optimum heat activation temperature was dependent upon the species of spore-former. Whereas our isolates were heat activated at 80°C for 12 min, this could not have been optimized for these 2 *rpoB* AT. Further research is needed to determine the optimized heat activation temperature for these isolates. Finally, the environmental conditions after heat activation have been shown to influence spore-former germination. For example, a study in the Netherlands characterized *B. weihenstephanensis* heat-activated spores' ability to germinate at 5, 10, 12, 20, and 30°C, and found that at higher germination temperatures more heat-activated spores were able to germinate (Garcia et al., 2010). In our study, the spores were heat activated at 80°C for 12 min, followed by incubation at 6°C over the shelf life, mimicking slightly abusive refrigeration conditions. Perhaps incubation of

the heat-activated spores at temperatures greater than 6°C would have resulted in activation of germination; however, further research is needed to confirm this hypothesis for *Paenibacillus* species.

Maximum Growth Rate Has Greatest Influence on Predicted Concentrations of Spore-Formers

The best- and worst-case scenario analysis indicated that, among the parameters tested, maximum growth rate had the greatest effect on predicted concentrations of psychrotolerant spore-formers in milk. This suggests that further research to characterize growth rates in fluid milk for a diversity of psychrotolerant spore-formers may have the largest effect on improving our predictive model. Whereas no other sensitivity analyses for psychrotolerant spore-formers exist to our knowledge, the importance of maximum growth rate estimates on final model outputs has been demonstrated by previous studies of other gram-positive organisms, such as *Listeria monocytogenes* (Pradhan et al., 2009). For example, Pradhan et al. (2009) reported that using specific maximum growth rates for different deli meats (as opposed to a generic deli meat maximum growth rate) influenced the model outcome of estimates of number of listeriosis cases. Similarly, in our model, we included specific maximum growth rates by *rpoB* AT. By using specific maximum growth rates instead of a universal maximum growth rate for all psychrotolerant spore-formers, we are able to account for our observation that different psychrotolerant spore-formers have different abilities to germinate and grow in fluid milk.

After the maximum growth rate parameter, the initial concentration of psychrotolerant spore-formers in bulk tank raw milk and lag phase were the next most sensitive parameters in the model. Best-case scenarios of N_0 lowered the percent of half-gallons spoiled at 21 d (>4.3 Log_{10} cfu/mL) from 66% in the baseline model to 39 (low) and 16% (high). This finding confirms the conclusions of Huck et al. (2007), who reported raw milk as an important source of spore-forming spoilage bacteria. The importance of initial spore concentrations has also been demonstrated for other parts of the dairy continuum, such as at the dairy farm (Vissers et al., 2007). Vissers et al. (2007) modeled the concentration of *Bacillus* spores in raw milk as a result of farm-level management decisions and found that the initial sources of spores (soil and feed) had the greatest effect on model predictions. In our study, the lag phase was less important for model predictions, with best-case scenarios of lag phase lowering the percent of half-gallons spoiled (>4.3 Log_{10} cfu/mL) from 66% in the baseline model to 57 (low) and 46% (high). The importance of modeling lag phase has been explored in other studies (Pradhan et al., 2010). In their study, Pradhan et al. (2010) expanded the original US Food and Drug Administration—Food Safety and Inspection Service *L. monocytogenes* risk assessment model by including lag phase as a model parameter (the original model assumed no lag phase); their sensitivity analysis indicated that lag phase duration during the production to retail segment had a considerable effect on model outcomes. Although our model did not separate the distribution chain by stage and considered lag phase estimates at a constant temperature of 6°C (to compare model outcomes to actual estimates from VSL), we found that lag phase estimates were not as influential on model outcomes compared with maximum growth rate estimates or the initial concentration of psychrotolerant spore-formers in bulk tank raw milk. However, including lag phase in our model will simplify future modifications to our model, which would allow for separation of the production to retail and retail to consumption phase of distribution.

Importantly, our sensitivity analysis directs future work toward obtaining more accurate estimates for maximum growth rate over other model parameters, such as lag phase or subtype frequency data, which is often more time-intensive to obtain sufficient data. Previous studies have also demonstrated the importance of storage temperature and storage time in sensitivity analyses (Pradhan et al., 2010; Latorre et al., 2011); future model enhancements, such as including temperature distributions over the supply chain, can be added to simulate more realistic storage conditions.

Refrigeration Is a Powerful Control Measure to Extend Fluid Milk Shelf Life

The what-if analyses conducted showed refrigeration at 4°C had a dramatic effect on lowering the mean concentration of psychrotolerant spore-formers in simulated half-gallons. Specifically, our what-if simulations of lowering the refrigeration temperature from 6 to 4°C indicated that only 9% of half-gallons of milk would be spoiled (>20,000 cfu/mL) by 21 d when stored at 4°C, compared with the initial 66% of half-gallons spoiled by 21 d when stored at 6°C. This translates to an extension of average shelf life (time to reach >20,000 cfu/mL) by 9 d by lowering the storage temperature from 6 to 4°C. McMeekin et al. (2008) described temperature as an important factor that determined the rate of spoilage of food. Indeed, this has been demonstrated previously in fluid milk for a variety of organisms (Chandler and McMeekin, 1985; Griffiths et al., 1987; Rosso et al., 1996; Schaffner et al., 2003; Elwell and Barbano, 2006; Rysstad and Kolstad, 2006; Pradhan et al., 2010). For example, a study in Tasmania found that lowering the

refrigeration temperature from 4 to 2°C extended the shelf life of pasteurized fluid milk contaminated with psychrotolerant, gram-negative nonspore-forming rods by 3.5 d (Chandler and McMeekin, 1985). Moreover, a Monte Carlo simulation model for United States fluid milk found that lowering refrigeration temperature from 6.5 to 4.4°C reduced the fraction of milk samples spoiled (defined in that study as $>10^7$ cfu/mL) due to psychrotolerant gram-negative bacteria after 14 d from 67 to 28% spoiled (Schaffner et al., 2003). For gram-positive organisms, such as *L. monocytogenes*, Pradhan et al. (2010) found that restricting the storage temperature distribution to <7°C was the most influential control measure to reduce listeriosis-associated deaths. As distribution chains extend and consumers demand higher-quality products, this is one intervention that can be employed to ensure high-quality fluid milk with extended shelf life (Institute of Medicine and National Research Council, 2015); however, implementation of this intervention can be challenging. Although the storage temperature of fluid milk is tightly controlled at the farm and processing level, domestic refrigerator storage temperature is highly variable (EcoSure, 2007). Consumer education about proper refrigeration temperatures would be needed to fully implement this intervention (Uçar and Özçelik, 2013). Another tool to aid in implementation of this intervention is time-temperature indicators. These indicators could be placed on fluid milk packages to help inform consumers if the product has been above a certain temperature for an amount of time that would lead to product spoilage (Koutsoumanis and Gougouli, 2015). Overall, storage temperature plays an important role in the shelf life of fluid milk. Further research on implementation of tools such as time-temperature indicators at the retail and consumer level is needed to inform how to effectively ensure tight control of storage temperature for the entirety of the distribution chain.

The second what-if analysis evaluated the effect of microfiltration on raw milk to reduce the initial contamination concentration of psychrotolerant spore-formers. Previous studies have experimentally demonstrated the use of microfiltration to extend fluid milk shelf life (Elwell and Barbano, 2006; Schmidt et al., 2012; Doll et al., 2017). For example, Elwell and Barbano (2006) microfiltered raw skim milk with a ceramic 1.4-μm membrane, and achieved an average 3.79 Log_{10} cfu/mL reduction of the total bacterial count in the permeate. Whereas spore counts of the permeate were reported to be below their detection limit of 25 cfu/mL, initial spore concentrations were not reported and effects of microfiltration on spore counts could thus not be quantified for that study (Elwell and Barbano, 2006). Elwell and Barbano (2006) also reported that 50% of microfiltered, pasteurized skim milk samples had total bacteria counts <20,000 cfu/mL after storage at 6.1°C for 92 d. Whereas our model only considered contamination due to psychrotolerant spore-formers, our estimate is that 65% of microfiltered milk samples have psychrotolerant spore-former counts <20,000 cfu/mL at 24 d of storage at 6°C; this suggests reduced spoilage similar to what was experimentally determined by Elwell and Barbano (2006). Similarly, Doll et al. (2017) characterized the efficiency of psychrotolerant spore-former removal from extended shelf life milk during microfiltration with a ceramic 1.4-μm membrane and found microfiltration accounts for an average reduction of psychrotolerant spore-former count of 2.2 Log_{10} MPN, with a range of 0.6 to 3.1 Log_{10} MPN. Our what-if scenario only considered contamination due to psychrotolerant spore-formers and used a 2.2-log reduction of psychrotolerant spore-formers due to microfiltration. We estimated that the shelf life [time (days) to reach a mean total bacterial count >20,000 cfu/mL] of microfiltered milk contaminated with psychrotolerant spore-formers is 25 d when stored at 6°C, an extension of 4 d from the baseline scenario without microfiltration.

Importantly, our what-if analyses and sensitivity analyses demonstrate the usefulness of predictive models for the dairy industry. What-if analyses can be used to quickly estimate outcomes of different processing decisions before having to implement a costly change at any part of the dairy continuum. Likewise, sensitivity analyses can be used to inform where future research should focus to improve model outcomes.

CONCLUSIONS

Control of psychrotolerant spore-formers in the fluid milk-processing continuum is essential to produce high-quality fluid milk with extended shelf life. Our study identified growth parameter data that are needed to reliably predict the shelf life of fluid milk due to psychrotolerant spore-formers, as well as processing decisions and supply chain interventions the dairy industry can employ to reduce spoilage by psychrotolerant spore-formers. Overall, our results lay a foundation for developing new tools to better predict and ultimately prevent dairy spoilage due to psychrotolerant spore-formers.

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