SPORE WARS:

TOOLS TO IDENTIFY, PREDICT, AND PREVENT DAIRY SPOILAGE

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Ariel Jean Buehler

August 2018

SPORE WARS: TOOLS TO IDENTIFY, PREDICT AND PREVENT DAIRY SPOILAGE

Ariel Jean Buehler, Ph. D.

Cornell University 2018

Microbial spoilage is an important aspect of food loss and can occur in products that have been heat-treated and are stored refrigerated, such as dairy products. Routes of contamination for dairy spoilage organisms include presence in raw materials and survival during processing (generally Gram-positive sporeformers) and post-processing contamination (caused by Gram-negative bacteria, yeast and molds). Given the multiple contamination pathways across the dairy processing continuum, a holistic approach is required to address dairy spoilage. To identify, predict, and prevent dairy spoilage, the studies reported here focused on (i) the application of modern molecular approaches to understand the types of fungi in dairy products and facilitate source tracking along the processing continuum in a standardized method, (ii) the development of a stochastic model and a challenge study protocol to allow industry to better evaluate spoilage control strategies for post-pasteurization fungal contamination and assess the value of these strategies quantitatively, and (iii) the development of a stochastic model to understand the effect of sporeformer contamination over the entire processing continuum and quantitatively assess the effect of spoilage control strategies. Our data revealed that dairy-relevant fungi represent a broad diversity over multiple phyla. Molecular subtyping

approaches, namely ITS sequencing, are a useful tool for fungal identification. Moreover, we demonstrated that ITS sequencing can be used for fungal contamination source tracking along the processing continuum, especially for over-represented subtypes. In our stochastic model based on mold post-pasteurization contamination of yogurt, we estimated consumer exposure to visible mold based on a proof-of-concept approach using air plate samples to estimate initial mold contamination rates. This model estimated that 550 ± 25.2 consumers would be exposed to visible mold growth for every 1 million cups of yogurt produced when no fungal inhibitor was used in the yogurt formulation. Our challenge study protocol developed a method for industry to better evaluate novel spoilage control strategies, such as protective cultures, and revealed that the two protective cultures we evaluated retarded mold, but not yeast growth in Greek yogurt. Finally, our second stochastic model provided a way to model spoilage due to psychrotolerant sporeformers in fluid milk throughout the processing continuum, from the dairy farm to the end of shelf-life and predicted that the mean concentration of psychrotolerant sporeformers in fluid milk at 21 days of storage at 6°C is 4.54 ± 1.71 Log_{10}CFU/mL. Our model also revealed ways to quantitatively assess intervention strategies (e.g., microfiltration) to reduce dairy spoilage through the use of what-if scenarios. Overall, these studies broaden our understanding of dairy spoilage organisms. The combination of molecular subtyping and stochastic modeling represents powerful tools the dairy industry can adopt to (i) achieve more accurate estimates of product spoilage and (ii) tailor spoilage control strategies based on data-driven evidence, thus providing a roadmap to reduce microbial spoilage of dairy products.

BIOGRAPHICAL SKETCH

Ariel was born and raised in Knoxville, Tennessee. Her parents exposed Ariel to the joys and trials of food production through home gardening in Tater Valley, Tennessee at a young age. Ariel earned a Bachelor of Science degree in Food Science from the University of Tennessee as a Haslam Scholar, where she completed a Scholars thesis investigating the survival of *Escherichia coli* O157:H7 on microgreens. Ariel's love for the food industry grew during her time as an undergraduate, where she interned for Kraft Foods Research and Development in Glenview, Illinois for three summers. At Kraft Foods, Ariel experienced the creativity of food science by translating research to products consumers demand and enjoy. Ariel began her graduate studies at Cornell University under the guidance of Dr. Martin Wiedmann in 2014. Ariel tailored her graduate experience to focus on real-world applications for the food industry. Ariel had the opportunity to travel to Hyderabad, India and Beijing, China to explore value addition in the dairy and produce industries. Ariel also applied her dairy spoilage research to the food industry directly as an intern for General Mills in Minneapolis, Minnesota, working on dairy quality innovation for Yoplait. After graduating, Ariel will return to General Mills as a research scientist, where she will work to ensure the safety and quality of foods for consumers in the United States and worldwide.

For my parents, who have endlessly supported me on this journey

ACKNOWLEDGMENTS

"A Ph.D. is a marathon, not a sprint" was the truest advice I received before starting graduate school. I had many people by my side during this journey, and I would like to thank them personally here.

First, I would like to thank my committee chair and advisor, Dr. Martin Wiedmann, for his guidance throughout graduate school. Martin allowed me to tailor my graduate experience to focus on industry-specific research projects and equipped me with a unique skill set to be an effective industry scientist. I would also like to thank my minor advisors, Dr. Yrjö Gröhn and Dr. Dennis Miller. Dr. Gröhn was always available to discuss project intricacies and give life advice along the way. I have Dr. Miller to thank for allowing me to go to China to help develop an edamame value-added snack and teaching me nutrition lessons to live by.

My racemates along the marathon route of graduate school were my labmates of the Food Safety Lab and Milk Quality Improvement Program and my classmates at Cornell. Labmates Sam Reichler, Genevieve Sullivan, Rachel Miller, and Yichang Liu made Saturdays spent inoculating Greek yogurt fun, and my classmates Pedro Menchik, Beth Burzynski, and Emily Griep exposed me to a broader realm of food science and enology. I have our lab managers, Sherry Roof and Nicole Martin, and our media support team, Maureen Gunderson and Alan Bitar, to thank for their endless support on my research projects.

I had many cheerleaders by my side for the journey. Friends at Cornell, lovingly known as Tri-Delt, L'oriel, and Cookies and Crème, helped me through the trials and triumphs. Laura Carrol (LC) supplied many nights of rosè and laughter, Lory

Henderson (LOH) was the best lunch date, Sheena Hilton was lip-sync extraordinaire, and Liz Buerman, Rachel Allison, and Madeline Bee showed me how to have fun in upstate New York. The Cornell Symphony viola section and Bethel Grove Bible Church worship team kept my creative side going and provided an outlet for playing the viola during graduate school.

Finally, my family served as my pacemaker for the race. My parents, David and Beth, were always a phone call away, and encouraged me constantly. My brother, Eddie, provided brotherly advice as he finished his Ph.D. on the West coast. My boyfriend, Kevin, endlessly supported me to the finish line of graduate school and served as my audience for countless practice presentations.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Worldwide food loss must be reduced to meet the nutritional demands of a global population expected to reach 9.8 billion people by 2050 (United Nations, 2016). Food loss occurs throughout the value chain from initial agricultural production to the consumer-consumption level (Gustavsson et al., 2011). In low-income countries, the majority of food loss occurs on the farm and transport/processing level. In contrast, in medium-to-high income countries the majority of food loss occurs at the retail and consumer level, where an estimated 30-40% of the available food supply goes uneaten (Godfray et al., 2010, Buzby et al., 2014). Microbial spoilage is a large contributor to food loss and can occur in products that have been both heat-treated and are stored refrigerated (Gram et al., 2002). Dairy products are one sector that fall into this category. An estimated 17% of dairy products are lost annually before consumption in the U.S.—likely due to noticeable spoilage or expired "use-by" dates (Buzby et al., 2014). In spite of the great emphasis on food safety research with federal research dollars aimed at reducing the burden of foodborne illness, relatively little emphasis has been spent on spoilage-based research (Snyder and Worobo, 2018). Thus, there is a great need for new methods to identify, predict, and reduce the prevalence of spoilage organisms to ensure food security globally.

Dairy products can be contaminated by spoilage organisms by two main routes: (i) presence in raw ingredients that survive during processing (generally Gram-positive, endospore-forming bacteria), and (ii) post-processing contamination (**PPC,** generally caused by Gram-negative bacteria, yeasts, and molds) (Huck et al., 2007a,

Kure et al., 2008). Gram-positive endospore-forming bacteria, hereafter referred to as sporeformers, include psychrotolerant organisms, such as *Bacillus* and *Paenibacillus,* and can contaminate and ultimately spoil products like fluid milk. PPC organisms include environmental contaminants present in the processing environment, such as Gram-negative bacteria, yeast and molds, and can contaminate and spoil fluid milk as well as cultured dairy products such as cheese and yogurt (Dogan and Boor, 2003, Garnier et al., 2017).

Given all of the possible routes of contamination in the farm to fork dairy processing continuum, rapid and accurate identification of dairy-relevant spoilage organisms along this continuum is critical. Molecular subtyping approaches have been applied to raw material contaminants, such as sporeformers, and have traced these organisms from the farm environment to the final product (Huck et al., 2007b, Ranieri and Boor, 2009). Molecular subtyping approaches have also been used to describe the diversity of Gram-negative bacteria as post-pasteurization contaminants (Martin et al., 2011, Masiello et al., 2016). However, for other post-pasteurization contaminants such as yeasts and molds, molecular subtyping approaches have been poorly researched. Traditional fungal identification methods are based on phenotypic classification and require extensive time and expertise for accurate identification (Pitkäranta et al., 2008). Thus, there is a need to (i) implement molecular subtyping approaches for dairy-associated fungi, (ii) to understand the diversity of fungal contaminants in dairy products based on molecular identification, and (iii) to understand the relationships among fungal organisms collected along the processing continuum. This effort will provide baseline data of fungi in dairy products rapidly and accurately and may also

facilitate source tracking of fungal contaminants. Thus, the goal of Chapter 2, "ITS Sequencing Reveals Considerable Fungal Diversity in Dairy Products" is to standardize methods to identify fungal contaminants in dairy products via the use of molecular subtyping approaches and apply these methods to provide a foundational understanding of the diversity of dairy-associated fungi. The goal of Chapter 3, "ITS Sequence-Based Characterization of Fungal Isolates from Multiple Yogurt Facilities—a Case Study" is to apply ITS subtyping to characterize fungal contaminants throughout the processing continuum in two yogurt process facilities to understand the relationships among these organisms within processing systems.

To control spoilage organisms and produce high quality dairy products, producers often employ interventions focusing on the production environment, processing parameters, the product formulation, or a combination of these tools. Production environment interventions include improved sanitation approaches (Dogan and Boor, 2003) and strict Good Manufacturing Practices (GMPs) (Te Giffel, 2003). Processing interventions, such as microfiltration or bactofugation, are technologies that improve the microbial quality of dairy products without the use of additional thermal processing (Te Giffel and Van Der Horst, 2004, Elwell and Barbano, 2006). Product formulation tools, often used in cultured dairy products, include traditional weak organic acid preservatives such as sorbic acid and benzoic acid, as well as biopreservatives, including protective cultures (Garnier et al., 2017). To understand which intervention will be most effective, there is a need to first understand (i) the impact at the consumer-level of having spoilage organisms in the final product at the time of consumption, and (ii) contamination patterns of spoilage organisms throughout

shelf-life. These two aims can be accomplished through the use of stochastic models. Stochastic models have been used in predictive microbiology to describe mean illness cases per year following exposure to foodborne pathogens (Pradhan et al., 2009, Pradhan et al., 2010, Pang et al., 2017). Similar modeling approaches, however, have been applied to the food spoilage realm less frequently, with a few models describing fluid milk and yogurt spoilage (Schaffner et al., 2003, Gougouli and Koutsoumanis, 2017). Thus, the development of stochastic models to describe consumer exposure to and contamination patterns of spoilage organisms is vital to quantitatively understand which intervention to employ on a producer-by-producer basis.

Before spoilage control strategies can be assessed, there is a need to establish a baseline exposure model to estimate the impact of product contamination at the consumer level. This effort will help producers assess the value of spoilage control strategies before implementation. Moreover, if spoilage control strategies are needed, there is a need for better methods to evaluate novel control strategies, such as protective cultures. Thus, Chapter 4, "Evaluation of Biopreservatives in Greek Yogurt to Inhibit Yeast and Mold Spoilage and Development of a Yogurt Spoilage Predictive Model" aims to (i) develop a probabilistic model to first estimate consumer exposure to visible mold contamination in yogurt (and thus determine if an intervention is necessary) and (ii) develop a challenge study protocol to evaluate novel spoilage control measures.

Beyond post-pasteurization contamination routes, there is also a need to understand spoilage contamination patterns throughout the processing continuum, especially for organisms that originate in the raw materials and can survive processing

regimes, such as Gram-positive sporeformers. This effort will help producers achieve an accurate estimate of shelf-life due to spoilage by these organisms as well as assess the effectiveness of intervention strategies before implementation. Thus, Chapter 5, "Psychrotolerant Sporeformer Growth Characterization for the Development of a Dairy Spoilage Predictive Model" aims to (i) better understand psychrotolerant sporeformer germination and growth behavior and (ii) develop a probabilistic model to enable better shelf-life estimations as well as assessment of intervention strategies.

Overall, the following chapters will expand on sophisticated tools the dairy industry can utilize to address spoilage in the 21st century. Specifically, the use of molecular subtyping of fungal organisms may lead to rapid and accurate surveillance of these organisms in processing facilities. Additionally, the development of stochastic models to understand consumer exposure to and contamination patterns of spoilage organisms may allow producers to quantitatively estimate (i) if an intervention is needed and (ii) which intervention to employ to achieve reduced spoilage in dairy products. In sum, these tools represent proactive approaches the dairy industry can apply to holistically address dairy spoilage.

REFERENCES

Buzby, J. C., H. Farah-Wells, and J. Hyman. 2014. The estimated amount, value, and calories of postharvest food losses at the retail and consumer levels in the United States. USDA-ERS Economic Information Bulletin.

Dogan, B. and K. J. Boor. 2003. Genetic diversity and spoilage potentials among Pseudomonas spp. isolated from fluid milk products and dairy processing plants. Appl. Environ. Microbiol. 69:130-138.

Elwell, M. W. and D. M. Barbano. 2006. Use of Microfiltration to Improve Fluid Milk Quality. J. Dairy Sci. 89:E20-E30.

Garnier, L., F. Valence, and J. Mounier. 2017. Diversity and Control of Spoilage Fungi in Dairy Products: An Update. Microorganisms 5(3):42.

Godfray, H. C. J., J. R. Beddington, I. R. Crute, L. Haddad, D. Lawrence, J. F. Muir, J. Pretty, S. Robinson, S. M. Thomas, and C. Toulmin. 2010. Food security: the challenge of feeding 9 billion people. Science 327:812-818.

Gougouli, M. and K. P. Koutsoumanis. 2017. Risk assessment of fungal spoilage: A case study of Aspergillus niger on yogurt. Food Microbiol. 65:264-273.

Gram, L., L. Ravn, M. Rasch, J. B. Bruhn, A. B. Christensen, and M. Givskov. 2002. Food spoilage—interactions between food spoilage bacteria. Int. J. Food Microbiol. 78:79-97.

Gustavsson, J., C. Cederberg, U. Sonesson, R. Van Otterdijk, and A. Meybeck. 2011. Global food losses and food waste. Food and Agriculture Organization of the United Nations.

Huck, J., B. Hammond, S. Murphy, N. Woodcock, and K. Boor. 2007a. Tracking spore-forming bacterial contaminants in fluid milk-processing systems. J. Dairy Sci. 90:4872-4883.

Huck, J. R., N. H. Woodcock, R. D. Ralyea, and K. J. Boor. 2007b. Molecular subtyping and characterization of psychrotolerant endospore-forming bacteria in two New York State fluid milk processing systems. J. Food Prot. 70:2354-2364.

Kure, C. F., E. Borch, I. Karlsson, J. P. Homleid, and S. Langsrud. 2008. Use of the selective agar medium CREAD for monitoring the level of airborne spoilage moulds in cheese production. Int. J. Food Microbiol. 122:29-34.

Martin, N., M. Ranieri, S. Murphy, R. Ralyea, M. Wiedmann, and K. Boor. 2011. Results from raw milk microbiological tests do not predict the shelf-life performance of commercially pasteurized fluid milk. J. Dairy Sci. 94:1211-1222.

Masiello, S., N. Martin, A. Trmčić, M. Wiedmann, and K. Boor. 2016. Identification and characterization of psychrotolerant coliform bacteria isolated from pasteurized fluid milk. J. Dairy Sci. 99:130-140.

Pang, H., E. Lambertini, R. L. Buchanan, D. W. Schaffner, and A. K. Pradhan. 2017. Quantitative Microbial Risk Assessment for Escherichia coli O157: H7 in Fresh-Cut Lettuce. J. Food Prot. 80:302-311.

Pitkäranta, M., T. Meklin, A. Hyvärinen, L. Paulin, P. Auvinen, A. Nevalainen, and H. Rintala. 2008. Analysis of fungal flora in indoor dust by ribosomal DNA sequence analysis, quantitative PCR, and culture. Appl. Environ. Microbiol. 74:233-244.

Pradhan, A. K., R. Ivanek, Y. T. Gröhn, R. Bukowski, I. Geornaras, J. N. Sofos, and M. Wiedmann. 2010. Quantitative risk assessment of listeriosis-associated deaths due to Listeria monocytogenes contamination of deli meats originating from manufacture and retail. J. Food Prot. 73:620-630.

Pradhan, A. K., R. Ivanek, Y. T. Gröhn, I. Geornaras, J. N. Sofos, and M. Wiedmann. 2009. Quantitative risk assessment for Listeria monocytogenes in selected categories of deli meats: impact of lactate and diacetate on listeriosis cases and deaths. J. Food Prot. 72:978-989.

Ranieri, M. L. and K. J. Boor. 2009. Short communication: bacterial ecology of high-temperature, short-time pasteurized milk processed in the United States. J. Dairy Sci. 92: 4833-4840.

Schaffner, D. W., J. McEntire, S. Duffy, R. Montville, and S. Smith. 2003. Monte Carlo simulation of the shelf life of pasteurized milk as affected by temperature and initial concentration of spoilage organisms. Food Protect. Trends 23:1014-1021.

Snyder, A. B. and R. W. Worobo. 2018. The incidence and impact of microbial spoilage in the production of fruit and vegetable juices as reported by juice manufacturers. Food Control 85:144-150.

Te Giffel, M. 2003. Good hygienic practice in milk processing. in Dairy Processing: Improving Quality. S. G, ed. CRC Press, Boca Raton, FL.

Te Giffel, M. and H. Van Der Horst. 2004. Comparison between bactofugation and microfiltration regarding efficiency of somatic cell and bacteria removal. Bulletin-International Dairy Federation 389:49-53.

United Nations, D. 2016. World population prospects: the 2015 revision. Population Division of the Department of Economic and Social Affairs of the United Nations Secretariat, New York.

CHAPTER 2

ITS SEQUENCING REVEALS CONSIDERABLE FUNGAL DIVERSITY IN DAIRY PRODUCTS*

*A. J. Buehler, R. L. Evanowski, N. H. Martin, K. J. Boor, and M. Wiedmann

Published in Journal of Dairy Science 2017, 100, 11; doi:10.3168/jds.2017-12635

ABSTRACT

Fungi are important spoilage organisms in dairy products. However, little is known about the diversity of naturally occurring spoilage fungi in raw milk and processed dairy products, at least partially due to the fact that classical fungal identification methods require considerable expertise. In order to gain further insight into the fungal diversity in the dairy system, we isolated fungi from raw milk, as well as raw and pasteurized milk cheese, and yogurt, using the selective Dichloran Rose Bengal Chloramphenicol agar. A total of 361 fungal isolates were obtained and further characterized by DNA sequencing of the internal transcribed spacer (ITS) region and the nuclear ribosomal large subunit (LSU) rRNA gene if needed. BLAST searches of the ITS region sequences against the Unite Database, and selected other databases if needed, allowed species identification of 183 isolates and only genus identification of 107 of the 346 isolates that allowed for successful ITS sequencing. The isolates characterized represented three phyla and 19 genera; the most common genera isolated were *Penicillium* (25% of isolates), *Debaryomyces* (18%) and *Candida* (9%). This study not only provides, through the use of modern molecular tools, a baseline understanding of the types of fungi in dairy products, but also confirms that ITS sequencing is a useful approach for identification of fungal organisms found in the dairy food chain.

INTRODUCTION

Dairy products have a long history of spoilage by yeasts and molds, but as shelf lives and distribution chains are extended these microorganisms have become increasingly problematic for the dairy industry. Some reports estimate that 5-10% of all food production is lost to fungal spoilage worldwide (Pitt and Hocking, 2009). While specific estimates for dairy product loss due to fungal spoilage do not appear to be available, cultured dairy products, such as yogurt and cheese, are well documented to be susceptible to spoilage by fungi because of the ability of many strains to grow at the low temperature and pH encountered in these products (Fröhlich-Wyder, 2003, Mayoral et al., 2005, Banjara et al., 2015). Additionally many cheeses have reduced water activity and high salt concentrations, which provides a unique niche for the growth of yeasts and molds (Gardini et al., 2006, Ledenbach and Marshall, 2009). Fungal spoilage organisms produce degradative enzymes that break down lipids, proteins, and carbohydrates leading to a variety of undesirable sensorial qualities (Ledenbach and Marshall, 2009). In addition, fungal spoilage is often easily visually detected by consumers; with broad use of social media, communication of fungal spoilage via pictures shared on social media is also increasingly common and has considerable potential to damage the reputation of dairy products (Newkirk et al., 2012).

Sources of yeast and mold contamination of dairy products typically appear to be the air and other environmental sources in processing facilities and other environments (e.g., aging facilities, retail) (Kure et al., 2001). Airborne mold has been reported to enter the processing environment from either the outdoor air via the

ventilation system or from moist niches present in the process environment (Kure et al., 2008). In the presence of elevated indoor moisture contents for a prolonged period of time, some fungi can grow and sporulate, resulting in further contamination in the indoor environment (Pitkäranta et al., 2008). Fungal environmental contamination in processing plants has previously been documented (Lund et al., 2003, Temelli et al., 2006). For example, Lund et al. (2003) collected swab and air samples from cheese production plants, the processing environment, and contaminated cheese products and identified the packaging environment and the coating step as the major points for fungal environmental contamination. In another study, Temelli et al. (2006) reported the cold room and production room air as the sources for fungal environmental contamination in Turkish white cheese. Based on these findings, air in the processing plant represents an important source of fungal contamination.

Yeasts and molds however are also commonly found in the dairy farm environment and can appear as natural contaminants in raw milk (Fleet, 1990, Lavoie et al., 2011, Atanassova et al., 2016). Most authors however suggest that fungi found in raw milk are typically heat sensitive and that raw milk thus is not an important (direct) source of fungi found in dairy products (Jacques and Casaregola, 2008). A few studies have surveyed the diversity of fungi in raw milk and reported that yeasts are more frequently isolated from raw milk than molds (Callon et al., 2007, Delavenne et al., 2011, Panelli et al., 2013). For example, Panelli et al. (2013) tested 40 bulk milk samples across the Italian Alps and most frequently isolated *Kluyveromyces marxianus*, *Atrotorquata lineata*, and *Candida* spp. Importantly, these studies identified some fungal species that had not previously been found in raw milk,

suggesting that the fungal diversity associated with raw milk remains to be fully understood.

Historically, methods used for identification of fungal microorganisms involved visual and labor intensive phenotypic characterization, which requires extensive training and experience to master (Pitkäranta et al., 2008). Standardized DNA sequencing methods, sometimes referred to as DNA barcoding, represent robust and rapid methods for fungal identification. Moreover, an accepted universal internal transcribed spacer (ITS) region barcode for fungi has been well documented to allow for reproducible and discriminatory DNA sequencing-based fungal identification (Mayoral et al., 2005, Schoch et al., 2012, Sulaiman et al., 2014).

The objective of this study was to (i) implement molecular characterization methods for dairy-associated fungi isolated from raw milk, raw and pasteurized milk cheeses, and yogurt products and to (ii) use these methods to provide initial insights into the fungal diversity associated with different dairy products.

MATERIALS AND METHODS

Sample Collection

Using a convenience sampling approach, samples of bulk tank raw milk, cheese, and yogurt were collected between April and September 2015 and used for isolation of yeast and mold (see Table 2.1 for details on samples collected). Bulk tank raw milk samples (300 mL each) were collected from 8 dairy farms in New York State. Raw and pasteurized cheese samples (14 and 33, respectively) were collected from producers, supermarkets, wholesale distributors, and specialty shops in NY,

excluding cheeses that clearly were intentionally inoculated with fungi (e.g., mold-ripened cheeses). Yogurt samples (n=30) representing plain, fruit, and other varieties were also conveniently collected from producers; none of the samples were visually spoiled at the time of collection. Samples were stored at 4°C until they were analyzed.

Table 2.1. Dairy products sampled for yeast and mold

Sample Type	Species	Origin	Raw/ Pasteurized Milk	Type	No. of samples	No. of Isolates obtained from[a]	
						direct plating	enrich-ments
Raw Milk	Cow	New York State Dairy Farms	Raw	Bulk tank	8	10	28
Cheese	Cow	USA	Pasteurized	Semi-hard	1	2	7
	Cow	New York, USA	Pasteurized	Soft, washed rind	1	1	0
	Cow/ Sheep	Italy	Pasteurized	Soft, washed rind	1	1	0
	Cow	USA	Raw	Hard, washed rind, brined	2	3	6
	Cow	USA	Raw	Semi-hard, natural rind	2	5	8
	Cow	USA	Raw	Hard, washed rind	1	0	4
	Cow	New York, USA	Raw	Soft, washed rind	2	7	16
	Goat	Portugal	Pasteurized	Hard	1	3	1
	Goat	Switzerland	Pasteurized	Hard	1	2	7
	Goat/ Sheep	France	Pasteurized	Hard	1	1	4
	Goat	Portugal	Pasteurized	Semi-hard	1	2	3
	Goat	Spain	Pasteurized	Semi-hard	1	1	2
	Goat	The Netherlands	Pasteurized	Semi-hard Gouda	1	2	3
	Goat	The Netherlands	Pasteurized	Semi-hard, natural	1	1	2

			rind			
Goat	Spain	Pasteurized	Semi-hard, washed rind	2	5	5
Goat	France	Pasteurized	Semi-hard, washed rind	1	1	6
Goat	Canada	Pasteurized	Brined	1	1	3
Goat	Canada	Pasteurized	Fresh	1	2	3
Goat	France	Pasteurized	Soft	1	1	1
Goat	Italy	Raw	Hard	1	2	4
Goat	Switzerland	Raw	Semi-hard	1	2	4
Sheep	Italy	Pasteurized	Hard	2	4	7
Sheep	Spain	Pasteurized	Hard	2	1	6
Sheep	Canada	Pasteurized	Hard, natural rind	1	1	5
Sheep	France	Pasteurized	Hard, natural rind	1	3	4
Sheep	Cyprus	Pasteurized	Semi-hard	1	2	2
Sheep	France	Pasteurized	Semi-hard	1	1	3
Sheep	Italy	Pasteurized	Semi-hard	2	4	5
Sheep	Italy	Pasteurized	Semi-hard, natural rind	2	3	7
Sheep	The Netherlands	Pasteurized	Semi-hard, natural rind	1	1	3
Sheep	France	Pasteurized	Semi-hard, washed rind	1	1	5
Sheep	France	Pasteurized	Brined	1	1	4
Sheep	Greece	Pasteurized	Brined	1	1	2
Sheep	New York, USA	Pasteurized	Fresh	1	1	0

	Sheep	Italy	Raw	Hard	1	3	5
	Sheep	USA	Raw	Hard, natural rind	1	2	5
	Sheep	Spain	Raw	Semi-hard	1	2	2
	Sheep	Spain	Raw	Semi-hard, natural rind	1	1	4
	Sheep	Spain	Raw	Soft	1	3	2
Yogurt	Cow	USA	Pasteurized		22	75	NA
	Cow	USA	Pasteurized	Added fruit	2	2	NA
	Cow	USA	Pasteurized	Added fruit and other	6	6	NA

[a]Detailed information on the number of isolates obtained from direct plating and enrichment of the different samples can be found in Supp. Table 2.2

Fungal Isolation

Raw milk samples were plated directly on dichloran rose bengal chloramphenicol agar (DRBC; Becton, Dickinson and Co., Sparks, MD). Additionally, two 200-mL enrichments were prepared, each consisting of 100 mL malt extract broth (MEB; Becton, Dickinson and Co., Sparks, MD), with 40 mg/L streptomycin sulfate (Sigma, St. Louis, MO) and 100 mL raw milk. Enrichments were prepared in sample bags and homogenized by hand for 30s before incubation; one enrichment was incubated at 25 ± 2°C for 72 h and one was incubated at 18 ± 2°C for 120 h. After these incubations, undiluted as well as serially diluted enrichments were spread plated on DRBC, followed by incubation at 25 ± 2°C for 120 h.

Cheese samples were tested by mixing approximately 20g of cheese with 20mL MEB (containing 40 mg/L streptomycin sulfate) in a sampling bag (prepared in duplicate), followed by homogenization at 260 rpm for 60s in a Stomacher 400 Circulator (Seward Ltd., United Kingdom). One homogenized enriched cheese sample was plated directly on DRBC, followed by incubation at 25 ± 2°C for 120 h. In addition, the two homogenized sample enrichments were incubated either at 25 ± 2°C for 72 h or at 18 ± 2°C for 120 h. After these incubations, undiluted enrichments were spread plated on DRBC, followed by incubation at 25 ± 2°C for 120 h.

Yogurt samples were diluted 1:10 in phosphate buffer solution (Weber Scientific, Hamilton, NJ) in a sampling bag, followed by homogenization at 260 rpm for 60s in a Stomacher 400 Circulator (Seward Ltd., United Kingdom). Each homogenized diluted yogurt sample was spread plated directly on DRBC and incubated at 25 ± 2°C for 120 h.

For all sample types, fungal colonies present on DRBC were visually examined, and colonies with distinct morphologies were selected and streaked for purity on malt extract agar (MEA; Becton, Dickinson and Co., Sparks, MD). For each sample, DRBC plates were evaluated separately to select unique phenotypes from both direct plating and enrichment for further characterization. Per sample between 0 and 10 isolates (average of 2 isolates) were collected from direct plating and between 0 and 11 isolates (average of 3 isolates) were collected from sample enrichments (Supplemental Table 2). All isolates were then frozen at -80°C in 15% glycerol. Isolate characteristics, including nucleotide sequences, and relevant sample information are available through the Food Microbe Tracker database at www.foodmicrobetracker.com (Vangay et al., 2013); http://www.foodmicrobetracker.com/search/display_list.aspx?refid=505 provides a direct link to all 361 isolates reported here.

ITS Sequencing

Isolates obtained as described above were characterized by DNA sequencing of the ITS region, using the ITS primers described by Schoch et al. (2012). Briefly, purified DNA for PCR was prepared for each isolate from 120 h cultures on MEA using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA). PCR conditions were slightly modified from Schoch et al. (2012) and consisted of (i) 1 cycle of 95°C for 5 min; (ii) 35 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min; (iii) 1 cycle of 72°C for 10 min, followed by a 4°C hold. PCR product amplification was confirmed by gel electrophoresis and PCR products were purified with the ExoSAP method (Affymetrix, Santa Clara, CA). Sanger sequencing with PCR primers was

performed at Cornell University's Life Sciences Core Laboratory Center (Ithaca, NY) using the ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). DNA sequences were assembled and proofread in Sequencher (version 5.3, Gene Codes Corporation, Ann Arbor, MI), and high-quality, double-stranded sequence data were used for further analysis.

Sequences were aligned using MUSCLE (Edgar, 2004) and ITS fragments, ranging in length from 251-nt to 724-nt, corresponding to two internal transcribed spacers and the 5.8S gene between them (hereafter referred to as the ITS region) were used for subsequent analyses.

LSU Sequencing

Isolates that could not be amplified using the ITS region primers were characterized by sequencing part of the gene that encodes the D1/D2 domain of the nuclear large subunit (LSU) (26S) ribosome, using primers NL1 and NL 4 (Kurtzman and Robnett, 1998). PCR conditions were slightly modified from Kurtzman and Robnett (1998) and consisted of (i) 1 cycle of 94°C for 5 min; (ii) 36 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 2 min; (iii) 1 cycle of 72°C for 10 min, followed by a 4°C hold. PCR products were purified and sequenced as described above for ITS sequencing.

AT Assignment

ITS allelic types (ATs) were assigned using BLAST. A unique ITS AT was assigned to every gene sequence that differed from any previously obtained sequence by one or more nucleotides. The first isolate of each new ITS AT was designated the reference strain for that AT.

Alignment, Tree Construction and Species Identification

For species identification, the ITS region sequences for each unique ITS AT were queried against ITS region sequences in the Unite database (Kõljalg et al., 2005). LSU sequences were queried against LSU sequences in Mycobank (Crous et al., 2004). An isolate with an identity score of ≥99.00% to exactly one type strain was assigned the species ID of that type strain. Isolates that had an identity score of ≥99.00% against more than one type strain in the same phyla were assigned the finest classification rank available (typically a genus designation; but in some cases only family designations could be assigned). Identity scores between 97.00% and 99.00% were assigned a phylum; no isolates showed identity scores lower than 97.00%.

For isolates that could not be assigned genus and species identifications from the UNITE database, Mycobank was used to allow for similarity searches against additional fungal databases. Isolates with an identity score of ≥99.00% to exactly one type strain were assigned the species ID of that type strain. For the genera *Penicillium*, *Mucor*, and *Geotrichum*, where known species complexes exist and database queries may result in unreliable identifications, ITS maximum-likelihood (ML) phylogenetic trees were constructed with sequences for type strains and isolates characterized in the study reported here. *Penicillium* type strains were acquired from the most recent verified reference database for the genus (Visagie et al., 2014). For the genera *Mucor* and *Geotrichum* no recent published verified reference databases are available. For these genera, the most recent publications providing DNA barcodes for type strains were hence used (De Hoog and Smith, 2004, Walther et al., 2013). Phylogenetic trees were constructed using the rapid maximum-likelihood algorithm RAxML (Stamatakis,

2006) with rapid bootstrapping and 100 bootstrap replicates (Supplemental Figures 2.1-2.3). Species level identification was only assigned to isolates if the ITS sequence representing a specific allelic type (AT) associated with a given isolates clearly clustered with a ITS sequence of a single type specimen with a bootstrap value of >50. The same approach to construct phylogenetic trees was also used to construct an ITS ML phylogenetic tree with all the ITS ATs identified in the study reported here (Figure 2.1).

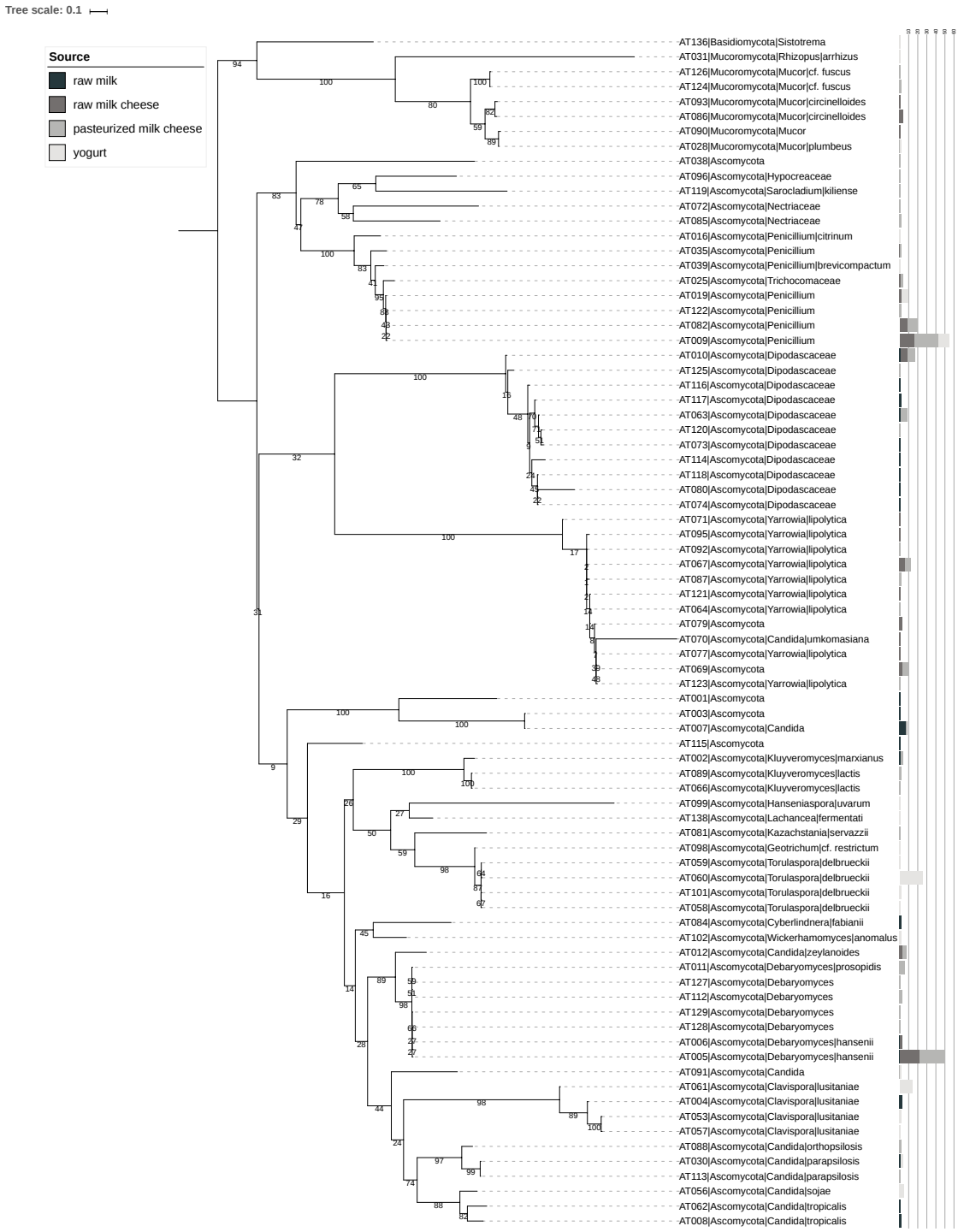


Figure 2.1. Midpoint-rooted maximum-likelihood (ML) phylogenetic tree of ITS region sequences from fungal species isolated from raw milk (black), raw milk cheese (dark grey), pasteurized milk cheese (medium grey), and yogurt (light grey). Numerical values indicate the percentage of bootstrap replications (out of 100 total replications) that support the corresponding node. Species identification of ATs was based on ITS region sequence analyses as described in Materials and Methods. ATs that could not be identified to the species level with a ≥99.00% identity score were assigned the finest classification rank.

RESULTS AND DISCUSSION

ITS Sequencing Provides for Rapid and Reliable Characterization of Yeasts and Molds, and Reveals Ascomycetes are Major Contributors of Dairy Product Fungal Contamination

A total of 361 isolates obtained from raw milk and different dairy products as described above were characterized by ITS PCR amplification and sequencing; 346 isolates yielded ITS products that could be successfully sequenced. The remaining 15 isolates, which all did not yield PCR products with primers ITS4 and ITS5, were characterized by sequencing part of the gene that encodes D1/D2 domain of the nuclear large subunit (LSU) (26S) rRNA. Thirteen of these isolates were successfully characterized by sequencing the D1/D2 domain, whereas two isolates could not be successfully sequenced using either ITS or LSU primers. The 359 isolates that could be characterized by either ITS or LSU sequencing represent the phyla *Ascomycota* (347 isolates), *Basidiomycota* (1 isolate) and *Mucoromycota* (11 isolates). It is important to note that the phylum-level classification of zygomycete fungi remains to be fully defined and clarified; while *Mucoromycota* represents the most current name for these organisms (Spatafora et al., 2016), the taxonomic classification of these organisms into phyla is likely to change.

Overall, the ITS sequence data for the 346 dairy-associated isolates from raw milk, raw and pasteurized milk cheeses, and yogurt yielded 81 unique ITS ATs. Using the Unite database (as well as Mycobank as needed), all 346 isolates that yielded ITS sequence data could be identified to at least the phylum level; 183 isolates could be assigned a species (based on an identity score of ≥99.00% against ITS region

sequences in the Unite Database) and 107 could be assigned a genus, but not a species. The most common AT (AT9, 55 isolates) was classified as a *Penicillium* species, while the second most common AT (AT5, 50 isolates) was classified as *Debaryomyces hansenii*. Importantly though, ITS AT-based species identifications should be interpreted carefully as current uncertainties as well as likely future changes in fungal taxonomy will affect ITS sequencing based species identification. Furthermore, ITS sequencing does not provide high resolution power for species complexes such as the *Penicillium camemberti* clade, *Mucor circinelloides,* and *Geotrichum*. Additional characterization methods, further discussed below, are necessary to confidently assign species names for these organisms.

Overall, our data indicate that ITS sequencing provides for straight-forward initial characterization of the majority of fungal isolates obtained from dairy products as 96% of isolates yielded sequence data suitable for searches against the Unite ITS database with 107 and 183 of isolates identified to the genus and species level. Although we were able to find one large study that characterized 610 yeast and mold isolates from raw milk and raw milk cheeses (Lavoie et al., 2011), our study represents the largest molecular study characterizing yeast and mold isolates across diverse dairy products. Most other previous studies (Sulaiman et al., 2014, Banjara et al., 2015, Garnier et al., 2017) that used ITS sequencing for characterization of dairy isolates included less than 175 isolates. Importantly, our findings are consistent with previous studies that have shown that "ITS barcoding" provides for a standardized ITS AT nomenclature that can transcend taxonomic changes. In addition, our data showed considerable AT diversity among the dairy isolates characterized. We also found that

ITS AT data can sometimes differentiate subtypes within a given species, for example, *Clavispora lusitaniae* included 4 different ATs. This indicates that ITS-based AT data (even for isolates that cannot be identified to the genus or species level) can be used for isolate characterization, which can help dairy producers assess whether isolates obtained at different times or locations (e.g., in ingredients and finished product) may or may not have a common source. While this may in some cases allow for exclusion of a source, more sensitive subtyping methods are needed to more definitively identify contamination sources. Specifically, even though ITS sequencing has high reliability for initial characterization and identification (given appropriate databases), it does not provide as high resolution power as other characterization and subtyping techniques (Stielow et al., 2015). Furthermore, intragenomic heterogeneities involving the ITS region have been documented in different fungal species such as *Geotrichum candidum* (Alper et al., 2011) and other yeast species including *Candida glabrata, Candida tropicalis*, *Pichia norvegensis*, *and Saccharomyces cerevisiae* (Zhao et al., 2015), potentially complicating ITS-based AT and species assignment. Importantly, sequencing different regions such as protein coding genes may provide higher resolution at the species level. For example, Garnier et al. (2017) characterized 41 unique fungal species from 175 French yogurt and cheese isolates by using ITS sequencing as a preliminary step, followed by sequencing of protein-coding genes (e.g. partial Beta-tubulin gene and partial elongation factor 1-alpha) for higher resolution power. If further discrimination beyond the species levels (i.e., subtyping) is required for fungal organisms, fragment analysis techniques such as amplified fragment length polymorphism (AFLP), microsatellites, and random amplified

polymorphic DNA (RAPD) may be appropriate tools (Almeida and Araujo, 2013, Saghrouni et al., 2013).

Ascomycetous Yeasts are Primary Fungal Contaminants in Raw Milk

The 38 fungal isolates obtained from raw milk samples (Table 2.2) all represented yeast, with no molds identified. For 4 isolates, the ITS PCR did not yield an amplicon; LSU sequencing was successful for 3 of these isolates, which were classified as *Pichia*, *Candida*, and *Ascomycota*. The 34 isolates with ITS sequence data represented 21 different ITS ATs; the most common AT among these isolates (AT7; 7 isolates) was classified as *Candida*. The remaining 20 ATs found among the raw milk isolates each represented 3 or fewer isolates. Interestingly, one isolate was identified with LSU sequence data as the yeast *Pichia rhodanensis*, a species that has not been previously reported as being isolated from raw milk [other *Pichia* species have been reported from raw milk though (Cocolin et al., 2002, Lavoie et al., 2011, Panelli et al., 2013)]. The most common genera identified among the 23 isolates with genus level identification were *Candida*. The predominance of yeast isolates was not surprising as a recent review suggest that yeast populations of 10^1 to 10^3 cfu/ml of raw milk are typical (Fleet, 2011). The fact that *Candida* was a common genus identified among raw milk isolates is also consistent with previous reports (Cocolin et al., 2002, Lavoie et al., 2011). For example, Lavoie et al. (2011) found *Candida* species in 44% of the 111 raw milk samples collected from farms in Quebec, Canada. While *Candida* species have been linked to bovine mastitis (Watts, 1988, dos Santos and Marin, 2005), it is likely that yeasts such as *Candida* are introduced into raw milk from the dairy farm environment as *Candida* species are widely distributed in the environment

and air (Torkar and Vengušt, 2008). In a study of 16 dairy farms in France, Vacheyrou et al. (2011) found that the same yeast species isolated from 9 stable and 3 milking parlor environments were also isolated from 14 of the farms' bulk tank raw milk. Importantly, *Candida* species are heat sensitive and are easily killed during processing; studies indicate that most *Candida* strains, at 10^5 cells/mL, can survive 20 min at 55°C but not 10 min at 62.5°C (Put et al., 1976).

Table 2.2. Unique ITS ATs isolated from raw milk, cheese, and yogurt

Genus and species (or finest classification rank possible)[1]	ITS AT	No. of isolates with a given AT obtained from				Total no. of isolates
		raw milk	raw milk cheese	past. milk cheese	yogurt	
Ascomycota						
Ascomycota[3]	1	1				1
Ascomycota[3]	3	1				1
Ascomycota[3]	38			1		1
Ascomycota[3]	69		3	6		9
Ascomycota[3]	79		3			3
Ascomycota[3]	115	1				1
Ascomycota[3]	137				1	1
Candida orthopsilosis	88			2		2
Candida parapsilosis	30	2			2	4
Candida parapsilosis	113			1		1
Candida sojae	56				5	5
Candida spp.	7	7		1		8
Candida spp.	91			1	1	2
Candida tropicalis	8	2				2
Candida tropicalis	62	1				1
Candida umkomasiana	70		2			2
Candida zeylanoides	12		3	4		7
Clavispora lusitaniae	4	3				3
Clavispora lusitaniae	53				2	2
Clavispora lusitaniae	57				1	1
Clavispora lusitaniae	61				14	14
Cyberlindnera fabianii	84	2				2
Debaryomyces hansenii	5	1	21	28		50
Debaryomyces hansenii	6	1	2			3
Debaryomyces prosopidis	11			6		6
Debaryomyces spp.	112			3		3
Debaryomyces spp.	127			1		1
Debaryomyces spp.	128			1		1
Debaryomyces spp.	129			1		1
Dipodascaceae[4]	10	1	8	8		17
Dipodascaceae[4]	63	1		7		8
Dipodascaceae[4]	73	1				1
Dipodascaceae[4]	74	1				1
Dipodascaceae[4]	80	1				1
Dipodascaceae[4]	114	1				1
Dipodascaceae[4]	116	1				1
Dipodascaceae[4]	117	2				2
Dipodascaceae[4]	118	1				1

Dipodascaceae[4]	120			1		1
Dipodascaceae[4]	125			1		1
Geotrichum cf. *restrictum*[2]	98				1	1
Hanseniaspora uvarum	99				1	1
Hypocreaceae[4]	96			1		1
Kazachstania servazzii	81			1		1
Kluyveromyces lactis	66			1		1
Kluyveromyces lactis	89			2		2
Kluyveromyces marxianus	2	2		2		4
Lachancea fermentati	138				1	1
Nectriaceae[4]	72			1		1
Nectriaceae[4]	85			2		2
Penicillium brevicompactum	39				1	1
Penicillium citrinum	16				1	1
Penicillium spp.	9		16	27	12	55
Penicillium spp.	19		2		7	9
Penicillium spp.	35		1	1		2
Penicillium spp.	82		9	11		20
Penicillium spp.	122			2		2
Sarocladium kiliense	119			1		1
Trichocomaceae[4]	25		2	2		4
Torulaspora delbrueckii	58				1	1
Torulaspora delbrueckii	59				1	1
Torulaspora delbrueckii	60				25	25
Torulaspora delbrueckii	101				1	1
Wickerhamomyces anomalus	102				2	2
Yarrowia lipolytica	64			1		1
Yarrowia lipolytica	67		6	6		12
Yarrowia lipolytica	71		1			1
Yarrowia lipolytica	77		1			1
Yarrowia lipolytica	87			2		2
Yarrowia lipolytica	92			1		1
Yarrowia lipolytica	95		1			1
Yarrowia lipolytica	121		1			1
Yarrowia lipolytica	123			1		1
Basidiomycota						
Sistotrema spp.	136				1	1
Mucoromycota						
Mucor circinelloides	86		4			4
Mucor circinelloides	93		1			1
Mucor cf. *fuscus*[2]	124			2		2
Mucor cf. *fuscus*[2]	126			1		1
Mucor plumbeus	28				1	1
Mucor spp.	90		1			1
Rhizopus arrhizus	31				1	1

[1]Column subheaders (e.g., *Ascomycota*, *Basidiomycota*, and *Mucoromycota*) represent phyla
[2] cf. = short for the Latin "confer" ("compare with"); signifies ATs that resemble the given named species, but where identification represents considerable uncertainty.
[3]These ITS ATs could only be identified to the phylum level
[4]Represents a family-level classification

Debaromyces Represents a Major Contributor to Fungal Microflora of Raw Milk Cheese and Pasteurized Milk Cheese

Overall, 90 yeasts and molds isolated from 14 raw milk cheese samples and 150 yeasts and molds isolated from 33 pasteurized milk cheese samples (Table 2.1) were initially characterized by ITS sequencing. A total of 47 different ITS ATs were identified among the 240 isolates. Among the 90 raw milk cheese isolates, the most common ATs were classified as *Debaryomyces hansenii* (AT5; 21 isolates), *Penicillium* spp. (AT9; 16 isolates), *Penicillium* spp. (AT82; 9 isolates), and *Dipodascaceae* spp. (AT10; 8 isolates) (Table 2.2). The remaining 16 ATs found among the raw milk cheese isolates each represented 6 or fewer isolates. Among the 150 pasteurized milk cheese isolates, the most common ATs were classified as *Debaryomyces hansenii* (AT5; 28 isolates), *Penicillium* spp. (AT9; 27 isolates), *Penicillium* spp. (AT82; 11 isolates), and *Dipodascaceae* spp. (AT10; 8 isolates) (Table 2.2). The remaining 32 ATs found among the pasteurized milk cheese isolates each represented 7 or fewer isolates. Consistent with our results, Wolfe et al. (2014) previously reported isolation of a considerable diversity of fungal organisms from the surfaces of different cheeses. When studying 137 cheese rinds from 10 different countries, this group identified 10 fungal genera that were consistent across all samples. While we elected to not include cheeses that were clearly inoculated with

fungal organisms (e.g., brie, blue cheese, bloomy rind cheeses, where the rind is intentionally inoculated with fungal organisms), we still reported considerable fungal diversity (at least 9 genera across 47 cheese samples). It is possible however that some cheeses included in our study reported here were deliberately inoculated through the washed rind process. Fungal organisms such as *Debaryomyces hansenii*, *Geotrichum candidum*, and *Yarrowia lipolytica* (all of which were isolated from cheeses in our study reported here) can be included in the brine solution that is used to wash the cheese rind or may be directly sprayed to the cheese's surface (Petersen et al., 2002, Bokulich and Mills, 2013). These organisms can be desirable in cheese and provide proteolytic and lipolytic enzymes as well as aroma compounds during the ripening process (Fleet, 1990). Thus, the frequency of the isolates from cheese reported below may include contaminants as well as deliberately inoculated organisms.

Fisher's exact test showed that there was no significant difference in the distribution of ATs among raw and pasteurized milk cheese samples ($p>0.05$); for this test ATs that occurred <5 times (combined frequency for raw and pasteurized cheese isolates) were combined into a single category ("uncommon ATs"). Consequently, results for raw and pasteurized milk cheese data are discussed combined below. Importantly, the observation that yeast and mold AT distribution does not differ between raw and pasteurized milk cheese samples suggests that contamination with these organisms predominantly occurs from the processing environment, consistent with the fact that the genera isolated here from cheeses are typically heat sensitive. For example, Wallace and Tanner (1931) showed that the conidia of *Penicillium* species are killed after heating for 5 minutes at 60°C.

Among the raw and pasteurized milk cheese fungal isolates, the most common ATs were identified as *D. hansenii* (AT5; 49 isolates), *Penicillium* spp. (AT9; 43 isolates), and *Penicillium* spp. (AT82; 20 isolates). The frequent occurrence of *D. hansenii* in a variety of cheeses is consistent with previous studies (Gardini et al., 2006, Capece and Romano, 2009, Padilla et al., 2014, Atanassova et al., 2016). For example, Capece and Romano (2009) sampled the curd and rind of two "Pecorino di Filiano" cheeses throughout each cheeses' ripening time of 120 days and reported that 96% of cheese samples tested positive for *D. hansenii*. The occurrence of *D. hansenii* can be attributed to the ability of this species to grow in extreme environments such as high salt concentrations, low temperature, and low pH (Gori et al., 2012), and its ability to metabolize lactic and citric acids (Capece and Romano, 2009). *D. hansenii* has also been isolated from brine solutions, raw milk, and dairy environments (Gori et al., 2012). In another study, Mounier et al. (2006) isolated the same *D. hansenii* strain from the surface of an Irish smear-ripened cheese as well as from the brine solution, processing equipment, and the hands of the workers in the dairy plant. These results mirror our results of frequent isolation of *D. hansenii* and suggest that *D. hansenii* can colonize the surface of washed-rind cheeses. The *Penicillium* section Fasciculata, series *Camemberti* clade includes dairy-relevant species such as *P. camemberti* and *P. commune*. These species are typically distinguished by their phenotypic characteristics; *Penicillium commune* has blue-green conidia while *P. camemberti* has white conidia. While *P. camemberti* is a desired organism in mold-ripened cheeses, *P. commune* has been previously reported as one of the most frequently occurring spoilage molds in cheese; dairy isolates classified into this species have also been

shown to rapidly grow at refrigeration temperatures (Lund et al., 1995, Kure et al., 2001, Hayaloglu and Kirbag, 2007). Growth of *P. commune* on cheese has been reported to result in discoloring of the surface and the production of off-flavors (Lund et al., 2003). Overall, *Penicillium* species (e.g., *P. commune, P. biforme*) closely related to AT 9 and AT 82, which were found here to be common among raw and pasteurized milk cheeses isolates, have previously been shown to be able to cause product defects that consumers can detect (Lund et al., 1995, Pitt and Hocking, 2009, Giraud et al., 2010). Prevention of contamination with these fungi (e.g., from environmental and raw material sources) is thus important to prevent quality issues with raw milk and pasteurized milk cheeses.

Torulaspora delbrueckii Represents a Major Contributor to Fungal Contamination in Yogurt

In total, 59 yeasts and 24 molds were isolated from 30 yogurt samples (Table 2.1). Twenty-two different ITS ATs were identified among these 83 isolates (Table 2.2). The most common ATs represented *Torulaspora delbrueckii* (AT60; 25 isolates), *Clavispora lusitaniae* (AT61; 14 isolates), *Penicillium* spp. (AT9; 12 isolates), and *Penicillium* spp. (AT19; 7 isolates). The remaining 18 ATs found among the yogurt isolates each represented 5 or fewer isolates.

Consistent with common identification of yeasts (e.g., *Candida* and *Kluyveromyces*) among yogurt isolates, yeasts are well documented as a major cause of spoilage in yogurt as the low pH of the product selects for their growth (Rohm et al., 1992, Mataragas et al., 2011, Krisch et al., 2014). For example, Suriyarachchi and

Fleet (1981) reported that among 128 yogurt samples, the yeasts *Candida famata* and *Kluyveromyces marxianus* were isolated from 16% and 9% of samples, respectively, and represent the most frequently isolated species in their study. Likewise, Green and Ibe (1987) reported similar findings for 100 yogurt samples, with *Clavispora lusitaniae, K. marxianus,* and *Candida krusei* isolated from 65, 48, and 51 of samples, respectively.

Previous studies have reported the presence of *T. delbrueckii* in dairy products such as cheese (Westall and Filtenborg, 1998); however, to our knowledge our study here is the first report of the presence of *T. delbrueckii* in yogurt. This finding could be attributed to the different methods used to identify the fungal isolates here and in previous studies. For example, Suriyarachchi and Fleet (1981) used physiological tests such as carbohydrate fermentation, nitrogen assimilation, and casein hydrolysis to characterize yeast isolates and then identified the isolates to species level with the use of a standard key for yeast identification. The standard key used in Suriyarachchi and Fleet's study contained only 434 yeast species. In contrast, the UNITE database, which we used to query ITS region sequences, contains sequence identification information for over 24,000 ascomycetes, many of which would be classified as yeasts (Kõljalg et al., 2005). Consequently, it is possible that *T. delbrueckii* were previously isolated, but were not identified as this species. Importantly, this illustrates the advantages of using ITS sequencing, along with fungal sequence databases with high quality reference ITS sequences, such as UNITE, for fungal characterization and identification.

The occurrence of molds in yogurt has not been widely reported. In one study,

Sulaiman et al. (2014) documented *Rhizomucor variabilis* in 15 recalled Greek yogurt samples. In another study, Snyder et al. (2016) characterized *Mucor circinelloides* spoilage in yogurt. In this study, we document the presence of *Penicillium*, *Mucor*, *Rhizopus*, and *Sistotrema* in yogurt. The occurrence of these genera in yogurt samples could suggest contamination from environmental sources, consistent with previous studies. Moist environments commonly found in dairy plants can support the growth of molds, which can lead to mold growth on floors, ceilings, walls, and drains (Sørhaug, 2011). Once in the dairy environment, mold spores can become airborne and contaminate the product (Salustiano et al., 2003). For example, Fleet reported fungi may be present in the processing environment and can enter the product during packaging (1990). Moreover, Snyder et al. (2016) hypothesized that *M. circinelloides* contamination in yogurt represented post-processing contamination as the species is heat sensitive. Thus, these studies and our study reported here suggest that fungal contaminants may be present in the dairy processing environment and may contaminate the product after heating.

CONCLUSION

Our data show that ITS sequencing is a useful and standardized tool for fungal classification, facilitated by a high PCR and sequencing success rate across a broad range of fungi as well as a large fungal sequence database (UNITE) with high quality reference ITS sequences. However additional databases and phylogenetic analyses are sometimes needed to identify fungal isolates from milk and dairy products. On-going realignments of fungal taxonomy however can represent a challenge for meaningful identification of some isolates based on ITS sequences. Importantly however, ITS

sequence data transcends taxonomic changes and isolate identification based on ITS data can easily be revised and updated as taxonomic changes are made. Through the use of DNA sequence-based approaches, we also demonstrated that fungi in dairy represent a broad diversity over multiple phyla. Overall, our data represent a step in developing new tools and approaches to better control dairy product spoilage due to fungal contaminants.

ACKNOWLEDGEMENTS

This publication was made possible through a gift from Chobani Inc. (New Berlin, NY), supporting innovative research in dairy quality. We thank them for their generous contribution and commitment to food innovation and quality.

REFERENCES

Almeida, L. A. and R. Araujo. 2013. Highlights on molecular identification of closely related species. Infection, Genetics and Evolution 13:67-75.

Alper, I., M. Frenette, and S. Labrie. 2011. Ribosomal DNA polymorphisms in the yeast Geotrichum candidum. Fungal Biol. 115:1259-1269.

Atanassova, M., C. Fernández-Otero, P. Rodríguez-Alonso, I. Fernández-No, J. Garabal, and J. Centeno. 2016. Characterization of yeasts isolated from artisanal short-ripened cows' cheeses produced in Galicia (NW Spain). Food Microbiol. 53:172-181.

Banjara, N., M. J. Suhr, and H. E. Hallen-Adams. 2015. Diversity of yeast and mold species from a variety of cheese types. Curr. Microbiol. 70:792-800.

Bokulich, N. A. and D. A. Mills. 2013. Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. Appl. Environ. Microbiol. 79:5214-5223.

Callon, C., F. Duthoit, C. Delbès, M. Ferrand, Y. Le Frileux, R. De Crémoux, and M.-C. Montel. 2007. Stability of microbial communities in goat milk during a lactation year: Molecular approaches. Syst. Appl. Microbiol. 30:547-560.

Capece, A. and P. Romano. 2009. "Pecorino di Filiano" cheese as a selective habitat for the yeast species, Debaryomyces hansenii. Int J. Food Microbiol. 132:180-184.

Cocolin, L., D. Aggio, M. Manzano, C. Cantoni, and G. Comi. 2002. An application of PCR-DGGE analysis to profile the yeast populations in raw milk. Int. Dairy J. 12:407-411.

Crous, P. W., W. Gams, J. A. Stalpers, V. Robert, and G. Stegehuis. 2004. MycoBank: an online initiative to launch mycology into the 21st century. Stud. Mycol. 50:19-22.

De Hoog, G. S. and M. T. Smith. 2004. Ribosomal gene phylogeny and species delimitation in Geotrichum and its teleomorphs. Stud Mycol 50:489-515.

Delavenne, E., J. Mounier, K. Asmani, J.-L. Jany, G. Barbier, and G. Le Blay. 2011. Fungal diversity in cow, goat and ewe milk. Int. J. Food Microbiol. 151:247-251.

dos Santos, R. d. C. and J. M. Marin. 2005. Isolation of Candida spp. from mastitic bovine milk in Brazil. Mycopathologia 159:251-253.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32:1792-1797.

Fleet, G. 1990. Yeasts in dairy products. J. Appl. Bacteriol. 68:199-211.

Fleet, G. H. 2011. Yeast spoilage of foods and beverages. Pages 53-63 in The Yeasts. 5 ed. Elsevier, London, UK.

Fröhlich-Wyder, M.-T. 2003. Yeasts in dairy products. Pages 209-237 in Yeasts in Food. Woodhead Publishing, Boca Raton, FL.

Gardini, F., R. Tofalo, N. Belletti, L. Iucci, G. Suzzi, S. Torriani, M. Guerzoni, and R. Lanciotti. 2006. Characterization of yeasts involved in the ripening of Pecorino Crotonese cheese. Food Microbiol. 23:641-648.

Garnier, L., F. Valence, A. Pawtowski, L. Auhustsinava-Galerne, N. Frotté, R. Baroncelli, F. Deniel, E. Coton, and J. Mounier. 2017. Diversity of spoilage fungi associated with various French dairy products. Int. J. Food Microbiol. 241:191-197.

Giraud, F., T. Giraud, G. Aguileta, E. Fournier, R. Samson, C. Cruaud, S. Lacoste, J. Ropars, A. Tellier, and J. Dupont. 2010. Microsatellite loci to recognize species for the cheese starter and contaminating strains associated with cheese manufacturing. Int. J. Food Microbiol. 137:204-213.

Gori, K., L. M. Sørensen, M. A. Petersen, L. Jespersen, and N. Arneborg. 2012. Debaryomyces hansenii strains differ in their production of flavor compounds in a cheese-surface model. Microbiologyopen 1:161-168.

Green, M. D. and S. N. Ibe. 1987. Yeasts as primary contaminants in yogurts produced commercially in Lagos, Nigeria. J. Food Prot. 50:193-205.

Hayaloglu, A. and S. Kirbag. 2007. Microbial quality and presence of moulds in Kuflu cheese. Int. J. Food Microbiol. 115:376-380.

Jacques, N. and S. Casaregola. 2008. Safety assessment of dairy microorganisms: the hemiascomycetous yeasts. Int. J. Food Microbiol. 126:321-326.

Kõljalg, U., K. H. Larsson, K. Abarenkov, R. H. Nilsson, I. J. Alexander, U. Eberhardt, S. Erland, K. Høiland, R. Kjøller, and E. Larsson. 2005. UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. New Phytol. 166:1063-1068.

Krisch, J., J. Csanádi, and C. Vágvölgyi. 2014. Fungi in and on dairy products. Pages 159-169 in Progress in Mycological Research: Fungi From Different Substrates. CRC Press, London, UK.

Kure, C. F., E. Borch, I. Karlsson, J. P. Homleid, and S. Langsrud. 2008. Use of the selective agar medium CREAD for monitoring the level of airborne spoilage moulds in cheese production. Int. J. Food Microbiol. 122:29-34.

Kure, C. F., Y. Wasteson, J. Brendehaug, and I. Skaar. 2001. Mould contaminants on Jarlsberg and Norvegia cheese blocks from four factories. Int. J. Food Microbiol. 70:21-27.

Kurtzman, C. P. and C. J. Robnett. 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie Van Leeuwenhoek 73:331-371.

Lavoie, K., M. Touchette, D. St-Gelais, and S. Labrie. 2011. Characterization of the fungal microflora in raw milk and specialty cheeses of the province of Quebec. Dairy Sci Technol 92:455-468.

Ledenbach, L. H. and R. T. Marshall. 2009. Microbiological spoilage of dairy products. Pages 41-67 in Compendium of the Microbiological Spoilage of Foods and Beverages. Springer, New York, NY.

Lund, F., O. Filtenborg, and J. Frisvad. 1995. Associated mycoflora of cheese. Food Microbiol. 12:173-180.

Lund, F., A. B. Nielsen, and P. Skouboe. 2003. Distribution of Penicillium commune isolates in cheese dairies mapped using secondary metabolite profiles, morphotypes, RAPD and AFLP fingerprinting. Food Microbiol. 20:725-734.

Mataragas, M., V. Dimitriou, P. N. Skandamis, and E. H. Drosinos. 2011. Quantifying the spoilage and shelf-life of yoghurt with fruits. Food Microbiol. 28:611-616.

Mayoral, M. B., R. Martín, A. Sanz, P. E. Hernández, I. González, and T. García. 2005. Detection of Kluyveromyces marxianus and other spoilage yeasts in yoghurt using a PCR-culture technique. Int. J. Food Microbiol. 105:27-34.

Mounier, J., S. Goerges, R. Gelsomino, M. Vancanneyt, K. Vandemeulebroecke, B. Hoste, N. Brennan, S. Scherer, J. Swings, and G. Fitzgerald. 2006. Sources of the adventitious microflora of a smear-ripened cheese. J. Appl. Microbiol. 101:668-681.

Newkirk, R. W., J. B. Bender, and C. W. Hedberg. 2012. The potential capability of social media as a component of food safety and food terrorism surveillance systems. Foodborne Pathog. Dis. 9:120-124.

Padilla, B., P. Manzanares, and C. Belloch. 2014. Yeast species and genetic heterogeneity within Debaryomyces hansenii along the ripening process of traditional ewes' and goats' cheeses. Food Microbiol. 38:160-166.

Panelli, S., E. Brambati, C. Bonacina, and M. Feligini. 2013. Diversity of fungal flora in raw milk from the Italian Alps in relation to pasture altitude. Springerplus 2:405.

Petersen, K. M., S. Westall, and L. Jespersen. 2002. Microbial Succession of *Debaryomyces hansenii* Strains During the Production of Danish Surfaced-Ripened Cheeses. J. Dairy Sci. 85:478-486.

Pitkäranta, M., T. Meklin, A. Hyvärinen, L. Paulin, P. Auvinen, A. Nevalainen, and H. Rintala. 2008. Analysis of fungal flora in indoor dust by ribosomal DNA sequence analysis, quantitative PCR, and culture. Appl. Environ. Microbiol. 74:233-244.

Pitt, J. I. and A. D. Hocking. 2009. Fungi and Food Spoilage. 3 ed. Springer, New York, NY.

Put, H., J. De Jong, F. Sand, and A. Van Grinsven. 1976. Heat resistance studies on yeast spp. causing spoilage in soft drinks. J. Appl. Bacteriol. 40:135-152.

Rohm, H., F. Eliskases-Lechner, and M. Bräuer. 1992. Diversity of yeasts in selected dairy products. J. Appl. Bacteriol. 72:370-376.

Saghrouni, F., J. Ben Abdeljelil, J. Boukadida, and M. Ben Said. 2013. Molecular methods for strain typing of Candida albicans: a review. J. Appl. Microbiol. 114:1559-1574.

Salustiano, V. C., N. J. Andrade, S. C. C. Brandão, R. M. C. Azeredo, and S. A. K. Lima. 2003. Microbiological air quality of processing areas in a dairy plant as evaluated by the sedimentation technique and a one-stage air sampler. Braz. J. Microbiol. 34:255-259.

Schoch, C. L., K. A. Seifert, S. Huhndorf, V. Robert, J. L. Spouge, C. A. Levesque, W. Chen, and F. B. Consortium. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc. Natl. Acad. Sci. U.S.A. 109:6241-6246.

Snyder, A. B., J. J. Churey, and R. W. Worobo. 2016. Characterization and control of Mucor circinelloides spoilage in yogurt. Int. J. Food Microbiol. 228:14-21.

Sørhaug, T. 2011. Spoilage molds in dairy products. Pages 780-784 in Encyclopedia of Dairy Sciences. 2 ed. Academic Press, San Diego, CA.

Spatafora, J. W., Y. Chang, G. L. Benny, K. Lazarus, M. E. Smith, M. L. Berbee, G. Bonito, N. Corradi, I. Grigoriev, and A. Gryganskyi. 2016. A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. Mycologia 108:1028-1046.

Stamatakis, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688-2690.

Stielow, J. B., C. A. Lévesque, K. A. Seifert, W. Meyer, L. Iriny, D. Smits, R. Renfurm, G. J. M. Verkley, M. Groenewald, D. Chaduli, A. Lomascolo, S. Welti, L. Lesage-Meessen, A. Favel, A. M. S. Al-Hatmi, U. Damm, N. Yilmaz, J. Houbraken, L. Lombard, W. Quaedvlieg, M. Binder, L. A. I. Vaas, D. Vu, A. Yurkov, D. Begerow, O. Roehl, M. Guerreiro, A. Fonseca, K. Samerpitak, A. D. van Diepeningen, S. Dolatabadi, L. F. Moreno, S. Casaregola, S. Mallet, N. Jacques, L. Roscini, E. Egidi, C. Bizet, D. Garcia-Hermoso, M. P. Martín, S. Deng, J. Z. Groenewald, T. Boekhout, Z. W. de Beer, I. Barnes, T. A. Duong, M. J. Wingfield, G. S. de Hoog, P. W. Crous, C. T. Lewis, S. Hambleton, T. A. A. Moussa, H. S. Al-Zahrani, O. A. Almaghrabi, G. Louis-Seize, R. Assabgui, W. McCormick, G. Omer, K. Dukik, G. Cardinali, U. Eberhardt, M. de Vries, and V. Robert. 2015. One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. Persoonia : Molecular Phylogeny and Evolution of Fungi 35:242-263.

Sulaiman, I. M., E. Jacobs, S. Simpson, and K. Kerdahi. 2014. Molecular identification of isolated fungi from unopened containers of greek yogurt by DNA sequencing of internal transcribed spacer region. Pathogens 3:499-509.

Suriyarachchi, V. R. and G. H. Fleet. 1981. Occurrence and growth of yeasts in yogurts. Appl. Environ. Microbiol. 42:574-579.

Temelli, S., Ş. Anar, C. Sen, and P. Akyuva. 2006. Determination of microbiological contamination sources during Turkish white cheese production. Food Control 17:856-861.

Torkar, K. G. and A. Vengušt. 2008. The presence of yeasts, moulds and aflatoxin M 1 in raw milk and cheese in Slovenia. Food Control 19:570-577.

Vacheyrou, M., A.-C. Normand, P. Guyot, C. Cassagne, R. Piarroux, and Y. Bouton. 2011. Cultivable microbial communities in raw cow milk and potential

transfers from stables of sixteen French farms. Int. J. Food Microbiol. 146:253-262.

Vangay, P., E. B. Fugett, Q. Sun, and M. Wiedmann. 2013. Food microbe tracker: a web-based tool for storage and comparison of food-associated microbes. J. Food Prot. 76:283-294.

Visagie, C. M., J. Houbraken, J. C. Frisvad, S. B. Hong, C. H. W. Klaassen, G. Perrone, K. A. Seifert, J. Varga, T. Yaguchi, and R. A. Samson. 2014. Identification and nomenclature of the genus Penicillium. Stud. Mycol. 78:343-371.

Wallace, G. and F. Tanner. 1931. Effect of heat on mold spores. Exp. Biol. Med. 28:970-972.

Walther, G., J. Pawłowska, A. Alastruey-Izquierdo, M. Wrzosek, J. Rodriguez-Tudela, S. Dolatabadi, A. Chakrabarti, and G. de Hoog. 2013. DNA barcoding in Mucorales: an inventory of biodiversity. Persoonia-Molecular Phylogeny and Evolution of Fungi 30(1):11-47.

Watts, J. L. 1988. Etiological agents of bovine mastitis. Vet. Microbiol. 16:41-66.

Westall, S. and O. Filtenborg. 1998. Spoilage yeasts of decorated soft cheese packed in modified atmosphere. Food Microbiol. 15:243-249.

Wolfe, Benjamin E., Julie E. Button, M. Santarelli, and Rachel J. Dutton. 2014. Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. Cell 158:422-433.

Zhao, Y., C.-C. Tsang, M. Xiao, J. Cheng, Y. Xu, S. K. Lau, and P. C. Woo. 2015. Intra-genomic internal transcribed spacer region sequence heterogeneity and molecular diagnosis in clinical microbiology. Int. J. Mol. Sci. 16:25067-25079.

SUPPLEMENTAL MATERIAL

Supplemental Table 2.1. Taxonomic Classification of all ITS ATs identified among the isolates characterized.

Phylum	Family	Genus	Species[1]	ITS AT	Taxonomic comments[2]
Basidiomycota	*Hydnaceae*	*Sistotrema*	na	136	
Mucoromycota	*Mucoraceae*	*Mucor*	cf. *fuscus*[3]	124	Clusters with *Mucor fuscus*
Mucoromycota	*Mucoraceae*	*Mucor*	cf. *fuscus*[3]	126	Clusters with *Mucor fuscus*
Mucoromycota	*Mucoraceae*	*Mucor*	na	90	Clusters within *Murcor sinensis* and *Mucor racemosus* species complex
Mucoromycota	*Mucoraceae*	*Mucor*	*plumbeus*	28	Identified via Mycobank
Mucoromycota	*Mucoraceae*	*Rhizopus*	*arrhizus*	31	
Mucoromycota	*Mucoraceae*	*Mucor*	*circinelloides*	93	Clusters within *Mucor circinelloides* species complex
Mucoromycota	*Mucoraceae*	*Mucor*	*circinelloides*	86	Clusters within *Mucor circinelloides* species complex
Ascomycota[4]	na	na	na	1	
Ascomycota[4]	na	na	na	3	
Ascomycota[4]	na	na	na	137	
Ascomycota	*Trichocomaceae*	na	na	25	Identified via Mycobank
Ascomycota[4]	na	na	na	38	
Ascomycota	*Dipodascaceae*	*Yarrowia*	*lipolytica*	64	Identified via Mycobank
Ascomycota[4]	na	na	na	69	
Ascomycota	*Nectriaceae*	na	na	72	Identified via Mycobank
Ascomycota	*Dipodascaceae*	na	na	73	Clusters within Galactomyces/Geotrichum clade
Ascomycota	*Dipodascaceae*	na	na	74	Clusters within Galactomyces/Geotrichum clade
Ascomycota	*Dipodascaceae*	*Yarrowia*	*lipolytica*	77	Identified via Mycobank
Ascomycota[4]	na	na	na	79	
Ascomycota	*Dipodascaceae*	na	na	80	Clusters within Galactomyces/Geotrichum clade
Ascomycota	*Dipodascaceae*	*Geotrichum*	cf. *restrictum*[3]	98	Clusters with *Geotrichum restrictum*
Ascomycota	*Dipodascaceae*	na	na	114	Clusters within *Galactomyces*/*Geotrichum* clade
Ascomycota[4]	na	na	na	115	
Ascomycota	*Dipodascaceae*	na	na	116	Clusters within *Galactomyces*/*Geotrichum* clade
Ascomycota	*Dipodascaceae*	na	na	117	Clusters within *Galactomyces*/*Geotrichum* clade
Ascomycota	*Dipodascaceae*	na	na	118	Clusters within *Galactomyces*/*Geotrichum* clade
Ascomycota	*Dipodascaceae*	na	na	120	Clusters within *Galactomyces*/*Geotrichum* clade
Ascomycota	*Dipodascaceae*	*Yarrowia*	*lipolytica*	123	Identified via Mycobank
Ascomycota	*Dipodascaceae*	na	na	125	Clusters within *Galactomyces*/*Geotrichum* clade
Ascomycota	*Saccharomycetaceae*	*Candida*	*orthopsilosis*	88	
Ascomycota	*Saccharomycetaceae*	*Candida*	*parapsilosis*	30	
Ascomycota	*Saccharomycetaceae*	*Candida*	*parapsilosis*	113	
Ascomycota	*Saccharomycetaceae*	*Candida*	*sojae*	56	
Ascomycota	*Saccharomycetaceae*	*Candida*	na	7	
Ascomycota	*Saccharomycetaceae*	*Candida*	na	91	
Ascomycota	*Saccharomycetaceae*	*Candida*	*tropicalis*	8	
Ascomycota	*Saccharomycetaceae*	*Candida*	*tropicalis*	62	
Ascomycota	*Saccharomycetaceae*	*Candida*	*zeylanoides*	12	
Ascomycota	*Metschnikowiaceae*	*Clavispora*	*lusitaniae*	4	
Ascomycota	*Metschnikowiaceae*	*Clavispora*	*lusitaniae*	53	

Ascomycota	*Metschnikowiaceae*	*Clavispora*	*lusitaniae*	57	
Ascomycota	*Metschnikowiaceae*	*Clavispora*	*lusitaniae*	61	
Ascomycota	*Debaryomycetaceae*	*Debaryomyces*	*hansenii*	5	
Ascomycota	*Debaryomycetaceae*	*Debaryomyces*	*hansenii*	6	
Ascomycota	*Debaryomycetaceae*	*Debaryomyces*	*prosopidis*	11	
Ascomycota	*Debaryomycetaceae*	*Debaryomyces*	na	112	
Ascomycota	*Debaryomycetaceae*	*Debaryomyces*	na	127	
Ascomycota	*Debaryomycetaceae*	*Debaryomyces*	na	128	
Ascomycota	*Debaryomycetaceae*	*Debaryomyces*	na	129	
Ascomycota	*Dipodascaceae*	na	na	10	Clusters within *Galactomyces*/*Geotrichum* clade
Ascomycota	*Dipodascaceae*	na	na	63	Clusters within *Galactomyces*/*Geotrichum* clade
Ascomycota	*Saccharomycodaceae*	*Hanseniaspora*	*uvarum*	99	
Ascomycota	*Hypocreaceae*	na	na	96	
Ascomycota	*Saccharomycetaceae*	*Kazachstania*	*servazzii*	81	
Ascomycota	*Saccharomycetaceae*	*Kluyveromyces*	*lactis*	66	
Ascomycota	*Saccharomycetaceae*	*Kluyveromyces*	*lactis*	89	
Ascomycota	*Saccharomycetaceae*	*Kluyveromyces*	*marxianus*	2	
Ascomycota	*Saccharomycetaceae*	*Lachancea*	*fermentati*	138	
Ascomycota	*Nectriaceae*	na	na	85	
Ascomycota	*Trichocomaceae*	*Penicillium*	*brevicompactum*	39	Clusters with *Penicillium brevicompactum*
Ascomycota	*Trichocomaceae*	*Penicillium*	*citrinum*	16	Clusters with *Penicillium citrinum*
Ascomycota	*Trichocomaceae*	*Penicillium*	na	9	Clusters within section *Fasciculata*, series *Camemberti* clade
Ascomycota	*Trichocomaceae*	*Penicillium*	na	82	Clusters within section *Fasciculata*, series *Camemberti* clade
Ascomycota	*Trichocomaceae*	*Penicillium*	na	19	Clusters within section *Fasciculata*, series *Camemberti* clade
Ascomycota	*Trichocomaceae*	*Penicillium*	na	35	Clusters within section *Aspergilloides, Penicillium glabrum* clade
Ascomycota	*Trichocomaceae*	*Penicillium*	na	122	Clusters within section *Fasciculata*, series *Camemberti* clade
Ascomycota	*Saccharomycetaceae*	*Candida*	*umkomasiana*	70	Identified via Mycobank
Ascomycota	*Saccharomycetaceae*	*Cyberlindnera*	*fabianii*	84	
Ascomycota	*Saccharomycetaceae*	*Sarocladium*	*kiliense*	119	
Ascomycota	*Saccharomycetaceae*	*Torulaspora*	*delbrueckii*	58	
Ascomycota	*Saccharomycetaceae*	*Torulaspora*	*delbrueckii*	59	
Ascomycota	*Saccharomycetaceae*	*Torulaspora*	*delbrueckii*	60	
Ascomycota	*Saccharomycetaceae*	*Torulaspora*	*delbrueckii*	101	
Ascomycota	*Wickerhamomyceteae*	*Wickerhamo-myces*	*anomalus*	102	
Ascomycota	*Dipodascaceae*	*Yarrowia*	*lipolytica*	67	
Ascomycota	*Dipodascaceae*	*Yarrowia*	*lipolytica*	71	
Ascomycota	*Dipodascaceae*	*Yarrowia*	*lipolytica*	87	
Ascomycota	*Dipodascaceae*	*Yarrowia*	*lipolytica*	92	
Ascomycota	*Dipodascaceae*	*Yarrowia*	*lipolytica*	95	
Ascomycota	*Dipodascaceae*	*Yarrowia*	*lipolytica*	121	

[1]na = classification not assigned

[2]AT were classified based on sequence similarity scores against the Unite database ITS reference sequences, unless specified otherwise; “Clusters with” indicates that ITS phylogenies were used to confirm and refine classification.

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

[4] these isolates could only be identified to the phylum level

Supplemental Table 2.2. Samples collected for yeast and mold isolation and number of isolates obtained from direct plating or enrichments of each sample.

Sample no.[1]	Species	Origin	Raw/Pasteurized Milk	Type	No. of Isolates obtained from	
					Direct plating	Enrich-ments
Raw Milk						
NA	Cow	NYS Dairy Farm	Raw	Bulk tank	1	2
NA	Cow	NYS Dairy Farm	Raw	Bulk tank	1	3
NA	Cow	NYS Dairy Farm	Raw	Bulk tank	1	3
NA	Cow	NYS Dairy Farm	Raw	Bulk tank	1	2
NA	Cow	NYS Dairy Farm	Raw	Bulk tank	1	2
NA	Cow	NYS Dairy Farm	Raw	Bulk tank	2	5
NA	Cow	NYS Dairy Farm	Raw	Bulk tank	2	11
NA	Cow	NYS Dairy Farm	Raw	Bulk tank	1	0
Cheese						
Al-184	Cow	USA	Pasteurized	Semi-hard	2	7
Al-200	Cow	New York, USA	Pasteurized	Soft, washed rind	1	0
Al-194	Cow/ Sheep	Italy	Pasteurized	Soft, washed rind	1	0
Al-197	Cow	USA	Raw	Hard, washed rind, brined	1	4
Al-198	Cow	USA	Raw	Hard, washed rind, brined	2	2
Al-199	Cow	USA	Raw	Semi-hard, natural rind	2	4

Al-179	Cow	USA	Raw	Semi-hard, natural rind	3	4
Al-181	Cow	USA	Raw	Hard, washed rind	0	4
Al-196	Cow	New York, USA	Raw	Soft, washed rind	5	8
Al-209	Cow	New York, USA	Raw	Soft, washed rind	2	8
Al-210	Goat	Portugal	Pasteurized	Hard	3	1
Al-193	Goat	Switzerland	Pasteurized	Hard	2	7
Al-195	Goat/ Sheep	France	Pasteurized	Hard	1	4
Al-236	Goat	Portugal	Pasteurized	Semi-hard	2	3
Al-267	Goat	Spain	Pasteurized	Semi-hard	1	2
Al-212	Goat	The Netherlands	Pasteurized	Semi-hard Gouda	2	3
Al-205	Goat	The Netherlands	Pasteurized	Semi-hard, natural rind	1	2
Al-221	Goat	Spain	Pasteurized	Semi-hard, washed rind	2	2
Al-214	Goat	Spain	Pasteurized	Semi-hard, washed rind	3	3
Al-220	Goat	France	Pasteurized	Semi-hard, washed rind	1	6
Al-261	Goat	Canada	Pasteurized	Brined	1	3
Al-202	Goat	Canada	Pasteurized	Fresh	2	3
Al-203	Goat	France	Pasteurized	Soft	1	1
Al-233	Goat	Italy	Raw	Hard	2	4
Al-234	Goat	Switzerland	Raw	Semi-	2	4

				hard		
Al-226	Sheep	Italy	Pasteurized	Hard	2	4
Al-265	Sheep	Italy	Pasteurized	Hard	2	3
Al-269	Sheep	Spain	Pasteurized	Hard	0	3
Al-270	Sheep	Spain	Pasteurized	Hard	1	3
Al-204	Sheep	Canada	Pasteurized	Hard, natural rind	1	5
Al-206	Sheep	France	Pasteurized	Hard, natural rind	3	4
Al-266	Sheep	Cyprus	Pasteurized	Semi-hard	2	2
Al-228	Sheep	France	Pasteurized	Semi-hard	1	3
Al-229	Sheep	Italy	Pasteurized	Semi-hard	1	4
Al-260	Sheep	Italy	Pasteurized	Semi-hard	3	1
Al-207	Sheep	Italy	Pasteurized	Semi-hard, natural rind	1	5
Al-224	Sheep	Italy	Pasteurized	Semi-hard, natural rind	2	2
Al-271	Sheep	The Netherlands	Pasteurized	Semi-hard, natural rind	1	3
Al-192	Sheep	France	Pasteurized	Semi-hard, washed rind	1	5
Al-237	Sheep	France	Pasteurized	Brined	1	4
Al-231	Sheep	Greece	Pasteurized	Brined	1	2
Al-254	Sheep	New York, USA	Pasteurized	Fresh	1	0
Al-235	Sheep	Italy	Raw	Hard	3	5
Al-201	Sheep	USA	Raw	Hard, natural rind	2	5
Al-227	Sheep	Spain	Raw	Semi-hard	2	2

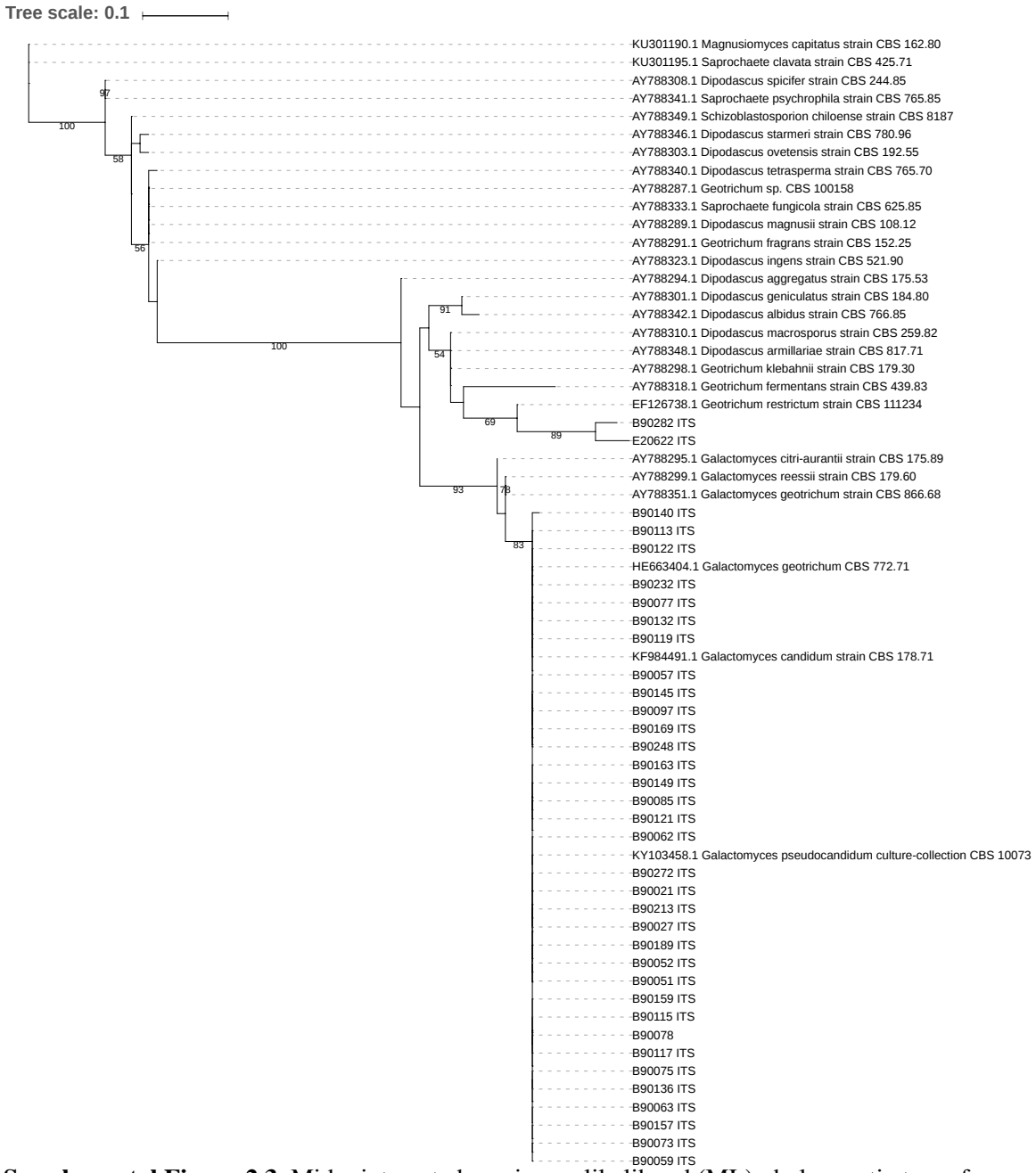
Al-223	Sheep	Spain	Raw	Semi-hard, natural rind	1	4
Al-225	Sheep	Spain	Raw	Soft	3	2
Yogurt						
NA	Cow	USA	Pasteurized		3	NA
NA	Cow	USA	Pasteurized		2	NA
NA	Cow	USA	Pasteurized		2	NA
NA	Cow	USA	Pasteurized		1	NA
NA	Cow	USA	Pasteurized		1	NA
NA	Cow	USA	Pasteurized		2	NA
NA	Cow	USA	Pasteurized		2	NA
NA	Cow	USA	Pasteurized		1	NA
NA	Cow	USA	Pasteurized		10	NA
NA	Cow	USA	Pasteurized		4	NA
NA	Cow	USA	Pasteurized		1	NA
NA	Cow	USA	Pasteurized		10	NA
NA	Cow	USA	Pasteurized		8	NA
NA	Cow	USA	Pasteurized		2	NA
NA	Cow	USA	Pasteurized		1	NA
NA	Cow	USA	Pasteurized		3	NA
NA	Cow	USA	Pasteurized		4	NA
NA	Cow	USA	Pasteurized		8	NA
NA	Cow	USA	Pasteurized		2	NA
NA	Cow	USA	Pasteurized		4	NA
NA	Cow	USA	Pasteurized		2	NA
NA	Cow	USA	Pasteurized		2	NA
NA	Cow	USA	Pasteurized	Added fruit	1	NA
NA	Cow	USA	Pasteurized	Added fruit	1	NA
NA	Cow	USA	Pasteurized	Added fruit and other	1	NA
NA	Cow	USA	Pasteurized	Added fruit and other	1	NA
NA	Cow	USA	Pasteurized	Added fruit and other	1	NA
NA	Cow	USA	Pasteurized	Added	1	NA

				fruit and other		
NA	Cow	USA	Pasteurized	Added fruit and other	1	NA
NA	Cow	USA	Pasteurized	Added fruit and other	1	NA

[1]NA = no sample number available
[2]NA = enrichments were not performed for yogurt samples. All isolates from yogurt samples were obtained from direct plating

Supplemental Figure 2.1. Midpoint-rooted maximum-likelihood (ML) phylogenetic tree of ITS region sequences of type specimens in the genus *Penicillium* (representing all *Penicillium* type specimens identified in Visagie et al. (2014)) as well as 94 isolates obtained here that were initially classified into the genus *Penicillium*. Numerical values indicate the percentage of bootstrap replications (out of 100 total replications) that support the corresponding node. Isolates were further classified into *Penicillium* sections and series based on Visagie et al. (2014). Species level identification was only assigned to isolates if the ITS sequence representing the allelic type (AT) associated with a given isolate clearly clustered with a ITS AT sequence of a single type specimen with a bootstrap value of >50.

Supplemental Figure 2.2. Midpoint-rooted maximum-likelihood (ML) phylogenetic tree of ITS region sequences of type specimens in the genus Mucor (representing all type specimens identified in Walther et al. (2013)) as well as 11 isolates obtained here that were initially classified into the genus *Mucor*. Numerical values indicate the percentage of bootstrap replications (out of 100 total replications) that support the corresponding node. Species level identification was only assigned to isolates if the ITS sequence representing the allelic type (AT) associated with a given isolate clearly clustered with a ITS AT sequence of a single type specimen with a bootstrap value of >50.



Supplemental Figure 2.3. Midpoint-rooted maximum-likelihood (ML) phylogenetic tree of ITS region sequences of type specimens in the genus *Geotrichum* (representing all type specimens identified in De Hoog and Smith (2004)) as well as 36 isolates that were initially classified into the genus *Geotrichum*. Numerical values indicate the percentage of bootstrap replications (out of 100 total replications) that support the corresponding node. Species level identification was only assigned to isolates if the ITS sequence representing the allelic type (AT) associated with a given isolate clearly clustered with a ITS AT sequence of a single type specimen with a bootstrap value of >50.

CHAPTER 3

ITS SEQUENCE-BASED CHARACTERIZATION OF FUNGAL ISOLATES FROM MULTIPLE YOGURT FACILITIES—A CASE STUDY*

*A. J. Buehler, M. Wiedmann, and N. H. Martin

Prepared for submission to Journal of Dairy Science

ABSTRACT

Fungal spoilage remains a significant issue in dairy product quality, especially for cultured dairy products such as yogurt. Fungal contamination can occur throughout the processing continuum, from the dairy farm environment to the finished product processing environment. To facilitate source tracking of fungal contaminants in the dairy processing environment, we obtained fungal isolates collected from raw materials (e.g., fruit preparations, added ingredients), in-process product samples, environmental samples (e.g., air plates, equipment surfaces), and finished product samples collected from two yogurt processing facilities. ITS PCR amplification and sequencing allowed us to classify the 852 isolates from these two facilities into 200 unique ITS allelic types (ATs), representing the phyla including Ascomycota (742 isolates), Basidiomycota (97 isolates), and Mucoromycota (13 isolates). Thirty ITS ATs were isolated from both facilities, whereas 62 and 108 ITS ATs were isolated only from either facility A or B, respectively. Nine ITS ATs were each represented by more than 20 isolates; these ATs comprised 53% of the 852 isolates. The considerable diversity of fungal isolates even in a single facility illustrates the challenge associated with controlling fungal contamination of dairy products. ITS barcoding, however, did show promise for facilitating source tracking of fungal contamination sources, particularly for ITS ATs over-represented in a given facility. For example, we found evidence for equipment specific reservoirs for two ATs (14 and 219) in facility B. While ITS sequencing can provide initial subtype information that can help trace fungal contamination along the processing continuum, our data also suggest that developing and implementing sampling plans that comprehensively capture yeast and

mold diversity in a given processing facility remains a considerable challenge.

Key words: yogurt, yeast, mold, internal transcribed spacer (ITS) sequencing

SHORT COMMUNICATION

Fungal organisms are well-documented agents that cause dairy product spoilage (Ledenbach and Marshall, 2009, Garnier et al., 2017). Global estimates on food loss suggest that 5-10% of the food supply is lost due to fungal spoilage (Pitt and Hocking, 2009). Dairy products, including cultured dairy products such as yogurt, are particularly susceptible to fungal spoilage. Many fungal organisms are able to grow at refrigerated temperatures and at the low pH encountered in yogurt products (Fleet, 2011, Snyder et al., 2016); many of these fungal organisms also produce lipolytic and proteolytic enzymes that result in physical degradation and undesirable sensory characteristics in the product (Suriyarachchi and Fleet, 1981, Fleet and Mian, 1987, Ledenbach and Marshall, 2009). For example, a study in Australia characterized yeasts found in yogurts purchased from retail and found that the most frequently isolated species, *Torulopsis candida* and *Kluyveromyces fragilis*, were able to hydrolyze casein and produce bitter flavors (Suriyarachchi and Fleet, 1981). Fungal spoilage of yogurt can also lead to visible defects such as bloated containers and surface mycelial growth (Snyder et al., 2016). These fungal spoilage defects represent a significant business risk in the age of social media, where consumers can readily share pictures of dairy product quality defects (Newkirk et al., 2012).

Fungal contamination can occur throughout the dairy processing continuum. Fungal organisms have been isolated from the dairy farm environment (Lavoie et al., 2011, Atanassova et al., 2016) and in the processing facility (Kure et al., 2003, Lund et al., 2003, Kure et al., 2008). While fungal organisms have been reported from raw milk (Lavoie et al., 2011, Buehler et al., 2017), these organisms are not typically heat

resistant and thus not a main source of fungal contamination. The processing facility environment, especially after pasteurization, represents a common source for fungal organisms to contaminate dairy products (Kure et al., 2003, Kure et al., 2004). In addition, ingredients used in yogurts (e.g., fruit preparations) may also be a source of fungal contaminants, typically yeasts (Suriyarachchi and Fleet, 1981). While previous studies have tracked fungal organisms in cheese processing facilities (Kure et al., 2003, Lund et al., 2003, Mounier et al., 2006, Temelli et al., 2006), we are not aware of any published studies that used molecular methods to elucidate transmission routes and sources of fungal organisms in yogurt processing facilities. DNA-barcoding methods can allow for the resolution of subtypes within fungal species and can provide tools for tracing sources of contamination throughout the processing continuum. The internal transcribed spacer region (ITS) is the universal barcode for fungi and has been described to allow for discriminatory DNA-sequencing based fungal identifications (Mayoral et al., 2005, Schoch et al., 2012, Hibbett and Taylor, 2013). ITS subtyping for fungal isolates represents one way the dairy industry can trace these organisms through the production continuum. Therefore, the objective of this study was to use ITS DNA sequencing as a molecular subtyping tool to determine the relationships among fungal organisms collected from two yogurt processing facilities along the processing continuum. This study was specifically conducted using fungal isolates obtained as part of routine industry monitoring programs. While we appreciate the potential shortcomings of this approach (e.g., lack of standardized set of sampling sites), this approach allowed us to obtain a large set of isolates and provided data on the potential implementation of barcoding approaches as part of existing

routine surveillance programs already implemented by industry, which make this contribution particularly appropriate for the *"Dairy Industry Today"* section.

Isolates were obtained from raw material samples, finished product samples, in-process product samples, and environmental samples that were collected from two yogurt processing facilities, hereafter referred to as facilities A and B, in the United States. Samples were collected over three years (2015-2017) as part of routine monitoring in each facility. Raw material samples included fruit preparations and other added ingredients; raw milk was not included as a raw material sample as dairy-relevant fungi are typically heat sensitive and thus raw milk would not represent a direct source of fungal contamination (Garnier et al., 2017). Finished product samples were collected as entire containers. In-process product samples were collected post-pasteurization and included product samples from fermentation "maturation" tanks and from separators. Environmental samples were collected using sponge-sticks (3M, St. Paul, MN), swab-samplers (3M), and air plate sampling using the sedimentation method (Dyer et al., 2004) on dichloran rose bengal chloramphenicol agar (DRBC; Becton, Dickinson and Co., Sparks, MD). For sponge and swab environmental samples, as well as raw material samples, isolates were obtained using standard methods on Petrifilm Yeast and Mold Count Plates (3M) and subsequently streaked for purity on DRBC agar (Becton, Dickinson and Co.) or potato dextrose agar (PDA; Becton, Dickinson and Co.). For finished product samples, isolates were obtained using standard methods on DRBC agar (Becton, Dickinson and Co.) or PDA agar (Becton, Dickinson and Co.). The obtained fungal isolates were then characterized by DNA sequencing of the ITS region, using the ITS primers and thermocycling

conditions described in Buehler et al. (2017). ITS allelic types (AT) were assigned using EMBOSS Needle v.6.6.0.0 (Rice et al., 2000). A unique ITS AT was assigned for every ITS region sequence that differed by ≥ 1 nucleotides from any previously obtained sequence either described in Buehler et al. (2017) or in this study. The first isolate of each new ITS AT was designated as the reference strain for that AT; ITS AT designations are consistent with those reported by Buehler et al. (2017). Species identification was performed as described in Buehler et al. (2017), using the UNITE database (https://unite.ut.ee/analysis.php) to query ITS region sequences. Isolate characteristics and relevant sample information can be found at www.foodmicrobetracker.com (Vangay et al., 2013).

All statistical analysis was performed in R v. 3.3.2 (R Core Team, 2013). The frequency distributions of ITS ATs between processing facilities and among all four sample types within processing facilities were compared using chi-square tests of independence. As chi-square tables included >20% of cells with expected values < 5, Monte Carlo simulations were used to estimate P values. For pairwise chi-square test comparisons of the distribution of ITS ATs between individual sample types within a single processing facility (e.g., the distribution of ITS ATs between in-process product samples and finished product samples in facility A), P values were estimated using Monte Carlo simulations and were then adjusted using the Benjamini and Yekutieli (2001) method for multiple comparisons. To test for over-representation of ITS ATs in facility A or B, pairwise Fisher exact tests were used to compare the frequency of a given ITS AT (for ATs isolated >10 times) to the frequency of all other ITS ATs.

Overall, 852 isolates were characterized by ITS PCR amplification and

sequencing; these isolates were classified into 200 unique ITS ATs. The isolates were classified into three phyla including Ascomycota (742/852 isolates), Basidiomycota (97/852 isolates), and Mucoromycota (13/852 isolates). The 303 isolates collected from facility A represented 92 ITS ATs, while the 549 isolates collected from facility B represented 138 ITS ATs. Thirty ITS ATs were isolated from both facilities, whereas 62 and 108 ATs were isolated only from either facility A or B, respectively. Nine ITS ATs (AT 60, AT 145, AT 30, AT 101, AT 51, AT 102, AT 61, AT 219, and AT 59) were each represented by more than 20 isolates; these ATs comprised 53% of the 852 isolates (data for all 48 ITS ATs represented by 3 or more isolates is shown in Figure 3.1). ITS ATs represented by only one isolate (n=129) comprised 15% of the 852 isolates. The remaining 32% of the 852 isolates were represented by ITS ATs containing ≥ 2 and ≤ 20 isolates. A chi-square test of independence revealed that there was a significant difference in the distribution of ITS ATs between facilities A and B ($P < 0.05$). Moreover, pairwise Fisher exact tests [comparing the frequency of a given ITS AT (for ATs that occurred >10 times) to the frequency of all other ITS ATs] showed that ITS AT 30, AT 60, AT 101, AT 155, and AT 209 were over-represented in facility A while ITS AT 14, AT 61, AT 145, and AT 219 were over-represented in facility B; hence results for facilities A and B are discussed separately below.

ITS rDNA Allelic Type	Facility A					Facility B					
Sample type	Raw material	In-process	Environmental	Finished product	Total # of isolates	Raw material	In-process	Environmental	Finished product	Total # of isolates	Classification
2		1			1		1	4	6	11	*Kluyveromyces*
9			1		1	1	1	2	7	11	*Penicillium*
14(B)							1	11		12	*Ascomycota*
19								4	2	6	*Penicillium*
21		1			1	1		3		4	*Penicillium*
23								3		3	*Aspergillus*
25						2		1	4	7	*Ascomycota*
26			1		1			3	1	4	*Basidiomycota*
27								4		4	*Penicillium*
29			1		1			1	1	2	*Penicillium*
30(A)		4	20	5	29		6	8	5	19	*Candida*
35						3	1			4	*Penicillium*
38			3		3	2		5		7	*Ascomycota*
39			2		2	1				1	*Penicillium*
51		5	2	4	11	5		11	13	29	*Ascomycota*
53						1			3	4	*Clavispora*
56									5	5	*Candida*
58			1	2	3				1	1	*Torulaspora*
59				6	6				15	15	*Torulaspora*
60(A)	1	2	4	51	58	2	3	2	61	68	*Torulaspora*
61(B)				3	3			1	31	32	*Clavispora*
67			1		1			4		4	*Yarrowia*
98				1	1				7	7	*Ascomycota*
99	1			6	7				1	1	*Hanseniaspora*
101(A)			1	35	36				10	10	*Torulaspora*
102				12	12	1			22	23	*Wickerhamomyces*
121			4		4						*Yarrowia*
135									3	3	*Metschnikowia*
143								1	3	4	*Candida*
145(B)			2		2				69	69	*Meyerozyma*
147								1	5	6	*Torulaspora*
155(A)		7	11	1	19						*Crypotococcus*
159							3			3	*Ascomycota*
163			1		1			3		3	*Cladosporium*
174			2		2			2		2	*Ascomycota*
184		3	4		7						*Candida*
208			2		2			1		1	*Trichosporon*
209(A)		3	11		14						*Crypotococcus*
219(B)				1	1		1	26	2	29	*Pleurostoma*
223							3		2	5	*Filobasidium*
227			2		2			1		1	*Aspergillus*
228								3		3	*Crypotococcus*
238				3	3						*Torulaspora*
245								3		3	*Acremonium*
250								3		3	*Trichoderma*
257			2		2			5		5	*Apiotrichum*
263								4		4	*Pleurostoma*
288		2			2			1		1	*Rhizopus*

Figure 3.1. Internal transcribed spacer (ITS) allelic type (AT) matrix displaying ITS ATs collected a total of three or more times from raw material samples, in-process product samples, environmental samples, or finished product samples from two yogurt facilities. Classification represents genera, with the exception of ITS ATs 14, 25, 26, 38, 51, 98, 159, and 174, where phyla are displayed. This matrix includes 48 ITS ATs, the remaining 152 ATs were all isolated < 3 times. Upper case letter of A or B in parenthesis after that AT designation marks ATs that were identified as over-represented in facility A or B, respectively.

In facility A, the most common ITS ATs were identified as *Torulaspora delbrueckii* (AT 60, 58 isolates), *T. delbrueckii* (AT 101, 36 isolates), and *Candida parapsilosis* (AT 30, 29 isolates). Interestingly, the 3 most common ITS ATs as well as the 5 ITS ATs over-represented in facility A were all classified as yeasts. Molds were however represented among the remainder of isolates from facility A and represented 42/303 isolates from this facility. This observed over-representation of yeast contaminants as compared to mold contaminants could suggest a focus on implementing yeast control strategies throughout this facility, while mold control strategies could be focused locally where mold contamination risk is elevated, such as in the filling and packaging area (Garnier et al., 2017) or areas where mold contaminants are found. In facility A, only 1 mold ITS AT from environmental sources (ITS AT 38) was obtained 3 times (the other 36 environmental mold isolates from facility A were obtained ≤ 2 times); this low repeated isolation of mold ITS ATs further complicates the use of these data for source tracking.

Among the four sample types in facility A (raw material samples, in-process product samples, environmental samples, and finished product samples), the majority of isolates (270/303 isolates) were collected from environmental samples or finished product samples. The distribution of ITS ATs differed among all sample types ($P < 0.05$, chi-square test). Pairwise chi-square tests for the association between individual sample types (in-process product, environmental, and finished product samples; raw material samples were not included as only 3 isolates were collected from these samples) and the distribution of ITS ATs revealed that the distribution of ITS ATs was significantly different ($P < 0.05$) between in-process product samples and finished

product samples as well as between environmental samples and finished product samples (Figure 3.2). The distribution of ITS ATs between environmental samples and in-process product samples was not significantly different ($P \geq 0.05$), suggesting the possibility of an environmental transmission route for in-process product contamination (Figure 3.2). While the significant difference observed for the distribution of ITS ATs between environmental and finished product samples could suggest that sources other than the environment could significantly contribute to finished product contamination, these findings may also reflect incomplete sampling of the environment which is likely to occur in routine sampling programs in processing facilities, further illustrating the challenge of tracking fungal spoilage organisms along the processing continuum. In addition, the limited routine sampling of raw materials in facility A impacted our ability to identify potential sources of yeast, which have been previously suspected to be found in fruit preparations used in yogurt manufacturing (Suriyarachchi and Fleet, 1981). However, sterile collection of fruit preparation samples from large sealed totes does represent a challenge, which potentially contributes to the low number of raw material samples collected.

		Facility A			
	Sample Type	Raw material N=3	In-process N=30	Environmental N=125	Finished product N=145
Facility B	Raw material N=31		ND[1]	ND	ND
	In-process N=25	44.1 (<0.05)		62.5 (≥0.05)	124 (<0.05)
	Environmental N=187	149 (≥0.05)	131 (≥0.05)		221 (<0.05)
	Finished product N=306	224 (<0.05)	199 (<0.05)	397 (<0.05)	

Figure 3.2. Association between sample type and ITS AT within each yogurt processing facility. Numbers represent chi-square test statistic, followed by P values in parentheses. As chi-square tables included >20% of cells with expected values <5, Monte Carlo simulations were used to estimate P values. P values were then adjusted for multiple comparisons using the Benjamini and Yekutieli (2001) method. Numbers in white boxes represent comparisons between sample type and ITS AT within facility A, while numbers in grey boxes represent comparisons between sample type and ITS AT within facility B. N represents the total number of isolates collected from the specific sample type in a given facility (column for facility B and row for facility A). ND indicates where analyses were not performed due to small sample size.

The ITS AT data generated for facility A also illustrates the potential value of these data for source tracking, which we initially assessed by focusing on ITS ATs over-represented in a given facility as these ITS ATs are less likely to simply represent common subtypes. Among the 5 ITS ATs over-represented in facility A, isolates characterized as ITS AT 101 were obtained during 6 months in 1 year and were predominantly identified from a product with a specific fruit prep ("fruit prep A"). Overall, 30 out of the 35 ITS AT 101 finished product isolates were from products with "fruit prep A," suggesting either the raw material or specific line or equipment used for this yogurt as a source. Interestingly, a second ITS AT (AT 60) that was over-represented in facility A (and isolated there over 2 years), was also the second most common AT found in facility B. While repeat isolation in a given facility of more widely distributed ITS ATs such as this AT may provide some potentially valuable hypotheses on contamination sources, subtyping methods with greater resolution power [e.g., sequencing protein-coding genes, fragment analysis techniques, microsatellites, and random amplified polymorphic DNA (Almeida and Araujo, 2013, Saghrouni et al., 2013)] may be needed to facilitate reliable source tracking.

In facility B, the most common ITS ATs were identified as *Meyerozyma guilliermondii* (AT 145, 69 isolates), *T. delbrueckii* (AT 60, 68 isolates), and *Clavispora lusitaniae* (AT 61, 32 isolates). The 3 most common ITS ATs as well as the 4 ITS ATs over-represented in facility B were all yeasts except for ITS AT 219 (*Pleurostoma richardsiae*), which was over-represented in facility B. ITS AT 219 was only found once among the isolates from facility A. In facility B, this AT was isolated 26 times from environmental samples, but only once and twice from in-process

product and finished product samples, respectively, suggesting the potential for an environmental reservoir or source in facility B (also supported by the fact that 8 of the 26 environmental AT 219 isolates were obtained from samples associated with a specific piece of equipment). These findings further illustrate the value of ITS barcoding for source tracking. The 3 most common ITS ATs in facility B represented 169 isolates; interestingly 161 of these isolates were collected from finished product samples. Infrequent isolation of yeasts from other sample types could be a consequence of sampling strategies and sample site selection, which may have biased results towards the recovery of molds. As yeast contamination is typically associated with added ingredients or improper cleaning and sanitation (Garnier et al., 2017), intensive sampling of raw materials (or sites impacted by raw materials) or internal equipment areas that are challenging to clean and sanitize may facilitate yeast isolation, while collection of air samples may bias sampling towards isolation of molds. Hence the nature of routine environmental sampling plans needs to be carefully considered when assessing yeast and mold contamination patterns and subtype data.

Among the four sample types, the majority of isolates (493/549) in facility B were collected from finished products and environmental samples. The distribution of ITS ATs differed among all sample types (raw material, in-process product, environmental, finished product) collected from facility B ($P < 0.05$, chi-square test). Pairwise chi-square tests for the association between ITS ATs and individual sample types showed that ITS AT distributions for (i) raw materials, (ii) in-process product, and (iii) environmental samples were significantly different ($P < 0.05$) from the distribution of ITS ATs from finished product samples (Figure 3.2), which as

discussed for facility A, may reflect incomplete sampling of these different sample types, but may also be explained by multiple sources contributing to finished product contamination.

Similar to facility A, a qualitative assessment of ITS ATs over-represented in facility B illustrates the potential value of these data for source tracking. In addition to suggesting a possible specific source of *Pleurostoma richardsiae* (ITS AT 219) in facility B (as discussed above), our data suggested that ITS AT 14 (isolated only in facility B), may have a specific reservoir as 8/12 isolates with this AT were isolated from air nozzles. In addition, for ITS AT 145, 31/69 finished product isolates were from product produced on a single specific line, which could indicate a line specific issue or possibly, but less likely, an issue associated with a specific type of product predominately manufactured on this line. Finally, for ITS AT 61, which was represented by 32 isolates from facility B (as opposed to 3 isolates from facility A), 22 isolates were obtained from two different lines (10 and 12 isolates), with two yogurt types (with fruit preps B and C) produced on both lines. This example illustrates how subtype data can be valuable to suggest specific directions for root cause analyses, even if the subtype data does not necessarily point to a specific source.

Overall, our data revealed that routine monitoring programs for fungal contamination in dairy processing facilities capture considerable fungal diversity, with 200 different subtypes (ITS ATs) found across two facilities. Moreover, we showed that ITS sequencing provides initial subtype information that can help identify likely fungal contamination sources or at least help develop specific hypotheses for root cause analysis efforts targeting fungal contamination issues, demonstrating the value

of these methods for industry. However, our data also further indicate the presence of common and widely distributed ITS subtypes, suggesting a need for more discriminatory subtyping methods to definitively identify contamination sources in some cases. This is also supported by a previous study that found the same ITS ATs in different dairy products (e.g., cheese and yogurt) (Buehler et al., 2017). As fungal contamination of yogurt as well as other dairy products will likely continue to represent a challenge, particularly considering that multiple routes for fungal contamination exist along the processing continuum (e.g., added ingredients, environmental contamination), industry will need to continue to implement improved control strategies for these spoilage organisms, using a holistic systems approach. Our data suggest that robust approaches to identify and control fungal spoilage should represent a combination of (i) raw material sampling, (ii) well designed environmental sampling programs targeting likely sources of both yeast and mold contamination, with collection of appropriate sampling site metadata, and (iii) use of subtyping methods, including methods with improved resolution over ITS barcoding when needed.

ACKNOWLEDGEMENTS

The authors would also like to thank the staff of the Milk Quality Improvement Program (Cornell University, Ithaca, NY), specifically Rachel Evanowski and Sherry Roof, for assistance with this study.

REFERENCES

Almeida, L. A. and R. Araujo. 2013. Highlights on molecular identification of closely related species. Infect. Genet. Evol. 13:67-75.

Atanassova, M., C. Fernández-Otero, P. Rodríguez-Alonso, I. Fernández-No, J. Garabal, and J. Centeno. 2016. Characterization of yeasts isolated from artisanal short-ripened cows' cheeses produced in Galicia (NW Spain). Food Microbiol. 53:172-181.

Benjamini, Y. and D. Yekutieli. 2001. The control of the false discovery rate in multiple testing under dependency. Ann. Stat. 29:1165-1188.

Buehler, A., R. Evanowski, N. Martin, K. Boor, and M. Wiedmann. 2017. Internal transcribed spacer (ITS) sequencing reveals considerable fungal diversity in dairy products. J. Dairy Sci. 100:8814-8825.

Dyer, R., J. Frank, B. Johnson, and P. Hickey. 2004. Microbiological tests for equipment, containers, water, and air. Pages 325-340 in Standard Methods for Examination of Dairy Products Vol. 17. 16 ed. American Public Health Association, Washington, DC.

Fleet, G. H. 2011. Chapter 5 - Yeast Spoilage of Foods and Beverages A2 - Kurtzman, Cletus P. Pages 53-63 in The Yeasts (Fifth Edition). J. W. Fell and T. Boekhout, ed. Elsevier, London.

Fleet, G. H. and M. A. Mian. 1987. The occurrence and growth of yeasts in dairy products. Int. J. Food Microbiol. 4:145-155.

Garnier, L., F. Valence, and J. Mounier. 2017. Diversity and Control of Spoilage Fungi in Dairy Products: An Update. Microorganisms 5:42.

Hibbett, D. S. and J. W. Taylor. 2013. Fungal systematics: is a new age of enlightenment at hand? Nat. Rev. Microbiol. 11:129.

Kure, C., I. Skaar, A. Holst-Jensen, and E. Abeln. 2003. The use of AFLP to relate cheese-contaminating Penicillium strains to specific points in the production plants. Int. J. Food Microbiol. 83:195-204.

Kure, C. F., E. Borch, I. Karlsson, J. P. Homleid, and S. Langsrud. 2008. Use of the selective agar medium CREAD for monitoring the level of airborne spoilage moulds in cheese production. Int. J. Food Microbiol. 122:29-34.

Kure, C. F., I. Skaar, and J. Brendehaug. 2004. Mould contamination in production of semi-hard cheese. Int J. Food Microbiol. 93:41-49.

Lavoie, K., M. Touchette, D. St-Gelais, and S. Labrie. 2011. Characterization of the fungal microflora in raw milk and specialty cheeses of the province of Quebec. Dairy Sci. Technol. 92:455-468.

Ledenbach, L. H. and R. T. Marshall. 2009. Microbiological spoilage of dairy products. Pages 41-67 in Compendium of the Microbiological Spoilage of Foods and Beverages. Springer, New York, NY.

Lund, F., A. B. Nielsen, and P. Skouboe. 2003. Distribution of Penicillium commune isolates in cheese dairies mapped using secondary metabolite profiles, morphotypes, RAPD and AFLP fingerprinting. Food Microbiol. 20:725-734.

Mayoral, M. B., R. Martín, A. Sanz, P. E. Hernández, I. González, and T. García. 2005. Detection of Kluyveromyces marxianus and other spoilage yeasts in yoghurt using a PCR-culture technique. Int. J. Food Microbiol. 105:27-34.

Mounier, J., S. Goerges, R. Gelsomino, M. Vancanneyt, K. Vandemeulebroecke, B. Hoste, N. Brennan, S. Scherer, J. Swings, and G. Fitzgerald. 2006. Sources of the adventitious microflora of a smear-ripened cheese. J. Appl. Microbiol. 101:668-681.

Newkirk, R. W., J. B. Bender, and C. W. Hedberg. 2012. The potential capability of social media as a component of food safety and food terrorism surveillance systems. Foodborne Pathog. Dis. 9:120-124.

Pitt, J. I. and A. D. Hocking. 2009. Fungi and Food Spoilage. 3 ed. Springer, New York, NY.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rice, P., I. Longden, and A. Bleasby. 2000. EMBOSS: the European molecular biology open software suite. 16:276-277.

Saghrouni, F., J. Ben Abdeljelil, J. Boukadida, and M. Ben Said. 2013. Molecular methods for strain typing of Candida albicans: a review. J. Appl. Microbiol. 114:1559-1574.

Schoch, C. L., K. A. Seifert, S. Huhndorf, V. Robert, J. L. Spouge, C. A. Levesque, W. Chen, and F. B. Consortium. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc. Natl. Acad. Sci. U.S.A. 109:6241-6246.

Snyder, A. B., J. J. Churey, and R. W. Worobo. 2016. Characterization and control of Mucor circinelloides spoilage in yogurt. Int. J. Food Microbiol. 228:14-21.

Suriyarachchi, V. R. and G. H. Fleet. 1981. Occurrence and growth of yeasts in yogurts. Appl. Environ. Microbiol. 42:574-579.

Temelli, S., Ş. Anar, C. Sen, and P. Akyuva. 2006. Determination of microbiological contamination sources during Turkish white cheese production. Food Control 17:856-861.

Vangay, P., E. B. Fugett, Q. Sun, and M. Wiedmann. 2013. Food microbe tracker: a web-based tool for storage and comparison of food-associated microbes. J. Food Prot. 76:283-294.

CHAPTER 4

EVALUATION OF BIOPRESERVATIVES IN GREEK YOGURT TO INHIBIT YEAST AND MOLD SPOILAGE AND DEVELOPMENT OF A YOGURT SPOILAGE PREDICTIVE MODEL*

*A. J. Buehler, N. H. Martin, K. J. Boor, and M. Wiedmann

Submitted to Journal of Dairy Science, May 18, 2018.

ABSTRACT

Dairy products, including cultured dairy products like cheese and yogurt, are susceptible to fungal spoilage. Traditionally, additives such as potassium sorbate have been used to control fungal spoilage; however, with consumer demand for clean-label products, other strategies to control fungal spoilage (e.g., biopreservatives) are increasingly being used in dairy formulations. In order to help the dairy industry better evaluate biopreservatives for control of fungal spoilage, we developed a challenge study protocol, which was applied to evaluate two protective cultures for their ability to control yeast and mold spoilage of Greek yogurt. Greek yogurt formulated with and without protective cultures was inoculated with a cocktail consisting of five yeasts and one mold to yield inoculum levels of 10^1 and 10^3 CFU/g of yogurt. The inoculated yogurts were stored at 7°C and fungal counts as well as time to visible growth, on the yogurt surface, of mycelium mold colonies or yeast, were determined over shelf-life. While fungal concentrations increased to spoilage levels ($\geq 10^5$ CFU/g) in all yogurt formulations at both inoculum levels by day 23 of storage at 7°C, no surface mold was observed over 76 days in any of the products formulated with protective cultures. Control yogurts without biopreservatives however all showed surface mold by day 23. In order to allow industry to better evaluate the business impact of improved control of surface mold growth that can be achieved with protective cultures, we developed a Monte Carlo simulation model to estimate consumer exposure to visible mold growth in yogurt formulated without fungal inhibitors. Our model showed that initial mold contamination rate has the largest effect on the model outcome, indicating that accurate data on contamination rates are important for use of these models. When air

plates were used, in a proof-of-concept approach, to estimate initial contamination rates in a small yogurt manufacturing operation, our model predicted that 550 ± 25.2 consumers would be exposed to visible mold growth for every one million cups of yogurt produced. With initial contamination rate data for individual facilities, this model could be used by industry to estimate the number of consumers exposed to visible mold spoilage and could allow industry to better assess the value of mold control strategies.

Key words: protective culture, yeast, mold, yogurt

INTRODUCTION

Fungal spoilage of food accounts for an estimated 5-10% of food loss worldwide (Pitt and Hocking, 2009). Dairy products, including cultured dairy products like cheese and yogurt, are susceptible to fungal spoilage (Suriyarachchi and Fleet, 1981, Kure et al., 2004, Ledenbach and Marshall, 2009, Garnier et al., 2017a). Fungal contamination can occur throughout the dairy processing continuum, from the dairy farm environment to the finished product processing environment (Kure et al., 2001, Temelli et al., 2006, Vacheyrou et al., 2011). While it has been reported that raw milk is a source for natural fungal contaminants (Lavoie et al., 2011, Atanassova et al., 2016, Buehler et al., 2017), these organisms are typically not heat resistant and thus not the main route for fungal contamination of dairy products (Jacques and Casaregola, 2008, Garnier et al., 2017b). The processing environment, specifically after pasteurization, represents the most common source for fungal contamination of dairy products. Moreover, the processing facility air is a common transmission route for fungal contaminants (Temelli et al., 2006, Beletsiotis et al., 2011, Radha and Nath, 2014). For example, during a one year prospective study of fungal air contamination in a yogurt plant in Greece, the molds *Penicillium* spp. and *Cladospordium* spp. represented the most commonly isolated species from the plant's indoor air samples (Beletsiotis et al., 2011). Yeast contamination can also be airborne (Beletsiotis et al., 2011), but more often originates from surfaces, supplier ingredients (e.g., fruit preparations), and the production environment (Penney et al., 2004, Mayoral et al., 2005, Bokulich and Mills, 2013).

Traditional methods for controlling fungal spoilage of yogurt include using

food additives such as potassium sorbate (Davidson et al., 2013). As consumers demand clean-label food products, the food industry is challenged to produce the same quality products with the same shelf-life without the use of traditional food additives (Zink, 1997, Devlieghere et al., 2004). One way to achieve a clean-label food product with a similar shelf-life to traditional products is through the use of biopreservatives (Schnürer and Magnusson, 2005, Crowley et al., 2013b). Biopreservatives are defined as added microorganisms or their metabolites or the combination of both that aid in shelf-life extension of food (Holzapfel et al., 1995). Examples of biopreservatives include lactic acid bacteria (e.g., protective cultures), bacteriocins, and natural enzyme systems (e.g., lactoperoxidase) (Stiles, 1996). The use of protective cultures, in particular, has been applied to many food sectors: baking (Gerez et al., 2009, Muhialdin et al., 2011, Garofalo et al., 2012), dairy (Delavenne et al., 2013, Cheong et al., 2014), and fruits and vegetables (Sathe et al., 2007, Crowley et al., 2013a). In yogurt, in particular, research has focused on strain-specific fungal inhibition, using model yogurt systems; however, limited research exists on the use of protective cultures in Greek yogurt (Delavenne et al., 2013, Lačanin et al., 2017). Greek yogurt production differs from traditional Swiss-style yogurt production in that Greek yogurt is centrifugally separated to concentrate the curd and remove the acid whey following fermentation. While the additional processing steps for Greek yogurt do not typically expose the product directly to the processing environment, there is still a need for control strategies to protect the product from fungal contamination during production, especially in filling and packaging, when the product is exposed to the processing environment. Including antifungal biopreservatives in the formulation could be one

way to protect the product from fungal contamination in a way that is acceptable to consumers.

Estimates of yogurt spoilage due to fungal contamination have the potential to help yogurt producers determine and assess the effectiveness of quality interventions to protect their product. Stochastic modeling approaches have been used extensively in food safety applications, most often to estimate mean illness cases per year following consumption of a particular contaminated food [e.g., listeriosis cases from deli meats (Pradhan et al., 2009, Pradhan et al., 2010), illness cases from consumption of fresh-cut lettuce contaminated with *Escherichia coli* O157:H7 (Pang et al., 2017), salmonellosis cases from consumption of pistachios (Lambertini et al., 2017)]; however, stochastic modeling approaches have been applied to the dairy quality realm less frequently, with only a few models focusing on fluid milk spoilage (Schaffner et al., 2003) and yogurt spoilage (Gougouli and Koutsoumanis, 2017). A recent study (Gougouli and Koutsoumanis, 2017) reported a simulation model that estimated the time to visible mold growth; however, this model did not account for variation in the initial contamination rate. While estimates of initial fungal contamination rate at the processing facility may be difficult to obtain, there is a need for a baseline understanding of these values to inform spoilage control strategies and to accurately predict the number of consumers exposed to visible mold growth in yogurt. In addition, there is a need to develop and use simulation models to allow for quantitative assessment of intervention strategies, such as protective cultures, before implementation. Thus, the objectives of this study are to (1) evaluate the effectiveness of protective cultures to inhibit yeast and mold growth in yogurt and (2) develop a

baseline stochastic model to estimate consumer exposure to yogurt with visible mold growth when no fungal inhibitor is employed in the yogurt formulation.

MATERIALS AND METHODS

Isolate Selection

Based on the isolates characterized in Buehler et al. (2017) and information listed in Food Microbe Tracker [www.foodmicrobetracker.com (Vangay et al., 2013)], six isolates were selected to represent a broad range of dairy-relevant fungal spoilage organisms. The selected isolates represent 5 yeast organisms and 1 mold organism (Table 4.1). Identification of the isolates to the genus level was completed in a previous study and was based on ITS rDNA sequencing (Buehler et al., 2017).

Table 4.1. Genus and species identification, isolation source, and ITS allelic type (AT) for the 6 study isolates

Genus	Species	FSL ID[a]	Source	ITS AT
Candida	*parapsilosis*	E2-0454	yogurt	30
Clavispora	*lusitaniae*	E2-0451	yogurt	61
Kloeckera	*apiculata*	E2-0456	yogurt	99
Metschnikowia	*pulcherrima*	E2-0608	yogurt	135
Penicillium	*commune*	E2-0427	yogurt	9
Torulaspora	*delbrueckii*	E2-0444	yogurt	60

[a]FSL ID – Cornell University Food Safety Lab isolate designation.

Cocktail preparation

The 5 yeast organisms (*Candida parapsilosis, Clavispora lusitaniae, Kloeckera apiculata, Metschnikowia pulcherrima,* and *Torulaspora delbrueckii*) were streaked for isolation on dichloran rose bengal chloramphenicol agar (**DRBC**; Becton, Dickinson and Co., Sparks, MD), followed by incubation at 25°C for 72 h. Single yeast colonies were inoculated into 9mL of malt extract broth (**MEB**; Becton,

Dickinson and Co.), followed by incubation at 30°C in a shaking incubator (New Brunswick Scientific, Edison, NJ) at 140 rpm for 48 h. The yeast organisms were then enumerated by spiral plating on DRBC, using an Autoplate 5000 (Advanced Instruments Inc., Norwood, MA). DRBC plates were incubated at 25°C for 72 h. Following incubation, colonies were counted with the QCount Automated Colony Counter (Advanced Instruments Inc.). Inoculated MEB solutions were stored at 4°C until cultures were enumerated.

The mold isolate, *Penicillium commune*, was streaked for isolation on DRBC, followed by incubation at 25°C for 72 h. To prepare a *P. commune* mycelium suspension, 5mL of phosphate buffer solution (**PBS**, Weber Scientific, Hamilton, NJ) was added to the DRBC plate and a sterile inoculation loop was used to dislodge mycelium growth into the PBS. A sterile cotton swab was then used to collect approximately half of the mycelium suspension and subsequently inoculate a lawn on a DRBC plate, which was then incubated at 25°C for 72 h. Following incubation, the mycelium growth was harvested into 5 mL of PBS as described above and used for preparation of the cocktail. The concentration of *P. commune* in the harvested mycelium suspension was estimated based on enumeration of *P. commune* mycelium suspensions from the initial DRBC plate and was confirmed after preparation of the cocktail. For enumeration, mycelium suspensions were serially diluted in PBS and spread plated on DRBC, followed by incubation at 25°C for 72 h. Colonies were counted with the QCount Automated Colony Counter (Advanced Instruments Inc.).

Two cocktails were prepared to achieve final concentrations of 10^1 and 10^3

CFU yeast and mold per g yogurt. To prepare the 10^1 CFU/g of yogurt inoculum, once each individual yeast and mold solution had been quantified, each solution was diluted in PBS to reach a concentration of 1.7 x 10^4 CFU/mL. To prepare the 10^3 CFU/g of yogurt inoculum, each solution was diluted in PBS to reach a concentration of 1.7 x 10^6 CFU/mL. The individual diluted solutions were then blended together in equal amounts to create the final cocktails. The cocktails were used within 2 h after blending. Both final cocktails were enumerated on DRBC to ensure the target concentration was achieved.

Preparation of Greek yogurt

Plain, nonfat Greek yogurt was produced at Cornell University's Food Processing and Development Laboratory (Ithaca, NY). Commercially available *Lactobacillus delbrueckii* subspp. *bulgaricus* and *Streptococcus thermophilus* strains as well as commercially available protective cultures were used in fermentation. Two protective cultures, A and B, were evaluated independently at three concentrations in the yogurt formulation: (i) the manufacturer's recommended concentration (100% A, 100% B), (ii) 5% less than the manufacturer's recommended concentration (95% A, 95% B), and (iii) 10% less than the manufacturer's recommended concentration (90% A, 90% B). The following yogurt formulations were prepared: (i) a control (i.e., with no protective culture), (ii) 3 batches of yogurt with protective culture A at the three levels mentioned above, and (iii) 3 batches of yogurt with protective culture B at the three levels mentioned above. The yogurt was separated, using a centrifugal separator, to 9.5% protein after the fermented product reached a pH in the range of 4.55-4.70. Following separation, the product was filled into 6-oz cups, sealed with a foil lid, and

cooled to 4°C. The product was then stored at 4°C until inoculation, a maximum of 4 d.

Inoculation and Subsequent Enumeration of Greek Yogurts

Yogurts were inoculated with 100µL of a yeast and mold cocktail (for final concentrations of 10^1 or 10^3 CFU/g of yogurt) or sterile PBS (negative control) from the bottom of the cup with a sterile 20-gauge needle to ensure the headspace of the yogurt remained intact. Immediately following inoculation, the hole produced from the needle was sealed with sterile, adhesive foil. The inoculated yogurts were then stored at 7°C until enumeration. Two yogurts of each formulation variable and inoculum concentration were enumerated on day 0 (inoculation day), day 9, and day 23. In addition, yogurts inoculated with 10^1 CFU/g and sterile PBS were enumerated on day 60 and day 76. To enumerate the day 0, 9, 23, 60, and 76 yogurts, yogurt samples were diluted 1:10 in PBS, followed by vigorous shaking for 10 s. Each diluted yogurt sample was spread plated directly on DRBC and incubated at 25°C for 120 h. For day 0, one 100-mL enrichment was prepared for each yogurt sample inoculated at 10^1 CFU/g, consisting of 90 mL MEB and 10g yogurt. Enrichments were prepared in sample bags and homogenized at 260 rpm for 60 s in a Stomacher 400 Circulator (Seward Ltd., United Kingdom) and incubated at 25°C for 72 h. Following incubation, enrichments were spread plated on DRBC, followed by incubation at 25°C for 120 h. A QCount Automated Colony Counter (Advanced Instruments Inc.) was used to enumerate colonies.

Time to Visible Fungal Growth

Time to visible fungal growth was visually estimated by opening 2 yogurts inoculated at 10^1 CFU/g yogurt of each yogurt formulation on day 0, 9, 23, and then every 3 days from day 34 to day 76 of incubation at 7°C. Briefly, the foil lid was removed from the cup, and the yogurt surface was visually inspected for fungal growth (either mycelium colonies or yeast). A picture of the surface of the sample was taken before discarding the sample.

Model Development

Model Assumptions. In this model, all of the initial mold contamination in the yogurt was assumed to be the mold *P. commune*. Previous studies of fungal diversity have found that *P. commune* is frequently isolated from the yogurt production facility (Gougouli et al., 2011, Buehler et al., 2017). Growth of *P. commune* was adjusted to changes in storage temperature over time by assuming instantaneous adaptation of growth rate when temperature changed; lag phase was cumulative over storage time, similar to previous approaches (Koutsoumanis, 2001, Gougouli and Koutsoumanis, 2010).

Model Parameters. A simulation model estimating the number of yogurt cups with visible *P. commune* growth when opened by consumers for a nationally distributed yogurt was programmed in R v. 3.3.2 (R Core Team, 2013). Model parameters included initial contamination rate ($\mathbf{C_0}$), time from production to retail ($\mathbf{S_R}$), retail case storage time ($\mathbf{S_C}$), domestic refrigerator storage time ($\mathbf{S_D}$), storage temperature from production to consumer purchase ($\mathbf{T_C}$), domestic refrigerator storage temperature ($\mathbf{T_D}$), mycelium growth rate as a function of temperature ($\boldsymbol{\mu_T}$), lag time as a function of temperature ($\boldsymbol{\lambda_T}$), mycelium colony diameter ($\mathbf{D_s}$), and visible mycelium

colony to consumer ($\mathbf{V_C}$) (Table 4.2).

Table 4.2. Variables used in Monte Carlo simulation of number of consumers exposed to visible mycelium growth in yogurt

Description	Units	Variable name	Description and details[1]	Source
Mold contamination rate	Percent	C_0	Binomial distribution (n =1,000,000, p = (1/1,800))	Based on experimental data reported in this study
Time from production to retail	Hours	S_R	Triangle distribution (min=192, max=792, most likely=240)	Expert elicitation
Retail case storage time	Hours	S_C	Triangle distribution (min=2, max=240, most likely=24)	Expert elicitation
Domestic refrigerator storage time	Hours	S_D	Triangle distribution (min=24, max=240, most likely=120)	Expert elicitation
Production to consumer purchase storage temperature	°C	T_C	Uniform distribution (min=1, max=5)	Pradhan et al. (2010)
Domestic refrigerator storage temperature	°C	T_D	Truncated Normal distribution (min=0, max=17.22, mean=3.26, sd=2.62)	EcoSure (2007)
Mycelium growth rate as a function of temperature for *Penicillium commune*	mm/ hour	μ_T	Cardinal model with inflection described by Rosso et al. (1993): $\mu_T = \frac{\mu_{opt}(T-T_{max})(T-T_{min})^2}{(T_{opt}-T_{min})[(T_{opt}-T_{min})(T-T_{opt})-(T_{opt}-T_{max})(T_{opt}+T_{min}-2T)]}$ Where: $\begin{cases} \mu_{opt} = 0.257; \\ T_{max} = 29.8; \\ T_{min} = -7.6; \\ T_{opt} = 19.5 \end{cases}$	Gougouli et al. (2011)
Lag time as a function of temperature for *Penicillium commune*	Hours	λ_T	Cardinal model with inflection described by Rosso et al. (1993): $\frac{1}{\lambda_T} = \frac{(1/\lambda_{opt})(T-T_{max})(T-T_{min})^2}{(T_{opt}-T_{min})[(T_{opt}-T_{min})(T-T_{opt})-(T_{opt}-T_{max})(T_{opt}+T_{min}-2T)]}$ Where: $\begin{cases} 1/\lambda_{opt} = 0.04; \\ T_{max} = 30.1; \\ T_{min} = -6.3; \\ T_{opt} = 23.8 \end{cases}$	Gougouli et al. (2011)
Mycelium colony diameter as a function of	mm	D_S	$D_S = \begin{cases} 0, S \le \lambda_T \\ \mu_T(S-\lambda_T), S > \lambda_T \end{cases}$	Baert et al. (2008), Gougouli et

which stage of supply chain				al. (2011)
Visible mycelium growth to consumer	N/A	V_C	$V_c = \begin{cases} 0, D_S < 3mm \\ 1, D_s \geq 3mm \end{cases}$	Gougouli et al. (2011), Burgain et al. (2013)

[1]μ_{opt} represents growth rate (mm/h) at the optimum temperature (ºC); λ_{opt} represents lag time (h) at the optimum temperature (ºC); T_{min}, T_{max}, and T_{opt} represent theoretical minimum, maximum and optimum growth temperatures (ºC). T represents time (h)

Initial contamination rate was estimated by collecting air-plate samples in the yogurt packaging room of the dairy plant at Cornell University (Ithaca, NY) according to Standard Methods for the Examination of Dairy Products (Dyer et al., 2004). Briefly, two DRBC plates were placed on the plexiglass guard of the yogurt filling and packaging machine where yogurt was filled into cups; this location was deemed to represent the best proxy for air contamination that would be deposited into open yogurt cups. The plates were left uncovered for 15 minutes and then were covered and subsequently incubated at 25°C for 120 hours. This was repeated for a total of 3 yogurt processing days. These plates yielded a total of 3 mold colonies over the 3 days. As this represents 45 min (3 d x 15 min/d) of air exposure over the surface area of two plates, we estimated the contamination rate to be 3 mold spores per 45 min (or 1 spore per 15 min or 900 s). To determine the initial contamination rate, expert opinion was elicited to estimate the amount of time yogurt cups are exposed to air after being filled with product for a large-scale yogurt production operation. This value was estimated as 0.5 s. With 1 mold spore deposition per 900 s, this yields an estimate of 1 mold contamination event per 1,800 cups of yogurt.

Expert opinion was solicited to estimate the model parameters of S_R, S_C, and S_D. T_C was obtained from Pradhan et al. (2009), and T_D was obtained from EcoSure (2007). μ_T and λ_T were modeled using the cardinal model with inflection described by Rosso et al. (1993). Values for μ_T and λ_T for *P. commune* in yogurt were obtained from Gougouli et al. (2011). The minimum colony size consumers can detect with a naked eye was defined as 3mm; this estimate has been used in previous studies (Gougouli et al., 2011, Burgain et al., 2013).

Model Simulations. The simulation model predicted the number of consumers exposed to visible mycelium growth in yogurt based on: (i) the yogurt distribution chain, (ii) storage temperatures at the producer to consumer purchase level and in the domestic refrigerator, (iii) initial mold contamination rates, and (iv) growth characteristics of *P. commune* in yogurt. Each iteration resulted in a prediction of how many consumers were exposed to mycelium growth ≥ 3mm. The simulation of 1 million cups of yogurt contaminated, then distributed to consumers, stored in domestic refrigerators, and finally opened by consumers was repeated 100 times.

Sensitivity Analysis

A best- and worst-case scenario analysis was used to determine quantitatively the most important parameters affecting consumer exposure to visible mold in the model (Zwietering and Van Gerwen, 2000). The impact of eight major aspects of yogurt spoilage due to mold contamination were evaluated using a best- and worst-case scenario analysis. Best-case scenarios were defined as changes that would reduce consumer exposure to mold in yogurt (e.g., lower initial contamination rate) while worst-case scenarios were defined as changes that would increase consumer exposure to mold in yogurt. The 8 major aspects of mold contamination evaluated were: (i) initial contamination rate, C_0, (ii) time from production to retail, S_R, (iii) retail case storage time, S_C, (iv) domestic refrigerator storage time, S_D, (v) domestic refrigerator storage temperature, T_D, (vi) storage temperature from production to consumer purchase, T_C, (vii) mycelium growth rate, μ_T, and (viii) lag time, λ_T. For C_0, the worst-case scenario was a 0.25 increase of the mean of the numerator to 1.25/1800 (low) and a 0.5 increase of the mean of the numerator to 1.5/1800 (high); the best-case scenario

was a 0.25 decrease of the mean of the numerator to 0.75/1800 (low) and 0.5 decrease of the mean of the numerator to 0.5/1800 (high). For S_R, S_C, and S_D, the worst-case scenario was calculated as a 20% (low) and 40% (high) increase in storage time; the best-case scenario was calculated as a 20% (low) and 40% (high) decrease in storage time. For T_D, the worst-case scenario was calculated as increasing the temperature mean 1 (low) and 2 (high) degrees C; the best-case scenario was calculated as decreasing the temperature mean 1 (low) and 2 (high) degrees C. For T_C, the worst-case scenario was calculated as increasing the maximum temperature 1 (low) and 2 (high) degrees C; the best-case scenario was calculated as decreasing the maximum temperature 1 (low) and 2 (high) degrees C. For μ_T, the worst-case scenario was a 20% (low) and 40% (high) increase; the best-case scenario was a 20% (low) and 40% (high) decrease. For λ_T, the worst-case scenario was a 20% (low) and 40% (high) decrease; the best-case scenario was a 20% (low) and 40% (high) increase. Best- and worst-case sensitivities were calculated as the difference between the number of consumers opening yogurt with visible mold growth for each aspect above independently and the baseline model, where all eight aspects were set to their original values. Ultimately, this sensitivity analysis helped to identify which model parameters have the most influence on whether a consumer opens a cup of yogurt with visible mold growth.

What-if Analysis

Two what-if scenarios were used to demonstrate the impact of the distribution chain and interventions yogurt processors and consumers might employ to reduce the number of yogurt cups with visible mold growth: having a regional distribution chain

and lowering the storage temperature. For scenario one, a regional yogurt producer was surveyed to obtain estimates of a regional distribution chain. Time from production to retail was modeled with a triangle distribution with a minimum of 24 h, maximum of 168 h, and most likely value of 120 h. Retail case storage time was modeled with a triangle distribution with a minimum of 24 h, maximum of 672 h, and most likely value of 336 h.

For scenario two, the storage temperature was lowered, applied in combination by (i) reducing the mean domestic refrigerator temperature by 2°C and (ii) limiting the maximum storage temperature during production to consumer purchase to 3°C.

Statistical Analysis

All statistical analysis was performed in R v. 3.3.2 (R Core Team, 2013). Microbiological count data were log-transformed before performing analyses on fungal numbers by day of shelf-life. Count data were analyzed separately by final inoculum level (either 10^1 or 10^3CFU/g of yogurt). A two-way ANOVA for each inoculum level (10^1CFU/g or 10^3CFU/g) was used to assess the effects of protective culture use, day of shelf-life, and the interaction between protective culture use and day of shelf-life. The data for yogurts formulated with protective cultures ("protective culture count") were standardized to the mean of the control count by inoculum level (either 10^1CFU/g or 10^3CFU/g) for each day of shelf-life by taking the log of the ratio of the protective culture count (CFU/g) to the mean of the control count (CFU/g). The standardized count served as the response.

RESULTS AND DISCUSSION

In order to improve industry's ability to control yeast and mold spoilage in

yogurt, we developed a challenge study protocol that was used to evaluate yeast and mold growth in yogurts formulated with and without protective cultures and challenged with yeast and mold cocktails, simulating low (10^1 CFU/g yogurt) and high (10^3 CFU/g yogurt) levels of post-pasteurization fungal contamination. This challenge study indicated that the two protective cultures evaluated controlled surface mold growth, but not yeast growth over 76 days of yogurt shelf-life at 7°C. In yogurts formulated without protective cultures, surface mold growth was visible within 23 days of storage. A predictive model was then developed to determine consumer exposure to visible mold in yogurt formulated without fungal inhibitors. While this model provides a tool that can be used by individual facilities to predict consumer exposure to visible mold growth, plant specific data, particularly with regard to initial mold contamination frequency will be required for accurate assessment of mold growth. Combined, the data and tools presented here can be used to facilitate improved control of fungal spoilage in yogurt and will allow industry to better assess the value and impacts of different control strategies aimed at reducing yogurt spoilage due to yeasts and molds.

A fungal yogurt spoilage challenge study approach indicates that protective cultures evaluated control surface mold growth, but not yeast growth over 76 days of yogurt shelf-life.

In order to develop a challenge study protocol we inoculated Greek yogurt, formulated with and without protective cultures, with a yeast and mold cocktail (containing 5 yeast and 1 mold strain) at final concentrations of 10^1 and 10^3 CFU/g yogurt. When incubated at a mild abuse temperature of 7°C, yogurt with no protective

cultures inoculated with 10^1 CFU of yeast and mold per g showed mean total yeast and mold numbers of 4.7 Log_{10}CFU/g at day 9, with numbers between 4.9 Log_{10}CFU/g and 6.5 Log_{10}CFU/g observed on days 23, 60, and 76 (Figure 4.1). From a practical standpoint, yeast spoilage of yogurt is noticeable when counts reach 10^5-10^6 CFU/g (Suriyarachchi and Fleet, 1981), hence in our model, Greek yogurt reached "spoilage levels" of yeast and mold by day 23 of storage at 7°C, well short of the 45 to 60 day target shelf-life typical for large US yogurt manufacturers. For the Greek yogurt formulations inoculated at 10^3 CFU/g yogurt, growth to spoilage levels (10^5-10^6 CFU/g) occurred by day 9 at a storage temperature of 7°C (Figure 4.1). Visible surface mold growth was evaluated for yogurts inoculated at 10^1 CFU/g yogurt and revealed that for yogurts formulated without protective cultures, visible mycelium growth occurred by day 23 of storage at 7°C, while yogurts formulated with protective cultures only displayed yeast growth over the entire 76 d shelf-life. For future efforts, our data suggest that a single low-level inoculum (e.g., 10^1 CFU/g or possibly lower) is sufficient for challenge study protocols. While previous challenge studies for mold used surface inoculation with mold spores (Delavenne et al., 2013, Lačanin et al., 2017), our data suggest that, at least for plain yogurt, spoilage due to mold surface growth can also be assessed by inoculating with a sterile needle into the bottom of closed yogurt cups. Our needle inoculation method could also be applied to other yogurt formulations and could potentially even be used to target specific areas in the yogurt. For example, fruit on the bottom yogurt varieties typically have a high sugar fruit preparation at the base of the yogurt cup (Chandan and O'Rell, 2013). Needle inoculation could be used to directly inject spoilage organisms into the fruit and yogurt

interphase to investigate contamination through added ingredients; however, further work would be needed to test this approach.

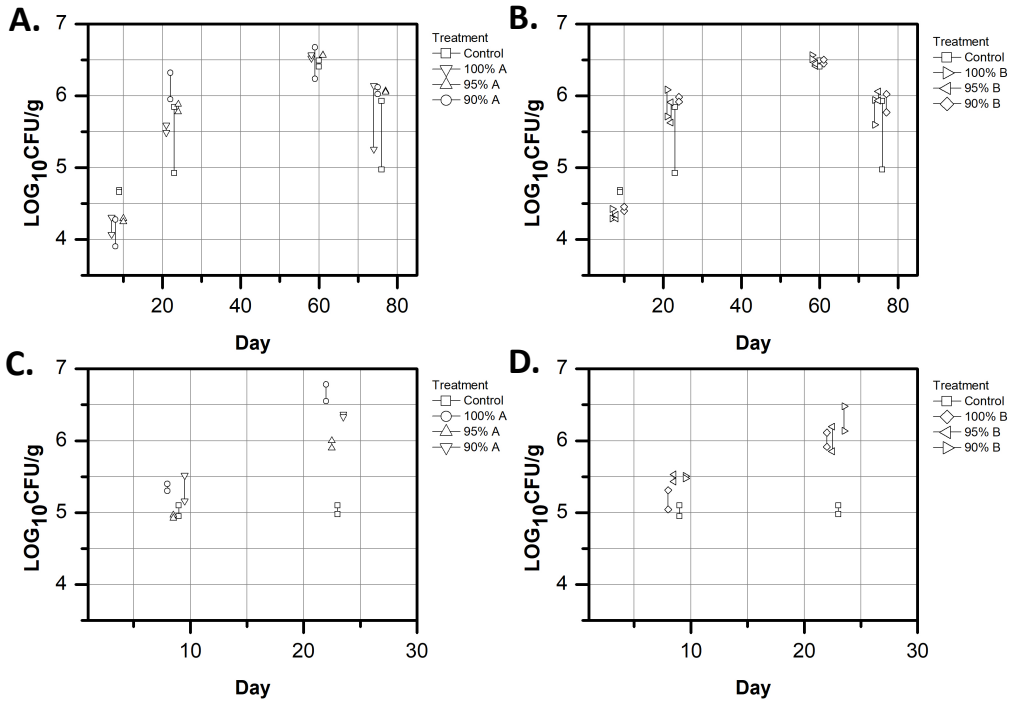


Figure 4.1. Concentration of yeast and mold in Greek yogurt by day of storage at 7°C. Symbols represent yeast and mold concentration from one cup of yogurt sampled. Two cups were sampled per treatment variable. Yogurts were inoculated with a cocktail consisting of five yeasts and one mold to yield inoculum levels of 10^1 and 10^3 CFU/g of yogurt. (A.) Yogurts inoculated at 10^1 CFU/g with and without protective culture A over 76 days. (B.) Yogurts inoculated at 10^1 CFU/g with and without protective culture B over 76 days. (C.) Yogurts inoculated at 10^3 CFU/g with and without protective culture A over 23 days. (D.) Yogurts inoculated at 10^3 CFU/g with and without protective culture B over 23 days.

Assessment of total fungal numbers in yogurt formulated with and without protective cultures and inoculated at 10^1 CFU/g on day 9, showed between 0.57 and 1.3 log lower counts for products formulated with 90, 95, and 100% of the recommended protective culture levels (with no significant differences between protective cultures A and B and different use levels; Table 4.3). For day 23, 60, and 76, counts for products formulated with protective cultures A and B (across use levels) were generally numerically higher than counts for the control without protective cultures (except for one sample, the difference was always less than 1 log); only two samples with protective cultures showed minimally lower counts (0.05 and 0.11 log). The effect of protective culture use, day of shelf-life and the interaction between protective culture use and day of shelf-life for yogurt samples inoculated with 10^1 CFU of yeast and mold per g were compared with a two-way ANOVA (Table 4.3). A nested F-test was performed to compare the full factorial model, with both day effects and protective culture effects, to the simple model with only day effects; the nested F-test statistic for this comparison was 1.20 (p value = 0.3204). Overall, our data indicate that, at an inoculum level of 10^1 CFU/g, the protective cultures evaluated clearly had no effect on fungal counts on day 23 or after. While there may be a small effect on fungal counts earlier in shelf-life, further experiments (with larger sample numbers) would be required to test this hypothesis, if this information is deemed relevant considering a common yogurt shelf-life of 45 to 60 days.

Table 4.3. Protective culture and day effect on yeast and mold concentrations (Log_{10}CFU/g) in Greek yogurt inoculated at 10^1 CFU/g stored at 7°C for 76 days.

Protective culture	Day	Mean Difference from Control[a]	Confidence Interval	
			Lower	Upper
100% A	9	-1.11AB	-1.76	-0.45
100% B	9	-0.73 ABCD	-1.38	-0.08
95% A	9	-0.93 ABC	-1.59	-0.28
95% B	9	-0.82 ABCD	-1.47	-0.17
90% A	9	-1.34 A	-1.99	-0.68
90% B	9	-0.57 ABCD	-1.22	0.09
100% A	23	-0.11 ABCDE	-0.76	0.54
100% B	23	0.71 BCDE	0.06	1.36
95% A	23	0.55 BCDE	-0.10	1.20
95% B	23	0.41 ABCDE	-0.24	1.06
90% A	23	1.26 E	0.61	1.91
90% B	23	0.83 CDE	0.17	1.48
100% A	60	0.23 ABCDE	-0.43	0.89
100% B	60	0.20 ABCDE	-0.45	0.85
95% A	60	0.26 ABCDE	-0.39	0.91
95% B	60	-0.05 ABCDE	-0.70	0.61
90% A	60	0.01 ABCDE	-0.64	0.66
90% B	60	0.06 ABCDE	-0.59	0.71
100% A	76	0.06 ABCDE	-0.60	0.71
100% B	76	0.22 ABCDE	-0.43	0.87
95% A	76	0.88 CDE	0.23	1.53
95% B	76	0.75 CDE	0.09	1.40
90% A	76	0.92 DE	0.27	1.57
90% B	76	0.52 BCDE	-0.14	1.17

[a]Results are summarized by the mean difference from control [log_{10}(Protective Culture Count/Mean Control Count)] for 2 cups of yogurt sampled per treatment. Means with the same letter are not statistically different from each other (overall α=0.05, Tukey's method for multiple comparisons)

For yogurt inoculated at 10^3 CFU/g, fungal counts were only assessed through day 23 as all products formulated with protective cultures exceeded 4.9 and 5.9 Log_{10}CFU/g on days 9 and 23, respectively. In addition, most products formulated with protective cultures showed visible spoilage (e.g., puffed containers) by day 23. Interestingly, for the yogurt samples inoculated at 10^3 CFU/g, the two-way ANOVA analysis (Table 4.4) indicated significant main effects for protective culture use, with a F-test statistic of 7.33 (p value <0.01) as well as for day of shelf-life, with a F-test statistic of 211.69 (p value <0.01). Surprisingly, yogurt formulated with protective cultures (across different use levels) generally showed >2 log higher counts as compared to products without protective cultures. As it is unlikely that protective cultures enhance fungal (and specially yeast) growth, it is more likely that at these high inoculum levels interactions between yeast and mold affects yeast growth. For example, yeasts may show improved growth when mold growth is suppressed due to protective cultures; this effect of protective cultures on mold growth was observed here, as detailed in the subsequent paragraph. While these findings further support that high inoculum levels (10^3 CFU/g) do not provide a valuable model for evaluating antifungal biopreservatives, future experiments with pure yeast strains or "yeast only" cocktails may be valuable to further address this observation.

Table 4.4. Protective culture and day effect on yeast and mold concentrations (Log_{10}CFU/g) in Greek yogurt inoculated at 10^3 CFU/g stored at 7°C for 23 days.

Protective Culture	Day	Mean Difference from Control[a]	Confidence Interval	
			Lower	Upper
100% A	9	0.74 AB	0.20	1.28
100% B	9	0.35 A	-0.20	0.89
95% A	9	-0.20 A	-0.74	0.35
95% B	9	1.04 ABC	0.50	1.59
90% A	9	0.72 AB	0.17	1.26
90% B	9	1.07 ABC	0.52	1.61
100% A	23	3.74 E	3.20	4.29
100% B	23	2.24 CD	1.70	2.78
95% A	23	2.09 BCD	1.54	2.63
95% B	23	2.27 CD	1.72	2.81
90% A	23	3.02 DE	2.47	3.56
90% B	23	2.92 DE	2.37	3.46

[a]Results are summarized by the mean difference from control [log_{10}(Protective Culture Count/Mean Control Count)] for 2 cups of yogurt sampled per treatment. Means with the same letter are not statistically different from each other (overall α=0.05, Tukey's method for multiple comparisons)

While yogurts inoculated at 10^3 CFU/g were only evaluated through day 23, yogurts inoculated at 10^1 CFU/g were evaluated on days 0, 9, 23, and then every 3 d from day 34-day 76 for visual appearance of surface mold growth. For the yogurts formulated without protective cultures, visual mycelium growth was first recorded on day 23 (in both Greek yogurts opened), all 18 cups of Greek yogurt formulated without protective cultures that were sampled on subsequent time points also displayed mycelium growth on the surface. In contrast, Greek yogurts formulated with protective cultures at all tested concentrations (i.e., 100% A, 100% B, 95% A, 95% B, 90%, 90% B; n=48) failed to display visible mycelium growth during the 76 d of observation; however, there were visible indications of yeast growth (e.g., gas bubble production, off-white surface slime) on the surface of these yogurts (Figure 4.2). This finding suggests that while the fungal counts increase over time in all yogurt formulations, these increases were due to yeast counts and not mold counts in the yogurts formulated with protective cultures, while the increase in fungal counts in the yogurts formulated without protective cultures were most likely due to yeast and mold counts. Importantly, this demonstrates that the two protective cultures, at all use levels we evaluated, were effective at inhibiting mycelium mold growth on the surface of the yogurt.

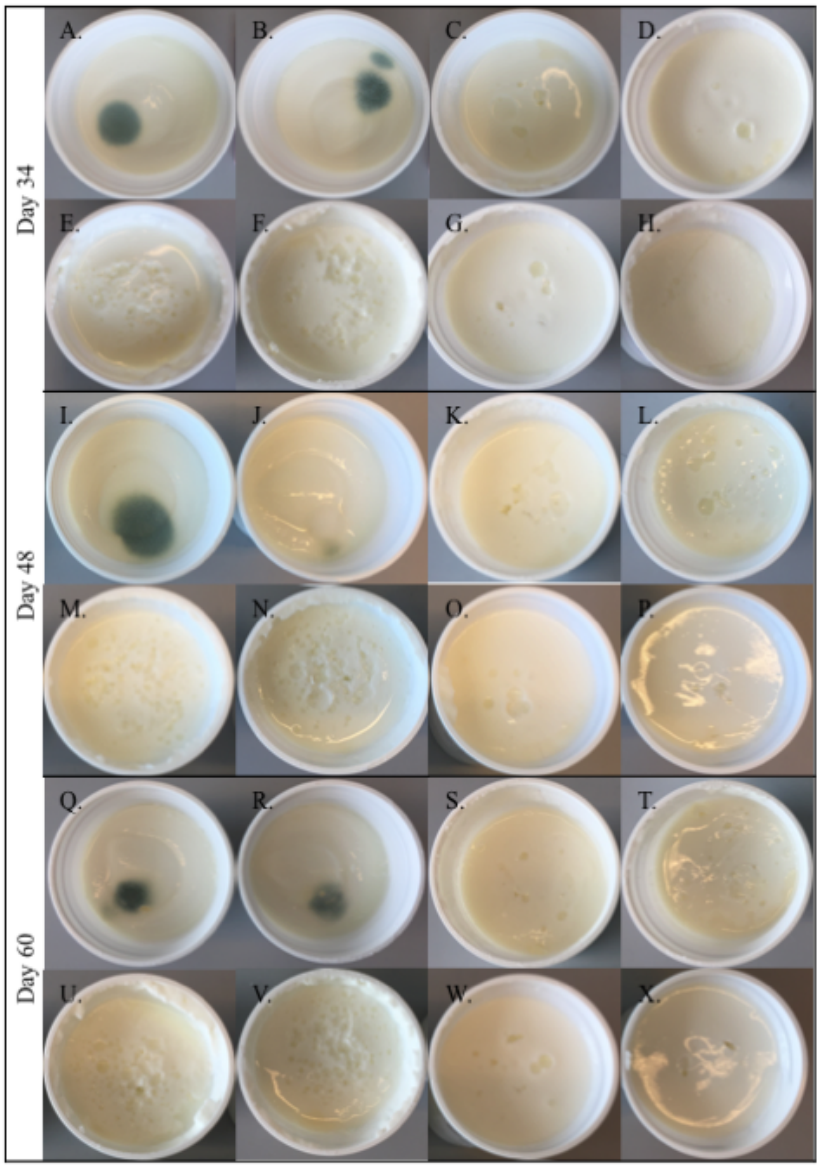


Figure 4.2. Time to visible growth of yeast and mold on the surface of Greek yogurt inoculated with a cocktail consisting of five yeasts and one mold to yield an inoculum level of 10^1 CFU/g over storage at 7°C on days 34, 48, and 60. Panels A, B, I, J, Q, and R were formulated without protective cultures. Panels C, D, K, L, S, and T were formulated with 100% protective culture A. Panels E, F, M, N, U, and V were formulated with 95% protective culture A. Panels G, H, O, T, W, and X were formulated with 90% protective culture A.

Initial contamination rate of yogurt with mold is the most important source of variability that affects the model estimate of consumers exposed to visible mold in yogurt.

In order to estimate the impacts and benefits of different mold control strategies (such as protective cultures effective against mold), we developed a Monte Carlo simulation model to estimate how many consumers are exposed to visible mold in yogurt per 1 million cups produced. This model used ten parameters to describe various aspects of the distribution (e.g., time from production to retail, consumer storage time, see Table 4.2 for details), assuming a national distribution chain with a 60 day shelf-life. Estimates for initial mold contamination rate were not available or accessible and were hence estimated by performing air plating for mold in a small commercial yogurt production facility. These experiments yielded an estimate of 1 mold contamination event per 1,800 yogurt cups; this estimate was deemed realistic in discussions with industry. While this estimate is based on a small number of data collected in a single facility, this approach allowed us to develop a proof-of-concept Monte Carlo simulation model to estimate consumer exposure to visible mold contamination in yogurt. The Monte Carlo simulation model using this initial contamination rate, along with the other parameters detailed in Table 4.2, yielded an estimate of 550 ± 25.2 consumers that would be exposed to visible mold on the surface of yogurt per 1 million cups produced (Figure 4.3). As this simulation approach can be used to estimate the number of consumers that will likely be exposed to visible mold growth in yogurt under a set of (current) production practices, it can also be used to help processors assess the value of spoilage control strategies by

estimating the reduction of consumer exposures to mold that can be achieved with different interventions. Ultimately, the value of this model for processors could be improved, if the model could be refined to include as an output the number of mold-related consumer complaints per 1 million cups produced. However, future efforts are needed to understand the relationship between consumer complaints and consumer exposure to fungal spoilage in yogurt. Previous reports of consumer complaint behavior for food products indicate low rates of reporting dissatisfaction directly to the manufacturer (Quelch and Ash, 1980, Richins and Verhage, 1985). For example, a study in Canada surveyed consumers about relative satisfaction about food products purchased and found that while around 36% of consumers reported dissatisfaction with a food product, only 2% formally complained to the manufacturer (Quelch and Ash, 1980). While this report could be used to estimate that 18 consumer exposures to visible mold are needed to trigger one consumer complaint, complaint rates may have changed considerably with the advent of social media platforms. Future research on current consumer complaint behavior for food products would be helpful to further enhance our simulation model.

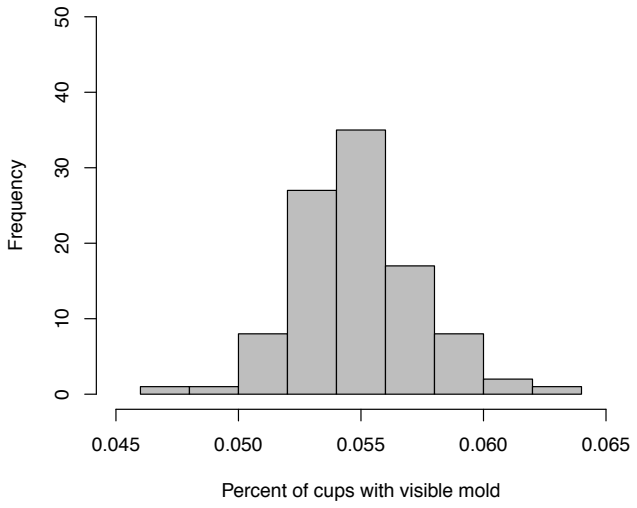


Figure 4.3. Histogram of the simulated percent of consumers exposed to visible mycelium mold growth per 1 million yogurt cups produced without the use of fungal inhibitors. Monte Carlo simulations comprised 100 iterations of 1 million cups of yogurt, and were based on ten model parameters: (i) initial mold contamination rate in the production facility, (ii) time from production facility to retail facility, (iii) retail case storage time, (iv) domestic refrigerator storage time, (v) temperature from production to consumer purchase, (vi) temperature during domestic refrigerator storage, (vii) lag time of *Penicillium commune* as a function of temperature, (viii) mycelium growth rate of *P. commune* as a function of temperature, (ix) mycelium colony diameter as a function of stage of supply chain, and (x) visible mycelium growth to consumer.

Importantly, best- and worst-case sensitivity analyses revealed that the initial contamination rate is the most important parameter that drives the model outcome of consumers exposed to visible mold in yogurt (Figure 4.4). Best-case scenarios of C_0 lowered the number of consumers exposed to visible mold in yogurt from 550 ± 25.2 in the baseline model to 411 ± 21.1 (low) and 275.11 ± 16.9 (high). This finding is similar to other studies that also indicated the importance of initial contamination rate at the processing level (Samson et al., 2010, Snyder et al., 2016). While our model estimated the initial mold contamination rate based on air plate sampling of a packaging room, the sensitivity analysis highlights the importance of the baseline mold contamination rate estimates to allow a given facility to accurately estimate, using our model described here, consumer exposure to visible mold (and hence the value of different mold control strategies). Approaches to estimating initial mold contamination rates could include (i) air plating approaches, such as those described here, or (ii) end of shelf-life or stress test observations for visible mold contamination in commercially packaged yogurt; the latter approach however would require large sample sizes (likely > 1,000 cups) due to the low-level nature of mold contamination in most yogurt facilities (MacBean, 2009). Thus, air plate sampling to obtain initial mold contamination rates may represent the most feasible first-step processors can take in understanding the baseline mold contamination rate on a facility-specific basis.

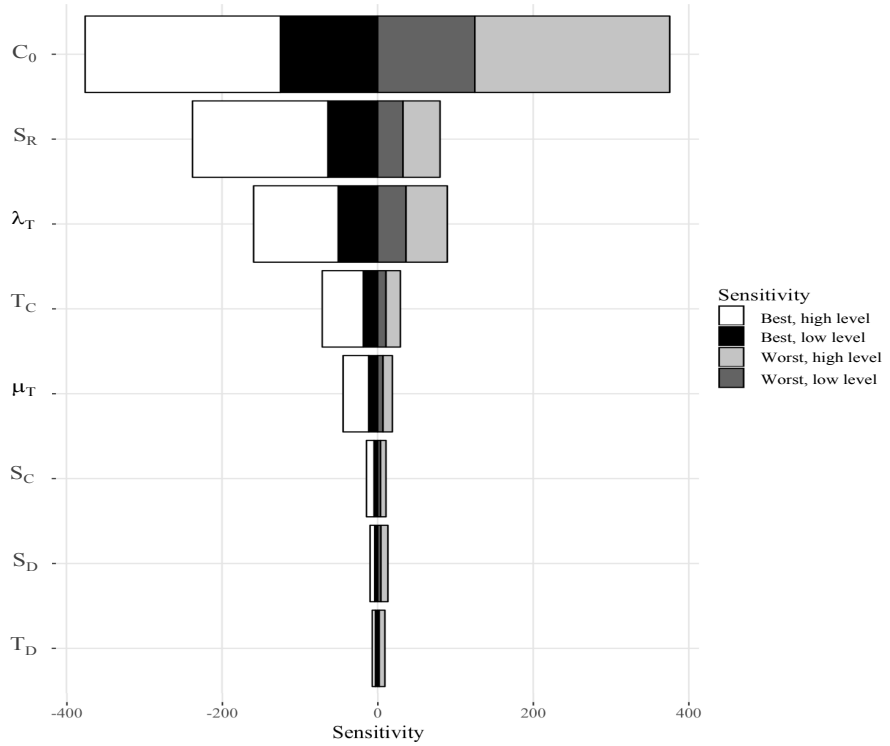


Figure 4.4. Best-case (white and black) and worst-case (gray) scenarios of the number of consumers exposed to visible mycelium mold (≥ 3mm) in yogurt at the time of domestic consumption out of one million yogurt cups produced for a nationally distributed yogurt. Parameters displayed represent the most important factors affecting the estimated number of consumers exposed to visible mold: C_0 represents the initial mold contamination rate, S_R represents the time (h) for the yogurt cups to leave the production facility and arrive at the retail facility, λ_T (lag time) represents the time (h) where the mycelium growth rate equals 0, T_C represents the temperature from production to consumer purchase, μ_T represents *Penicillium commune's* mycelium growth rate, T_D represents the temperature in the consumer's refrigerator, S_C represents the time (h) the yogurt cups stay at retail before being bought by the consumer, and S_D represents the time (h) the yogurt cups are in the consumer's refrigerator before being opened. Best- and worst-case scenarios were calculated as the difference between the mean number of consumers exposed to visible mold when opening a cup of yogurt for each input value and the baseline model. Worst-case scenarios were calculated as a 0.25 (low) and 0.5 (high) increase of the numerator of the mean of C_0, 20% (low) and 40% (high) increase of S_R, S_C, S_D, and, μ_T, 20% (low) and 40% (high) decrease of λ_T, 1-degree C (low) and 2-degrees C (high) increase of the mean of T_D, and 1-degree C (low) and 2-degrees C (high) increase of the maximum value of T_C. Best-case scenarios were calculated similarly, with a 0.25 (low) and 0.5 (high) decrease of the numerator of the mean of C_0, 20% (low) and 40% (high) decrease of S_R, S_C, S_D, and, μ_T, 20% (low) and 40% (high) increase of λ_T, 1-degree C (low) and 2-degrees C (high) decrease of the mean of T_D, and 1-degree C (low) and 2-degrees C (high) decrease of the maximum value of T_C.

Best- and worst-case sensitivity analyses indicate that "time from production to retail" (which encompasses warehouse storage and transportation) was the next most sensitive parameter in the model. Best-case scenarios of S_R lowered the number of consumers exposed to visible mold in yogurt from 550 ± 25.2 in the baseline model to 534 ± 24.9 (low) and 493 ± 24.0 (high). The importance of the distribution chain for controlling microbial contaminants has also been demonstrated for other refrigerated foods (Pradhan et al., 2010). For example, a study that evaluated the effect of storage time from retail to consumer, found that when the storage time was shortened from 28 d to 16 d, the mean number of listeriosis-associated deaths per year were reduced between 24% and 57%, depending on the retail-meat formulation [with or without growth-inhibitors (Pradhan et al., 2010)]. Our results indicate that improved data on the production to retail supply chain, as well as possibly a refined model that separates this time into (i) "warehouse storage" and (ii) transportation time could further improve model estimates, particularly if accurate estimates on initial contamination frequency are available.

Monte Carlo models can provide quantitative estimates on the effect of different interventions on mold spoilage

The Monte Carlo simulation model developed here also provides the opportunity to assess how different interventions can reduce consumer exposure to visible mold spoilage, using so called "what-if" scenarios. While modeling the impact of using a protective culture would seem a logical what-if scenario to evaluate with this model, this scenario would have provided limited insights as both protective cultures used here reduced visible mold spoilage to an undetectable level (none of the

inoculated yogurts formulated with protective cultures showed visible mold growth). As a proof-of-concept, we thus selected two scenarios for "what-if analyses" (Table 4.5). The first scenario evaluated was the effect of a shortened distribution chain (i.e., "having a regionally-distributed yogurt") on consumer exposure to visible mold. This scenario involved changes in two parameters, based on expert opinion from a smaller regional yogurt processor, including (i) shortening the time from production to retail and (ii) increasing the retail case storage time (presumably reflecting reduced product turn-over as compared to more popular national brands). These changes had a minimal effect on the number of consumers exposed to visible mold with an estimate of 532 ± 24.3 consumers exposed to visible mold per 1 million cups of yogurt produced (versus 550 ± 25.2 consumer exposures in the base model) (Figure 4.5). While these results suggest limited changes in outcome when the simulated distribution chain was changed to reflect a specific smaller regional yogurt manufacturer, specific facilities that use this model should collect and use data specific to their supply chain (in addition to an accurate estimate of initial contamination rates) in order to provide the most accurate outcome estimates.

Table 4.5. Summary of what-if scenario analysis outcomes

What-if condition	Number of consumers exposed to visible mold per 1 million cups of yogurt produced	
	Mean	Standard deviation
Initial condition	550	25.2
Regional distribution chain: S_R[1]: Triangle distribution (min=24, max=168, most likely=120) S_C[2]: Triangle distribution (min=24, max=672, most likely=336)	532	24.3
Lower storage temperature by (i) lowering the mean domestic refrigerator temperature 2°C and (ii) limiting the production to consumer purchase storage temperature to a maximum of 3°C	473	23.7

[1]S_R represents time (h) for yogurt cups to leave production facility and arrive at retail facility.
[2]S_C represents time (h) that yogurt cups are at retail facility until purchased by consumer.

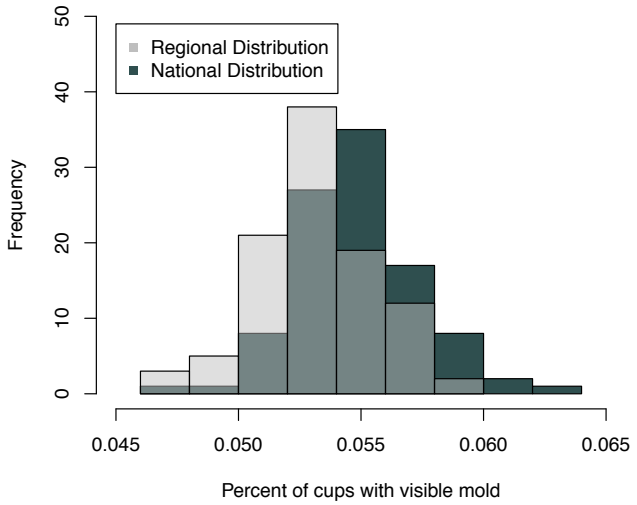


Figure 4.5. Histograms of the simulated percent of consumers exposed to visible mycelium mold growth in yogurt per 1 million cups of yogurt produced for a nationally distributed yogurt (dark grey) compared to a regionally distributed yogurt (light grey).

The second “what-if” scenario run here assessed the effect of lowering the storage temperature of yogurt throughout the distribution chain. For this “what-if” scenario we (i) limited the maximum storage temperature from production to consumer purchase to 3°C and (ii) lowered the mean domestic refrigerator temperature by 2°C. These changes, which were applied in combination, lowered the mean number of consumers exposed to visible mold growth to 473 ± 23.7 consumers per 1 million cups of yogurt produced (Figure 4.6). Importantly, these two “what-if” scenarios showed limited effects on the outcome (i.e., consumer exposure to visible mold) when different changes to the distribution chain were simulated, which is consistent with our sensitivity analysis, which showed that initial contamination rate had the largest effect on outcomes. While storage and distribution temperatures close to 0°C may reduce consumer exposure to visible mold, these scenarios were not modelled here since they were (i) deemed unrealistic and (ii) would require more specific data on mold growth at extremely low temperatures, particularly since some studies show that certain molds including *Penicillium* can grow at temperatures as low as -4°C (Mislivec and Tuite, 1970).

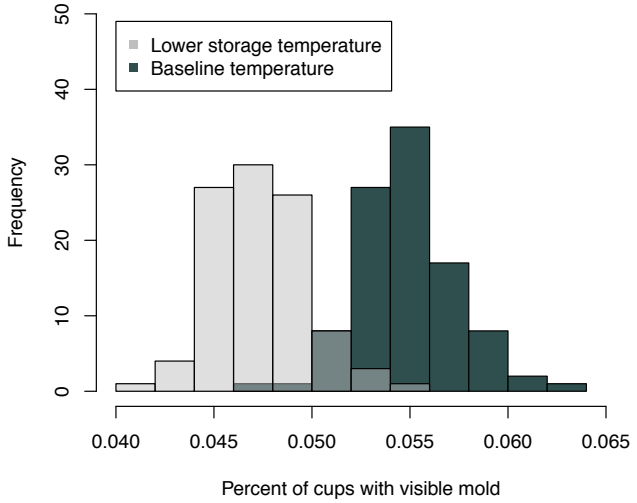


Figure 4.6. Histograms of the simulated percent of consumers exposed to visible mycelium mold growth in yogurt per 1 million cups of yogurt produced assuming no change in storage temperature over shelf-life of yogurt (dark grey) compared to lowering the mean domestic refrigerator temperature 2°C and limiting the production to consumer purchase storage temperature to a maximum of 3°C (light grey).

Overall, our data support that, in addition to the use of preservatives or protective cultures, reducing initial mold contamination represents the most effective approach to reducing mold spoilage. Reducing initial contamination levels however typically requires a number of specific approaches and strategies that differ in the contributions to reducing mold contamination between processing facilities and even within processing facilities (e.g., between days or seasons). Key strategies to reducing mold contamination include good manufacturing practices (GMPs), validated sanitation standard operating procedures (SSOPs), air filtration systems, and aseptic packaging conditions (Sofos, 1993, Salustiano et al., 2003, Tamime and Robinson, 2007). For example, a study in a yogurt processing facility in Brazil reported a significant decrease in the yeast and mold counts following implementation of GMPs and SSOPs (Cusato et al., 2013). This processing facility specifically reported that food handler training led to substantial changes in food handlers' behavior towards food quality; most food handlers did not understand basic routes of contamination before the systems implementation (Cusato et al., 2013). Airborne transmission of mold spores also plays an important role in mold contamination in the processing facility. Hence, implementation of air filtration systems in the filling areas or use of enclosed filling cabinets supplied with high efficiency particulate air (HEPA)-filtered air represent key strategies to reduce mold contamination (Nauth, 2004). The microbiological quality of packaging materials also plays a role in contamination levels in the processing facility. Sterilizing packaging materials before they are filled with product could help lower the initial contamination rate as well. Further research on the reduction of the initial contamination rate that each of these interventions

achieves is needed to fully assess which measure to employ as well as an evaluation of facility-specific design concerns. Due to the complexity of mold transmission in processing facilities and the associated complexity of interventions, implementation of specific environmental control strategies could not be assessed with our Monte Carlo simulation model and would require considerably more complex agent-based models.

CONCLUSION

Our study reported here provides a set of data and tools that will be valuable as the dairy industry is faced with external pressures (e.g., the "clean label trend") that require novel approaches to control spoilage and in particular fungal spoilage of yogurt. While our specific data suggest that the two protective cultures evaluated did not effectively control yeast growth at initial contamination levels of 10^1 CFU/g, this finding is not necessarily representative of all protective cultures. In addition, future efforts may yield other biopreservatives that can control both yeast and mold growth, as supported by some studies that reported characterization of lactic acid bacteria strains that inhibit both yeast and mold (Delavenne et al., 2013). The challenge study protocol and the Monte Carlo simulation model reported here will provide a valuable set of tools that can be used for evaluation of specific biopreservative strategies of interest, with the ability to quantify spoilage reduction through the use of simulation models with facility and supply chain specific input data. The simulation model should also be easily expandable to other organisms (e.g., yeast) and other dairy commodities where yeast and mold spoilage is a major concern (e.g., shredded cheese).

ACKNOWLEDGEMENTS

The authors would like to thank the staff of the Food Processing and

Development Laboratory (Cornell University, Ithaca, NY) for help in yogurt production. This publication was made possible through a gift from Chobani Inc. (New Berlin, NY), supporting innovative research in dairy quality. We thank them for their generous contribution and commitment to food innovation and quality.

REFERENCES

Atanassova, M., C. Fernández-Otero, P. Rodríguez-Alonso, I. Fernández-No, J. Garabal, and J. Centeno. 2016. Characterization of yeasts isolated from artisanal short-ripened cows' cheeses produced in Galicia (NW Spain). Food Microbiol. 53:172-181.

Baert, K., F. Devlieghere, L. Bo, J. Debevere, and B. De Meulenaer. 2008. The effect of inoculum size on the growth of Penicillium expansum in apples. Food Microbiol. 25:212-217.

Beletsiotis, E., D. Ghikas, and K. Kalantzi. 2011. Incorporation of microbiological and molecular methods in HACCP monitoring scheme of molds and yeasts in a Greek dairy plant: A case study. Procedia Food Sci. 1:1051-1059.

Bokulich, N. A. and D. A. Mills. 2013. Facility-specific “house” microbiome drives microbial landscapes of artisan cheesemaking plants. Appl. Environ. Microbiol. 79:5214-5223.

Buehler, A., R. Evanowski, N. Martin, K. Boor, and M. Wiedmann. 2017. Internal transcribed spacer (ITS) sequencing reveals considerable fungal diversity in dairy products. J. Dairy Sci. 100:8814-8825.

Burgain, A., M. Bensoussan, and P. Dantigny. 2013. Effect of inoculum size and water activity on the time to visible growth of Penicillium chrysogenum colony. Int. J. Food Microbiol. 163:180-183.

Chandan, R. C. and K. R. O'Rell. 2013. Manufacture of various types of yogurt. Pages 263-295 in Manufacturing Yogurt and Fermented Milks. Ramesh C. Chandan and A. Kilara, ed. John Wiley & Sons, Inc., Ames, Iowa.

Cheong, E. Y., A. Sandhu, J. Jayabalan, T. T. K. Le, N. T. Nhiep, H. T. M. Ho, J. Zwielehner, N. Bansal, and M. S. Turner. 2014. Isolation of lactic acid bacteria with antifungal activity against the common cheese spoilage mould Penicillium commune and their potential as biopreservatives in cheese. Food Control 46:91-97.

Crowley, S., J. Mahony, and D. van Sinderen. 2013a. Broad-spectrum antifungal-producing lactic acid bacteria and their application in fruit models. Folia Microbiol. 58:291-299.

Crowley, S., J. Mahony, and D. van Sinderen. 2013b. Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. Trends Food Sci. Technol. 33:93-109.

Cusato, S., A. H. Gameiro, C. H. Corassin, A. S. Sant'Ana, A. G. Cruz, J. d. A. F. Faria, and C. A. F. de Oliveira. 2013. Food safety systems in a small dairy

factory: Implementation, major challenges, and assessment of systems' performances. Foodborne Pathog. Dis. 10:6-12.

Davidson, P. M., T. M. Taylor, and S. E. Schmidt. 2013. Chemical preservatives and natural antimicrobial compounds. Pages 765-801 in Food Microbiol. American Society of Microbiology.

Delavenne, E., R. Ismail, A. Pawtowski, J. Mounier, G. Barbier, and G. Le Blay. 2013. Assessment of lactobacilli strains as yogurt bioprotective cultures. Food Control 30:206-213.

Devlieghere, F., L. Vermeiren, and J. Debevere. 2004. New preservation technologies: Possibilities and limitations. Int. Dairy J. 14:273-285.

Dyer, R., J. Frank, B. Johnson, and P. Hickey. 2004. Microbiological tests for equipment, containers, water, and air. Pages 325-340 in Standard Methods for Examination of Dairy Products 17 ed. American Public Health Association, Washington, DC.

EcoSure. 2007. EcoSure 2007 cold temperature database. Accessed Oct. 15, 2017. http://foodrisk.org/exclusives/EcoSure.

Garnier, L., F. Valence, and J. Mounier. 2017a. Diversity and Control of Spoilage Fungi in Dairy Products: An Update. Microorganisms 5:42.

Garnier, L., F. Valence, A. Pawtowski, L. Auhustsinava-Galerne, N. Frotté, R. Baroncelli, F. Deniel, E. Coton, and J. Mounier. 2017b. Diversity of spoilage fungi associated with various French dairy products. Int. J. Food Microbiol. 241:191-197.

Garofalo, C., E. Zannini, L. Aquilanti, G. Silvestri, O. Fierro, G. Picariello, and F. Clementi. 2012. Selection of Sourdough Lactobacilli with Antifungal Activity for Use as Biopreservatives in Bakery Products. J. Agric. Food Chem. 60:7719-7728.

Gerez, C. L., M. I. Torino, G. Rollán, and G. Font de Valdez. 2009. Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. Food Control 20:144-148.

Gougouli, M., K. Kalantzi, E. Beletsiotis, and K. P. Koutsoumanis. 2011. Development and application of predictive models for fungal growth as tools to improve quality control in yogurt production. Food Microbiol. 28:1453-1462.

Gougouli, M. and K. P. Koutsoumanis. 2010. Modelling growth of Penicillium expansum and Aspergillus niger at constant and fluctuating temperature conditions. Int. J. Food Microbiol. 140:254-262.

Gougouli, M. and K. P. Koutsoumanis. 2017. Risk assessment of fungal spoilage: A case study of Aspergillus niger on yogurt. Food Microbiol. 65:264-273.

Holzapfel, W., R. Geisen, and U. Schillinger. 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. International J. Food Microbiol. 24:343-362.

Jacques, N. and S. Casaregola. 2008. Safety assessment of dairy microorganisms: the hemiascomycetous yeasts. Int. J. Food Microbiol. 126:321-326.

Koutsoumanis, K. 2001. Predictive modeling of the shelf life of fish under nonisothermal conditions. Appl. Environ. Microbiol. 67:1821-1829.

Kure, C. F., I. Skaar, and J. Brendehaug. 2004. Mould contamination in production of semi-hard cheese. Int J. Food Microbiol. 93:41-49.

Kure, C. F., Y. Wasteson, J. Brendehaug, and I. Skaar. 2001. Mould contaminants on Jarlsberg and Norvegia cheese blocks from four factories. Int. J. Food Microbiol. 70:21-27.

Lačanin, I., J. Mounier, A. Pawtowski, M. Dušková, J. Kameník, and R. Karpíšková. 2017. Assessment of the antifungal activity of Lactobacillus and Pediococcus spp. for use as bioprotective cultures in dairy products. World J. Microbiol. Biotechnol. 33:188.

Lambertini, E., J. Barouei, D. W. Schaffner, M. D. Danyluk, and L. J. Harris. 2017. Modeling the risk of salmonellosis from consumption of pistachios produced and consumed in the United States. Food Microbiol. 67:85-96.

Lavoie, K., M. Touchette, D. St-Gelais, and S. Labrie. 2011. Characterization of the fungal microflora in raw milk and specialty cheeses of the province of Quebec. Dairy Sci. Technol. 92:455-468.

Ledenbach, L. H. and R. T. Marshall. 2009. Microbiological spoilage of dairy products. Pages 41-67 in Compendium of the Microbiological Spoilage of Foods and Beverages. Springer, New York, NY.

MacBean, R. D. 2009. Packaging and the shelf life of yogurt. Pages 147-148 in Food Packaging and Shelf Life: A Practical Guide. G. L. Robertson, ed. CRC Press, Boca Raton, FL.

Mayoral, M. B., R. Martín, A. Sanz, P. E. Hernández, I. González, and T. García. 2005. Detection of Kluyveromyces marxianus and other spoilage yeasts in yoghurt using a PCR-culture technique. Int. J. Food Microbiol. 105:27-34.

Mislivec, P. B. and J. Tuite. 1970. Temperature and relative humidity requirements of species of Penicillium isolated from yellow dent corn kernels. Mycologia:75-88.

Muhialdin, B. J., Z. Hassan, and S. K. Sadon. 2011. Antifungal Activity of Lactobacillus fermentum Te007, Pediococcus pentosaceus Te010, Lactobacillus pentosus G004, and L. paracasi D5 on Selected Foods. J. Food Sci. 76.

Nauth, K. R. 2004. Yogurt. Page 158 in Handbook of Food and Beverage Fermentation Technology. Vol. 134. Y. H. Hui, L. Meunier-Goddik, J. Josephsen, W.-K. Nip, and P. S. Stanfield, ed. CRC Press, New York.

Pang, H., E. Lambertini, R. L. Buchanan, D. W. Schaffner, and A. K. Pradhan. 2017. Quantitative Microbial Risk Assessment for Escherichia coli O157: H7 in Fresh-Cut Lettuce. J. Food Prot. 80:302-311.

Penney, V., G. Henderson, C. Blum, and P. Johnson-Green. 2004. The potential of phytopreservatives and nisin to control microbial spoilage of minimally processed fruit yogurts. Innov. Food Sci. Emerg. Technol. 5:369-375.

Pitt, J. I. and A. D. Hocking. 2009. Fungi and Food Spoilage. 3 ed. Springer, New York, NY.

Pradhan, A. K., R. Ivanek, Y. T. Gröhn, R. Bukowski, I. Geornaras, J. N. Sofos, and M. Wiedmann. 2010. Quantitative risk assessment of listeriosis-associated deaths due to Listeria monocytogenes contamination of deli meats originating from manufacture and retail. J. Food Prot. 73:620-630.

Pradhan, A. K., R. Ivanek, Y. T. Gröhn, I. Geornaras, J. N. Sofos, and M. Wiedmann. 2009. Quantitative risk assessment for Listeria monocytogenes in selected categories of deli meats: impact of lactate and diacetate on listeriosis cases and deaths. J. Food Prot. 72:978-989.

Quelch, J. A. and S. B. Ash. 1980. Consumer satisfaction with food products. Food Policy 5:313-318.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Radha, K. and L. S. Nath. 2014. Studies on the air quality in a dairy processing plant. Ind. J. Vet. & Anim. Sci. Res 43:346-353.

Richins, M. L. and B. J. Verhage. 1985. Seeking redress for consumer dissatisfaction: The role of attitudes and situational factors. J. Consum. Policy 8:29-44.

Rosso, L., J. Lobry, and J. Flandrois. 1993. An unexpected correlation between cardinal temperatures of microbial growth highlighted by a new model. J. Theor. Biol. 162:447-463.

Salustiano, V. C., N. J. Andrade, S. C. C. Brandão, R. M. C. Azeredo, and S. A. K. Lima. 2003. Microbiological air quality of processing areas in a dairy plant as evaluated by the sedimentation technique and a one-stage air sampler. Braz. J. Microbiol. 34:255-259.

Samson, R. A., J. Houbraken, U. Thrane, J. C. Frisvad, and B. Andersen. 2010. Detection and isolation of indoor fungi. Pages 10-19 in Food and Indoor Fungi. CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands.

Sathe, S. J., N. N. Nawani, P. K. Dhakephalkar, and B. P. Kapadnis. 2007. Antifungal lactic acid bacteria with potential to prolong shelf-life of fresh vegetables. J. Appl. Microbiol. 103:2622-2628.

Schaffner, D. W., J. McEntire, S. Duffy, R. Montville, and S. Smith. 2003. Monte Carlo simulation of the shelf life of pasteurized milk as affected by temperature and initial concentration of spoilage organisms. Food Prot. Trends 23:1014-1021.

Schnürer, J. and J. Magnusson. 2005. Antifungal lactic acid bacteria as biopreservatives. Trends Food Sci. Technol. 16:70-78.

Snyder, A. B., J. J. Churey, and R. W. Worobo. 2016. Characterization and control of Mucor circinelloides spoilage in yogurt. Int. J. Food Microbiol. 228:14-21.

Sofos, J. N. 1993. Current microbiological considerations in food preservation. Int. J. Food Microbiol. 19:87-108.

Stiles, M. E. 1996. Biopreservation by lactic acid bacteria. Antonie Van Leeuwenhoek 70:331-345.

Suriyarachchi, V. R. and G. H. Fleet. 1981. Occurrence and growth of yeasts in yogurts. Appl. Environ. Microbiol. 42:574-579.

Tamime, A. Y. and R. K. Robinson. 2007. Background to Manufacturing Practice. Pages 118-120 in Yoghurt: Science and Technology. Elsevier, Boca Raton, FL.

Temelli, S., Ş. Anar, C. Sen, and P. Akyuva. 2006. Determination of microbiological contamination sources during Turkish white cheese production. Food Control 17:856-861.

Vacheyrou, M., A.-C. Normand, P. Guyot, C. Cassagne, R. Piarroux, and Y. Bouton. 2011. Cultivable microbial communities in raw cow milk and potential

transfers from stables of sixteen French farms. Int. J. Food Microbiol. 146:253-262.

Vangay, P., E. B. Fugett, Q. Sun, and M. Wiedmann. 2013. Food microbe tracker: a web-based tool for storage and comparison of food-associated microbes. J. Food Prot. 76:283-294.

Zink, D. L. 1997. The impact of consumer demands and trends on food processing. Emerg. Infect. Dis. 3:467.

Zwietering, M. and S. Van Gerwen. 2000. Sensitivity analysis in quantitative microbial risk assessment. Int J. Food Microbiol. 58:213-221.

CHAPTER 5

PSYCHROTOLERANT SPOREFORMER GROWTH CHARACTERIZATION FOR THE DEVELOPMENT OF A DAIRY SPOILAGE PREDICTIVE MODEL*

*A. J. Buehler, N. H. Martin, K. J. Boor, and M. Wiedmann

Published in Journal of Dairy Science 2018, doi: 10.3168/jds.2018-14501

ABSTRACT

Psychrotolerant sporeforming bacteria represent a major challenge regarding microbial spoilage of fluid milk. These organisms can survive most conventional pasteurization regimes and subsequently germinate and grow to spoilage levels during refrigerated storage. In order to improve predictions of fluid milk shelf-life and assess different approaches to control psychrotolerant sporeforming 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 sporeforming bacteria. We characterized 14 psychrotolerant sporeformers 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 24h 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 HTST-pasteurized fluid milk. Monte Carlo simulations predicted that 66% of half-gallons (1,900 mL) of high temperature, short time (**HTST**) 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}MPN/mL (e.g., microfiltration) can extend fluid milk shelf-life by four days (end of shelf-life was defined here as the first day when the mean total bacterial count exceeds 20,000 CFU/mL). This study not only provides a baseline understanding of the growth rates of psychrotolerant sporeformers 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). While microbial spoilage can occur due to post-processing 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 sporeformers) 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 can 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 this 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 (**PMO**) 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 PMO 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 sporeformers 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, while *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 Bacillales species linked to fluid milk spoilage (e.g., *B. weihenstephanensis, P. odorifer, P. peoriae,* and *V. 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 sporeformers 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 sub-lethal heat treatments, such as those used in HTST

pasteurization (Setlow, 2014, Moir and Cooper, 2016). Upon germination, sporeformers 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 sporeformers when stored at 6°C (Ranieri and Boor, 2009). While some studies have characterized psychrotolerant sporeformers for their ability to grow at refrigeration temperatures, there is a general lack of information on specific growth rates and parameters for psychrotolerant sporeformers (Ivy et al., 2012). Understanding specific growth parameters of psychrotolerant sporeformers is a first step to facilitate development and implementation of better control strategies to reduce psychrotolerant sporeformer growth in fluid milk.

Many factors, including initial spore concentration in raw milk, sporeformer frequency in raw milk and their corresponding growth rates can influence the ultimate shelf-life of fluid milk contaminated with psychrotolerant sporeformers. 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 this study were to (1) understand the germination and growth characteristics of psychrotolerant sporeforming *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 sporeforming 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 5.1) (Trmčić et al., 2015). In addition to these 10 isolates, we also included: (i) one isolate representing *B. wiedmannii* (a newly described species that has been reported to grow at low temperatures) and (ii) three isolates representing *Psychrobacillus* [since 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, 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; AT 513 was however 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 SNPs. 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 Microbe Tracker Database at www.foodmicrobetracker.com (Vangay et al., 2013).

Table 5.1. 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 sporeformers.

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 1,000X total magnification and spores were identified as unstained structures within the cell. For isolates for which there were no spores confirmed, AK Agar #2 plates were placed in the incubator for an additional two weeks. 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 phosphate buffer solution (**PBS**, Weber Scientific, Hamilton, NJ), and subsequently washed three times by centrifugation at 11,710 x *g* for 15 min. Following washing, 5mL distilled water and 5mL 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 pre-cooled 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 germination and lag phase and then every two days during exponential and stationary phase until three time points were taken during 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). BHI plates were incubated at each isolate's optimum growth temperature for 48 h. Following incubation, colonies were counted with the QCount Automated Colony Counter (Advanced Instruments Inc.).

Growth model

Cell density measurements were fitted to a three-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, four growth parameters including lag phase (d), maximum growth rate ($\boldsymbol{\mu}_{max}$, Log_{10}CFU/mL/d), initial cell density (N_0, Log_{10}CFU/mL), and maximum cell density (N_{max}, Log_{10}CFU/mL) were calculated for each isolate (Table 5.2).

To characterize the growth parameters as a function of temperature, the square root model for maximum growth rate ($\boldsymbol{\mu}_{\mathbf{max}}$,) was used (Ratkowsky et al., 1983). According to this model, $\boldsymbol{\mu}_{\mathbf{max}}$ and lag time (*Lt*) are expressed as

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

$$\sqrt{\frac{1}{Lt}} = a(T - T_0) \qquad (2)$$

where $\boldsymbol{\mu}_{\mathbf{max}}$ is the exponential growth rate (Log_{10}CFU/mL/d), *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 sporeformers in fluid milk, and *Lt* is the lag time (d). For this model, the value for T_0 was estimated as -3.62°C based on growth curves of *Paenibacillus odorifer* (*rpoB* allelic type (**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, $\boldsymbol{\mu}_{\mathbf{max}}$ 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 $\boldsymbol{\mu}_{\mathbf{max}}$ 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:

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

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

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 5.2) were used in the simulation model to predict growth of psychrotolerant sporeformers in fluid milk that was stored at 4°C.

Table 5.2. Growth parameters of psychrotolerant sporeformers in skim milk broth

Genus	species	rpoB AT	Lag (d) at		μ_{max} (Log_{10}CFU/ mL/d)[1] at		N_{max} (Log_{10}CFU / mL)[1]
			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.	23	46.2	29.0	NA[3]	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_0, initial cell density; 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]Not applicable. These isolates failed to germinate and grow in skim milk broth.

Predictive Model Development

Model Assumptions. In this model, bulk tank raw milk from the farm was assumed to be the only source of psychrotolerant sporeformers in the system. Previous studies have demonstrated that psychrotolerant sporeformers are ubiquitous in the dairy farm environment and enter the milk supply through bulk tank raw milk (Christiansson et al., 1999, Te Giffel et al., 2002, Masiello et al., 2014). When studying the growth of various psychrotolerant sporeformers in milk, we assumed each simulated half-gallon of milk (1,900 mL) was only contaminated with one subtype. This is a simplifying assumption common in predictive microbiology (Malakar et al., 2003). Concentrations of psychrotolerant sporeformers over shelf-life were determined assuming a constant storage temperature of 6°C. This assumption was made in order 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 5.3) including: (i) initial farm bulk tank raw milk psychrotolerant sporeformer concentration (**N_0**), (ii) farm bulk tank raw milk psychrotolerant sporeformer *rpoB* AT frequency (**F**), (iii) maximum growth rate by subtype (**μ_{max}**), (iv) lag phase by subtype (**t_{lag}**), and (v) maximum microbial population by subtype (**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 sporeformers obtained from 99 farms across New York State was retrieved from Masiello et al. (2014) to estimate F

(Table 5.4). Growth characteristics (μ_{max}, t_{lag}, and N_{max}) were determined as described in the previous section (Table 5.2). To assign growth characteristics to psychrotolerant sporeformer *rpoB* ATs isolated at the bulk tank farm level, but for which no growth parameters were available, a *rpoB* region maximum-likelihood (ML) phylogenetic tree was constructed with sequences for the isolates characterized in the study reported here as well as sequences representing all *rpoB* ATs found among the psychrotolerant sporeformer 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 5.1). 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).

Table 5.3. Variables used in Monte Carlo simulation of the shelf-life of pasteurized milk

Description	Units	Variable name	Description and details[1]
Initial microbial population	Log_{10}MPN/mL	N_0	Modeled as lognormal (-0.72, 0.99) distribution
Psychrotolerant sporeformer *rpoB* AT frequency	Percent	F	Frequency table based on data reported by Masiello et al. (2014)
Maximum growth rate	Log_{10}CFU/mL/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 $t \geq t_{max}$: $N_t = N_{max}$

[1] μ_{max}, maximum growth rate (Log_{10}CFU/mL/d); N_0, initial cell density (Log_{10}MPN/mL); N_{max}, maximum cell density (Log_{10}CFU/mL); N_t, cell density at time t (Log_{10}CFU/mL); t_{lag}, time (d) when lag phase ends; t_{max}, time (d) when maximum cell density is reached.

Table 5.4. Numbers and prevalence of psychrotolerant sporeformer *rpoB* allelic types (AT) obtained from spore-pasteurized bulk tank milk samples collected from 99 New York State farms over one year[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 ATs that resemble the given named species, but where identification represents considerable uncertainty.

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 sporeformers in a half-gallon of milk (1,900 mL) stored at 6°C from 14 d to 24 d of shelf-life based on initial sporeformer concentration in the farm bulk tank raw milk, frequency of psychrotolerant sporeformers in farm bulk tank raw milk, and growth characteristics of psychrotolerant sporeformers as inputs. Each iteration resulted in the prediction of a value for the initial concentration of psychrotolerant sporeformers in a half-gallon of milk stored at 6°C. This value was then traced over the milk's shelf-life, from 14 d 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 sporeformer concentrations (Zwietering and Van Gerwen, 2000); best-case scenarios were generally defined as changes that would reduce finished product spoilage (e.g., reduced initial spore levels) while worst-case scenarios were defined as those that increased fluid milk spoilage. The effects of 5 major aspects of psychrotolerant sporeformer contamination and growth parameters contributing to spoilage of fluid milk were evaluated using best- and worst-case scenario analysis. The five aspects of psychrotolerant sporeformer growth and contamination considered were: (i) the initial farm-level bulk tank milk contamination concentration, N_0, Buchanan psychrotolerant sporeformer growth parameters including (ii) t_{lag} and (iii) $\boldsymbol{\mu}_{max}$, and the frequency of the two most prevalent psychrotolerant sporeformer subtypes, (iv) *rpoB* AT 15 and (v) *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 $\boldsymbol{\mu}_{max}$, the worst-case scenario was calculated by increasing $\boldsymbol{\mu}_{max}$ 20% (low) and 40% (high); the best-case scenario was calculated by decreasing $\boldsymbol{\mu}_{max}$ 20% (low) and 40% (high). The frequency of *rpoB* ATs 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 two ATs represented subtypes with relatively low $\boldsymbol{\mu}_{max}$ 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 sporeformer concentrations for each aspect independently and the baseline model, where all five aspects were set to their original values. Ultimately, these scenarios helped to identify aspects of psychrotolerant sporeformer 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 sporeformers. The control strategies considered were: (i) lower refrigeration temperature during shelf-life storage and (ii) 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 5.2) (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 as Doll et al. (2017) described a 2.2 log-reduction on average of psychrotolerant sporeformers after microfiltration of milk in Germany.

Model Validation. Simulated model counts at 14 d were compared to actual counts on 14 d obtained experimentally from commercial market milk that was sampled across New York State from October 2016-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 standard plate count (**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 (**CVTA**, Becton, Dickinson and Co.), were classified as showing evidence of Gram-negative post pasteurization contamination and were excluded from the dataset 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 to 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 11/14 isolates. Three *Psychrobacillus* isolates (*rpoB* ATs 147, 321, and 564) failed to sporulate after three weeks 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 5.2. Briefly, for these isolates lag phases at 6°C ranged from 1.9 d to 18.2 d, maximum growth rate at 6°C ranged

from 0.6 to 1.5 Log_{10}CFU/mL/d, and maximum cell density ranged from 5.8 to 7.6 Log_{10}CFU/mL.

Initial Psychrotolerant Sporeformer Populations in Bulk Tank Milk

MPN data for psychrotolerant sporeformer 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 sporeformer populations at the bulk tank level and to fit a log-normal distribution, which was used as an input for our model. Since 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/mL 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 5.1). This corresponds roughly to 1 spore per 5 mL of bulk tank milk. Our observation of low levels of psychrotolerant sporeformers in bulk tank milk is in agreement with previous studies (Mayr et al., 1999, McGuiggan et al., 2002, Doll et al., 2017).

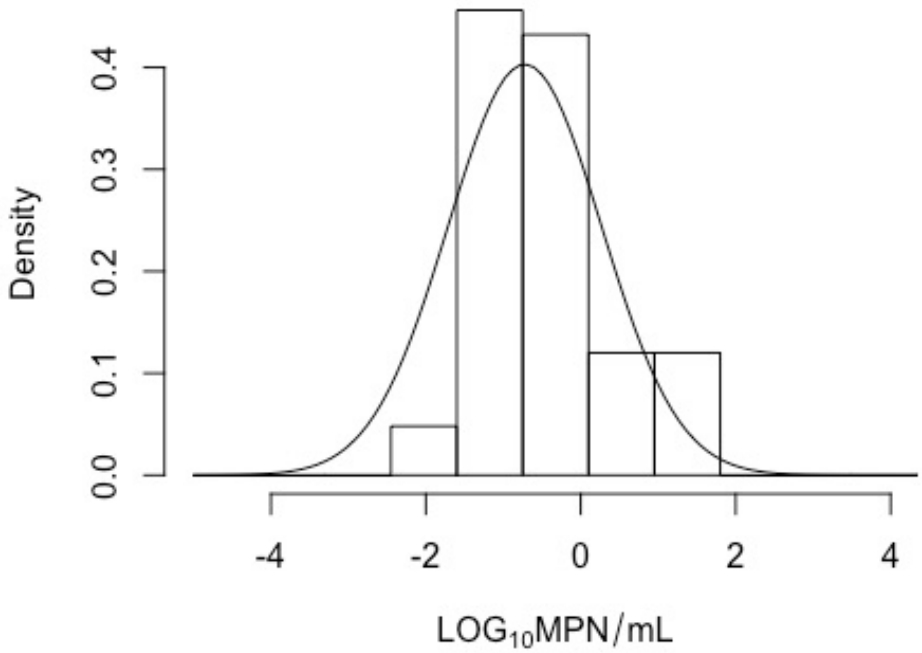


Figure 5.1. Initial bulk tank raw milk psychrotolerant sporeformer populations based on data reported by Masiello et al. (2014) and the simulated log normal distribution of initial bulk tank raw milk psychrotolerant sporeformer populations with a mean of -0.72 Log_{10}MPN/mL and a standard deviation of 0.99 Log_{10}MPN/mL.

Distribution of Simulated Concentrations of Psychrotolerant Sporeformers 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) by 20 d of storage, and 83% of simulated half-gallons of fluid milk reached > 20,000 CFU/mL by 24 d (Figure 5.2). The mean concentration of psychrotolerant sporeformers 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 d 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 (Figure 5.2).

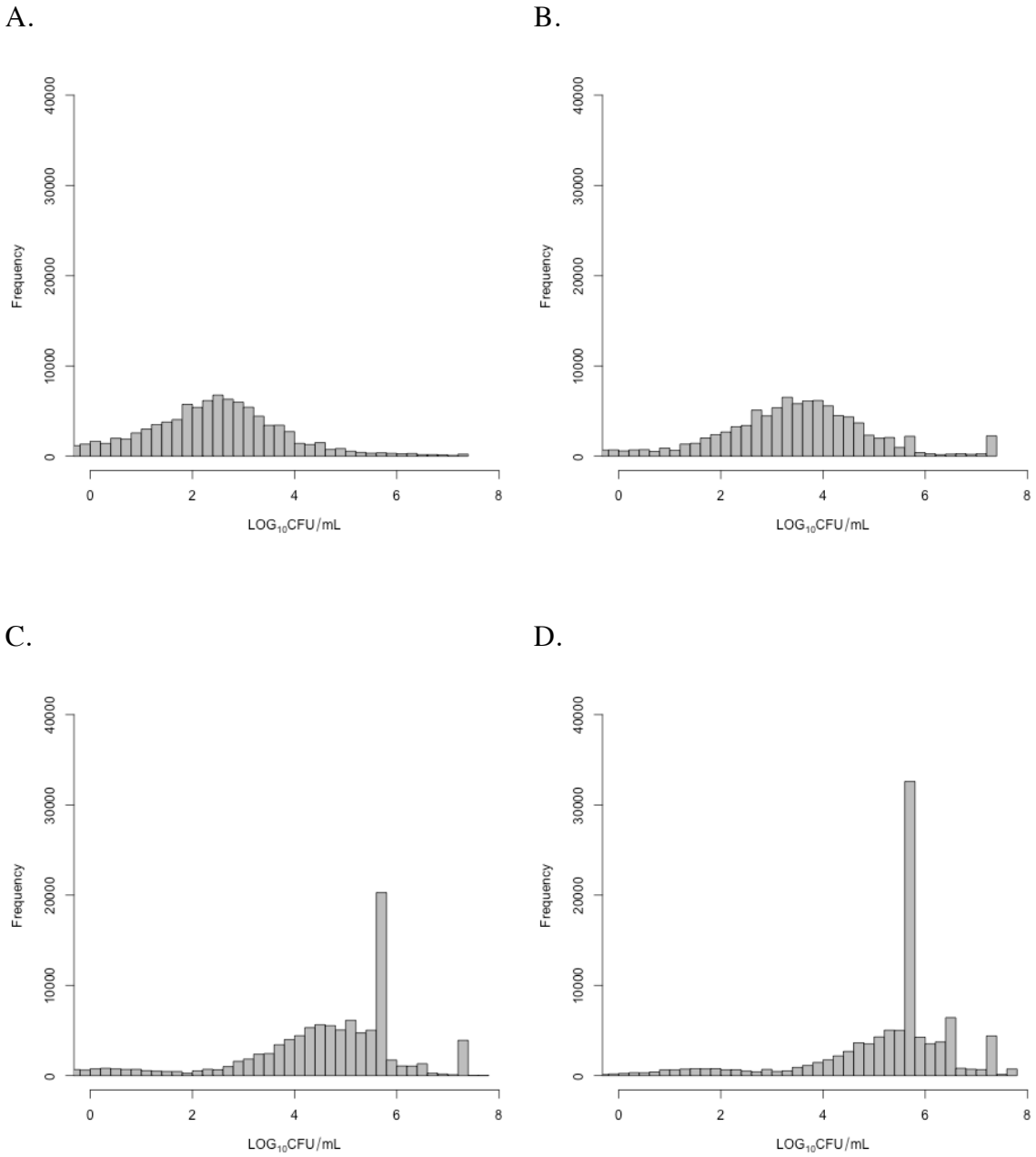


Figure 5.2. Histograms of the simulated concentration of psychrotolerant sporeformers in fluid milk per half-gallon over shelf-life when stored at 6°C at (A) 14 d; (B) 17 d; (C) 21 d, and (D) 24 d. Monte Carlo simulations comprised 100,000 iterations, and were based on five model parameters: (i) initial farm bulk tank raw milk psychrotolerant sporeformer concentration, (ii) farm bulk tank raw milk psychrotolerant sporeformer *rpoB* allelic type (AT) frequency, (iii) maximum growth rate by subtype, (iv) lag phase by subtype, and (v) maximum microbial population by subtype. For each the 21 d and 24 d histogram, the prominent bar at 5.8 Log_{10}CFU/mL (around 24,000 and 39,000 out of 100,000 iterations, respectively, for 21 d and 24 d) can be explained by the fact that

the second most frequent *rpoB* AT (AT 3) has an 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 $\boldsymbol{\mu}_{max}$ 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).

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 impact on the model output (Figure 5.3). 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 day 21 of simulated storage as compared to a base concentration (4.54 ± 1.71 Log_{10}CFU/mL). The lag phase parameter had a moderate impact 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* ATs 3 and 15 had very little impact on the model output. Increasing *rpoB* AT 15 to 50% (from an estimate of 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.

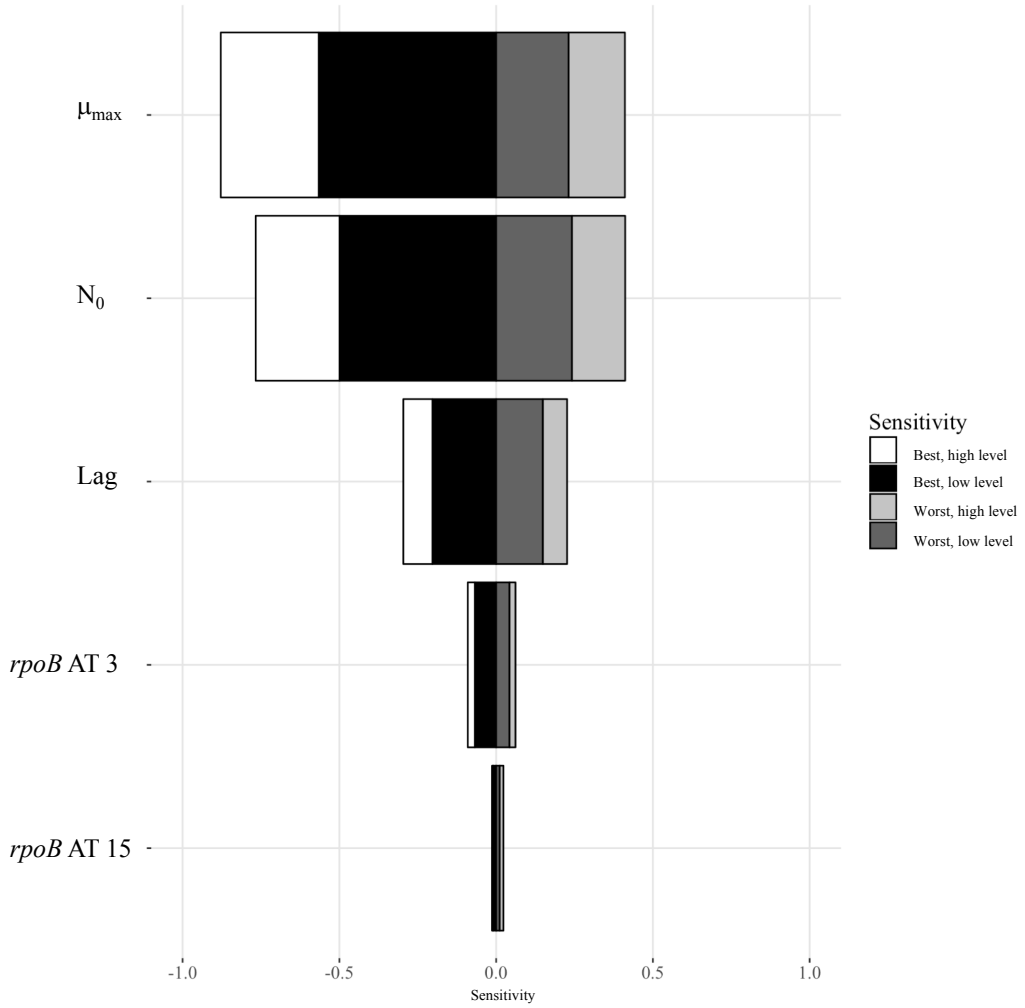


Figure 5.3. Sensitivity analyses assessing the effects of best-case (white and black) and worst-case (gray) scenarios on the percent of half-gallons of fluid milk that exceed 4.3 Log_{10}CFU/mL on day 21 of refrigerated storage at 6°C for different aspects of psychrotolerant sporeformer growth: μ_{max} represents the maximum growth rate, N_0 represents the farm bulk tank raw milk psychrotolerant sporeformer concentration, lag represents the time (d) where the growth rate equals 0, and *rpoB* ATs 3 and 15 represent the respective frequencies of these allelic types (ATs). 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* ATs 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* ATs 15 and 3 independently to 40% (low) and 50% (high).

Model Validation

To assess the simplifying assumptions made in the development of this model, it is important to evaluate 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 sporeformers to the distribution of the simulated counts (Figure 5.4). 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 versus 1.10 Log_{10}CFU/mL for simulated).

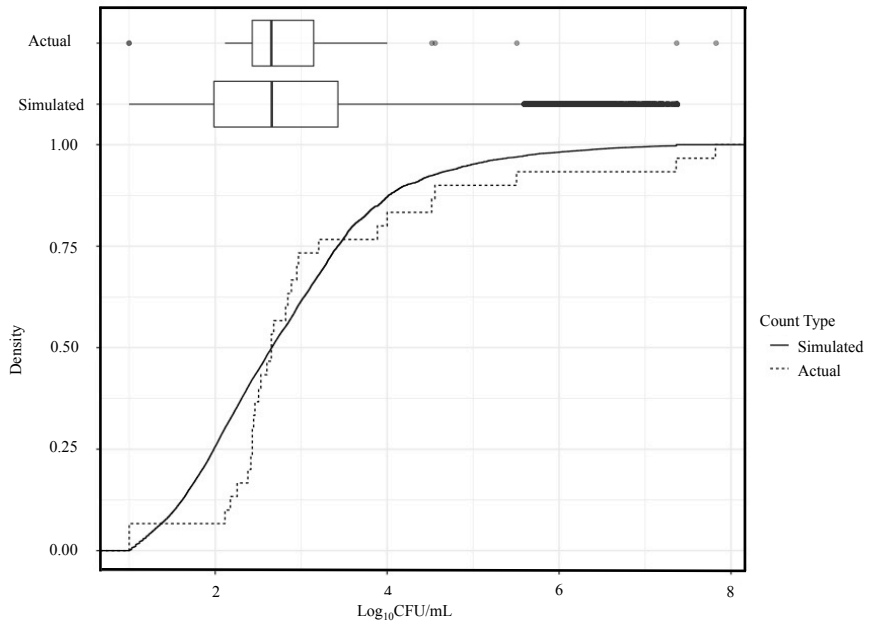


Figure 5.4. Empirical cumulative probability distributions and corresponding boxplots of the simulated concentration of psychrotolerant sporeformers (Log_{10}CFU/mL) in fluid milk per half-gallon at 14 d of storage at 6°C (solid line) and actual concentrations of presumptive psychrotolerant sporeformers 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 the 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 x the interquartile range. The lower whisker extends similarly, to the smallest value no further than 1.5 x the interquartile range. Data beyond the end of the whiskers is plotted individually as dots. The median is the line in the box.

What-If Analyses

The predicted effects of refrigeration temperature and microfiltration on fluid milk spoilage by psychrotolerant sporeformers are shown in Table 5.5. Lowering the refrigeration temperature by two degrees Celsius during storage had a dramatic effect on the percent of samples spoiled [9% versus 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 sporeformers on day 21 of simulated storage at 4°C was 2.37 ± 1.52 Log_{10}CFU/mL, compared to an original mean concentration of 4.54 ± 1.71 Log_{10}CFU/mL for storage at 6°C for 21 d (Figure 5.5). Moreover, the shelf-life of simulated fluid milk half-gallons, defined by the time (d) 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 sporeformer population by 2.2 Log_{10}MPN/mL) was estimated to lower the mean concentration of psychrotolerant sporeformers 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 5.6). This extended the shelf-life of the simulated fluid milk half-gallons (defined as the time (d) 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.

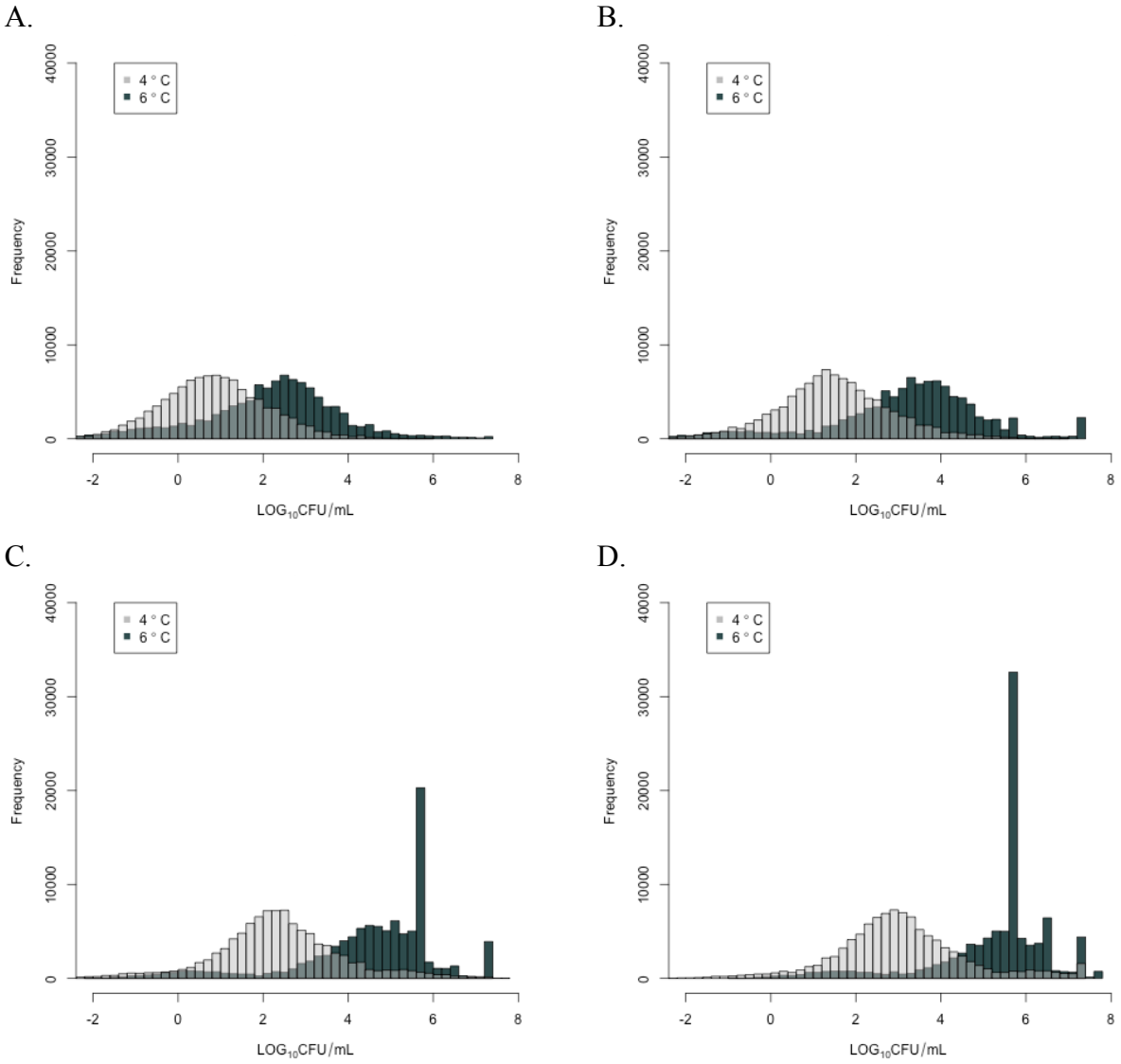


Figure 5.5. Histograms of the simulated concentration of psychrotolerant sporeformers (Log_{10}CFU/mL) in fluid milk per half-gallon assuming storage of milk at 4°C (light gray) compared to storage of milk at 6°C (dark gray) over shelf-life at (A) 14 d; (B) 17 d; (C) 21 d, and (D) 24 d.

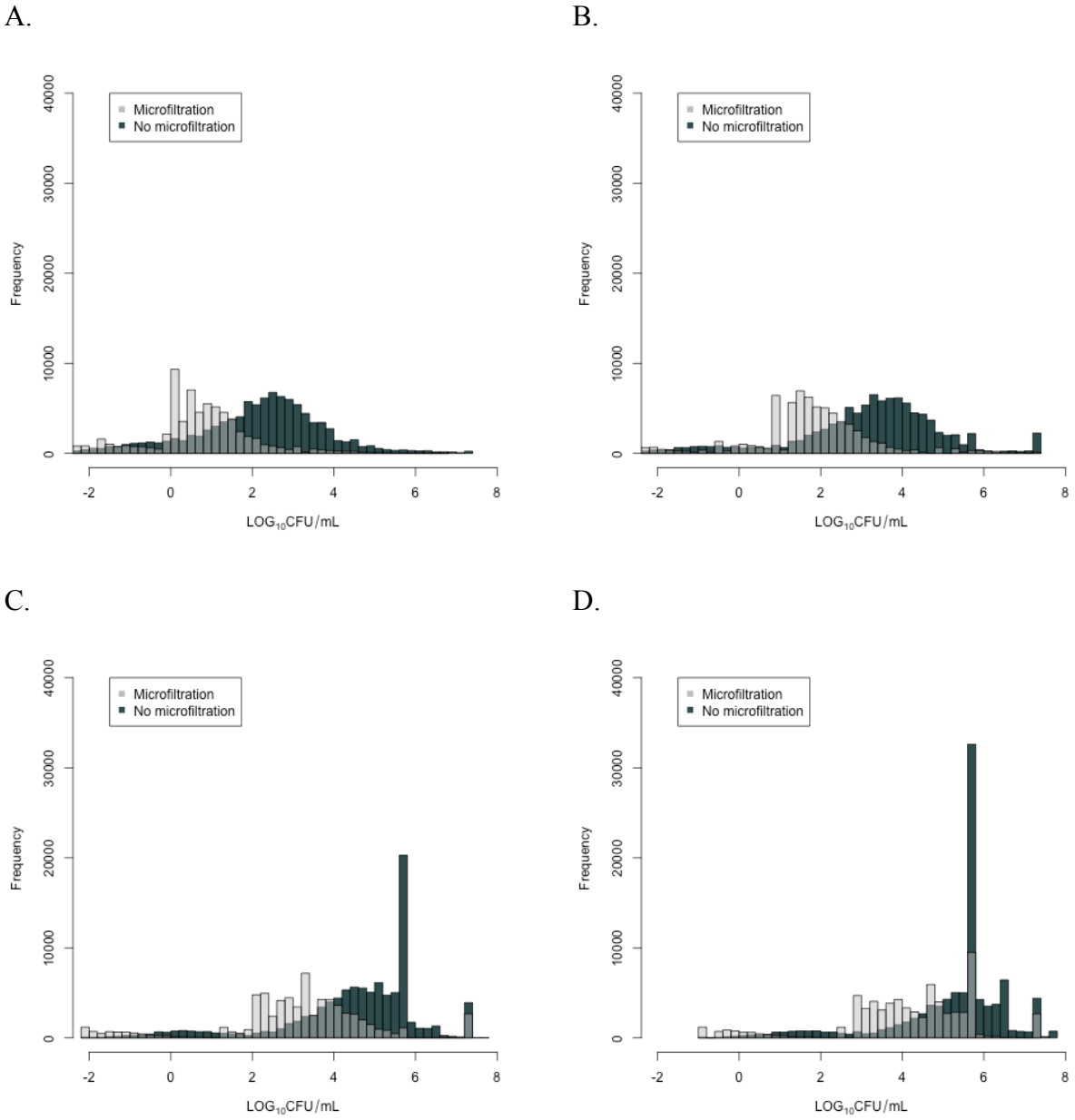


Figure 5.6. Histogram of the simulated concentration of psychrotolerant sporeformers (Log_{10}CFU/mL) in fluid milk per half-gallon (light grey) assuming microfiltration of raw milk (yielding a 2.2 Log_{10}MPN/mL reduction of spore numbers) (light gray) compared to no treatment of raw milk (dark gray) over shelf-life at (A) 14 d; (B) 17 d; (C) 21 d, and (D) 24 d.

Table 5.5. Summary of what-if scenario analysis outcomes

What-if condition	Storage temperature	Concentration at 21 d (Log_{10}CFU/mL)		Fraction of half-gallon containers that exceed 4.3 Log_{10}CFU/mL at day 21
		Mean	Standard deviation	
Initial condition	6°C	4.54	1.71	66%
Lower refrigeration temperature	4°C	2.37	1.52	9%
Lower initial raw milk contamination levels by 2.2 Log_{10}MPN/mL via microfiltration	6°C	3.03	1.83	13%

DISCUSSION

This study characterized psychrotolerant sporeformer growth patterns and subsequently developed a predictive model to estimate the concentration of psychrotolerant sporeformers 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 impact the concentration of psychrotolerant sporeformers in fluid milk over its shelf-life. Importantly, this 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.

Dairy relevant psychrotolerant sporeformers differ in their ability to sporulate and germinate.

Among the 14 sporeformer isolates tested, all three isolates that belong to the genus *Psychrobacillus* (*rpoB* ATs 564, 147, 321) failed to sporulate under the laboratory conditions used (i.e., growth on AK#2 agar over 3 weeks). This is consistent with a number of previous reports that achieving successful sporulation of wildtype sporeformer isolates can be challenging (Duncan and Strong, 1968, Cazemier et al., 2001, 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). While *Psychrobacillus* isolates were included in our isolate set to capture the diversity of psychrotolerant sporeformer 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 9/33 isolates from soil, manure, and bedding pack samples (Huck et al., 2008). The three 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 2/209 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 thus 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 sporeformers only sporulated on specific media, and Garcia et al. (2010) indicated that sporulation rates may be affected by temperature.

Among the 11 sporeformers for which spore preparations were successfully obtained, two *Paenibacillus* isolates [representing *P. amylolyticus* s.l., and *P. glucanolyticus* (*rpoB* ATs 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). While we did not observe germination and growth for these isolates, isolates with these *rpoB* ATs are rarely found in raw milk; *rpoB* AT 23 was only isolated once

during a cross-sectional study of the frequency of psychrotolerant sporeformers 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 5.4). This suggests that these *rpoB* ATs may exist as vegetative cells in the farm environment rather than as spores; thus 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 a number of factors, including: (i) sporulation conditions that yielded spores with reduced ability to germinate; (ii) heat activation step conditions that did not facilitate germination; and (iii) environmental conditions after heat activation that did not facilitate germination. Sporulation conditions have previously been shown to affect the ability of sporeformers to germinate (Raso et al., 1998a, Raso et al., 1998b, Black et al., 2005, 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 to *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 sporeformers germinate or not (Vary and Halvorson, 1965, Levinson and Hyatt, 1970, Ghosh et al., 2009). Ghosh et al. (2009) reported that there was an optimum heat activation temperature, dependent upon the species of sporeformer. While our isolates were heat activated at 80°C for 12 min, this could not have been optimized for these two *rpoB* ATs. 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 sporeformer germination. For example, a study in The Netherlands characterized *Bacillus weinhenstephanesis* 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 the greatest influence on predicted concentrations of psychrotolerant sporeformers in fluid milk

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 sporeformers in milk. This suggests that further research to characterize growth rates in fluid milk for a diversity of psychrotolerant sporeformers may have the largest impact on improving our predictive model. While no other sensitivity analyses for psychrotolerant sporeformers 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 sporeformers, we are able to account for our observation that different psychrotolerant sporeformers have different abilities to germinate and grow in fluid milk.

After the maximum growth rate parameter, the initial concentration of psychrotolerant sporeformers 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), relating to raw milk as an important source of sporeforming 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 impact on model predictions. In our study reported here, 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 FDA-FSIS *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. While our model did not separate the distribution chain by stage and considered lag phase estimates at a constant temperature of 6°C (in order to compare model outcomes to actual estimates from VSL), we found that lag phase estimates were not as influential on model outcomes compared to maximum growth rate estimates or the initial concentration of psychrotolerant sporeformers in bulk tank raw milk. However, including lag phase in our model will simplify future modifications to our model that would allow for separation of the production to retail and retail to consumption phase of distribution.

Importantly, our sensitivity analysis directs future work towards 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 sporeformers in simulated half-gallons. Specifically, our what-if simulations of lowering the refrigeration temperature from 6°C 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 to 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°C 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°C to 2°C extended the shelf-life of pasteurized fluid milk contaminated with psychrotolerant, Gram-negative non-sporeforming 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°C 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% spoiled 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. While 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 sporeformers. 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. While 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. While our model only considered contamination due to psychrotolerant sporeformers, our estimate is that 65% of microfiltered milk samples have psychrotolerant sporeformer 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 sporeformer 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 sporeformer count of 2.2 Log_{10}MPN, with a range of 0.6 Log_{10}MPN to 3.1 Log_{10}MPN reduction of psychrotolerant sporeformers. Our what-if scenario only considered contamination due to psychrotolerant sporeformers, and used a 2.2 log reduction of psychrotolerant sporeformers due to microfiltration. We estimate that the shelf-life (time (d) to reach a mean total bacterial count > 20,000 CFU/mL) of microfiltered milk contaminated with psychrotolerant sporeformers 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.

CONCLUSION

Control of psychrotolerant sporeformers 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 sporeformers, as well as processing decisions and

supply chain interventions the dairy industry can employ to reduce spoilage by psychrotolerant sporeformers. Overall, our results lay a foundation for developing new tools to better predict and ultimately prevent dairy spoilage due to psychrotolerant sporeformers.

ACKNOWLEDGEMENTS

This publication was supported by the New York State Milk Promotion Advisory Board through the New York State Department of Agriculture and Markets (Brooklyn, NY). The authors thank members of Cornell University Milk Quality Improvement Program for laboratory support.

REFERENCES

Bartoszewicz, M., B. M. Hansen, and I. Swiecicka. 2008. The members of the Bacillus cereus group are commonly present contaminants of fresh and heat-treated milk. Food Microbiol. 25:588-596.

Baty, F. and M. Delignette-Muller. 2017. nlsMicrobio: data sets and nonlinear regression models dedicated to predictive microbiology. Accessed Mar. 10, 2017. https://cran.r-project.org/web/packages/nlsMicrobio/nlsMicrobio.pdf.

Black, E. P., K. Koziol-Dube, D. Guan, J. Wei, B. Setlow, D. E. Cortezzo, D. G. Hoover, and P. Setlow. 2005. Factors influencing germination of Bacillus subtilis spores via activation of nutrient receptors by high pressure. Appl. Environ. Microbiol. 71:5879-5887.

Buchanan, R., R. Whiting, and W. Damert. 1997. When is simple good enough: a comparison of the Gompertz, Baranyi, and three-phase linear models for fitting bacterial growth curves. Food Microbiol. 14:313-326.

Buzby, J. C., H. Farah-Wells, and J. Hyman. 2014. The estimated amount, value, and calories of postharvest food losses at the retail and consumer levels in the United States. USDA-ERS Economic Information Bulletin.

Carey, N., S. Murphy, R. Zadoks, and K. Boor. 2005. Shelf lives of pasteurized fluid milk products in New York State: A ten-year study. Food Protect. Trends 25:102-113.

Cazemier, A., S. Wagenaars, and P. Ter Steeg. 2001. Effect of sporulation and recovery medium on the heat resistance and amount of injury of spores from spoilage bacilli. J. Appl. Microbiol. 90:761-770.

Chandler, R. and T. McMeekin. 1985. Temperature function integration and the prediction of the shelf-life of milk. Aust. J. Dairy Technol. 40:10.

Checinska, A., A. Paszczynski, and M. Burbank. 2015. Bacillus and other spore-forming genera: variations in responses and mechanisms for survival. Annu Rev Food Sci Technol. 6:351-369.

Christiansson, A., J. Bertilsson, and B. Svensson. 1999. Bacillus cereus spores in raw milk: factors affecting the contamination of milk during the grazing period. J. Dairy Sci.:305-314.

Delignette-Muller, M. L. and C. Dutang. 2015. fitdistrplus: An R package for fitting distributions. J. Stat Softw. 64:1-34.

Dogan, B. and K. J. Boor. 2003. Genetic diversity and spoilage potentials among Pseudomonas spp. isolated from fluid milk products and dairy processing plants. Appl. Environ. Microbiol. 69:130-138.

Doll, E. V., S. Scherer, and M. Wenning. 2017. Spoilage of microfiltered and pasteurized extended shelf life milk Is mainly induced by psychrotolerant spore-forming bacteria that often originate from recontamination. Front Microbiol. 8.

Duncan, C. L. and D. H. Strong. 1968. Improved medium for sporulation of Clostridium perfringens. Appl. Microbiol. 16:82-89.

Durak, M. Z., H. I. Fromm, J. R. Huck, R. N. Zadoks, and K. J. Boor. 2006. Development of molecular typing methods for Bacillus spp. and Paenibacillus spp. isolated from fluid milk products. J. Food. Sci. 71:M50-M56.

EcoSure. 2007. EcoSure 2007 cold temperature database. Accessed Jan. 5, 2018. http://foodrisk.org/exclusives/EcoSure.

Elwell, M. W. and D. M. Barbano. 2006. Use of Microfiltration to Improve Fluid Milk Quality. J. Dairy Sci. 89:E20-E30.

FDA. 2015. Standards for grade "A" milk and/or milk products. Pages 32-35 in Grade "A" Pasteurized Milk Ordinance. US Department of Health and Human Services,

Public Health Service, Washington, DC.

Frank, J. and A. Yousef. 2004. Tests for groups of microorganisms. Pages 227-248 in Standard Methods for the Examination of Dairy Products. Vol. 17. H.M. Wehr and J. F. Frank, ed. Am. Public Health Assoc., Washington, DC.

Fromm, H. I. and K. Boor. 2004. Characterization of pasteurized fluid milk shelf-life attributes. J. Food. Sci. 69:M207-M214.

Gaillard, S., I. Leguérinel, and P. Mafart. 1998. Model for combined effects of temperature, pH and water activity on thermal inactivation of Bacillus cereus spores. J. Food. Sci. 63:887-889.

Garcia, D., M. v. der Voort, and T. Abee. 2010. Comparative analysis of Bacillus weihenstephanensis KBAB4 spores obtained at different temperatures. Int. J. Food Microbiol. 140:146-153.

Ghosh, S., P. Zhang, Y.-q. Li, and P. Setlow. 2009. Superdormant spores of Bacillus species have elevated wet-heat resistance and temperature requirements for heat activation. J. Bacteriol. 191:5584-5591.

Griffiths, M., J. Phillips, and D. Muir. 1987. Effect of low-temperature storage on the bacteriological quality of raw milk. Food Microbiol. 4:285-291.

Hoxey, E., C. Soper, and D. Davies. 1985. Biological indicators for low temperature steam formaldehyde sterilization: effect of defined media on sporulation, germination index and moist heat resistance at 110dEC of Bacillus strains. J. Appl. Microbiol. 58:207-214.

Huck, J., B. Hammond, S. Murphy, N. Woodcock, and K. Boor. 2007. Tracking spore-forming bacterial contaminants in fluid milk-processing systems. J. Dairy Sci. 90:4872-4883.

Huck, J., M. Sonnen, and K. Boor. 2008. Tracking heat-resistant, cold-thriving fluid milk spoilage bacteria from farm to packaged product. J. Dairy Sci. 91:1218-1228.

Institute of Medicine and National Research Council. 2015. Overview of the U.S. Food System. in A Framework for Assessing Effects of the Food System. MC Nesheim, M Oria, and P. Yih, ed. The National Academies Press, Washington, D.C.

Ivy, R. A., M. L. Ranieri, N. H. Martin, H. C. den Bakker, B. M. Xavier, M. Wiedmann, and K. J. Boor. 2012. Identification and characterization of psychrotolerant sporeformers associated with fluid milk production and processing. Appl. Environ. Microbiol. 78:1853-1864.

Kantor, L. S., K. Lipton, A. Manchester, and V. Oliveira. 1997. Estimating and addressing America's food losses. Food Review 20:2-12.

Kent, D., K. Chauhan, K. Boor, M. Wiedmann, and N. Martin. 2016. Spore test parameters matter: Mesophilic and thermophilic spore counts detected in raw milk and dairy powders differ significantly by test method. J. Dairy Sci. 99:5180-5191.

Koutsoumanis, K. P. and M. Gougouli. 2015. Use of time temperature integrators in food safety management. Trends Food Sci. Technol. 43:236-244.

Latorre, A. A., A. K. Pradhan, J. A. S. Van Kessel, J. S. Karns, K. J. Boor, D. H. Rice, K. J. Mangione, Y. T. Gröhn, and Y. H. Schukken. 2011. Quantitative risk assessment of listeriosis due to consumption of raw milk. J. Food Prot. 74:1268-1281.

Levinson, H. S. and M. T. Hyatt. 1970. Effects of Temperature on Activation, Germination, and Outgrowth of Bacillus megaterium Spores. J. Bacteriol. 101:58-64.

Logan, N. A. and P. De Vos. 2009. Genus I. Bacillus Cohn 1872. Pages 113-114 in Bergey's Manual of Systematic Bacteriology: Volume 3: The Firmicutes. Vol. 3. P. Vos, G. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K.-H. Schleifer, and W. Whitman, ed. Springer Science & Business Media, New York.

Magnusson, M., A. Christiansson, and B. Svensson. 2007. Bacillus cereus spores during housing of dairy cows: factors affecting contamination of raw milk. J. Dairy Sci. 90:2745-2754.

Malakar, P., G. Barker, M. Zwietering, and K. Van't Riet. 2003. Relevance of microbial interactions to predictive microbiology. Int. J. Food Microbiol. 84:263-272.

Martin, N., N. Carey, S. Murphy, M. Wiedmann, and K. Boor. 2012. A decade of improvement: New York State fluid milk quality. J. Dairy Sci. 95:7384-7390.

Masiello, S., N. Martin, R. Watters, D. Galton, Y. Schukken, M. Wiedmann, and K. Boor. 2014. Identification of dairy farm management practices associated with the presence of psychrotolerant sporeformers in bulk tank milk. J. Dairy Sci. 97:4083-4096.

Mayr, R., I. Eppert, and S. Scherer. 1999. Incidence and identification of psychrotrophic (7 degrees C-tolerant) Bacillus spp in German HTST pasteurized milk. Milchwissenschaft 54:26-30.

McGuiggan, J., D. R. McCleery, A. Hannan, and A. Gilmour. 2002. Aerobic spore-forming bacteria in bulk raw milk: factors influencing the numbers of psychrotrophic, mesophilic and thermophilic Bacillus spores. Int. J. Dairy Technol. 55:100-107.

McMeekin, T., J. Bowman, O. McQuestin, L. Mellefont, T. Ross, and M. Tamplin. 2008. The future of predictive microbiology: strategic research, innovative applications and great expectations. Int. J. Food Microbiol. 128:2-9.

Minh, H. N. T., A. Durand, P. Loison, J.-M. Perrier-Cornet, and P. Gervais. 2011. Effect of sporulation conditions on the resistance of Bacillus subtilis spores to heat and high pressure. Appl. Microbiol. Biotechnol. 90:1409-1417.

Moir, A. and G. Cooper. 2016. Spore Germination. Pages 217-236 in The Bacterial Spore: from Molecules to Systems. Vol. 3. Driks A. and P. Eichenberger, ed. ASM Press, Washington, DC.

Nicolaï, B. and J. Van Impe. 1996. Predictive food microbiology: a probabilistic approach. Math Comput. Simul. 42:287-292.

Popescu, A.-A., K. T. Huber, and E. Paradis. 2012. ape 3.0: New tools for distance-based phylogenetics and evolutionary analysis in R. Bioinformatics 28:1536-1537.

Pradhan, A. K., R. Ivanek, Y. T. Gröhn, R. Bukowski, I. Geornaras, J. N. Sofos, and M. Wiedmann. 2010. Quantitative risk assessment of listeriosis-associated deaths due to Listeria monocytogenes contamination of deli meats originating from manufacture and retail. J. Food Prot. 73:620-630.

Pradhan, A. K., R. Ivanek, Y. T. Gröhn, I. Geornaras, J. N. Sofos, and M. Wiedmann. 2009. Quantitative risk assessment for Listeria monocytogenes in selected categories of deli meats: impact of lactate and diacetate on listeriosis cases and deaths. J. Food Prot. 72:978-989.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ranieri, M., J. Huck, M. Sonnen, D. Barbano, and K. Boor. 2009. High temperature, short time pasteurization temperatures inversely affect bacterial numbers during refrigerated storage of pasteurized fluid milk. J. Dairy Sci. 92:4823-4832.

Ranieri, M. L. and K. J. Boor. 2009. Short communication: bacterial ecology of high-temperature, short-time pasteurized milk processed in the United States. J. Dairy Sci. 92.

Ranieri, M. L., R. A. Ivy, W. R. Mitchell, E. Call, S. N. Masiello, M. Wiedmann, and K. J. Boor. 2012. Real-time PCR detection of Paenibacillus spp. in raw milk to predict shelf life performance of pasteurized fluid milk products. Appl. Environ. Microbiol. 78:5855-5863.

Raso, J., G. Barbosa—Canovas, and B. Swanson. 1998a. Sporulation temperature affects initiation of germination and inactivation by high hydrostatic pressure of Bacillus. J. Appl. Microbiol. 85:17-24.

Raso, J., M. M. Góngora-Nieto, G. V. Barbosa-Cánovas, and B. G. Swanson. 1998b. Influence of several environmental factors on the initiation of germination and inactivation of Bacillus cereus by high hydrostatic pressure. Int. J. Food Microbiol. 44:125-132.

Ratkowsky, D., R. Lowry, T. McMeekin, A. Stokes, and R. Chandler. 1983. Model for bacterial culture growth rate throughout the entire biokinetic temperature range. J. Bacteriol. 154:1222-1226.

Rosso, L., S. Bajard, J. Flandrois, C. Lahellec, J. Fournaud, and P. Veit. 1996. Differential growth of Listeria monocytogenes at 4 and 8 C: consequences for the shelf life of chilled products. J. Food Prot. 59:944-949.

Rysstad, G. and J. Kolstad. 2006. Extended shelf life milk—advances in technology. Int. J. Dairy Technol. 59:85-96.

Schaffner, D. W., J. McEntire, S. Duffy, R. Montville, and S. Smith. 2003. Monte Carlo simulation of the shelf life of pasteurized milk as affected by temperature and initial concentration of spoilage organisms. Food Protect. Trends 23:1014-1021.

Schmidt, V. S. J., V. Kaufmann, U. Kulozik, S. Scherer, and M. Wenning. 2012. Microbial biodiversity, quality and shelf life of microfiltered and pasteurized extended shelf life (ESL) milk from Germany, Austria and Switzerland. Int. J. Food Microbiol. 154:1-9.

Setlow, P. 2006. Spores of Bacillus subtilis: their resistance to and killing by radiation, heat and chemicals. J. Appl. Microbiol. 101:514-525.

Setlow, P. 2014. Germination of spores of Bacillus species: what we know and do not know. J. Bacteriol. 196:1297-1305.

Stamatakis, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688-2690.

Te Giffel, M. t., A. Wagendorp, A. Herrewegh, and F. Driehuis. 2002. Bacterial spores in silage and raw milk. Antonie van Leeuwenhoek 81:625-630.

Trmčić, A., N. H. Martin, K. J. Boor, and M. Wiedmann. 2015. A standard bacterial isolate set for research on contemporary dairy spoilage. J. Dairy Sci. 98:5806-5817.

Uçar, A. and A. Ö. Özçelik. 2013. Individuals' Knowledge and Practices of the Cold Chain. Ecol. Food Nutr. 52:116-129.

Vaerewijck, M., P. De Vos, L. Lebbe, P. Scheldeman, B. Hoste, and M. Heyndrickx. 2001. Occurrence of Bacillus sporothermodurans and other aerobic spore-forming species in feed concentrate for dairy cattle. J. Appl. Microbiol. 91:1074-1084.

Vangay, P., E. B. Fugett, Q. Sun, and M. Wiedmann. 2013. Food microbe tracker: a web-based tool for storage and comparison of food-associated microbes. J. Food Prot. 76:283-294.

Vary, J. and H. Halvorson. 1965. Kinetics of germination of Bacillus spores. J. Bacteriol. 89:1340-1347.

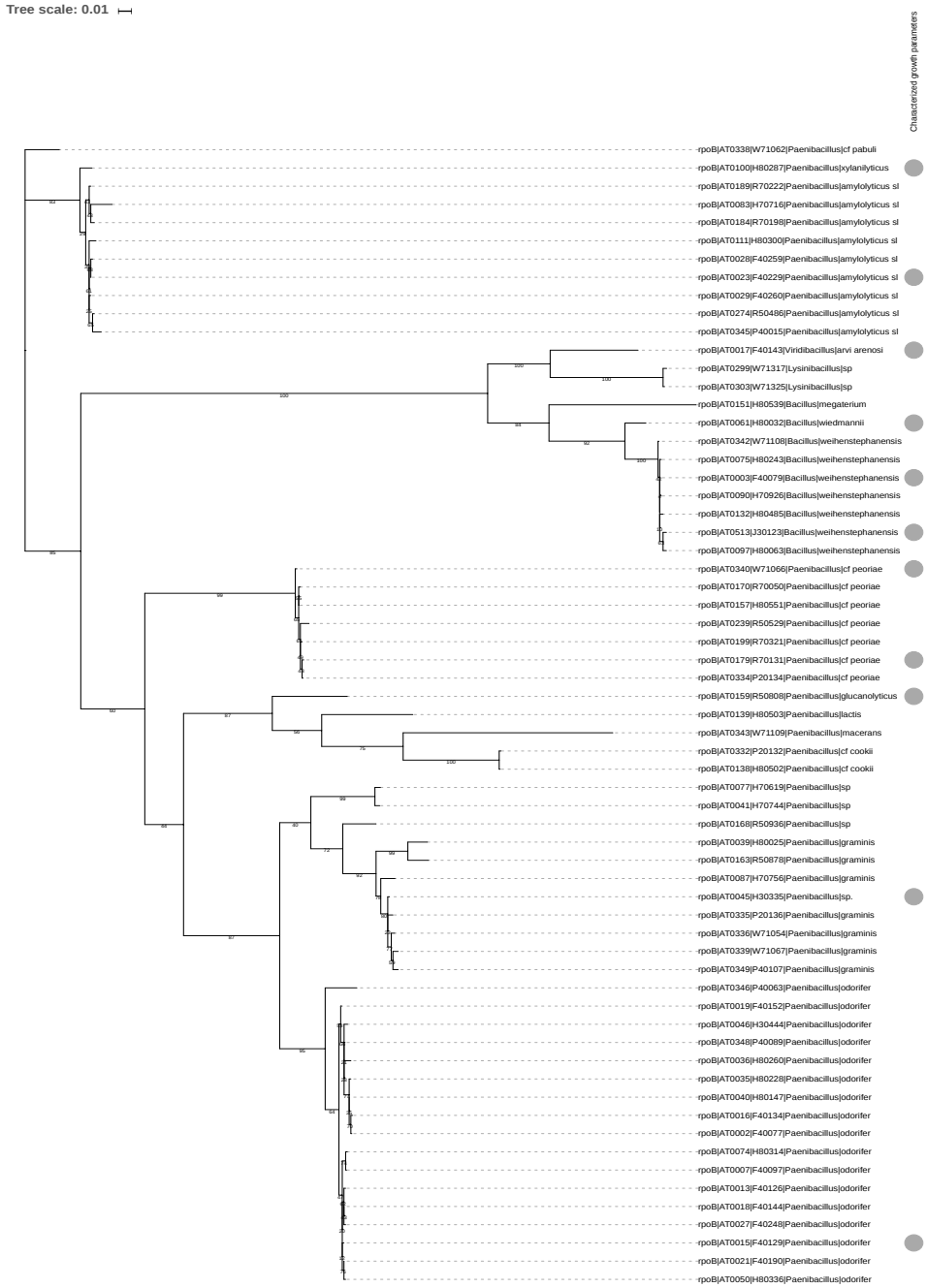
Vissers, M., M. Te Giffel, F. Driehuis, P. De Jong, and J. Lankveld. 2007. Predictive modeling of Bacillus cereus spores in farm tank milk during grazing and housing periods. J. Dairy Sci. 90:281-292.

Wilcox, R. 2005. Kolmogorov–Smirnov test. Encyclopedia of Biostatistics 4. Accessed Sept. 25, 2017. https://doi.org/10.1002/0470011815.b2a15064.

Zwietering, M. and S. Van Gerwen. 2000. Sensitivity analysis in quantitative microbial risk assessment. Int. J. Food Microbiol. 58:213-221.

Zwietering, M. H., J. C. de Wit, and S. Notermans. 1996. Risk Analysis and Production of Safe Food Application of predictive microbiology to estimate the number of Bacillus cereus in pasteurised milk at the point of consumption. Int. J. Food Microbiol. 30:55-70.

SUPPLEMENTAL MATERIAL



Supplemental Figure 5.1. Midpoint-rooted maximum-likelihood (ML) phylogenetic tree of partial *rpoB* sequences from psychrotolerant sporeformers characterized in this study as well as psychrotolerant sporeformers identified in raw milk by Masiello et al (2014). Numerical values indicate the percentage of bootstrap replications (out of 100 total replications) that support the corresponding node. Labels indicate the prefix *rpoB* followed by *rpoB* allelic type (AT) (e.g., AT0338), Cornell Food Safety Lab isolate designation (e.g., W71062), genus (e.g., *Paenibacillus*), and species (e.g., cf *pabuli*). The scale bar represents the genetic distance between isolates. Grey circles to the right of labels indicate isolates for which growth parameters were experimentally determined.

APPENDIX 1

Sensitivity analysis calculation

For the best-case, worst-case sensitivity analysis, the frequency of *rpoB* ATs 3 and 15 were evaluated after the frequency table (Table 5.4) was converted to the corresponding *rpoB* ATs with the closest pairwise distance with available growth parameters. This resulted in frequencies of 30.2% and 30.8% for *rpoB* AT 3 and *rpoB* AT 15, respectively. For the best-case sensitivity at a low-level change, the individual probability of *rpoB* AT 3 was increased to 40% while the other *rpoB* AT probabilities remained the same. As the total probabilities for all *rpoB* ATs with available growth parameters now summed to 1.098, each of the individual *rpoB* AT probabilities (including *rpoB* AT 3) decreased proportionally. Thus, the probability of *rpoB* AT 3 for the best-case sensitivity at a low-level change was 36.4% (rather than 40%). For the best-case sensitivity at a high-level change for *rpoB* AT 3, the individual probability of *rpoB* AT 3 was increased to 50%, while the other *rpoB* AT probabilities remained constant. The total probabilities for all *rpoB* ATs with available growth parameters (including *rpoB* AT 3) then summed to 1.198, and thus the probability of *rpoB* AT 3 for a best-case sensitivity at a high-level change was 41.7% (rather than 50%). For the best-case sensitivity for *rpoB* AT 15 at a low-level change, the individual probability of *rpoB* AT 15 was increased to 40%, while the other *rpoB* AT probabilities remained the same. The total probabilities for all *rpoB* ATs with available growth parameters (including *rpoB* AT 15) summed to 1.092, and thus the probability of *rpoB* AT 15 for a best-case sensitivity at a low-level change was 36.6% (rather than 40%). For the best-case sensitivity for *rpoB* AT 15 at a high-level change,

the individual probability of *rpoB* AT 15 was increased to 50%, while the other *rpoB* AT probabilities remained the same. The total probabilities for all *rpoB* ATs with available growth parameters (including *rpoB* AT 15) summed to 1.192, and thus the probability of *rpoB* AT 15 for the best-case sensitivity at a high-level change was 41.9% (rather than 50%).

For the worst-case sensitivity of *rpoB* AT 3 at a low-level change, the individual probability of *rpoB* AT 3 was decreased to 20% while the other *rpoB* AT probabilities remained the same. The total probabilities for all *rpoB* ATs with available growth parameters (including *rpoB* AT 3) summed to 0.898, resulting in a probability for *rpoB* AT 3 for a worst-case sensitivity low-level change of 22.3% (rather than 20%). For the worst-case sensitivity of *rpoB* AT 3 at a high-level change, the individual probability for *rpoB* AT 3 was decreased to 10% while the other *rpoB* AT probabilities remained the same. The total probabilities for all *rpoB* ATs with available growth parameters (including *rpoB* AT 3) summed to 0.798, resulting in a probability for *rpoB* AT 3 for the worst-case sensitivity at a high-level change of 12.5% (rather than 10%). For a worst-case sensitivity of *rpoB* AT 15 at a low-level change, the individual probability of *rpoB* AT 15 was decreased to 20% while the other *rpoB* AT probabilities remained the same. The total probabilities for all *rpoB* ATs with available growth parameters (including *rpoB* AT 15) summed to 0.892, resulting in a probability of *rpoB* AT 15 for worst-case sensitivity at a low-level change of 22.4% (rather than 20%). For the worst-case sensitivity of *rpoB* AT 15 at a high-level change, the individual probability for *rpoB* AT 15 was decreased to 10% while the other *rpoB* AT probabilities remained the same. The total probabilities for

all *rpoB* ATs with available growth parameters (including *rpoB* AT 15) summed to 0.792, resulting in a probability for *rpoB* AT 15 for a worst-case sensitivity at a high-level change of 12.6% (rather than 10%).

CHAPTER 6

CONCLUSION

Microbial spoilage represents a significant issue for dairy product quality, especially as the dairy industry aims to produce high quality dairy products with extended shelf-lives. Microbial spoilage of dairy products is further complicated given the multiple contamination pathways that exist along the processing continuum. In order to address microbial dairy spoilage and equip the dairy industry with tools to produce high-quality products, a holistic approach is required. Thus, the overall research aim of this work was to provide the dairy industry with sophisticated, data-driven tools to inform management and intervention decisions to produce high-quality dairy products that can reach more consumers.

In the first study, we applied molecular subtyping approaches to understand the diversity of dairy-relevant spoilage fungi. These tools have been previously applied to other dairy-associated spoilage organisms, including Gram-positive sporeformers and Gram-negative coliforms; however, molecular subtyping for dairy-relevant fungi is under-researched. Through our characterization and identification work, we demonstrated that ITS sequencing provides rapid and standardized fungal identification information. Moreover, ITS sequencing facilitates source tracking throughout the production continuum. One main challenge that remains for ITS sequence characterization of dairy-relevant spoilage fungi is the low-resolution power of the ITS region for a few dairy-associated species complexes, including the *Penicillium camemberti* clade, *Mucor circinelloides*, and *Geotrichum* species. Further characterization work of these species complexes using protein coding genes such as

β-tubulin paired with the use of curated reference databases that include these region sequences is needed to fully understand dairy-associated fungal diversity. Furthermore, since our data suggested that one of the main routes of fungal contamination in dairy products is through the environment, future work should focus in depth on the processing environment as a source of fungal contaminants. For example, research is needed to characterize the fungal diversity by type of equipment or processing line, to provide information to prioritize interventions. Overall, this work demonstrated that ITS sequencing for dairy-relevant fungal contaminants is a useful tool to achieve a baseline understanding of the diversity of dairy-relevant fungal organisms.

In the second study, we used ITS sequencing to characterize fungal contaminants collected during routine industry monitoring programs throughout the processing continuum in two yogurt processing facilities, including in raw materials, in-process product samples, environmental samples, and finished product samples. We characterized a broad diversity of fungal contaminants representing 3 phyla, highlighting the challenges of source tracking fungal spoilage organisms. Our data suggested that routine monitoring programs paired with ITS barcoding can be used for initial source tracking efforts and root cause analysis; however, our data also revealed the presence of common ITS subtypes where greater discriminatory subtyping methods are needed to definitively identify contamination sources.

In the third study, we focused on yeast and mold spoilage of Greek yogurt and developed a challenge study protocol to allow industry to better evaluate the effectiveness of spoilage control strategies, namely protective cultures. We also

enhanced industry's ability to assess the value of spoilage control strategies, such as protective cultures, by developing a stochastic model based on mold post-pasteurization contamination to estimate consumer exposure to visible *Penicillium commune* growth on the surface of Greek yogurt. These two tools, used in combination, are one way the dairy industry can (i) assess spoilage control strategies quantitatively through the use of our stochastic model, and (ii) implement an appropriate strategy based on processor-specific needs and performance in specific products with a challenge study evaluation. Our stochastic model that developed a framework to assess consumer exposure to visible mold in yogurt could be expanded to yeast spoilage as well with specific yeast growth characteristics in yogurt and initial yeast contamination rates. Future work could focus on estimation of initial yeast contamination rates at the processing level as yeast contamination routes often differ from mold contamination routes in the processing facility. Overall, the stochastic consumer-exposure model and challenge study protocol represent significant steps the dairy industry can take to use data-driven tools to inform decision making to produce high quality, long shelf-life dairy products.

In the fourth study, we expanded our stochastic model to the entire processing continuum to estimate the shelf-life of fluid milk spoiled by psychrotolerant sporeformers. Our work revealed that under current HTST processing regimes, an estimated 66% of fluid milk half-gallons will exceed the Pasteurized Milk Ordinance's regulatory limits by day 21 of shelf-life. We used sensitivity and scenario analyses to identify the most influential model parameters—maximum growth rate and refrigeration temperature. While our model evaluated shelf-life at a constant

temperature of 6°C (in order to compare our model to commercially spoiled samples held over shelf-life at 6°C), future model refinements could incorporate the temperature distributions encountered throughout the supply chain. Expanding the model to incorporate a range of storage temperatures would help in understanding consumer exposure to psychrotolerant spoilage in fluid milk.

Overall, the combination of molecular subtyping with stochastic modeling represents a significant step for the dairy industry to move towards precision food quality. These two tools can help processors target spoilage control strategies based on molecular subtype differences among spoilage organisms that may be found in specific facilities. Moreover, stochastic modeling allows processors to predict the impact of spoilage events and determine which intervention to employ on a facility-specific basis. These tools can be expanded beyond the dairy products studied here as well. For example, the yogurt consumer exposure model could be expanded to shredded cheese, where mold contamination is common. The psychrotolerant sporeformer model could be expanded to other sporeformers in cheese (e.g., *Clostridium tyrobutyricum*) or fruit juice (e.g., *Alicyclobacillus acidoterrestris*). Importantly, with the use of facility-specific inputs, these tools provide data-driven information for processors to reduce microbial spoilage in a precise, targeted approach.

In sum, these studies expand our understanding of dairy spoilage organisms. Specifically, we demonstrate the utility of ITS sequencing for fungal identification and source tracking and have developed the framework for stochastic spoilage models to inform intervention and management decisions. This work provides the dairy industry

with novel data-driven tools to produce high quality dairy products with extended shelf-life for more consumers.