Development of tools to improve the microbiological safety of high-risk cheese varieties

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page Number

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LIST OF SYMBOLS, ABBREVIATIONS, AND DEFINITIONS

~	Circa
≈	Approximately equal to
APC	Aerobic plate count
AU	Arbitrary unit
A_w	Water activity
Bac^+ / Bac^-	Bacteriocin-producing / non-producing
BHI	Brain Heart Infusion (broth / agar)
CDC	Centers for Disease Control and Prevention
CFR	Code of Federal Regulations
CFU	Colony forming unit
CI	Confidence interval
CM	Cultured milk bacterial fermentate
CSV	Cultured sugar-vinegar blend bacterial fermentate
DRT	Decimal reduction time; D-value
D-value	Time required for 1-log reduction at given temperature
ESL	Extended Shelf-Life
FDA	U.S. Food and Drug Administration
GRAS	Generally Recognized As Safe
Growth	≥1 log CFU/g increase from time zero count (except where otherwise specified)
Gr_{max}	Maximum growth rate
Growth rate	Rate of exponential growth in the log phase of a bacterial curve
HTST	High-temperature short-time (71.7°C for 15 s) pasteurization
HUS	Hemolytic Uremic Syndrome
IU	International unit
LAB	Lactic acid bacteria
Lag phase	Delay before the start of exponential growth
Lb.	*Lactobacillus*
Lc.	*Lactococcus*
MIC	Minimum inhibitory concentration

mM	Millimolar
MOX	Modified Oxford agar
MPN	Most Probable Number
MRS	de Man, Rogosa, Sharpe agar
NaL	Sodium lactate
NFDM	Non-fat dry milk
Nis^+ / Nis^-	Nisin-producing / non-producing
NSLAB	Non-starter lactic acid bacteria
PC	Protective culture
PCA	Plate count agar
Ped.	*Pediococcus*
PI	Prediction interval
PDO	Protected Designation of Origin
RH	Relative humidity
RTE	Ready-to-eat
S.O.I.	Standard of Identity
STEC	Shiga toxin-producing *Escherichia coli*
Str.	*Streptococcus*
Tg	Glass transition temperature
TS	Total solids
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSBYE	Tryptic soy broth with yeast extract
UHT	Ultra-High Temperature
USDA	U.S. Department of Agriculture
YM	Yeast and mold
Z-value	Temperature increase required for 10-fold reduction in D-value

ABSTRACT

Listeriosis attributed to natural cheese consumption is predominantly attributed to post-pasteurization contamination and/or the use of raw or improperly pasteurized cheesemilk. To control *Listeria monocytogenes* growth in fresh soft cheeses made with pasteurized milk (e.g. queso fresco or ricotta), formulation with organic acids and/or biopreservatives (e.g. protective cultures, bacterial fermentates) has been proposed. In cheese varieties made with raw milk, Shiga toxin-producing *Escherichia coli* (STEC, including *E. coli* O157:H7) represents an additional pathogen of concern. To reduce *L. monocytogenes* and STEC populations in raw milk intended for cheese manufacture, thermization (i.e. sub-pasteurization) has been proposed. However, temperature and time combinations necessary to achieve sufficient lethality of each pathogen have not been well characterized.

Our objectives for the proposed research were: 1) to determine combinations of pH, organic acid, and moisture which inhibit *L. monocytogenes* in a novel model soft cheese system (pH 5.25-6.00, 50-56% moisture, 1.25% salt; containing cream, micellar casein, water, lactose, salt, and organic acid); 2) to evaluate three commercial protective cultures (*Lc. lactis* subsp. *lactis* or *Lb. plantarum*; 10^6 CFU/g target) and three commercial bacterial fermentates (cultured milk or cultured sugar-vinegar; 0.5% or 1.0% level) for their antilisterial effectiveness in a permissive model cheese (pH 6.00, 56% moisture, 1.25% salt, made with lactic acid); and 3) to generate and validate decimal reduction times for *L. monocytogenes* and STEC in non-homogenized whole milk heated to thermization temperatures to establish treatments necessary for 3-log reductions of each pathogen.

L. monocytogenes refrigerated challenge studies of 8-10 weeks were conducted in Objectives 1 and 2 and thermal inactivation studies in Objective 3. *L. monocytogenes* growth

inhibition was found to be dependent on model cheese pH and organic acid used (propionic ≈ acetic > lactic > citric). Additional inhibition was observed using all bacterial fermentates and a single protective culture, with cultured sugar-vinegar fermentate found most efficacious among biopreservatives tested. 3-log decreases of *L. monocytogenes* and STEC at 65.6°C, 62.8°C, and 60.0°C were realized in 0:51, 1:39, 7:21 and 0:21, 0:51, 3:00 (min:s), respectively. Results from this work can be used by high-risk cheese manufacturers to improve the safety of their products.

SUMMARY

This dissertation is organized into six chapters. Chapters 1 and 2 serve to provide sufficient background for the following chapters. Chapter 1 reviews literature relevant to the research goals of this project. Chapter 2 is an invited review article titled "Heat-induced inactivation of microorganisms in milk and dairy products" to be submitted for a special issue of International Dairy Journal on heat-induced changes in milk. The chapter details methodology considerations for pathogen heat inactivation experiments and pertinent literature on the subject. My contributions to the manuscript were in providing the case study for heat inactivation of *L. monocytogenes* in cheese milk, compiling thermization definitions relevant to raw milk cheeses and associated predicted *L. monocytogenes* lethality (Table 2-4 and Figure 2-1), co-authoring "Importance of Understanding D-values and Z-values", and minor additions to "Mechanisms of Heat Inactivation or Resistance for Different Microorganisms of Dairy Relevance".

Chapter 3 is a manuscript to be submitted to the Journal of Dairy Science titled "Development and validation of D-values for *L. monocytogenes* and Shiga toxin-producing *Escherichia coli* in milk to reduce pathogen risks in unpasteurized milk cheeses." D-values and times to achieve a 3-log reduction of *L. monocytogenes* and STEC were generated and validated in whole milk at 3 thermization temperatures relevant to raw milk cheesemaking (60.0, 62.8 and 65.6°C). STEC D-values were found to be shorter than those for *L. monocytogenes*. A lower test temperature (57.2°C) was found insufficient in reducing *L. monocytogenes* within a practical thermization treatment time (30 min). Values were additionally compared against existing published D-values. Development of a statistical model for published *L. monocytogenes* D-values in whole milk was conducted by graduate student Michael Mays, Statistics, UW-Madison.

Chapter 4 is a research paper published in the Journal of Food Protection titled "Growth of *L. monocytogenes* in a model high-moisture cheese on the basis of pH, moisture, and acid type." pH (5.25-6.00) and organic acid (citric, lactic, acetic, or propionic) were found to affect *L. monocytogenes* growth at 4°C, while moisture had an insignificant effect at the levels tested (50 or 56%). Propionic acid model cheese experiments (Figure 4-4) were managed by undergraduate student Christie Cheng as part of her FRI Summer Scholar internship program. Dr. Dennis Seman, Meat Science, UW-Madison, assisted in statistical modeling of experimental datasets.

Chapter 5 is a research paper published in the Journal of Food Protection titled "Effect of commercial protective cultures and bacterial fermentates on *L. monocytogenes* growth in a refrigerated high-moisture model cheese." A permissive model cheese (pH 6.00, 56% moisture, made with lactic acid) was formulated with 6 biopreservatives at levels of 10^6 CFU/g protective culture or 0.5 or 1.0% bacterial fermentate and challenged with *L. monocytogenes* at 4°C, with fermentates showing better antilisterial activity than protective cultures at the refrigerated temperature. Following primary experiments that found a cultured sugar-vinegar blend fermentate (CSV-1) to inhibit *L. monocytogenes* growth for the full 8-week study when incorporated at a 1% level, a second set of experiments was conducted to investigate the combined effect of pH adjustment and addition of 0.5% CSV-1 to *L. monocytogenes* in model cheese. These secondary experiments (Figure 5-4) were managed by undergraduate student Kory Anderson.

The sixth and final chapter provides a general summary and directions for further research. Appendix 1 includes a generalized linear model of published *L. monocytogenes* growth in cheese challenge studies and Appendix 2 a final report by Michael Mays on the statistical model developed in Chapter 3.

CHAPTER 1:

Review of Relevant Literature

CHEESE STANDARDS OF IDENTITY

Cheese is a diverse food with >500 varieties currently recognized and 72 distinct cheese Standards of Identity (S.O.I.'s) defined in the U.S. Code of Federal Regulations (CFR) (Burkhalter 1981, FDA 2018). Federal S.O.I.'s for cheese were first published in 1938. Many popular cheeses in the U.S. including Brie, Camembert, Feta, Havarti, and Hispanic-style cheeses (e.g. queso fresco, queso blanco) do not have S.O.I.'s currently defined.

U.S. regulations for using raw or heat-treated milk in cheesemaking were issued in 1949 under 21 CFR Part 133. These regulations gave cheesemakers 2 options: 1) to pasteurize cheesemilk, or 2) to hold cheeses at a temperature of >2°C for ≥60 days, a practice known as the 60-day aging rule (Donnelly 2018). The CFR mandates certain cheese varieties (e.g. high-moisture (>50%), unripened cheeses) to be manufactured using pasteurized milk only, a process that inactivates all vegetative pathogens. Pasteurization standards are based on the destruction of *Coxiella burnetii* and require milk to be heated at 145°F (62.8°C) for 30 min or 161°F (71.7°C) for 15 sec except when containing more fat than found in fluid whole milk or additional solids (e.g. sugars); in these cases the specified temperature must be increased by 5°F (2.9°C) (Holsinger 1997). Table 1-1 outlines pasteurization and aging requirements for cheeses as specified in the CFR.

21 CFR 133.182 allows for manufacture of soft ripened cheeses from raw milk if aged ≥60 days. As surface mold-ripened cheeses such as Brie and Camembert do not have S.O.I.'s particular to them, these varieties can be legally made using raw milk given they comply with moisture requirements for the S.O.I. (<50% moisture) (Donnelly 2018). Due to renewed interest in artisanal and farmstead cheeses in recent years, production of raw milk varieties of these and other "non-standard" cheeses are anticipated to rise (D'Amico 2008).

Table 1-1. Code of Federal Regulations pasteurization and aging requirements for S.O.I. cheese varieties (FDA 2018, adapted from Johnson 1990).

21 CFR	Cheese Variety	Pasteurization Requirements	Minimum Aging
133.102	Asiago fresh and asiago soft cheese	None	60 days
133.103	Asiago medium cheese	None	6 months
133.104	Asiago old cheese	None	1 year
133.106	Blue cheese	None	60 days
133.108	Brick cheese	None	60 days at ≥2°C
133.109	Brick cheese for manufacturing	None	None
133.111	Caciocavallo siciliano cheese	None	90 days at ≥2°C
133.113	Cheddar cheese	None	60 days at ≥2°C
133.114	Cheddar cheese for manufacturing	None	None
133.116	Low sodium cheddar cheese	None	60 days at ≥2°C
133.118	Colby cheese	None	60 days at ≥2°C
133.119	Colby cheese for manufacturing	None	None
133.121	Low sodium colby cheese	None	60 days at ≥2°C
133.123	Cold-pack and club cheese	Made from pasteurized milk cheeses or cheeses aged ≥2°C for ≥60 days	None
133.124	Cold-pack cheese food	Made from pasteurized milk cheeses or cheeses aged ≥2°C for ≥60 days	None
133.125	Cold pack cheese food with fruits, vegetables, or meats	Made from pasteurized milk cheeses or cheeses aged ≥2°C for ≥60 days	None
133.127	Cook cheese, koch kaese	None (product heated to 82°C)	None
133.128	Cottage cheese	Pasteurization required	N/A
133.129	Dry curd cottage cheese	Pasteurization required	N/A

21 CFR	Cheese Variety	Pasteurization Requirements	Minimum Aging
133.133	Cream cheese	Pasteurization required	N/A
133.134	Cream cheese with other foods	Pasteurization required	N/A
133.136	Washed curd and soaked curd cheese	None	60 days at ≥2°C
133.137	Washed curd cheese for manufacturing	None	None
133.138	Edam cheese	None	60 days at ≥2°C
133.140	Gammelost cheese	None (product heated to 62.7°C)	None
133.141	Gorgonzola cheese	None	90 days
133.142	Gouda cheese	None	60 days at ≥2°C
133.144	Granular and stirred curd cheese	None	60 days at ≥2°C
133.145	Granular cheese for manufacturing	None	None
133.146	Grated cheeses	Made from pasteurized milk cheeses or cheeses aged ≥2°C for ≥60 days	None
133.148	Hard grating cheeses	None	6 months
133.149	Gruyere cheese	None	90 days
133.150	Hard cheeses	None	60 days at ≥2°C
133.152	Limburger cheese	None	60 days at ≥2°C
133.153	Monterey cheese and monterey jack cheese	Pasteurization required	N/A
133.154	High-moisture jack cheese	Pasteurization required	N/A
133.555	Mozzarella cheese and scamorza cheese	Pasteurization required	N/A
133.556	Low-moisture mozzarella and scamorza cheese	Pasteurization required	N/A
133.557	Part-skim mozzarella and scamorza cheese	Pasteurization required	N/A
133.558	Low-moisture part-skim mozzarella and scamorza cheese	Pasteurization required	N/A

21 CFR	Cheese Variety	Pasteurization Requirements	Minimum Aging
133.160	Muenster and munster cheese	Pasteurization required	N/A
133.161	Muenster and munster cheese for manufacturing	None	None
133.162	Neufchatel cheese	Pasteurization required	N/A
133.164	Nuworld cheese	None	60 days
133.165	Parmesan and reggiano cheese	None	10 months
133.181	Provolone cheese	None	60 days at ≥2°C
133.182	Soft ripened cheeses	None	60 days at ≥2°C
133.183	Romano cheese	None	5 months
133.184	Roquefort cheese, sheep's milk blue-mold, and blue-mold cheese from sheep's milk	None	60 days
133.185	Samsoe cheese	None	60 days at ≥2°C
133.186	Sap sago cheese	Milk "heated to boiling temperature"	5 months
133.187	Semisoft cheeses	None	60 days at ≥2°C
133.188	Semisoft part-skim cheeses	None	60 days at ≥2°C
133.189	Skim milk cheese for manufacturing	None	None
133.190	Spiced cheeses	None	60 days at ≥2°C
133.191	Part-skim spiced cheeses	None	60 days at ≥2°C
133.193	Spiced, flavored standardized cheeses	None	60 days at ≥2°C
133.195	Swiss and emmentaler cheese	None	60 days
133.196	Swiss cheese for manufacturing	None	60 days

INTRINSIC AND EXTRINSIC FACTORS RELATED TO MICROBIAL CHEESE SAFETY

Intrinsic (e.g. pH, salt, moisture, competing microflora) and extrinsic (e.g. storage time and temperature, packaging) factors are primarily dictated by cheese variety and contribute in varying extents to the microbial safety of cheese (Beard 2009). With the exception of cheeses involving extensive acidification or curd-cooking in manufacture (e.g. Feta, pasta filata, or Swiss-style cheeses), the safety of unpasteurized milk cheeses is primarily dictated by the microbiological quality of the milk itself and not the ability of the cheesemaking process to inactivate pathogens (Condron 2009, Donnelly 2018). Milk thermization, or sub-pasteurization, has been used as a means of reducing bacterial counts, especially psychrotrophic spoilage organisms, in milk intended for raw milk cheese manufacture. Several working definitions of cheesemilk thermization exist, with no established definition in U.S. regulations currently declared, though a general microbial reduction of 3 to 4 log CFU/ml is expected (CAC 2004) and a positive phosphatase test for the treated milk is observed (Eugster 2019). Table 2-4 lists published thermization definitions relevant to cheesemaking. Thermization provides some advantages of milk pasteurization, such as more consistent control of the cheesemaking process and more uniform cheese quality (Johnson 1990). However, major contributors to raw milk cheese flavor, texture, and sensory characteristics including lipoprotein lipase and enzymes of resident non-starter lactic acid bacteria (NSLAB) are inactivated by heating at temperatures near those of pasteurization, and thus their activity is minimal or absent in pasteurized milk cheeses (Driessen 1989). Cheeses made with raw milk ripen faster than those made from pasteurized milk, developing stronger flavors and odors than pasteurized milk cheeses of the same age (Beuvier 2004). Microbial populations of cheeses made with raw instead of thermized or

pasteurized milk have been found to contain approximately 3 log CFU/g more (10^8 vs. 10^5 CFU/g) total bacteria, predominantly made up of nonstarter *Lactobacilli* (Albenzio 2001, Coppola 1997). Additionally, milk heat treatments at temperatures greater than 65°C adversely affect the rennet coagulability of milk for cheesemaking (Fox 1997). It is for these changes that raw milk cheesemakers are hesitant to introduce or increase milk thermization parameters beyond mild treatments.

The safety of pasteurized milk cheeses relates more to prevention of post-processing contamination with environmental bacterial pathogens, particularly *Listeria monocytogenes*, where recontamination and subsequent growth in the product can occur (Paxson 2008). Cheeses with high moisture (>50%), low acid (pH ≥6), and low to moderate salt-in-moisture content are especially prone to contamination and post-processing growth of *L. monocytogenes* (Genigeorgis 1991). Changes in cheese physicochemical properties during aging, such as growth of surface mold accompanied by considerable increases in pH and proteolytic activity in surface mold-ripened varieties such as Brie and Camembert, can additionally lead to increasing risks for *L. monocytogenes* growth over the cheese aging period whether initially present in cheesemilk or introduced as a post-processing contaminant (D'Amico 2008, Sulzer 1991).

RISK ASSESSMENTS RELEVANT TO HIGH-RISK CHEESE VARIEITIES

Several risk assessments have been conducted that are pertinent to the cheese varieties included in this research project. Relevant findings from each assessment are summarized here.

FDA *L. MONOCYTOGENES* QUANTITATIVE RISK ASSESSMENT IN RTE FOODS

In 2003, the U.S. Food and Drug Administration (FDA) released a quantitative risk assessment investigating behavior of *L. monocytogenes* in RTE foods, including cheeses. Within the risk assessment, cheeses were categorized as follows (FDA 2003):

(1) Soft Fresh cheese, e.g. queso fresco and queso de crema; >50% moisture

(2) Soft Unripened cheese, e.g. cottage, cream and ricotta cheeses; >50% moisture

(3) Soft Ripened cheese, e.g. Camembert, Brie, mozzarella, Feta; >50% moisture

(4) Semi-Soft cheese, e.g. Brick and Provolone; >39% to ≤50% moisture

(5) Hard cheese, e.g. Cheddar and Parmesan; ≤39% moisture

(6) Process cheese, e.g. American and cold-pack cheeses; ≤43% moisture

Cheeses were evaluated for their relative exposure to *L. monocytogenes* based on serving size and frequency of consumption, contamination level and frequency, duration of home storage, and growth of the pathogen during home storage. The six cheese categories were additionally evaluated for relative risk ranking in causing listeriosis compared to other RTE foods based on two factors: risk per serving and risk per annum (Figure 1-1). “Soft Unripened” cheese was ranked as a *High Risk* food category. These cheeses have low rates of contamination, however, are consumed in high volume by a large proportion of the U.S. population, elevating their relative per annum risk for contamination. The FDA suggested in the assessment that research on *L. monocytogenes* behavior in cheeses should focus on soft unripened cheeses in order to prevent future outbreaks implicating cheese (FDA 2003). However, no listeriosis cases have been associated with cottage cheese to-date (Ostergaard 2014) and a preemptive recall of cream cheese over listeriosis concerns in 2018 resulted in no illnesses (FDA 2018b). In the 2001 draft *L. monocytogenes* RTE foods risk assessment, “Fresh Soft Cheese” and “Soft Ripened Cheese”

Figure 1-1. Two-dimensional matrix of ready-to-eat food categories based on cluster analysis of predicted per serving and per annum relative risk rankings for listeriosis (adapted from FDA 2003).

Decreased Risk per Annum →

↓ Decreased Risk per Serving

Clusters A and B **Very High Risk** **Clusters 1A, 1B** Deli Meats Frankfurters (not reheated)	**Clusters C and D** **High Risk** **Clusters 1C, 1D** Pâté and Meat Spreads Unpasteurized Fluid Milk Smoked Seafood	**Cluster E** **Moderate Risk** **Cluster 1E** No food categories	**Cluster 1**
High Risk **Clusters 2A, 2B** High Fat and Other Dairy Products Pasteurized Fluid Milk Soft Unripened Cheese	**Moderate Risk** **Clusters 2C, 2D** Cooked RTE Crustaceans	**Moderate Risk** **Cluster 2E** No food categories	**Cluster 2**
Moderate Risk **Clusters 3A, 3B** No food categories	**Moderate Risk** **Clusters 3C, 3D** Deli-type Salads Dry/Semi-dry Fermented Sausages Frankfurters (reheated) Fresh Soft Cheese Fruits Semi-Soft Cheese Soft Ripened Cheese Vegetables	**Low Risk** **Cluster 3E** Preserved Fish Raw Seafood	**Cluster 3**
Moderate Risk **Clusters 4A, 4B** No food categories	**Low Risk** **Clusters 4C, 4D** No food categories	**Very Low Risk** **Cluster 4E** Cultured Milk Products Hard Cheese Ice Cream and Other Frozen Dairy Products Processed Cheese	**Cluster 4**

were additionally classified as *High Risk* products, however, were classified as *Moderate Risk* in the published assessment in 2003. The FDA attributed this decline in assigned risk to decreased contamination rates due to higher levels of cheesemilk pasteurization among both categories, and suggested *L. monocytogenes* recontamination to be the primary means of *L. monocytogenes* exposure in the two cheese categories. “Hard Cheese,” including Cheddar and Parmesan, and

"Processed Cheese" categories were classified as *Very Low Risk* food categories. The FDA suggested that both food categories are subjected to bactericidal treatment, have very low contamination rates, and possess inherent characteristics that inactivate the pathogen (in the case of "Hard Cheese") or prevent its growth (in the case of "Processed Cheese"). The report suggested that without gross manufacturing errors, listeriosis outbreaks attributed to either food category were highly unlikely. Hard Cheeses were ranked 23rd out of 23 food categories for listeriosis risk in the assessment, with a relative per serving risk of disease from Deli Meats (ranked 1st of 23) almost 10,000,000-fold greater than the risk of disease predicted from Hard cheese consumption (FDA 2003).

FOOD STANDARDS AUSTRALIA / NEW ZEALAND MICROBIOLOGICAL RISK ASSESSMENT OF RAW MILK CHEESE

In a 2009 quantitative microbiological risk assessment of raw milk cheeses based on available published challenge studies, Food Standards Australia / New Zealand ascertained extra hard (<36% moisture) and Swiss-type raw milk cheeses to pose low to negligible risk to the public health and safety of the general populace in terms of STEC survival or growth in the cheeses, while raw milk Cheddar, Feta, and Camembert cheeses were found to pose high risks due to predicted survival and/or growth of STEC during cheesemaking. Conversely, extra hard cheeses, high-cook temperature Swiss-types, and Cheddar raw milk cheeses were found to pose low to negligible risk to the general populace in terms of *L. monocytogenes* survival or growth, while raw milk Swiss-type cheeses with low curd cooking temperature, blue cheeses, Feta, and Camembert cheeses were found to pose high risks to immunocompromised individuals due to likelihood of survival and/or growth of *L. monocytogenes* during cheesemaking. The authors

noted equivalency of challenge study results between bovine, caprine, and sheep's milk cheese with the exception of raw milk Cheddar made from sheep's milk, which was found to have a protective effect on *L. monocytogenes* survival (Condron 2009).

The regulators additionally evaluated the combined effects of thermization and aging, defined as 62°C treatment for 15 s and 90 days aging at 2°C under Australian regulations, on the survival of STEC and *L. monocytogenes*. The authors predicted thermization to result in an approximately 2 log decrease of STEC in raw milk while aging 90 days at 2°C would result in an additional 1 log reduction of STEC depending on cheese variety. The authors predicted *L. monocytogenes* reductions realized by thermization and aging to be less than those observed in STEC, with particular risk in *L. monocytogenes* growth during the aging process predicted in raw milk Camembert (Condron 2009).

FDA / HEALTH CANADA LISTERIOSIS FROM RAW MILK SOFT RIPENED CHEESE QUANTITATIVE RISK ASSESSMENT

A joint FDA and Health Canada 2015 quantitative risk assessment of *L. monocytogenes* in soft ripened raw milk cheeses concluded that a mild treatment reducing *L. monocytogenes* by 3 log CFU/ml in bulk raw milk before cheesemaking (e.g. via thermization) would reduce the mean listeriosis risk approximately 7.2-fold to 10-fold lower than the baseline estimates for soft ripened cheese made from raw milk. Even with this intervention, an approximately 7.4- to 11-fold higher risk than the baseline estimate for pasteurized milk soft ripened cheese was found by regulators. The assessment additionally found that a 4 log CFU/ml reduction in *L. monocytogenes* in raw milk would reduce the mean risk approximately 35-fold to 50-fold and a 5 log reduction 56-fold to 96-fold; these corresponded to a 1.7- to 2.0-fold or 1.1- to 1.2-fold

higher risk than the baseline estimate for pasteurized milk soft-ripened cheese (FDA 2015). The assessment additionally reported *L. monocytogenes* prevalence to be 0.6 to 0.7% among pasteurized milk soft ripened cheeses samples versus 3.2 to 4.7% among those made with raw milk (FDA 2015).

HIGH-RISK CHEESE VARIETIES

A disproportionate number of foodborne disease outbreaks related to cheese have implicated 2 cheese categories: 1) raw milk cheeses and 2) high-moisture, low-acid cheeses, especially fresh soft Hispanic-styles (Ibarra-Sanchez 2017, Langer 2012). This project focuses on pathogen control measures specific to each cheese category.

RAW MILK CHEESES

In the U.S., only 1.6% of the population was estimated to consume raw milk cheeses in 2017, however, popularity of these varieties has risen in recent years (Costard 2017). The number of artisan cheesemakers in the U.S. was found to have more than doubled between 2000 and 2011 to nearly 450, with 75% reportedly using unpasteurized milk for some or all of their cheeses (Paxson 2011, Roberts 2007). Most U.S. specialty cheese consumers are not concerned about potential health issues related to raw milk cheese consumption and purchase these cheeses for their perceived flavor complexity, and/or their belief that raw milk cheeses are produced more naturally or traditionally than their pasteurized milk counterparts (Licitra 2019). However, a doubling in consumption of raw milk cheeses by U.S. consumers was estimated by the Centers for Disease Control and Prevention (CDC) to potentially increase outbreak-related illnesses by 96%, as unpasteurized dairy products were found to cause 840 times more illnesses and 45 times

more hospitalizations than pasteurized dairy products (Costard 2017). A separate CDC review of U.S. outbreaks associated with raw milk products found cheese to be the causative agent in 27 of these outbreaks over the period 1993 to 2006 (Langer 2012). Australian regulators additionally reported that despite raw milk cheeses comprising less than 10% of total worldwide cheese production, outbreaks attributed to raw milk cheese represent nearly 70% of total cheese foodborne disease outbreaks (Condron 2009).

Raw milk cheeses make up a much larger share of total cheese production and consumption in Europe, where approximately 10% of cheese products are estimated to be made from raw milk (Panthi 2017). Raw milk cheeses make up a significant portion of total cheese production in France and Italy, and approximately half of cheeses produced in Switzerland are those made from raw or thermized milk (Eugster 2019). 186 European cheeses to-date have Protected Designation of Origin (PDO) status, with 39%, 8%, and 53% requiring cheese to be manufactured using raw milk only, pasteurized milk only, and raw or pasteurized milk, respectively (Licitra 2019). In a recent survey of 142 Belgian homestead cheese producers, 87% of Belgian homestead cheeses were found to be produced by raw milk (FASFC 2019). In Canada, it was estimated that raw milk cheese accounted for 15% of specialty cheese manufactured in 2005, or approximately 9% of all cheese produced (Condron 2009).

RAW MILK CHEESE REGULATIONS

Regulations for thermization and raw milk cheese aging vary between the U.S. and the rest of the world. Raw milk cheese under European legislation must be made with milk which has not been heated to more than 40°C or has not undergone treatment with an equivalent effect (Melini 2017), and thermizing is considered a heat treatment that distinguishes thermized milk

from raw milk for cheese manufacture (Paxson 2008). Certain European PDO cheeses, e.g. Camembert de Normandie, have no aging requirement and their safety is instead assessed through microbiological criteria for cheeses made from raw or thermized milk (D'Amico 2008). Recent Swiss guidelines for cheesemilk processing for alpine dairies suggest equivalency of thermization treatments of a) 65°C for 15 s, b) 60°C for 5 min, and c) 57°C for 30 min (Jakob 2015). Raw milk cheese production was illegal in Australia until 2012 (McIntyre 2015). An Australian thermization requirement of ≥62.5°C for ≥15 s coupled with ≥90 days aging at ≥2°C is required for unpasteurized milk cheese manufacture in the country (Condron 2009). A single Canadian province (Québec) began permitting the sale of raw milk soft ripened cheese with no aging requirement in 2008, while the rest of the country requires ≥60 days' aging at a temperature of ≥2°C (McIntyre 2015). The following raw milk cheese requirements were proposed by Canadian regulators in 1996: a) minimum thermization of ≥63°C for ≥16 s; b) cheese pH ≤5.5 and a_w ≤0.95 at the end of the manufacturing process; and c) storage ≥2°C for ≥60 days (Government of Canada 1996).

HIGH-MOISTURE, LOW-ACID CHEESES

Fresh cheeses can be classified as curd-style cheeses which do not undergo ripening and include varieties such as queso fresco, queso blanco, cottage cheese, fresh mozzarella, and ricotta, many of which fall within general ranges for having high moisture (>50%) and low acid (pH ≥6) (Genigeorgis 1991). Fresh cheeses are required under the CFR to be manufactured using pasteurized milk only (FDA 2018).

Fresh Hispanic-style cheeses represent approximately 80% of total cheeses consumed in Mexico and are the most consumed Latin-style cheese in the U.S. market (Saxer 2013). 287

million pounds of Latin-style cheese were produced in 2017, a 316% increase from 1997 (Awe 2017). The Hispanic-style fresh cheese segment of the U.S. cheese industry is currently comprised mostly of small and medium-sized operations (Kabuki 2004). Among Hispanic cheese varieties, queso fresco was found to have the largest volume share in U.S. retail multi-outlet and convenience stores at 62% in 2017, a 10.8% volume increase from the year prior (Awe 2017).

The fresh Hispanic-style cheese category is a heterogenous group of white, unripened soft cheeses with NaCl contents of 1 to 3% and shelf-lives of 45 to 70 days (Kabuki 2004). Hispanic-style cheeses are coagulated using rennet and may have organic acids added, including citric, acetic, or lactic acid. Lactic starter cultures are typically not used to produce Hispanic-style cheese (Bolton 1999). Among 100 commercial samples of Hispanic-style cheeses analyzed for analytical parameters, moisture, pH, and salt ranges were found to be 39.5 to 58.6% (mean 50.09%), 5.00 to 6.90 (mean 5.88) and 0.52 to 3.70% (mean 1.87%), respectively (Genigeorgis 1991b). Analytical values in commercial queso fresco samples were found to range from 41 to 59% moisture, pH 5.3 to 6.5, 17 to 21% protein, 18 to 29% fat, and 1 to 3% salt in one survey (Lourenco 2017). A separate survey of 64 commercial queso fresco samples produced in the U.S. and representing 9 brands found pH values to range from 6.62 to 6.86, moisture from 43.90 to 54.50%, and salt 1.53 to 2.01%, with a single cheese labeled as containing active starter culture and 2 samples formulated with potassium sorbate (Holle 2018). Acetic and citric acids were reported to be the most frequent acidulants used in queso blanco manufacture (Glass 1995), though other authors reported the use of acid whey and citrus fruit juices to be commonly added as direct acidulants to hot milk in queso blanco manufacture (Farkye 1995). In overall sensory acceptability scores, Farkye and colleagues found lactic acid-manufactured queso blanco to garner lower scores compared to cheeses made using acetic or citric acids (Farkye 1995).

Analytical values in commercial queso blanco samples were found to range from 45 to 55% moisture, pH 5.25 to 5.90, and 1.8 to 3% salt (Uhlich 2006).

Cottage cheese can be manufactured either with the use of mesophilic starter cultures (e.g. *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*) or via direct-set methodology utilizing the addition of organic acids (Ho 2016). The added cream dressing may be fresh or cultured using a diacetyl-producing culture, e.g. *Lc. lactis* subsp. *lactis* bv. diacetylactis. Typical analytical values for cottage cheese are ~80% moisture, 1 to 1.2% salt, and pH <5.5 (Ostergaard 2014). In commercial cottage cheese samples made with aroma-producing mesophilic cultures, an average pH value of 5.35 was found (Ostergaard 2015). Commercial cottage cheese samples produced in the U.K. were found to have pH values of 4.6 to 5.1 and salt 0.8 to 1.0% (w/w) with cream dressing forming 25 to 40% (w/w) of the final formula and a typical shelf-life of 2 weeks at temperatures ≤5ºC (Hicks 1991). Other researchers found pH values of cottage cheese to range from 4.75 to 5.30 and salt from 1 to 2% and suggested 3 weeks to be the maximum attainable shelf-life in unopened fresh cottage cheese under typical refrigeration (4 to 7ºC) conditions without added preservatives. Once opened, a decrease in shelf-life to 1 week was observed (Ho 2016).

Direct acidification is used in the manufacture of string and fresh mozzarella. Citric, acetic, and lactic acids have been reported among the most commonly used direct acidulants for fresh mozzarella cheesemaking (Tirloni 2019). Curd-stretching can occur at a higher pH in directly-acidified mozzarella than in mozzarella made with starter culture (e.g. pH 5.6 to 5.7 versus pH 5.15 to 5.35) (Johnson 2013). In a survey of 15 commercial mozzarella samples manufactured via direct acidification with citric acid, moisture and pH ranges of 48.47% to 67.41% and 5.43 to 6.48, respectively, were recorded (Tirloni 2019).

Acetic, citric, and lactic acids have been reported to be the main organic acids directly added during the manufacture of ricotta cheese, with citric being the most commonly used in ricotta manufacture. Ricotta cheeses have a typical pH of 6.0 to 6.5 and shelf-life of 20 to 40 days (Tirloni 2019b). Moisture, pH, and salt ranges in 8 commercial brands of ricotta were found to be 72.2% to 82.1%, 5.49 to 6.61, and 0.2% to 0.5%, respectively (Tirloni 2019b).

PATHOGENS OF INTEREST

L. monocytogenes, Shiga toxin-producing *Escherichia coli* (STEC, including *E. coli* O157:H7), *Salmonella* spp., and *Staphylococcus aureus* have been identified as primary pathogens of concern in natural cheeses (Donnelly 2018, Panthi 2017). The focus of this dissertation is on *L. monocytogenes* and STEC. Chapter 3 focuses on thermal treatments necessary for reduction of *L. monocytogenes* and STEC in milk intended for raw milk cheese. *L. monocytogenes* and STEC outbreaks and recalls implicating raw milk cheeses are listed in Table 1-2. Chapters 4 and 5 of this research study focus on control of *L. monocytogenes* growth in high-moisture, low-acid cheeses via formulation measures. Listeriosis outbreaks implicating these varieties have been reviewed by other authors (Ibarra-Sanchez 2017, Jackson 2018). Jackson and colleagues analyzed listeriosis outbreaks associated with soft cheese in the US over the time period of 1998-2014, finding two thirds of listeriosis outbreaks attributed to cheese to implicate soft Hispanic-styles, with 54% cases of listeriosis in soft cheeses attributed to Hispanic-styles (Jackson 2018).

Table 1-2. Outbreaks and recalls attributed to *Listeria monocytogenes* and Shiga toxin-producing *Escherichia coli* contamination in unpasteurized bovine milk cheeses (adapted from Verraes 2015).

Cheese	Isolate	Country	Year	Cases	Reference
Unpasteurized milk Vacherin mont d'or cheese	*L. monocytogenes*	Switzerland	1983	122 cases, 33 dead	Bille 1989*
Raw cow and goat milk farm fresh cheese	*E. coli* O119:B14 (*vtx2*)	France	1992	4 cases, 4 HUS, 1 death	Casenave 1993*
Farm raw milk cheese	*E. coli* O157 (PT28, *vtx2*)	Scotland	1994	22 cases, 1 HUS	Ammon 1997*
Raw milk Brie de meauw cheese	*L. monocytogenes* serovar 4b	France	1995	36 cases, 4 dead, 7 fetal/neonatal cases	Vaillant 1998*
Raw milk cheese	*E. coli* O110:H-	Germany	1996	3 cases, 0 HUS	Bockemuhl 1996*
Raw milk livarot, pont-l'eveque cheese	*L. monocytogenes* serovar 4b	France	1997	14 cases	Jacquet 1998*
Unaged raw Cheddar cheese curds**	*E. coli* O157:H7	US	1998	63 cases, 55 laboratory-confirmed, ,2 HUS, 24 hospitalized	CDC 2000*
Unpasteurized milk cheese	*E. coli* O157 (PT2, *vtx2*)	England	1998	10 cases, 1 HUS	CDSC 1998*
Unpasteurized farm cheese	*E. coli* O157	Scotland	1998	4 cases	Strachan 2006*
Fresh Mexican-style raw milk cheese**	*L. monocytogenes*	US	2000	13 cases, 11 pregnant women (5 stillbirths, 3 premature deliveries, 3 infected newborns)	MacDonald 2005*
Unpasteurized Gouda	*E. coli* O157:H7	Canada	2002	13 cases, 2 HUS	Honish 2005*
Unpasteurized queso fresco**	*L. monocytogenes*	US	2003	12 cases, 12 hospitalized, 1 dead	CDC 2014*
Unpasteurized queso fresco**	*E. coli* O157:H7	US	2004	3 cases, 2 hospitalized	CDC 2014*
Inadequately pasteurized Bocconcini fresh cheese**	*L. monocytogenes*	Canada	2004	1 case	News Ontario 2004
Raw milk cheese	*E. coli* O157:H7	Canada	2004	3 cases	MAPAQ 2004*
Unpasteurized queso fresco**	*L. monocytogenes*	US	2005	12 cases, 12 hospitalized, 0 dead	CDC 2014*
Raw milk Brie	*E. coli* O26, O80 (*stx2*, *eae*)	France	2005	6 HUS	INVS 2007*

Cheese	Isolate	Country	Year	Cases	Reference
Raw milk soft cheese	*L. monocytogenes*	Norway	2007	21 cases, 5 deaths	EFSA 2009
Raw milk cheeses	*L. monocytogenes*	Canada	2007	15 cases, 3 fetal/neonatal cases, 1 death	Powell 2008
Raw milk cheese	*E. coli* O157:H7	Canada	2008	16 cases	Gaulin 2012*
Unpasteurized Gouda	*E. coli* O157:H7, *L. monocytogenes*	US	2010	38 cases, 15 hospitalized, 1 HUS	McCollum 2012*
Aged raw milk Gouda	*E. coli* O157:H7	US	2010	38 cases, 1 HUS, 15 hospitalized	CDC 2014*
Unpasteurized milk cheeses	*E. coli* O157:NM(H-) (*stx1*, *stx2*)	US	2010	8 cases, 2 hospitalized	CDC 2014*
Raw milk Brie	*L. monocytogenes* 4b	France	2012	25 cases	Tourdjman 2015
Raw milk soft ripened cheeses	*L. monocytogenes*	Belgium	2011	No illnesses reported (product recalled)	FASFC 2012
Aged raw milk Gouda	*E. coli* O157:H7	Canada	2013	29 cases, 5 hospitalized, 1 death	Gill 2015
Aged raw milk cheese	*E. coli* O103	US	2013	3 cases	Marler 2013
Raw milk soft ripened cheeses	*L. monocytogenes*	US	2014	No illnesses reported (product recalled)	Powell 2014
Raw milk Cheddar	*L. monocytogenes*	US	2014	No illnesses reported (product recalled)	Chapman 2014
Raw milk surface-ripened cheese	*E. coli* O26:11	Canada	2014	No illnesses reported (product recalled)	Food Safety News 2014
Raw milk hard and soft ripened cheeses	STEC (unspecified)	Ireland	2015	No illnesses reported (product recalled)	FSAI 2015
Raw milk Camembert	*L. monocytogenes*	France	2016	2 cases	Powell 2016b
Raw milk blue cheese	*L. monocytogenes*	US	2016	No illnesses reported (product recalled)	Powell 2016
Raw milk soft cheese	*E. coli* O26 and *E. coli* O157:H7	Romania	2016	25 cases, 19 HUS, 3 deaths	EFSA 2016
Unpasteurized blue cheese	*E. coli* O157:H7	Scotland	2016	26 cases, 17 hospitalized, 1 death	Food Safety News 2018b
Raw milk cheeses	Non-O157 STEC (unspecified)	US	2016	7 cases, 1 hospitalized	Chapman 2016
Raw milk soft ripened cheese	*L. monocytogenes*	US	2017	8 cases, 6 hospitalized, 2 dead	CDC 2017
Raw milk Alpine cheese	*L. monocytogenes*	US	2017	No illnesses reported (product recalled)	Food Safety News 2017
Raw milk Monterey Jack	*L. monocytogenes*	US	2017	No illnesses reported (product recalled)	Food Safety

Cheese	Isolate	Country	Year	Cases	Reference
					News 2017b
Raw milk Cheddar	*L. monocytogenes*	Ireland	2017	No illnesses reported (product recalled)	FSAI 2017
Unpasteurized blue cheese	*L. monocytogenes*	Scotland	2017	No illnesses reported (product recalled)	FSS 2017
Raw milk soft washed rind, smear-ripened cheese	*E. coli* O26:H11 (*eae*, *stx2*)	France	2017	15 cases, 11 HUS, 1 death	Food Safety News 2018
Raw milk washed rind cheeses	*L. monocytogenes*	France	2018	No illnesses reported (product recalled)	FSANZ 2019
Raw milk Brie	*L. monocytogenes*	France	2018	No illnesses reported (product recalled)	Whitworth 2019
Raw milk soft ripened cheeses	STEC (unspecified)	Belgium	2018	No illnesses reported (product recalled)	Powell 2018
Raw milk semi-hard cheese	Pathogenic *E. coli*	Canada	2018	5 cases	Food Safety News 2018c
Raw milk Camembert	*E. coli* O26:H11	France	2018	No illnesses reported (product recalled)	FSAI 2018
Raw milk soft ripened cheeses	*L. monocytogenes*	US	2019	No illnesses reported (product recalled)	FDA 2019
Raw milk Brie	*L. monocytogenes*	France	2019	2 cases, 1 fetal, 1 death	Food Safety News 2019b
Raw milk soft-ripened cheese	*L. monocytogenes*	Canada	2019	No illnesses reported (product recalled)	Food Safety News 2019c
Raw milk Bethmale (Tomme) cheese	*L. monocytogenes* and *E. coli* O26:H11	France	2019	No illnesses reported (product recalled)	Food Safety News 2019
Washed-rind raw milk (Tomme) cheese	*L. monocytogenes*	US	2019	No illnesses reported (product recalled)	FDA 2019b
Raw milk soft ripened cheese	*E. coli* O111:H8	France	2019	No illnesses reported (product recalled)	Powell 2019
Raw milk soft ripened cheese	*E. coli* O26 (*stx2*, *eae*)	France	2019	18 cases, 17 HUS	Jones 2019

*See Verraes 2015 for primary reference citation except where otherwise indicated.
**Indicates illegally manufactured cheese per U.S. regulations due to inadequate pasteurization or aging.

LISTERIA MONOCYTOGENES

Listeria monocytogenes is a Gram-positive, facultatively anaerobic, non-spore-forming rod and the causative agent of listeriosis. The optimum growth temperature for the bacterium is

35 to 37°C, but it can grow over a wide range of temperatures (1 to 45°C) as well as pH values (pH 4.1 to 9.6) and in up to 13% salt (Yousef 2003, Horita 2018). The psychrotrophic nature of *L. monocytogenes* in conjunction with its tolerance to pH and salt stress and ability to form biofilms can lead to its persistence in food manufacturing environments, making control difficult for ready-to-eat (RTE) food manufacturers (Horita 2018). Sites of *L. monocytogenes* contamination in the cheese manufacturing environment can include starter cultures, brine, drains, floor, packaging material, cheese vats, shelves, cheese cloth, curd cutting knives, brushes, and coolers (Melo 2015).

An estimated 15 to 20% of the general population in developed countries is susceptible to listeriosis infection, made up mostly of the immunodeficient, pregnant, children, and the elderly (Arques 2015). Foodborne listeriosis carries an estimated 19% fatality rate in these individuals (Gahan 1996). Between 1983 and 2019, at least 41 *L. monocytogenes* cheese outbreaks world-wide were recorded, attributed most prominently to soft cheeses and mold-ripened soft cheeses (Wemmenhove 2019). One of the earliest reported U.S. listeriosis outbreaks occurred in 1985, implicating queso fresco and Cotija Hispanic-style cheeses and resulting in 142 illnesses, 28 deaths, and 20 fetal losses. In this outbreak, raw milk was inadvertently introduced into pasteurized cheesemilk. However, the majority of listeriosis soft cheese outbreaks in recent years have been linked to those made from pasteurized milk (Jackson 2018). In Europe, approximately half of total listeriosis outbreaks in recent years have been traced to dairy products, with the predominant *L. monocytogenes* serotype implicated in outbreaks being 4b (Melo 2015).

L. monocytogenes is an intracellular pathogen to humans and animals hosts and can exist intracellularly in monocytes. Because of its intracellular nature, studies investigating its thermal tolerance within bovine leukocytes were undertaken by several authors in order to test whether

its location within phagocytic cells confers additional protection against milk heat treatment (Bunning 1988, Doyle 1987, Lovett 1990). However, the intracellular position of *L. monocytogenes* was found unable to protect it from thermal inactivation and no significant difference in decimal reduction times was observed between intracellular and freely suspended *L. monocytogenes* in raw milk heated to temperatures 52.2°C to 71.7°C (Bunning 1986) or in sterile whole milk heated to temperatures 57.8°C to 74.4°C by two heating methodologies (Bunning 1988). *L. monocytogenes* has been shown to be more heat-tolerant than most other non-sporeforming pathogens. However, vat and High-Temperature Short-Time (HTST) pasteurization treatments have been shown to completely destroy *L. monocytogenes* given the raw milk is properly refrigerated before pasteurization, with 4.5 to 6.2 decimal reductions realized from pasteurization even under worst-case scenarios (Farber 1992).

The International Life Sciences Institute designated foods with the following properties as being high-risk for becoming vehicles of listeriosis: 1) having the potential for contamination with *L. monocytogenes*, 2) supporting the growth of *L. monocytogenes* to high CFU/g, 3) being RTE, 4) requiring refrigeration, and 5) having an extended storage life (ILSI 2015). The Codex Alimentarius Commission as well as the European Food Safety Authority have proposed that *L. monocytogenes* levels up to 100 CFU/g at the point of consumption do not pose a public health risk (EFSA 2007). However, a "zero-tolerance" policy for *L. monocytogenes* in RTE foods was established in 1989 in the U.S. (Shank 1996). The discrepancy exists due to the question of the infective dose of *L. monocytogenes*. Studies have suggested that the infective dose is much greater than 10,000 cells even for those most susceptible to infection, thus concluding that a level of up to 100 CFU/g *L. monocytogenes* in food at the time of consumption does not pose a risk to public health (FAO/WHO 2004). EU regulations allow for a maximum of 100 CFU/g *L.*

monocytogenes in RTE foods which do not support *L. monocytogenes* growth (pH ≤4.4 or a_w ≤0.92; pH ≤5.0 and a_w ≤0.94; or shelf-life of < 5 days). The criterion also applies to any RTE food if the manufacturer can validate that the product will not exceed 100 CFU/g throughout the product shelf-life, otherwise a "zero-tolerance" criterion, wherein absence in 5 x 25 g samples is validated, applies (Samelis 2017).

Several researchers have attempted to find a correlation between levels of coliform bacteria in raw milk products and safety without success, including Jackson and colleagues surveying U.S. raw milk samples for coliforms, *Bacillus cereus*, *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* spp., and finding coliform counts not to be an index of pathogen presence (Jackson 2012). Likewise, Limoges found no statistically significant association between generic *E. coli* levels of 10 and 100 CFU/g and presence of *L. monocytogenes* in domestic and imported cheeses (Limoges 2019). However, Margolles and colleagues found high levels of coliforms (4 to 5 log CFU/g) detected in several regional Spanish cheeses and found statistical association with high counts and the presence of *Listeria* spp. (Margolles 1996).

SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI*

Escherichia coli belong to the *Enterobacteriaceae* family of bacteria and are Gram-negative, aerobic, facultatively anaerobic, non-sporeforming rods that ferment lactose with the production of acid and gas. *E. coli* are capable of growing over a wide range of temperatures (7 to 46°C), pH (4.4 to 10.0), and at a_w values ≥0.95 (Donnelly 2018). The majority of *E. coli* are harmless to humans, however, some have acquired virulence factors, such as Shiga toxins, which can cause severe diarrheal diseases. Shiga toxin-producing *E. coli* (STEC) are *E. coli* strains that produce Shiga toxins (*stx1*, *stx2*) similar to those produced by *Shigella dysenteriae* type 1. In

addition to the *stx* gene, STEC can contain the *eae* gene which encodes for the intimin protein associated with attaching and effacing lesions in the intestinal cell wall (Bibbal 2015). More than 300 STEC serotypes have been identified, with some strains shown capable of surviving at pH values as low as 2.5 to 3.0. While not all STEC are human pathogens, some strains are capable of causing abdominal cramping, mild to bloody diarrhea, vomiting, and in serious cases Hemolytic Uremic Syndrome (HUS), especially in young and elderly populations (Perrin 2015). Pathogenic STEC include a broad range of O:H serovars, with serovars O26, O45, O103, O111, O121, O145, and O157 implicated in prior foodborne outbreaks. The infectious dose of *E. coli* O157:H7 and other pathogenic STEC is estimated to be very low, at <100 CFU (Donnelly 2018).

STEC can be isolated from the intestinal tract of dairy cattle and other warm-blooded animals, where infections begin with colonization to the intestinal mucosa. Cattle are the main animal reservoir of STEC and can carry STEC in their gastrointestinal tracts asymptomatically, shedding them in their feces. Raw milk can become contaminated with STEC via fecal material contact or via mastitic milk, especially if hygiene rules are not followed during milking or processing (Miszczycha 2013). *E. coli* cells leave the cow gut in stationary phase and are likely to contaminate raw milk in the stationary phase as well (Peng 2011), an important consideration given increased acid tolerance observed in *E. coli* O157:H7 cells in stationary phase (Arnold 1995). Because of their ability to ferment lactose, STEC can show strong reproduction in the first 24 hours of cheese manufacture before the carbohydrate is depleted by starter LAB. Semi-hard cheeses are ripened faster and often consumed earlier than hard cheeses because of their higher moisture content and can represent a higher risk to human health if complete inactivation of STEC is not achieved during the aging period (Eugster 2019). In a 2013 Canadian *E. coli* O157:H7 outbreak linked to unpasteurized milk Gouda, regulators isolated the outbreak strain in

an intact cheese wheel that had been aged 83 days but were unable to recover the strain in environmental samples from the manufacturing facility, suggesting the contamination source was likely the unpasteurized cheesemilk with the isolate surviving cheese manufacture and aging (Salazar 2020). In an *in vitro* screening of 15 generic *E. coli* and 26 STEC isolated from raw milk cheeses and 6 STEC isolated from cattle feces, Peng and colleagues found all tested strains capable of growing at pH and a_w values commonly found in semi-hard cheeses (pH 5.2, a_w 0.970), while reductions in the tested strains were observed at pH 4.5 or a_w 0.942 (Peng 2012).

STEC are completely destroyed by batch or HTST pasteurization within a wide margin of safety (Condron 2009). Peng and colleagues found 7 generic *E. coli* strains isolated from raw milk cheese able to survive heating at 55°C for 15 min, while none of the 32 tested STEC strains isolated from cheese or cattle feces were found able to survive (Peng 2012). Currently there are no published reports suggesting non-O157 STEC to have higher heat tolerance than O157 strains (King 2014). Australian regulators estimated heating of raw milk at 62°C for 15 s (the Australian minimum requirement for thermization specific to raw milk cheese) to result in an approximately 2 log decrease of STEC in raw milk (Condron 2009), though a separate report from the agency estimated <1 log to 5 log CFU/ml kill of STEC could be realized depending on the strain present, the type and composition of milk being heated, the number of organisms present, and the source of the reference data used to estimate kill (King 2014).

L. MONOCYTOGENES AND STEC INCIDENCE IN RAW MILK

U.S. federal regulations do not regulate the presence of pathogens in raw milk intended for cheese manufacture, only the presence of pathogens in the resulting cheese (Donnelly 2018). Incidence of *L. monocytogenes* in U.S. raw milk was estimated to range from 0.4 to 16% by

Melo and colleagues, while Salazar and colleagues reported *L. monocytogenes* prevalence ranges of 2.3 to 6.5% in unpasteurized U.S. bulk tank milk (Melo 2015, Salazar 2020). Worldwide, the prevalence of *L. monocytogenes* in raw milk has been estimated to range from 0 to 21.7%, however, reported maximum concentrations of *L. monocytogenes* present in raw milk samples are reportedly low (0.1 to 30 CFU/ml) (Wemmenhove 2019).

Prevalence of STEC in U.S. raw milk has been reported to range from 0 to 3%, while frequencies in Australian and European raw milk samples of 1 to 3% and 0 to 5.7%, respectively, have been reported (King 2014). In a French study, Bibbal and colleagues found 1.8% of dairy cows to harbor STEC strains O157:H7, O103:H2, O26:H11, O145:H28, or O111:H8 (Bibbal 2015). Reports on the concentration of STEC in raw milk are scarce, though levels are reportedly low. A survey of *E. coli* O157 and O26 in bulk raw milk in Italy reported concentrations from <0.3 to 1.4 MPN/ml in positive samples (Trevisani 2013). As STEC growth limits for temperature fall close to those of refrigeration, proper holding temperature of raw milk is critical to prevent STEC growth. Studies have found slow growth of STEC at 7°C (<1 log in 150 hours) while a slow decline in STEC was observed with raw milk held at <5°C (King 2014).

EXISTING META-ANALYSES OF *LISTERIA* SPP. AND *ESCHERICHIA COLI* THERMAL LETHALITY IN DAIRY PRODUCTS

Results from a compilation of existing published thermal death time experiments conducted in whole milk samples for *L. monocytogenes* and STEC are included in Chapters 2 and 3 (see Figures 2-1, 3-5, 3-6, and Tables 2-4, 3-S1, and 3-S2). Predictive models constructed from meta-analyses have been previously published by several authors for *L. monocytogenes* (Mackey 1989, Sorqvist 2003, van Asselt 2006, van Lieverloo 2011, van Lieverloo 2013),

Listeria spp. (Sorqvist 2003), and generic *E. coli* (Sorqvist 2003, van Asselt 2006) (Table 1-3). Limitations in the applicability of these predictive models to the current research objectives (i.e. to determine appropriate thermization treatments in cheesemilk for *L. monocytogenes* and STEC) are discussed in Chapters 2 and 3, with additional considerations given here.

260 data points for *L. monocytogenes* thermal inactivation in dairy were compiled by van Lieverloo and colleagues, however, these were analyzed with 154 data points from 3 other foodstuffs (cabbage and fruit juices, liquid egg, and beef gravy) as well 321 data points taken from microbiological media. Within their model, van Lieverloo and colleagues found milk, cream, and certain microbiological media to differ significantly ($P<0.05$) from 14 other heating menstrua; they did not find butter nor ice cream to differ significantly from the other heating menstrua. Additionally, between 93 data points taken from lab-scale pasteurizers with flow, 211 from low culture volumes in large volumes of pre-heated menstrua, 350 from low culture volumes in submerged glass capillary tubes or coils, and 81 from large culture volumes in glass vials heated in waterbaths, the authors found no significant difference ($P>0.05$) in D-values between heating methodologies (van Lieverloo 2011). Likewise, van Asselt and colleagues compiled 940 data points for *L. monocytogenes* inactivation, however, exact numbers of data points taken from dairy-specific datasets were not published. 22 non-dairy foodstuffs, 1 unknown, and 1 microbiological media dataset were included in their analysis, along with dairy data including butter, cream, ice cream, and milk products. The authors separately analyzed low a_w products (those with 10% NaCl or <0.92 a_w), as these gave statistically different D-values than the compiled dataset (van Asselt 2006). The authors additionally compiled 382 published *E. coli* D- values from dairy products (including butter, cream, ice cream, and milk), 23 non-dairy foodstuffs and microbiological media to construct a predictive model for *E. coli* inactivation by

Table 1-3. Literature-derived D-value equations relevant to dairy for *Listeria* spp. and *Escherichia coli*.

Organism	D-value equation	z-value	Dataset	Reference
L. monocytogenes	$\log D_{\text{all foods, s}} = 10.888 - 0.14519$ temperature (°C)	6.9°C	Unknown number of data points in various foods and microbiological media	Mackey 1989
	$\log D_{\text{milk heated in sealed tubes, s}} = 11.931 - 0.1635$ temperature (°C)	6.1°C	Unknown number of data points in raw or sterile whole, skim, and reconstituted dried milk; cream data omitted	
	$\log D_{\text{milk heated in slug flow heat exchanger, s}} = 10.126 - 0.1348$ temperature (°C)	7.4°C	Unknown number of data points in raw or sterile whole, skim, and reconstituted dried milk; cream data omitted	
E. coli	$\log D_s = 11.6471 - 0.16768$ temperature (°C)	6.0°C	332 data points in various foods and microbiological media	Sorqvist 2003
Listeria spp.	$\log D_{\textit{L. monocytogenes}, s} = 12.3787 - 0.17401$ temperature (°C)	5.7°C	474 data points in various foods and microbiological media	Sorqvist 2003
	$\log D_{\textit{L. innocua}, s} = 14.2559 - 0.20077$ temperature (°C)	5.0°C	36 data points in various foods and microbiological media	
	$\log D_{\textit{L. ivanovii}, \textit{seeligeri}, \text{and } \textit{welshimeri}, s} = 11.3419 - 0.15713$ temperature (°C)	6.4°C	24 data points in milk	

Organism	D-value equation	z-value	Dataset	Reference
E. coli	$\log D_{min}$ = -0.67 - (temperature (°C) - 70°C)/10.6°C	10.6°C	382 data points in various foods and microbiological media	van Asselt 2006
L. monocytogenes	$\log D_{min}$ = -1.06 - (temperature (°C) - 70°C)/7.0°C	7.0°C	940 data points in 22 non-dairy foods, microbiological media, butter, cream, ice cream, and milk	van Asselt 2006
L. monocytogenes	$\log D_{min}$ = 9.01 - 0.157 temperature + 0.167 pH + 0.090 log (sugar (%)) + 0.060 log (fat (%)) + 0.0060 (storage temperature (°C)) - 0.249 log (storage time (h)) ± 0.298	6.37°C	735 data points (260 in dairy, 154 in other foods, 321 in microbiological media)	van Lieverloo 2011
L. monocytogenes	$\log D_{milk\ alone,\ min}$ = 10.0 - 0.162 temperature (°C)	6.16°C	175 data points in milk	van Lieverloo 2013
	$\log D_{all\ fluids,\ min}$ = 9.07 - 0.148 temperature (°C)	6.78°C	807 data points (260 in dairy, 226 in other foods, 321 in microbiological media)	

heating (van Asselt 2006). Sorqvist compiled D-value data from 474 published D-values for *L. monocytogenes*, 32 for *L. innocua*, and 24 for *L. ivanovii*, *L. seeligeri*, and *L. welshimeri,* analyzing the three non-*monocytogenes* species together to form an equation predicting their decrease in milk. The authors additionally compiled 332 data points for *E. coli* thermal

inactivation in foodstuffs and microbiological media, with the exact number of dairy-specific data points not specified (Sorqvist 2003).

FATE OF *L. MONOCYTOGENES* AND STEC IN RAW MILK CHEESES

A significant body of published research on the behavior of *L. monocytogenes* and STEC in cheeses made from raw milk exists. A 10-fold increase in raw milk microorganisms is typically observed during the transition from milk to curd due to entrapment of bacteria in curd (Eugster 2019, Farrokh 2013). Challenge study results are summarized here, broken down by cheese category tested. Due to biosafety constraints, some challenge studies including *L. innocua* and/or generic *E. coli* are included as well. While this project focuses on pathogen control in bovine milk cheeses, caprine and ovine milk cheeses are additionally included, as prior assessments have indicated equivalent pathogen behavior between cheesemilk types with few exceptions (Condron 2009).

MULTIPLE CHEESE VARIETIES TESTED

In a 2020 study, Gerard and colleagues conducted *L. monocytogenes* challenge studies in 32 Belgian farm cheeses. The researchers found the risk of *L. monocytogenes* growth at 7 to 9°C to be low in Belgian fresh cheeses (produced by extended lactic acid acidification with shelf-life 7 to 19 days) if the cheese pH reached ≤5.0 by the end of the production process, regardless of whether the cheeses were made with pasteurized or raw milk. The authors additionally found *L. monocytogenes* populations to decrease in all semi-hard cheeses made from pasteurized milk, however, substantial intra- and inter-batch variation of *L. monocytogenes* growth potential was observed among semi-hard cheeses made with raw milk, with 4 of 9 raw milk semi-hard cheeses

showing >0.5 log CFU/g *L monocytogenes* growth in at least a single batch among triplicates tested (Gerard 2020). A separate study conducted *L. monocytogenes* growth studies in raw milk Belgian homestead cheeses, finding 7 of 8 soft cheeses capable of supporting up to 3 to 5 log CFU/g growth over their shelf-life, with growth attributed to high pH values in the soft cheeses tested. In durability studies testing *L. monocytogenes* behavior during cheese aging in 5 naturally contaminated cheese varieties, the researchers found the pathogen capable of growing in all cheeses with the exception of Feta (FASFC 2019).

Lahou and Uyttendaele inoculated *L. monocytogenes* onto cheese slicing surfaces or the cheese rind of 3 retail soft cheeses (white-molded raw cow's milk cheese, pasteurized cow's milk cheese with spicy herbs, and washed rind pasteurized cow and sheep's milk cheese) and 2 retail semi-hard cheeses (smear-ripened raw cow's milk cheese and natural-ripened raw cow's milk cheese) with growth of *L. monocytogenes* measured after 14 days storage at either 7 or 14ºC. Increased outgrowth of the pathogen was seen with inoculation on the cheese slicing surface more so than on the cheese rind. Before *L. monocytogenes* challenge studies were conducted, the researchers tested 60 retail cheese samples purchased in Belgium for presence and quantification of *L. monocytogenes*, finding approximately 5 log CFU/g *L. monocytogenes* in a white-molded soft-ripened raw milk cheese, a level above the EU limit of 2.0 log CFU/g for RTE foods. Among 16 raw, soft or semi-soft cheeses included in the survey, pH values ranged from 4.16 to 7.47, a_w from 0.9366 to 0.9926, NaCl from 0.58 to 3.67%, and LAB from 5.53 to 9.35 log CFU/g. Among 14 raw, semi-hard cheeses, pH values ranged from 5.13 to 5.98, a_w from 0.9372 to 0.9651, NaCl from 1.17 to 2.83%, and LAB from 6.29 to 9.02 log CFU/g (Lahou 2017).

Falardeau and colleagues investigated the growth of *L. monocytogenes* in raw (n = 4), pasteurized (n = 18), or thermolyzed (n = 1) milk soft ripened cheeses when inoculated at target 3 log CFU/g with storage at 8°C for 14 days. Average increases in *L. monocytogenes* over the storage period were 2.7 log CFU/g. The authors found significantly lower levels of *L. monocytogenes* growth in raw milk cheeses compared to pasteurized milk cheeses, averaging 1.5 and 2.9 log CFU/g *L. monocytogenes* growth, respectively; however, the lowest levels of *L. monocytogenes* growth were observed in two pasteurized milk cheeses. The authors found pH and a_w to be insignificantly correlated with *L. monocytogenes* growth. Additionally, alpha-diversity including microbial richness and evenness as measured by 16s rRNA sequencing of the V4 region of sample DNA were insignificantly correlated with *L. monocytogenes* growth (Falardeau 2020).

Masoud and colleagues monitored bacterial species through the use of pyrosequencing and qRT-PCR in Danish raw milk cheeses inoculated with non-pathogenic *E. coli* into the cheesemilk. The authors found *E. coli* populations in cheeses to be affected by cheese cooking temperature and consequent acidification rate (i.e. 39°C cook with 8 h acidification or 50°C cook with 4 h acidification) but not by ripening starter cultures used or the indigenous raw milk microbiota. The authors found *Lc. lactis*, *Str. thermophilus*, *Lb. casei*, and *Lb. rhamnosus* to be the main bacterial populations in raw milk and raw milk cheeses studied (Masoud 2012).

Ramsaran and colleagues manufactured raw milk Feta and Camembert cheeses using a commercial *Lc. lactis* subsp. *lactis* starter culture with or without the addition of a nisin-producing *Lc. lactis* subsp. *lactis* adjunct culture to assess the cheeses' ability to allow *L. monocytogenes* or *E. coli* O157:H7 growth when inoculated together into cheesemilk at a level of 10^4 CFU/ml. The researchers inoculated milk with 10^4 CFU/ml of a bioluminescent strain of

L. monocytogenes and *E. coli* O157:H7 and 1 to 2% (v/v) of the *Lc. lactis* subsp. *lactis* mesophilic starter culture and 3% of the nisin-producing adjunct before storing cheeses for 65 to 75 days at 2°C. Growth of the starter culture and concomitant decrease in cheese pH was noted to be inhibited in the presence of the nisin-producing adjunct, however, overall LAB counts were found not to significantly differ between treatment groups. The initial pH of raw milk Feta was approximately 5.1, rising to 5.25 over 75 days storage. *L. monocytogenes* numbers remained near approximately 5.75 log CFU/g throughout the 75-day storage period in raw milk Feta. Conversely, in pasteurized milk Feta manufactured with the starter culture only, *L. monocytogenes* numbers increased from approximately 6.00 log CFU/g at day 1 to 6.45 log CFU/g at day 55. In Camembert cheese, the authors found *L. monocytogenes* to increase 1.84, 1.82, and 2.08 log CFU/g from initial counts by day 65 in raw milk Camembert manufactured with starter only, raw milk Camembert manufactured with starter and nisin-producing *Lc. lactis* subsp. *lactis*, and pasteurized milk Camembert manufactured with starter only, respectively. The authors additionally noted that *L. monocytogenes* populations were approximately 1 log CFU/ higher at the surface of the tested Camembert samples versus in the interior of the cheeses, which they attributed to higher pH due to mold growth on the surface of the cheeses. *E. coli* O157:H7 was found to initially increase in all tested Feta cheeses until a minimum pH value was reached after 10 days' storage. *E. coli* O157:H7 counts declined by approximately 2 log CFU/g by day 20 in all cheeses, however, the authors found no significant decline thereafter, with *E. coli* O157:H7 counts at 55 to 75 days not differing statistically to those observed at 20 days' storage. Increases of approximately 2 log CFU/g *E. coli* O157:H7 were observed in all tested Camembert cheeses 24 hours after manufacture, followed by a general decrease in *E. coli* O157:H7 numbers. *E. coli* O157:H7 counts were found to be 0.80, 0.85, and 0.78 log CFU/g higher than initial inoculum

counts in raw milk Camembert manufactured with starter only, raw milk Camembert manufactured with starter and nisin-producing *Lc. lactis* subsp. *lactis*, and pasteurized milk Camembert manufactured with starter only, respectively, at the end of storage (Ramsaran 1998).

SWISS-STYLE CHEESES

Peng and colleagues studied the fate of 2 generic *E. coli* and 3 STEC strains during the manufacture and ripening of raw milk Swiss semi-hard cheese when inoculated individually into cheesemilk at target 1 or 3 log CFU/ml. The authors observed an increase in *E. coli* of 3.5 log CFU/g from raw milk to fresh cheese at day 1, attributed to concentration in the curd as well as growth during manufacture. During 16 weeks ripening, the authors observed log-linear declines in *E. coli* numbers, with increased survival observed in the generic *E. coli* versus the tested STEC strains. Following ripening, the authors found 6 and 13 of 16 total cheeses inoculated with either 1 or 3 log CFU/ml STEC, respectively, to contain >10 CFU/g of STEC; additionally, STEC detection following enrichment procedures was possible in almost all cheeses (Peng 2013).

In a separate study, Peng and colleagues inoculated cheesemilk with individual low (2 log CFU/ml target) and high (4 log CFU/ml target) inocula of generic *E. coli* strains of either high or low thermotolerance before Gruyere- (50.0% moisture, 1.6% salt, pH 5.6) and Appenzeller-type (52.1% moisture, 1.8% salt, rind pH 5.8, core pH 6.1) cheese manufacture. *E. coli* levels decreased in a log-linear fashion in the rind and core of Appenzeller-type cheese, with faster inactivation observed in core than rind samples and of heat-sensitive *E. coli* than heat-resistant. At the end of Appenzeller-type ripening, the authors found *E.coli* to be present at ≥1.3 log CFU/g in rind samples and detectable via enrichment in core samples. Conversely, *E. coli* were

quickly inactivated during Gruyere-type cheese manufacture, which was attributed to the higher cooking temperature used (Peng 2013b). Likewise, Ercolini and colleagues found curd cooking at 55°C for 20 min to result in a 1.1 log CFU/g reduction in *E. coli* O157:H7 in inoculated Grana cheese; however, no observed reduction was observed following the same treatment in Grana inoculated with *L. monocytogenes* (Ercolini 2005).

Bachmann and Spahr manufactured Tilsiter-type semi-hard cheese (day 1 pH 5.21, day 90 pH 5.78, 39.3% moisture, and 1.23% salt) from raw milk inoculated with target 4 to 6 log CFU/ml *Aeromonas hydrophila*, *Campylobacter jejuni*, generic *E. coli*, *L. monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella* spp., *S. aureus*, or *Yersinia enterocolitica*. The cheese, manufactured with a commercial lactic starter, was salted in 20% (w/v) brine and subsequently ripened at 11 to 13°C for 90 days following manufacture. At the age of commercial ripeness (90 days), the cheese was absent of each pathogen and its associated toxic metabolites with the exception of *L. monocytogenes*, which endured the manufacturing and ripening process and decreased <1 log CFU/g to approximately 3.5 log CFU/g in the cheese interior. The authors additionally noted extensive *L. monocytogenes* growth on the cheese surface, though *L. monocytogenes* surface population data was not reported (Bachmann 1995).

GOUDA, CHEDDAR, AND COLBY CHEESES

D'Amico and colleagues found 60 days aging to be insufficient in eliminating *E. coli* O157:H7 from raw milk Cheddar and Gouda when inoculated into cheesemilk at ~20 CFU/ml. The authors found changes in pathogen level observed during manufacture and aging to be insignificantly different between the two cheese varieties, with levels of *E. coli* O157:H7 reaching 25 and 5 CFU/g for Cheddar and Gouda, respectively, after 60 days aging and

remaining detectable by enrichment >270 days post-manufacture in both varieties (D'Amico 2010). The 60-day aging rule was additionally rebutted by Reitsma and colleagues in 1996, who found survival of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* Typhimurium beyond 60 days in unpasteurized milk Cheddar samples inoculated with each pathogen (Reitsma 1996).

Salazar and colleagues inoculated raw milk with 1 or 3 log CFU/ml *L. monocytogenes* or 1 log CFU/ml *E. coli* O157:H7 before manufacturing laboratory or pilot-plant-scale Gouda with storage at 10°C for up to 163 days. *L. monocytogenes*, when inoculated at a level of 1 log CFU/ml into cheesemilk, was found to survive ≥60 days aging cheese manufacture and aging in 4 of 5 trials, while 1 log CFU/ml *E. coli* O157:H7 survived ≥60 days in a single of 5 trials (Salazar 2020).

Lee and colleagues inoculated raw and pasteurized milk with one of three *L. monocytogenes* strains at a target of 3 to 4 log CFU/ml and 0.01% (w/v) of a mesophilic starter (*Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*) in order to manufacture 6 experimental Cheddar cheeses, with ripening conducted at 13 to 15°C. Significant *L. monocytogenes* reductions of approximately 1 log CFU/g were observed in 50 to 90 days and 25 to 55 days of ripening in raw and pasteurized milk cheeses, respectively. *L. monocytogenes* populations fell below detection limits in 120 to 160 days and 120 to 130 days in raw and pasteurized milk cheeses, respectively. The authors observed higher microbial richness in raw milk Cheddars (17 phylum, 196 genus, 312 species identified) than their pasteurized counterparts (12 phylum, 162 genus, 256 species identified). The authors additionally found the ratio of organic acid-producing bacteria (*Lactobacillus* spp., *Lactococcus* spp., *Streptococcus* spp., *Bifidobacterium* spp., and *Roseomonas* spp.) to be higher in pasteurized cheesemilk versus raw cheesemilk (6.5% and

4.8%, respectively) and attributed the observed differences in *L. monocytogenes* survival to this observation (Lee 2020).

Kornacki and colleagues inoculated pasteurized cheesemilk with 2 to 3 log CFU/ml enteropathogenic *E. coli* before manufacture of Colby-like cheeses. A single lot of cheese supported survival of 10^3 CFU/g for 12 weeks storage at 10°C. The authors additionally found high moisture, high pH lots of Colby to support growth of the organism up to levels $\geq 10^8$ CFU/g, with the organism remaining at this high level over 12 weeks storage (Kornacki 1982).

OTHER RAW MILK CHEESES

Perrin and colleagues inoculated raw milk with target 2 log CFU/ml of STEC O157:H7, O26:H11, O103:H2, or O145:H28 before manufacturing raw milk soft cheese using starter culture, added just after STEC inoculation. Following cheese manufacture, cheeses were ripened at 4°C for 14 days before subsequent storage at 8°C for 28 days. 24 hours post cheese manufacture, the authors found STEC populations to have risen to 4 to 5 log CFU/g before steadily decreasing during ripening and storage. The authors found *E. coli* O157:H7 to decrease faster than serotypes O26:H11, O103:H2, or O145:H28 during cheese ripening, while no statistically significant differences were observed among non-O157:H7 STEC-inoculated cheeses. Probability of survival in inoculated cheeses following 2 weeks ripening for serotypes O157:H7, O26:H11, O103:H2, and O145:H28 were found to be 1%, 34%, 37%, and 27%, respectively (Perrin 2015). Likewise, Miszczycha and colleagues found *E. coli* O157:H7 to grow and survive to a lesser extent in 4 cheese varieties inoculated with serotypes O157:H7, O26:H11, O103:H2, or O145:H28 (Miszczycha 2013).

Morgan and colleagues inoculated soft lactic cheeses made with raw goat milk and starter culture with *L. monocytogenes* via inoculation into the cheese milk before manufacture then storage at 2ºC for 42 days. The interior of the cheese was found to harbor approximately 3.3 log CFU/g *L. monocytogenes* on the day of manufacture, decreasing steadily to 1.5 log CFU/g by day 28 and remaining at this level until the study's end. Approximately 2.0 log CFU/g *L. monocytogenes* was recovered from the cheese surface on day 7, decreasing to 1.5 log CFU/g by day 14 and remaining at this level for the remainder of the study. Starting cheese pH (both interior and surface) was found to be 4.25. Interior cheese pH rose to 4.49 by day 14 and continually rose to a final 42-day pH of 5.23. By day 14, surface pH had risen to 5.71 and continued to rise to a maximum pH of 6.52 by day 42. Initial cheese moisture was found to be 60.9%, increasing slightly to 61.1% by day 7 and then steadily decreasing to 40.2% by day 42. Salt content of the cheese began at 0.06%, increased to 1.10% by day 7, was found to be 1.02% on day 21, and 0.92% by day 42 (Morgan 2001).

Margolles and others investigated the behavior of *L. monocytogenes* in Afuega'l Pitu cheese, an artisanal raw milk acid-coagulated Spanish soft cheese with an associated shelf-life of 3 to 30 days. Calf rennet and starter cultures (*Lc. lactic* subsp. *lactis*, *Lc. lactic* subsp. *lactis* bv. diacetylactis, and *Leuconostoc citreum*) were used to coagulate whole milk to an acidity of 0.716% by the end of the 16-h coagulation process. The authors found the pathogen unable to grow under refrigerated storage (5 to 6°C) over 7 days, which was attributed to the cheese's low pH value of ~4.10. The studied cheese was found to have a moisture content of 72.86% at day 2 after manufacture and salting, which fell to 58.15% (surface) and 61.89% (interior) after 7 days of ripening, and salt-in-moisture levels of 1.59% (surface) and 0.52% (interior) changed to 1.31% (surface) and 1.11% (interior) within the same interval (Margolles 1997).

Pinto and colleagues investigated the survival of *L. innocua* in Minas traditional Serro cheese, a Brazilian raw milk cheese manufactured with natural starter from the previous day's cheese, over 60 days ripening at 30ºC. The authors inoculated cheesemilk with 1, 2, or 3 log CFU/ml of a single strain of *L. innocua*, finding it unable to grow but to survive over the study period with observed reductions ranging from 1.07 to 1.66 log CFU/g. Initial pH value of cheeses were found to be 4.90, dropping to approximately 4.45 before rising to 4.85 during ripening. Initial salt levels were approximately 1.4 to 1.6% and rose to 2.3 to 2.4% by the end of storage (Pinto 2009).

Theodoridis and colleagues investigated the Greek cheese Chevre Metsovo for its ability to support the growth of *L. monocytogenes* during cheesemaking and ripening at 17ºC. Chevre Metsovo was manufactured using a mixture of pasteurized and raw goat's milk. Moisture of the cheese was found to be 35.51%, salt 3.01%, and pH at 90 and 120 days post-ripening of 5.18 and 5.16, respectively. Inactivation of *L. monocytogenes* was not observed, though no growth or a steady decline in numbers of *L. monocytogenes* was realized after an initial pH drop due to starter culture activity (Theodoridis 2006).

In studies investigating *L. monocytogenes* behavior during manufacture and ripening of laboratory-scale model raw and pasteurized milk cheeses, Schvartzman and colleagues inoculated 2.7 log CFU/ml *L. monocytogenes* and 0.02% commercial starter (*Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis* bv. diacetylactis, and *Leuc. mesenteroides* subsp. *cremoris*) into raw or pasteurized cheesemilk. The authors found 0.8 ppm L-lactic acid in pasteurized milk and 1 ppm L-lactic acid in raw milk, and observed raw cheesemilk to contain approximately 3 log CFU/ml higher background flora. This higher level of flora limited the growth and L-lactic acid production of starter bacteria in raw model cheeses and

lead to higher pH values throughout manufacture and ripening. At the end of cheesemaking (approximately 5 h), the authors observed L-lactic acid levels of 160 ppm and 70 ppm in pasteurized and raw milk cheeses, respectively. The authors observed no *L. monocytogenes* growth during cheesemaking in the raw milk model cheese, however, observed approximately 2 log CFU/g *L. monocytogenes* growth to a level of approximately 5.5 log CFU/g during cheesemaking in the pasteurized milk model cheese. Over the cheese ripening period, the authors observed approximately 2 log CFU/g *L. monocytogenes* growth to occur in the raw milk model cheese, while *L. monocytogenes* inactivation was observed in the pasteurized milk model cheese (Schvartzman 2011b).

FATE OF *L. MONOCYTOGENES* IN FRESH SOFT CHEESES

L. monocytogenes has been extensively studied for its ability to grow and/or survive in a variety of high-moisture cheese varieties relevant to Chapters 4 and 5 in this project. As *L. monocytogenes* represents a much larger post-processing risk than STEC in high moisture, low acid cheeses made with pasteurized milk (Ibarra-Sanchez 2017, Langer 2012), *L. monocytogenes* control measures only were focused on in this dissertation. The point in time of contamination (i.e. into cheesemilk or onto curd) was found to be insignificant ($p = 0.33$) in predicting *L. monocytogenes* growth in an analysis of 1103 cheese samples inoculated with the pathogen compiled from published studies; i.e. if cheese formulation was supportive of *L. monocytogenes* growth to high levels, the point at which *L. monocytogenes* was introduced into the sample was inconsequential, controlling for the approximate 10-fold increase realized in *L. monocytogenes* numbers in the transition from milk to curd if inoculated into the milk (Engstrom 2012, Appendix 1). Therefore, results from challenge studies in which *L. monocytogenes* was

inoculated into cheesemilk or onto cheese as a post-processing contaminant are included. Challenge study results are summarized here, categorized by cheese variety tested. Due to biosafety constraints, some challenge studies including *L. innocua* are included as well. Additionally, though this project focuses on pathogen control in bovine milk cheeses, caprine and ovine milk cheeses are included in data summaries.

MULTIPLE CHEESE VARIETIES TESTED

Cataldo and colleagues challenged retail Italian-style soft cheeses made with bovine milk for their ability to support the growth of a single strain of *L. monocytogenes* when acid-adapted (strain grown in Brain Heart Infusion broth [BHIB] adjusted with lactic acid to pH 5.1) or non-acid-adapted (strain grown in BHIB) before inoculation into cheeses at approximately 5.5 log CFU/g. Cheeses were stored at 4°C for 2 weeks, with enumeration of *L. monocytogenes* on days 1, 7, and 14. Non-acid adapted *L. monocytogenes* grew from an initial level of 5.5 log CFU/g after 1 day to 6.5 log CFU/g at 7 days before declining to 3.6 log CFU/g after 14 days storage at 4ºC in unripened stirred-curd mozzarella (pH 5.4 to 6.0, 3.0 to 4.5% NaCl), while cell numbers were 5.6, 6.6, and 4.0 log CFU/g, on days 1, 7, and 14, respectively, using the acid-adapted culture. In Crescenza (pH 5.0 to 5.6, 4.5 to 10% NaCl), non acid-adapted *L. monocytogenes* reduced from 4.8 to 4.0 to 4.0 log CFU/g, on days 1, 7, and 14, while the acid-adapted pathogen showed substantial growth (5.0 to 6.9 to 8.0 log CFU/g on days 1, 7, and 14). Ricotta (pH 6.2 to 6.5, 0.5 to 1.5% NaCl) showed substantial growth with both acid-adapted (5.5 to 8.0 to 4.0 log CFU/g on days 1, 7, and 14) and non acid-adapted cells (5.5 to 8.5 to 3.5 log CFU/g on days 1, 7, and 14). Moisture contents for the tested cheeses were not given (Cataldo 2007).

Genigeorgis and colleagues challenged market cheeses with *L. monocytogenes*, assessing their ability to support growth and/or survival of the pathogen at 4, 8, and 30ºC. The authors purchased 49 market cheeses representing 24 types and 28 brands and surface inoculated cheeses with approximately 4 log CFU/g cells of a 5-strain cocktail of *L. monocytogenes* before storing cheeses aerobically. Time 0 aerobic plate counts (APC) of samples were additionally tested in uninoculated samples. The authors found a highly significant effect of pH >5.5 and absence of starter culture to be correlated with *L. monocytogenes* growth. The authors found only 1 of 7 Hispanic-style soft cheeses to declare lactic acid starter on its label, and this cheese, like the other 6 Hispanic-style soft cheeses tested, supported growth of *L. monocytogenes*. 3 of 3 ricotta samples tested contained vinegar and were able to support growth of *L. monocytogenes* at all test temperatures. Ricotta was deemed the most supportive cheese of *L. monocytogenes* growth among varieties tested, with its low brine (<0.7%; brine % = [%NaCl/(water + %NaCl)]x100)), relatively high pH (5.9 to 6.1), and low level of competitive microflora (<2.0 log CFU/g) attributed to this designation. Three queso fresco samples were tested with the following characteristics: 1) pH 6.6, 6.6% brine, 6.34 log CFU/g APC; 2) pH 6.6, 4.5% brine, 6.80 log CFU/g APC; and 3) pH 6.5, 6.15% brine, 6.00 log CFU/g APC. Each supported growth at 30ºC, however, the first two showed modest declines at 4 and 8ºC while sample 3 grew at all three test temperatures. A queso ranchero sample (pH 6.2, 4.1% brine, 7.79 log CFU/g APC) showed growth at all three temperatures as well. Three queso panella samples were tested (pH 6.2 to 6.7, 2.5 to 3.95% brine, 6.4 to 7.87 log CFU/g APC), with each supporting growth of the pathogen under all test temperatures. Three ricotta samples were tested (pH 5.9 to 6.1, <0.6 to <0.7% brine, <2.0 log CFU/g APC), with one containing potassium sorbate and vinegar and the other 2 only vinegar. All 3 ricottas tested supported growth of *L. monocytogenes* at all three test

temperatures Five cottage cheese samples were tested, with a single cottage cheese containing acetic acid (pH 5.0, 1.16% brine, 5.0 log CFU/g APC, no starter culture) found unsupportive of growth at any of the three test temperatures. The other 4 samples had characteristics as follows: 1) pH 4.9, 1.00% brine, 6.41 log CFU/g APC, starter culture added; 2) pH 5.0, 1.04% brine, 7.12 log CFU/g APC, starter culture added; 3) pH 5.0, 1.14% brine, 6.21 log CFU/g APC, no starter culture; and 4) pH 5.1, 1.13% brine, 3.0 log CFU/g APC, no starter culture. Sample 1 was found to support growth of *L. monocytogenes* at 4 and 8ºC, while causing decline of the pathogen at 30ºC. Sample 2 supported growth only at 4ºC, sample 3 at 4 and 8ºC, and sample 4 supported growth at all three test temperatures, even with a lower inoculum level of 2.11 log CFU/g *L. monocytogenes*. The authors noted that an opened cottage cheese package contaminated with *L. monocytogenes* could reach a 1-log CFU/g growth threshold within the shelf-life of the product (assuming 15 to 20 days). Among cheeses unable to support growth of *L. monocytogenes*, cream cheese, Feta, and kasseri soft cheeses were included. The cream cheese tested had the following parameters: pH 4.8, <0.9% brine, and 3.3 log CFU/g APC. Imported Fetas had pH values 4.3 and 4.2, brines of 7.0 and 7.4%, and 7.12 and 7.07 log CFU/g APC. Domestic Fetas had pH values of 4.3 and 4.3, brines of 7.5 and 2.2%, and 5.0 and 7.14 log CFU/g APC. Imported Kasseri had a pH of 5.3, brine of 5.52%, and 7.19 log CFU/g APC, while domestic Kasseri had a pH of 4.8, 5.8% brine, and 5.25 log CFU/g APC. All cream, Feta, and kasseri cheeses tested had starter culture added and reduced *L. monocytogenes* numbers by ≥2.04 log CFU/g during storage (Genigeorgis 1991).

QUESO FRESCO

Leggett and colleagues monitored the growth and survival of *L. monocytogenes* in vacuum-packaged queso fresco inoculated into curd or onto slices with approximately 3 log CFU/g of a 5-strain cocktail of *L. monocytogenes* and stored at 4 and 10°C for up to 35 days. The researchers additionally monitored cheese pH over 41 days storage at both temperatures. Average composition for the authors' tested cheeses were 55.8% moisture, 3.15% lactose, 1.67% NaCl, and a_w 0.983. pH in cheese stored at 4°C was found to be 6.75 on day 1 and 6.62 on day 41, while pH in cheese stored at 10°C was found to be 6.56 on day 1 and 5.77 on day 41. APC was measured to be 3.54 and 3.37 log CFU/g after 1 day storage of cheeses at 4°C and 10°C, respectively. By day 28, APC had risen to 4.0 log CFU/g at 4°C and 8.07 log CFU/g at 10°C. Additionally, yeast and mold were detected in cheeses stored at 4°C after 28 days and after 56 days in cheeses stored at 10°C. The authors also tested whether differences between inoculation into the cheese curd vs. onto the slice surface of the manufactured queso fresco made an observable difference in *L. monocytogenes*. They found no significant difference ($P > 0.05$) from days 6 through 35. However, they did find *L. monocytogenes* counts to be consistently lower in cheeses stored at 4°C versus 10°C for 12 days of storage, regardless of inoculation method. By day 20 of storage, however, no difference was detectable, indicating refrigeration as a sole antilisterial measure to be ineffective in preventing outgrowth of the pathogen (Leggett 2012).

Lin and colleagues inoculated commercial queso fresco samples with 10 to 100 CFU/g of a 5-strain *L. monocytogenes* cocktail before storage at 4, 12, or 21ºC. The authors measured *Listeria* counts up to 12 weeks post-inoculation with both enrichment and enumeration. Growth was observed in all samples at all test temperatures, regardless of sampling site (surface, interior, or exudate) and inoculum level; however, higher *L. monocytogenes* counts were obtained from

surface and interior cheese samples than from exudate. pH value of the tested cheese was 6.2 to 6.6 initially (Lin 2006).

Holle and colleagues surveyed 64 retail samples of U.S.-produced queso fresco with pH values 6.62 to 6.86, moisture 43.90 to 54.50%, and salt 1.53 to 2.01%. A single cheese from the survey was labeled as containing active starter culture. The authors inoculated 8 surveyed samples with *L. monocytogenes* before storage at 4ºC for 14 days, with ≥2.5 log CFU/g *L. monocytogenes* growth observed in all tested cheeses over the storage period (Holle 2018).

Hariram and colleagues inoculated 8 commercial queso fresco samples with 2 to 3 log CFU/g *L. monocytogenes*. Inoculated cheeses were stored at 4°C or 7°C for 6 to 9 months. Cheeses showing >1 log CFU/g *L. monocytogenes* growth over the storage period included a single control cheese (pH 5.50, 52.3% moisture and stored at 4°C with an expected shelf-life of 3 months) and cheese containing 0.1% sorbic acid (pH 5.50, 56.8% moisture and stored at 4°C with an expected shelf-life of 8 months). Cheeses showing <1 log CFU/g *L. monocytogenes* growth ranged in pH from 5.15 to 5.85 and moisture from 48.4% to 57.7%. The authors noted that the use of starter cultures, maintaining target analyticals (pH 5.25 and moisture 49.6%), and/or application of 0.2% sorbic acid prevented the growth of *L. monocytogenes* in the tested queso fresco samples (Hariram 2020).

Lourenco and colleagues investigated GRAS antimicrobial treatments for their ability to control *L. monocytogenes* in queso fresco. Caprylic acid (0.29%), Nisaplin (0.05%), a sodium lactate (3%)/sodium diacetate (0.22%) mixture, a *Lc. lactis* subsp. *lactis* strain, monolaurin, and lactic acid (1.2%) were included. Queso fresco curds (pH 6.0) were inoculated with 4 log CFU/g *L. monocytogenes* and stored 3 weeks at 4ºC. The authors combined antimicrobial ingredients (individually and in mixtures) to 2.3 kg portions of manufactured cheese curds before hand-

mixing for 2 min in sealed bags. The bags were then inoculated with 4 log CFU/g *L. monocytogenes*, curds molded, and allowed to drain an additional 2 hours. Portions of queso fresco were then bagged and stored at 4ºC for up to 21 days. Control samples reached levels of approximately 7 to 8 log CFU/g over storage. Caprylic acid reduced the cheese pH from 6.0 to 5.8 while no change in pH was observed with the addition of Nisaplin. Both individually showed *L. monocytogenes* inhibition, with caprylic acid extending the lag phase until *L. monocytogenes* growth was observed to approximately 11 days while Nisaplin treatment caused an immediate decline in *L. monocytogenes* with recovery to inoculum levels by day 10. Nisaplin and caprylic acid caused >3 and >5 log CFU/g reduction in *L. monocytogenes* final counts, respectively, while all other individual treatments resulted in <1 log CFU/g reductions in final counts. Mixtures were more effective, with Nisaplin + caprylic acid delivering 6 log CFU/g reduction in *L. monocytogenes* final counts versus in control cheese. A >4 log CFU/g reduction in *L. monocytogenes* final counts versus controls was observed in queso fresco treated with lactic acid + sodium lactate/sodium diacetate. Sensory analysis of Nisaplin and caprylic acid treatments showed modest dislike versus untreated commercial queso fresco, while lactate/diacetate treatment resulted in a vinegar odor (Lourenco 2017).

OTHER HISPANIC-STYLE CHEESES

Naldini and colleagues inoculated Minas Frescal cheese, a Brazilian high-moisture cheese made either with a lactic culture or via direct acidification, with 4 to 5 log CFU/g *L. monocytogenes* before storage for 25 days at 5 or 10°C. The researchers added $CaCl_2$ and either 1% of a mesophilic, *Lc. lactis* subsp. *lactis* / *Lc. lactis* subsp. *cremoris* starter or 0.025% lactic acid to the cheesemilk before adding chymosin to coagulate the milk. Curd was salted with brine

at 2% (w/w milk). Cheese moisture contents ranged from 64.1% in the directly acidified cheeses to 65.8% in the lactic cultured cheeses. pH values for directly acidified were higher, at approximately 6.5 on day 1 and decreasing to 5.4 by the end of storage at 5°C. Meanwhile, pH in cheeses made using lactic cultures were found to be approximately 5.3 on day 1 and decreased to 4.6 by the end of storage at 5°C. *L. monocytogenes* in directly acidified cheese stored at 5°C increased from approximately 5.0 log CFU/g on day 1 to >7.0 log CFU/g by day 12 and a final value of 7.5 log CFU/g by the end of storage. In lactic cultured cheeses stored at 5°C, *L. monocytogenes* numbers rose from approximately 4.2 log CFU/g on day 1 to a maximum value of 4.7 log CFU/g on day 6 and remaining below this level through the end of the storage to a final value of 4.5 log CFU/g. Meanwhile LAB counts within the affected cheeses were noticeably lower in the directly acidified cheeses, at a starting level of approximately 5.5 log CFU/g and increasing to 7.5 log CF/g over the study, while in lactic cultured cheeses LAB counts remained near 8.6 log CFU/g for the duration of the study. With storage at 10ºC, *L. monocytogenes* numbers again rose to higher levels in the directly acidified cheese versus cheese made with the lactic culture, increasing 1.76 log CFU/g in the first 6 days of storage with an associated drop in pH from 6.39 to 5.46. Cheese made with lactic culture was unable to support growth of *L. monocytogenes* over the 25-day study and had a lower pH of approximately 5.4 which was reduced to 4.7 by day 25. LAB numbers in the lactic culture cheese remained near 9.0 log CFU/g throughout the study, while LAB in the directly acidified cheese increased from approximately 6.3 log CFU/g on day 1 to 7.8 log CFU/g by the end of the study (Naldini 2009). Pingitore and colleagues found Minas cheese manufactured with lactic acid and rennet able to support growth of *L. monocytogenes* from approximately 3.50 to 8.17 log CFU/g when stored at 8 to 10ºC for 12 days. LAB numbers were reported to remain near 1.00 log CFU/g throughout

storage in uninoculated control cheese. No measured pH or moisture data were given for the studied cheeses, however, a salt content of 2% added to the cheese curd was noted as well as a typical pH >5.0 reported for fresh Minas cheese (Pingitore 2012).

Solano-Lopez and Hernandez-Sanchez investigated the behavior of *L. monocytogenes* in the Mexican cheeses Manchego and Chihuahua. Mexican Manchego (unrelated to Spanish Manchego) cheese is a soft, 5-day ripened cheese made from pasteurized cow's milk, while Chihuahua is a semi-hard, 40-day ripened cheese made from pasteurized cow's milk and includes a cheddaring step in manufacture. The authors inoculated commercial pasteurized whole milk with 6.30 to 6.95 log CFU/ml of a single strain of *L. monocytogenes* before adding 1% (v/v) of a commercial lactic starter culture followed by $CaCl_2$ and rennet. Manchego cheese was ripened for 5 days and Chihuahua cheese for 6 weeks at 12ºC with 85% RH. No significant decrease in *L. monocytogenes* numbers was observed for either cheese over the ripening period. The cheddaring process employed in Chihuahua decreased the *L. monocytogenes* population an additional approximate 0.5 log CFU/g compared to the Manchego cheese, which the authors attributed to loss in the whey from cheddaring. Moisture, pH, lactic acid, and chloride levels in Manchego cheese were found to be 42.1%, 5.4, 1.06%, and 2.9%, respectively, while values in Chihuahua were found to be 36.4%, 5.8, 1.16%, and 2.9%, respectively (Solano-Lopez 2000).

COTTAGE CHEESE

Hicks and Lund inoculated three creamed cottage cheeses with approximately 4.6 log CFU/g of a single strain of *L. monocytogenes* before storing samples at 4, 8, or 12ºC for 14 days. In cheese 1, pH value was found to be 5.06 at time 0, with a final pH of 5.00 observed after 14 days at 4ºC. Titratable acidity began at 31.6% and reduced to 29.0%, while the lactic acid (0.22%

w/w) and citric acid (0.12% w/w) contents remained the same over storage at the same test temperature. In cheese 2, the pH remained relatively stable between time 0 and 14 days at 4ºC, changing from 4.69 to 4.67. Over the same period, titratable acidity rose from 48.4 to 48.7%, lactic acid from 0.38 to 0.76%, and citric acid from 0.07 to 0.19%. In cheese 3, the pH dropped from 4.75 to 4.60, while titratable acidity increased from 42.6 to 47.0%, lactic acid reduced from 0.63 to 0.47%, and citric acid decreased from 0.11 to 0.08%. *L. monocytogenes* was unable to grow in any of the challenged cheeses, with inactivation increased with decreasing pH and increasing lactic acid content. The authors additionally found that the number of LAB remained constant (approximately 7 log CFU/g) over storage at 4ºC in all samples (Hicks 1991).

El-Ziney and Debevere investigated the ability of creamed cottage cheese to support growth of *L. monocytogenes*. Creamed cottage cheese with pH 5.4, 74.5% moisture, 0.5% NaCl, and 0.28% lactic acid was found to support 0.4 log CFU/g growth (from an initial inoculum of 5 to 6 log CFU/g) over 21 days storage at 7ºC (El-Ziney 1998). Moir and colleagues investigated the ability of commercial creamed cottage cheese (pH 4.9 and 80 to 86% moisture) to support the growth of *L. monocytogenes* under an atmosphere of 40% (v/v) CO_2. The authors found *L. monocytogenes* unable to grow above initial counts of approximately 2.1 log CFU/g over the course of 30 days storage at 15ºC regardless of packaging method (air or 40% CO_2). APC of uninoculated cheeses was found to increase from approximately 6.0 log CFU/g at 5 days to 8.0 log CFU/g at 12 days, remaining at this elevated level through the 30-day study (Moir 1993).

Ryser and colleagues investigated the behavior of *L. monocytogenes* when introduced into the manufacturing process of cottage cheese. The authors manufactured the cheese via a short-set methodology in pilot-plant sized vats, using pasteurized skim milk fortified to 12% total solids with non-fat dry milk (NFDM) inoculated to contain 4 to 5 log CFU/ml of a single strain

of *L. monocytogenes* (Scott A or V7). Half of each batch was creamed while the other half remained uncreamed. 2 commercial starter cultures, both *Lc. lactis* subsp. *cremoris*, were used individually in cheese manufacture at a level of 5% (w/w) in replicate trials. Following manufacture and inoculation, samples were stored at 3ºC. Moisture contents ranged from 80.3 to 81.8% in creamed samples and 82.3 to 83.2% in uncreamed samples with respective fat levels of 4.0 to 4.5% and 0.4 to 0.8%. Moisture levels in both cheese types exceeded the CFR limit of 80% specified for cottage cheese. pH values of creamed cottage cheese at day 3 were 5.43, 5.32, 5.45, and 5.40 in trials 1 through 4, respectively, and at day 28, 5.26, 5.30, 5.02, and 5.25. pH values of uncreamed cottage cheese at day 3 were 5.18, 5.13, 5.22, and 5.12 in trials 1 through 4, respectively, and at day 28, 5.41, 5.68, 5.15, and 5.36. Following cooking of the curd (increasing temperature from 32.2°C to 57.2°C in 30 min), *L. monocytogenes* numbers reduced to below detectable limits (10 or 100 CFU/g) and remained at these levels for the remaining manufacturing steps. However, *L. monocytogenes* was not completely eliminated from the cheese, with 40 and 50% recovery observed via cold enrichment in 2 of the 4 trials. Regardless of whether the cheese was creamed or uncreamed, the pathogen was observed to survive the entire cheesemaking process in each trial, as counts were recovered from samples stored at 3ºC. Creamed cottage cheese samples recovered *L. monocytogenes* more often than uncreamed samples when stored at 3ºC, with recovery in numbers up to 110 CFU/g and 20 CFU/g, respectively, following 28 days storage (Ryser 1985).

Piccinin and Shelef researched the behavior of *L. monocytogenes* in commercial cottage cheese made with and without sorbate when inoculated at a level of 10^3 CFU/g with storage at 5, 10, or 20ºC. pH values for cheese made with sorbate and stored at 5ºC were 4.99 to 5.22 and 4.86 to 5.18 at 0 and 24 days, respectively, and for cheese made without sorbate 4.83 to 5.00 and 4.67

to 4.86 at 0 and 24 days, respectively. No *L. monocytogenes* growth was observed in any of the samples at temperatures of 5, 10, or 20ºC, and changes of -1.52 to -0.08 and -0.54 to +0.06 log CFU/g were observed in the cheeses made with and without sorbate, respectively, after 24 days storage at 5ºC. Aerobic plate counts were 2.94 to 4.36 and 2.53 to 5.60 log CFU/g at days 0 and 24, respectively, in cottage cheese made with sorbate, and 4.67 to 5.65 and 5.34 to 7.65 log CFU/g in cottage cheese made without sorbate (Piccinin 1995).

MOZZARELLA CHEESE

Tirloni and colleagues investigated the growth potential of *L. monocytogenes* in 33 brands of mozzarella cheese, with pH values of studied cheeses varying from 5.32 to 6.43. 27 mozzarella samples (cheeses 1-27) were manufactured using pasteurized cow's milk. 20 cheeses (cheeses 1-20) were produced via natural acidification using thermophilic starter cultures and cow rennet (cheeses 1-12, pH 5.68 to 6.23, moisture 59.46 to 70.65%) or direct acidification with citric acid (cheeses 13-20, pH 5.43 to 6.43, moisture 62.15 to 67.41%). 7 cheeses were classified as mozzarella for pizza (cheeses 21-27, pH 5.69 to 6.48, moisture 48.47 to 63.84); these were mainly manufactured via direct acidification with citric acid. 6 cheeses were manufactured using pasteurized buffalo milk and using microbial rennet (cheeses 28-33, pH 5.32 to 5.56, moisture 61.65 to 65.77%). All cheeses except those denoted as mozzarella for pizza were packaged with brine. Stated commercial shelf-lives varied from 25 to 35 days at 4ºC. The authors measured pH, moisture, a_w, and organic acids (lactic, citric, and acetic acids), estimating undissociated organic acid using the Henderson-Hasselbalch equation. Lactic, citric, and acetic acids in the water phase ranged from less than detectable (<16 mg/kg) to 14,709 mg/kg, less than detectable (<216 mg/kg) to 29,195 mg/kg, and less than detectable (<47 mg/kg) to 1,725 mg/kg, respectively, with

undissociated organic acid concentrations calculated in mozzarella cheese samples to range from 0.00 to 2.37 mM lactic, 0.00 to 0.38 mM citric, and 0.00 to 2.60 mM acetic (Tirloni 2019). Concentrations of undissociated acids were lower than previously published minimum inhibitory concentration (MIC) values for *L. monocytogenes* for each acid type, with MIC values for undissociated lactic, citric, and acetic acids found to be 5.0±0.5 mM, 19.0±6.5 mM, and 3.8±0.9 mM in BHIB by Wemmenhove and colleagues, and 5.1 to 14.7 mM, >24 mM, and >8.8 mM in ricotta by Tirloni and colleagues (Tirloni 2019b, Wemmenhove 2016). The authors found citric, lactic, and acetic acids to account for 45.5, 18.6, and 35.9%, respectively of the total organic acid content in the mozzarella samples. Citric acid levels decreased over storage, attributed to citrate use as a substrate in the Krebs cycle during metabolism of the natural or added microflora. The authors additionally measured APC, *Pseudomonas*, *Enterobacteriaceae*, yeasts and mold, *Lactobacilli*, *Lactococci*, and *Streptococci* at the beginning and end of declared shelf-life for each mozzarella cheese included in their study, finding background microflora to reach >5 log CFU/g in all products at the end of shelf-life. *Streptococci* and *Lactobacilli* were the predominant microflora at time 0, with 3.3 to 3.45 log CFU/g enumerated and reaching an average >7 log CFU/g over storage. A single mozzarella, manufactured via direct acidification with citric acid and having the most permissive pH and organic acid content among included cheeses, was chosen by the authors for challenge testing with *L. monocytogenes*. The authors inoculated the cheese brine with a 3-strain cocktail of *L. monocytogenes*, giving an approximately 2 to 3 log CFU/g *L. monocytogenes* starting inoculum. Inoculated samples were stored at 4, 9, 15, and 20ºC in duplicate trials, and the authors obtained a total of 21 *L. monocytogenes* growth curves. *L. monocytogenes* was able to grow in the inoculated mozzarella at all test temperatures, reaching final levels of >6 log CFU/g. Antagonistic activity by other

organisms was not witnessed by the authors under the tested substrate and temperature conditions, and the authors observed *L. monocytogenes* to grow faster than *Streptococci* and *Lactobacilli* in the challenged cheese (Tirloni 2019).

Menon and Garg sterilized commercial mozzarella cheese containing no preservatives via autoclaving at 121°C for 15 minutes before inoculating with approximately 4 log CFU/g *L. monocytogenes*. Samples were then stored at 30°C for 7 days and 7°C for 15 days. *L. monocytogenes* increased approximately 2 logs within one day at 30°C and reached a maximum of 7.38 to 7.43 log after 5 days storage. In samples stored at 7°C, *L. monocytogenes* increased to 5.28 to 5.30 log after 6 days storage, reaching maximum values of 5.78 to 6.57 after 12 days storage. No analytical values for the tested mozzarella samples were recorded in the study (Menon 2001).

Jayamanne and Samarajeewa added a single strain of *L. monocytogenes* and LAB starter cultures (2%, from day-old buffalo curd) to boiled and cooled buffalo milk and allowed the mix to ferment at ambient temperature (26ºC) for 18 h. Initial counts of *L. monocytogenes* and LAB were approximately 5.0 and 2.2 log CFU/ml, respectively. pH and titratable acidity were measured at 2 h intervals during fermentation. The authors observed LAB to reach a maximum level of 10^6 CFU/ml after 14 h fermentation with a parallel drop in pH from 6.8 to 4.1 and an increase in titratable acidity from 0 to 1.2%. The authors identified a critical pH value of 5.5, as no growth of *L. monocytogenes* was observed below pH 5.5 in the cultured samples and a steep decline in *L. monocytogenes* was observed. *L. monocytogenes* remained detectable in cultured samples through 14 h, after which levels of the organism were not detectable. The authors additionally inoculated 10^5 CFU/ml *L. monocytogenes* into buffalo milk with varying pH values (4.0, 4.5, 5.0, or 5.5) adjusted with lactic acid and enumerated at 12 h intervals over 96 h. The

authors observed the pathogen to decrease to undetectable levels after 48, 72, and 84 hours for pH values of 4.0, 4.5, and 5.0, respectively, while pH 5.5 milk showed a steady rise in numbers over the 96-h incubation period, increasing approximately 0.5 log CFU/ml (Jayamanne 2010).

Villani and colleagues inoculated a 4-strain cocktail of *L. monocytogenes* (containing strains Scott A, V7, OH, and CA) into the cheesemilk of unripened water buffalo mozzarella at levels of approximately 3 and 5 log CFU/ml. The authors acidified the cheesemilk with a natural whey starter and used liquid rennet to clot the curd. The pH of the cheese at the end of curd ripening was found to be 4.83 to 4.91. An approximately 2 log reduction was realized after hot water stretching of the curd at 95ºC. Storage of the cheese in a final conditioning liquid (pH 3.8 to 4.2) of skim whey from previous mozzarella manufacture supplemented with 1% NaCl completely eliminated *L. monocytogenes* within 24 hours or 48 hours in low and high inoculum mozzarella cheeses, respectively, with *L. monocytogenes* absence validated via enrichment procedures (Villani 1996).

RICOTTA CHEESE AND RELATED VARIETIES

Martins and colleagues investigated ricotta cheese (pH 5.47 and 53% moisture) made with acetic acid for its ability to support growth of *L. monocytogenes* during 28 days storage at 4°C. The pH value of the tested cheese increased from 5.47 to 6.63 over the study duration, which the authors attributed to proteolysis due to mold growth. Levels of *L. monocytogenes* increased from approximately 5 log CFU/g to 7 log CFU/g by the end of storage (Martins 2010).

Spanu and colleagues investigated traditional sheep's whey ricotta salata for its ability to support growth of *L. monocytogenes* when inoculated onto wheels of the cheese at a level of approximately 2 log CFU/g. The authors selected 66 ricotta salata cheese wheels from a local

cheesemaking plant and used a spray inoculum consisting of 3 strains of *L. monocytogenes*. Inoculated cheeses were vacuum packaged before storage at 4ºC for 6 months. pH values of cheeses on day 0 averaged 6.30 and fell to an average value of 5.88 by 6 months. Time 0 a_w values averaged 0.95 and decreased to an average of 0.94 after 6 months, while time 0 moisture was found to average 53.65% and decrease to 46.70% after 6 months. Levels of *L. monocytogenes* recovered from the rind of the cheese were 1.80 log CFU/g 6 hours post-inoculation and rose to 5.94 log CFU/g after 2 months storage. A maximum level of 7.24 log CFU/g *L. monocytogenes* was reached within 4 months, and at 6 months 6.80 log CFU/g *L. monocytogenes was* recovered from the cheese rind. Conversely, no *L. monocytogenes* was recovered within the cheese paste at 6 h, 2 months, 4 months, or 6 months. APC on the cheese rind were found in levels of 7.08 log CFU/g and 7.37 log CFU/g at the beginning and end of storage, respectively, remaining 6.27 log CFU/g at all sampling intervals. APC in the cheese paste were 4.65 log CFU/g at 6 hours, 3.81 log CFU/g at 2 months, 3.11 log CFU/g at 4 months, and 3.15 at 6 months (Spanu 2012).

Papageorgiou and colleagues investigated the growth of *L. monocytogenes* in the whey cheeses Mizithra (pH 6.50, 67.8 to 69.2% moisture, and 0.0% NaCl), Anthotyros (pH 6.41, 65.8 to 67.8% moisture, and 1.37 to 1.54% NaCl), and Manouri (pH 6.30, 50.6 to 53.4% moisture, 2.23 to and 2.40% NaCl) during storage at temperatures of 5, 12, and 22ºC for up to 38 days. The three cheese varieties studied contain no starter cultures and are traditionally made with whey recovered during Feta cheese production from the whey of ewe's or goat's milk, with the whey being heated to allow for denaturation and coagulation of the protein. Cheeses were inoculated to contain approximately 2.70 log CFU/g *L. monocytogenes*. pH values in all cheeses dropped to 4.97 to 5.30 over storage. The authors noted that in no trial among the cheeses or temperatures

tested was a pH value of <5.7 observed concurrently with *L. monocytogenes* growth, and that only when the pathogen reached stationary phase did the pH drop to <5.7. Maximum *L. monocytogenes* populations of 7.84 to 8.62 log CFU/g were reached after 24 to 30 days at 5ºC, with all three cheeses showing similar growth patterns and 1-log increases in *L. monocytogenes* observed after 4 to 5 days in all cheeses tested. Additionally, the authors found *L. monocytogenes* growth to be accelerated with increasing temperature (Papageorgiou 1996).

Tsiotsias and colleagues investigated *L. monocytogenes* behavior in Anthotyros, a soft whey cheese. Duplicate batches were produced in a dairy plant and samples of both irradiated and unirradiated cheese were inoculated with *L. monocytogenes* strain Scott A at a level of 5 to 6 log CFU/g before being vacuum-sealed and stored at either 4 or 10ºC for 42 days. Time 0 APC counts were 4.54 log CFU/g and yeasts 3.80 log CFU/g. In control cheeses these levels rose to approximately 6.0 and 4.5 log CFU/g, respectively, at 42 days storage at 4ºC. Control cheese day 0 analytical values were 65.0% moisture, 0.6% NaCl, and pH 6.4. After 42 days at 4ºC, these values changed to pH 6.3, 66.5% moisture, and 0.5% NaCl. *L. monocytogenes* was able to grow in control cheese from an initial level of 5.0 log CFU/g to 8.0 log CFU/g within 14 days, remaining at this elevated level for the remaining duration of the study (42 days total) at 4ºC. From an initial level of 5.0 log CFU/g, cheese stored at 10ºC supported growth to approximately 8.5 log CFU/g within 21 days, then reducing to approximately 7.5 log CFU/g when enumerated on days 28, 35, and 42 of storage (Tsiotsias 2002).

CREAM CHEESE AND RELATED VARIETIES

Smith-Palmer and colleagues investigated the effects of plant essential oils, including bay, clove, cinnamon, and thyme, in commercial full-fat and low-fat cream cheeses made with

no added preservatives in their antilisterial effectiveness. Concentrations of 0.1, 0.5, and 1.0% of each essential oil were mixed into a 1:10 dilution of cheese to phosphate buffered saline. This cheese mixture was then inoculated with a single strain of *L. monocytogenes* before storage at 4ºC for 14 days. *L. monocytogenes* was more readily inhibited in low-fat versus full-fat cream cheese mixtures. The authors found both clove and cinnamon oils to effectively inhibit *L. monocytogenes* at a 1% level. 1% cinnamon oil reduced *L. monocytogenes* numbers in the low-fat cheese mixture to ≤1.0 log CFU/ml after 3 days, while a slower decrease (to approximately 3.3 log CFU/ml within 3 days and 2 log CFU/ml by day 14) was observed in the full-fat cheese mixture. 1% clove oil was the only essential oil to reduce *L. monocytogenes* to ≤1.0 log CFU/ml in the full-fat cheese mixture (Smith-Palmer 2001).

Kagkli and others found *L. monocytogenes* unable to grow when inoculated into Katiki spreadable soft traditional Greek cheese of approximate pH 4.3 to 4.5, 75% moisture, and 1% NaCl and stored at temperatures of 5, 10, 15, or 20°C for up to 30 days. Conversely, resident LAB were able to grow in the affected cheese over the course of 15 days, with faster growth observed as temperature was increased from 5 to 20°C, and less LAB growth observed (0.5-log vs. 1.5-log) in cheese stored at 5°C versus those stored ≥10°C (Kagkli 2009). Panagou studied the behavior of *L. monocytogenes* in Katiki made with pasteurized goat's milk. Commercial samples were inoculated to give an initial population of 10^6 CFU/g *L. monocytogenes*, then stored under aerobic conditions at 5, 10, 15, and 20°C for up to 40 days. The authors found *L. monocytogenes* to decrease to ≤2 log CFU/g by the end of storage for all temperatures tested and found that increasing storage temperature accelerated the rate of *L. monocytogenes* deactivation. The decrease in *L. monocytogenes* populations was attributed to low pH and lactic acid starter

cultures used in the products. LAB counts increased from 6.2 to 6.5 log CFU/g to 8.0 to 8.2 log CFU/g, while pH decreased from 4.5 to 4.6 at day 0 to 4.2 to 4.3 at day 40 (Panagou 2008).

Theodoridis and colleagues investigated the Greek cheese Pichtogalo Chanion (soft white spreading cheese made using pasteurized ewe's and goat's milk) for its ability to support the growth of *L. monocytogenes* during cheesemaking and storage. Cheese samples had a moisture content of 64.45%, salt of 1.25%, and pH (on day 2) of 4.23. The authors used an equal volume of whole raw ewe's and whole raw goat's milk which was pasteurized before inoculation with 2.3 to 5.3 log CFU/ml of *L. monocytogenes* strain Scott A. *Lc. lactis* subsp. *lactis*, *Lb. casei* subsp. *pseudoplantarum*, *Lb. casei* subsp. *casei,* and *Lc. lactis* subsp. *lactis* bv. diacetylactis were isolated from Pichtogalo Chanion cheeses and used at levels of 1.3, 1.3, 1.0, and 0.4% (v/v milk), respectively, to make the cheese. After addition of starter cultures, approximately 7.50 log CFU/g of *Lactobacilli* and *Lactococci* in the milk were enumerated, and LAB populations remained high (approximately 8.75 log CFU/g) throughout storage of the cheese at 4ºC over 30 days. Rennet was added for curd coagulation and 1% salt added to the cheese before refrigeration. Eight trials were conducted, with a single trial using raw milk and no starter culture. The researchers found *L. monocytogenes* to be inactivated after 5 to 10 days of cheese storage at 4ºC in 6 trials, while inactivation was completed after 20 days in 2 trials (one trial with higher *L. monocytogenes* inoculum and one trial using raw milk and no starter culture). Total *L. monocytogenes* reductions realized were -2.74 to -5.59 log CFU/g (Theodoridis 2006).

Rogga and colleagues investigated the ability of Galotyri, a traditional Greek soft acid-curd spreadable cheese, to support aerobic growth of *L. monocytogenes* at 4ºC and 12ºC for 28 days. Commercial and artisan Galotyri made from ewe's milk with the addition of commercial and natural starter cultures, respectively, were used in the study. In addition, the authors

manufactured laboratory-scale Galotyri from raw ewe's milk supplemented with 3% (w/v) NaCl and 0.7% (w/v) of either the commercial industrial Galotyri or the artisan Galotyri. No rennet was used in making the laboratory-scale cheeses. Starting analytical values for each cheese were as follows: pH 3.80, 76.9% moisture, and 1.8% salt for commercial cheese; pH 4.00 75.8% moisture, and 1.8% salt for artisan cheese; pH 4.42, 74.0% moisture, and 2.9% salt for pilot-scale cheese made with commercial cheese as a starter; and pH 4.36, 77.5% moisture, and 3.1% salt for laboratory-scale cheese made with artisan cheese as a starter. The cheeses were inoculated with 3 (all cheeses) or 7 (commercial and artisan Galotyri only) log CFU/g of a 5-strain *L. monocytogenes* cocktail. The authors noted visible spoilage after 21 days at 4ºC and 7 days at 12ºC in most of the cheeses tested, with commercial and artisan cheeses being designated terminally spoiled after 21 days at 12ºC due to surface yeast growth. pH was noted to remain at initial values for 28 days in the commercial and artisan samples stored at 4ºC and for 14 days in samples stored at 12ºC. Laboratory-scale cheese pH values were noted to be near or slightly over 5.0 at the time of spoilage in samples stored at 12ºC. Time 0 counts of *L. monocytogenes* ranged from 2.8 to 3.4 log CFU/g in the lower inoculum targeted cheeses and from 6.8 to 6.9 log CFU/g in the higher inoculum targeted cheeses. No increases in *L. monocytogenes* numbers were observed over the study period, with final values of <1.0 to 2.6 log CFU/g observed in cheeses inoculated with target 3 log CFU/g *L. monocytogenes* and 1.2 to 1.9 log CFU/g *L. monocytogenes* in cheeses inoculated with target 7 log CFU/g *L. monocytogenes*. A more modest reduction in pathogen counts observed in the laboratory-cheese was attributed to a higher pH compared to the commercial and artisan varieties. The authors noted that independent of the initial inoculum level of *L. monocytogenes* a tailing effect was observed, wherein inactivation of a low contamination of *L. monocytogenes* within the studied cheese could not be assured (Rogga

2005). This finding was in agreement with those of Leuschner and Boughtflower who found *L. monocytogenes* to survive a laboratory-scale soft cheesemaking process even with an initial contamination of milk as low as 1 to 10 CFU/ml (Leuschner 2002).

OTHER FRESH SOFT CHEESES

Leuschner and Boughtflower investigated soft cheese inoculated with low levels (1 to 10 CFU/ml into the cheesemilk) of *L. monocytogenes*. Cheese was prepared by using a lyophilized dairy starter culture for direct inoculation into prewarmed retail pasteurized, non-homogenized whole milk to which vegetarian rennet was then added. Potassium sorbate was added at 2.5 ml/L milk to suppress mold growth. Curd coagulation was carried out after 1.5 h at 32ºC and whey drainage after 4 h at 45ºC. Set curd pH measured 6.5. Mashed curd with added whey produced a final soft cheese with pH of 5.4, which was stored in screw-cap containers. Within 24 h, cheese pH dropped to 4.5 and remained at this value for the 4-week duration of the study with storage at 4ºC. The pathogen was found unable to grow above levels of 55 CFU/g over the study period. The authors did not provide additional analytical parameters on the tested cheeses outside of pH value (Leuschner 2002).

Papageorgiou and Marth studied the behavior of *L. monocytogenes* when inoculated at a level of 3.70 log CFU/ml into the cheesemilk of Feta cheese. The authors found that the low pH (4.60) of the cheese following brining proved inhibitory to the pathogen for the length of storage (90 days) at 4ºC. Moisture ranged 53.89 to 55.67%, salt 2.21 to 2.50%, and a_w 0.974 to 0.976 (Papageorgiou 1989).

Gohil and colleagues investigated the ability of Labneh soft cheese to support the survival of approximately 4 log CFU/g *L. monocytogenes* inoculated onto the cheese surface

before aerobic storage. The authors obtained prepared Labneh, both salted (1.0%) and unsalted, from a local dairy. The authors pH adjusted the cheese (pH 3.8, 53% moisture) with 10% NaOH to pH 4.5. The authors found survival of the pathogen to be influenced mostly by pH ($p<0.05$) followed by salt ($p<0.05$) and temperature. *L. monocytogenes* was reduced to undetectable limits within 72 hours storage at 4 and 10ºC with Labneh at pH 3.8. Increased survival was observed with pH 4.5, especially in unsalted samples (Gohil 1996). Labneh soft cheese was also researched by Issa and Ryser, who investigated the behavior of *L. monocytogenes* in Labneh manufactured with starter culture (pH 3.99 to 4.21, 77.35 to 78.81% moisture, 1% NaCl, and 1.36 to 1.50% titratable acidity) when inoculated into the curd at the time of salting at a level of approximately 3.6 log CFU/g before storage at 6 and 20ºC. *L. monocytogenes* was able to persist but not grow for 15 days, while other pathogens tested (*Salmonella* spp. and *E. coli* O157:H7) were inactivated in that time (Issa 2000).

Ozturkoglu and colleagues monitored the behavior of *L. innocua* in Turkish white cheese made with starter culture. Analytical parameters for cheese inoculated with 2.75 log CFU/g *L. innocua* were as follows on day 0: pH 4.92, 5.96% salt, and 60.23% moisture. These changed to pH 6.00, 6.80% salt, and 54.87% moisture by day 45 of 4°C storage. In cheese inoculated with 6.98 log CFU/g *L. innocua*, day 0 values were pH 5.14, 6.20% salt, and 61.49% moisture. By day 45 of 4°C storage the values changed to pH 6.10, 6.98% salt, and 57.0% moisture. No growth of *L. innocua* was observed in the tested cheeses, which the authors attributed to the use of starter culture in cheese manufacture as well as the high salt content of the tested cheeses (Ozturkoglu 2006).

Olarte and colleagues inoculated Cameros fresh goat cheese with 4.40 to 4.53 log CFU/g *L. monocytogenes* before applying various modified atmosphere packaging treatments (aerobic

storage, 20% CO_2/80% N_2, 40% CO_2/60% N_2, or 100% CO_2) and storing for 28 days at 4°C. Cheese did not contain a starter culture and was made using pasteurized goat's milk. The researchers also monitored the growth of mesophiles and psychrotrophs in uninoculated control cheeses. All tested cheeses supported *L. monocytogenes* growth, regardless of packaging means. 100% CO_2 delayed time-to-1-log growth to approximately 10 days, while 1 log CFU/g of *L. monocytogenes* growth was observed in air-packaged cheese within approximately 3 days. Final levels of *L. monocytogenes* reached varied from approximately 6.7 log CFU/g in cheese stored in 100% CO_2 to 8.0 log CFU/g in the air-packaged sample, with increasing levels of CO_2 showing decreased levels of *L. monocytogenes* populations. Starting pH and moisture values for the cheese were pH 6.7 and 65.71%, respectively. A rapid reduction in pH to 6.1 was observed in cheese packaged in 100% CO_2 within 7 days, while in cheese stored aerobically, cheese pH remained at or slightly above 6.7 in the same storage period. pH values in cheeses packaged in 20% CO_2/80% N_2 or 40% CO_2/60% N_2 were found to be approximately 6.55 and 6.50, respectively, after 7 days' storage at 4°C. Final 28-day pH values in all samples reached approximately 6.0. More rapid moisture loss was additionally observed in cheese packaged in 100% CO_2, with a 5% loss observed within the first 7 days of storage and a final moisture loss of approximately 10% observed at the end of the 28-day storage period. Moisture losses for all other samples appeared similar over the study, despite a higher loss of approximately 6% in air-packaged samples versus a more modest loss of 4% observed in 20% CO_2/80% N_2 or 40% CO_2/60% N_2 after 28 days storage. Increased moisture loss observed in cheese packaged in 100% CO_2 was attributed to the rapid pH decline in these samples. Mesophilic plate count rose from approximately 4.5 to 8.0 log CFU/g and psychrotrophic plate count from approximately 1.0

to 8.0 log CFU/g in uninoculated control samples stored aerobically by the end of storage (Olarte 2002).

EXISTING *L. MONOCYTOGENES* GROWTH MODELS

Predictions from food safety models based on laboratory media can deviate markedly from food safety models developed using data from food systems. Still, relatively few predictive models exist for *L. monocytogenes* in foodstuffs. Reasoning for widespread use of laboratory media versus foods in development of *L. monocytogenes* behavior studies include utilization of a homogenous substrate, unvarying intrinsic factors, use of optical density measurements, and reduced labor needs versus food challenge studies (Schvartzman 2011). ComBase is an online quantitative model for food microbiology that predicts growth or inactivation of microorganisms. Datasets for ComBase include >60,000 records deposited from challenge studies conducted in broth and food systems, and include parameters such as temperature, pH, and a_w to predict behavior of foodborne pathogens (ComBase 2021). Lag phase duration can be described as the time required for cells to physiologically adjust to an environment prior to replication (Baranyi and Roberts 1994). Ostergaard and colleagues noted the difficulty in finding realistic lag phase times in food modeling, which they attributed primarily to the unknown physiological state of the bacterial cells at the time of product contamination. To mitigate this, the authors suggested omitting the lag phase (i.e. setting it at 0) and relying solely on growth rate prediction as an option in modeling foodborne pathogens (Ostergaard 2015). Other authors have suggested the use of logistic regression, which predict the probability of bacterial growth under specific conditions within a certain time. As opposed to kinetic models, logistic regression models provide quantitative information on growth limits versus rates of growth (Schvartzman 2011).

Still other authors have noted advantages of generalized regression over least-squares regression, including the ability to incorporate censored observations (where no growth was observed) (Legan 2004).

ComBase datasets include liquid laboratory culture media and may therefor result in different growth patterns than in a food system. Bacteria in media are not exposed to the solid structure, background microflora, lower O_2 diffusion, nor presence of enzymes, peptides, and/or organic acids that may be present in cheese (de Araujo 2017). Francois and colleagues found slightly faster *Listeria* spp. growth to occur in a broth system versus in liver pâté, though growth estimations were deemed to accurately fit data in this food system. The same finding was not, however, replicated in cooked ham, lettuce, or cabbage, where broth-based *Listeria* spp. growth data translated poorly to that in the tested products (Francois 2006). ComBase maximum growth rate (Gr_{max}) predictions with temperature, pH, and a_w conditions were generally fail-safe for predicting *L. monocytogenes* growth in 100 commercial samples of Coalho, a minimally-ripened semi-hard medium- to high-moisture cheese made with rennet and with or without starter culture. However, higher actual Gr_{max} values were found versus ComBase predictions in cheeses stored at 12ºC in most cases (de Araujo 2017). Additionally, ComBase was found to have a poor fit (R^2 = 0.37) with *L. monocytogenes* growth data in a soft model cheese system (Schvartzman 2011).

MODELS IN CHEESE

Schvartzman and colleagues developed a semi-soft laboratory-scale model cheese system made up of pasteurized milk, skim milk powder, NaCl, lactic acid, and rennet. The purpose of the authors' model was to predict the probability of growth initiation of *L. monocytogenes* during the early stages of cheesemaking, including acidification of the milk, coagulum formation,

cutting/breaking of the gel, cooking, and molding/pressing of the curd, with no ripening period included. The authors used a factorial design targeting 5 pH values (5.6 to 6.5), 4 a_w values (0.938 to 0.960) and 2 inoculation levels (1 to 20 CFU/ml and 500 to 1,000 CFU/ml), with each combination tested in 6 independent replicates. 8% (w/v) low-heat skim milk powder was added to pasteurized milk before pH adjustment with 10% (w/v) lactic acid or NaOH, while NaCl was added at levels of 0, 3, 4.5, 6, or 8% to achieve targeted water activities. Addition of skim milk powder was necessary for achieving formulations with low a_w and high pH targeted model cheeses, and therefore was used in all treatments. The authors used the factors temperature, pH, inoculation level, a_w, and lactic acid content to predict the amount of growth within 8 hours using the ComBase Modelling Toolbox. The growth prediction from this model was then compared with observed data and to predictions from their developed ordinary logistic regression model. The authors validated their model using the following combinations for model cheeses inoculated with a high or low inoculum of *L. monocytogenes*: a_w 0.959 / pH 6.38, a_w 0.963 / pH 6.43, a_w 0.967 / pH 5.84, a_w 0.969 / pH 6.44, and a_w 0.982 / pH 6.34. In their validation, an increase of ≥0.5 log CFU/g was considered *L. monocytogenes* growth initiation. Validation data was then used in validating logistic regression model predictions. The authors found 63% of the tested pH/a_w combinations to initiate growth during the 8 hours tested in the study. The authors found that at a low *L. monocytogenes* contamination level (1 to 20 CFU/ml), a_w values ≥0.975 always resulted in growth initiation. Conversely, in highly contaminated cheeses (inoculated with 500 to 1,000 CFU/ml *L. monocytogenes*), a critical a_w of approximately 0.965 was found (i.e. ≥0.965 resulted in growth initiation). The authors also found that when *L. monocytogenes* growth was observed, the extent of growth was a_w and contamination level dependent, with correlations of 0.74 and 0.63 for a_w for low and high contamination levels, respectively. pH and

log increase were found not to be positively correlated, with correlations of -0.03 and -0.08 for low and high contamination levels, respectively. The authors did note, however, that the relatively narrow range of pH values tested (5.6 to 6.5) might account for the limited effect of pH on *L. monocytogenes* growth found in their model. At low contamination levels, *L. monocytogenes* growth was always observed in model cheeses with a_w values of 0.972 to 0.996 (containing 0 to 3% NaCl), with 1.57 to 3.96 log CFU/g increases observed. In 93% of replicates, *L. monocytogenes* growth was not initiated in highly contaminated model cheeses with a_w values of 0.938 to 0.957. Cheeses of pH 5.6 with a_w 0.95 or 0.97 were not included in the authors' logistic regression, as the authors were unable to coagulate milk to form model cheeses under these conditions. In comparing *L. monocytogenes* challenge study data in model cheeses to ComBase predictions, the authors found ComBase to have a poor fit ($R^2 = 0.37$) versus observed data, while the fit with the authors' own ordinary logistic regression model had a good fit ($R^2 = 0.94$). ComBase predicted *L. monocytogenes* growth initiation (≥0.5 log CFU/g) in 57% of the model cheese formulations tested and correctly predicted growth initiation in 41% of model cheeses in which growth was observed. The authors noted that ComBase underestimated the observed amount of *L. monocytogenes* growth in 70% of model cheeses at a_w values of 0.98 to 0.99 (the typical a_w for cottage or soft cheese) while the model overestimated cases where cheese a_w values were approximately 0.96 to 0.97. The authors suggested that a reduction in the a_w of milk to ≤0.97 would reduce the probability of *L. monocytogenes* growth initiation within the cheesemaking process to 0.1 if contaminated at a low level (1 to 20 CFU/ml). Conversely, with a higher (500 to 1,000 CFU/ml) contamination level, a a_w value of ≤0.94 would be necessary to reduce the probability of *L. monocytogenes* growth initiation within the cheesemaking process to 0.1 (Schvartzman 2011).

Tirloni and colleagues investigated the growth of *L. monocytogenes* in 8 brands of ricotta cheese, developing 2 cardinal parameter models with terms for temperature (Model 1) and temperature and pH (Model 2). The authors additionally evaluated and recalibrated an existing *L. monocytogenes* growth model by Mejlholm and Dalgaard which included the effects of organic acid content (Model 3; Mejlholm 2009). The authors inoculated ricotta samples with 3 to 4 log CFU/g of a 3-strain *L. monocytogenes* cocktail. Starting pH values of ricotta samples were found to be 5.49 to 6.61, moisture values 72.2% to 82.1%, percent water-phase salt 0.20% to 0.60%, and a_w values 0.996 to 0.998. Starting acetic, citric, and lactic in the water phase of ricotta samples ranged from 660 to 2,021 ppm, 14,774 to 50,862 ppm, and <69.4 to 4,146 ppm, respectively. The authors additionally added individual organic acids to a single commercial ricotta cheese before *L. monocytogenes* inoculation and storage at 15.2ºC for 7 days. In this portion of the study, the authors adjusted cheese pH using HCl to ensure undissociated acid contents were maximized. With acetic acid added to ricotta (pH 4.95 to 5.27, 74.6% to 76.7% moisture, 37.3 mM to 174.4 mM, 77.5 mM to 117.3 mM, and 5.7 mM to 12.7 mM undissociated acetic, citric, and lactic acids in the water phase, respectively) 3 of 3 challenged cheeses showed no growth of *L. monocytogenes* over the test period. With citric acid added to ricotta (pH 4.90 to 4.99, 75.0% to 76.6% moisture, 4.9 mM to 7.6 mM, 1764.3 mM to 3914.8 mM, and 1.3 mM to 6.0 mM undissociated acetic, citric, and lactic acids in the water phase, respectively) 2 of 3 challenged cheeses showed no growth of *L. monocytogenes* over the test period. With lactic acid added to ricotta (pH 4.99 to 5.18, 74.5% to 75.8% moisture, 4.2 mM to 14.4 mM, 53.1 mM to 84.7 mM, and 35.2 mM to 213.6 mM undissociated acetic, citric, and lactic acids in the water phase, respectively) 1 of 3 challenged cheeses showed no growth of *L. monocytogenes* over the test period. The authors found concentrations of >8.8 mM, 24.0 mM, and 5.1 to 14.7 mM

undissociated acetic, citric, and lactic acids, respectively, to inhibit *L. monocytogenes* growth in ricotta. The authors found a much higher MIC for citric acid than literature values found in broth systems, which they attributed to studies using broth versus ricotta as a substrate. The authors found the existing *L. monocytogenes* model (Model 3), developed in meat and fish products with continuous variables temperature, pH, a_w, lactic acid, acetic acid, and citric acid and discrete variables for presence/absence of nitrite, phenol, CO_2, sorbic acid, diacetate, and benzoic acid, to underestimate *L. monocytogenes* growth in ricotta. The authors found combined and observed growth-inhibiting effects of acetic, citric, and lactic acids in ricotta to be much less pronounced than predicted, resulting in fail-dangerous predictions. The authors additionally applied their *L. monocytogenes* challenge data to *L. monocytogenes* growth models by Gougouli, Augustin, Rosshaug, and Ostergaard in ice cream, cheese, blue-white cheese, and cottage cheese with cultured cream dressing, respectively, finding all models to result in fail-dangerous estimates for *L. monocytogenes* growth in the studied ricotta samples (Augustin 2005, Gougouli 2008, Ostergaard 2014, Rosshaug 2012). All 4 existing studied models contained the continuous variable temperature, while some additional continuous variables pH, lactic acid, NaCl, a_w, and discrete variables presence/absence of sorbic acid, presence/absence of nitrite, and presence/absence of CO_2 were included in the existing models. The authors found 2 *L. monocytogenes* growth models developed in broth (Pathogen Modeling Program aerobic and anaerobic models with variables temperature, pH, NaCl, and presence/absence of nitrite) and 1 *L. monocytogenes* growth model developed in broth and food (ComBase with variables temperature, pH, and a_w) to overestimate the maximum growth rate of *L. monocytogenes* in ricotta. Conversely, the authors found *L. monocytogenes* growth models developed for fermented cheeses to underestimate growth rates in ricotta (Rosshaug 2012, Augustin 2005). Based on

temperature and pH, Tirloni and colleagues predicted time for 2 log CFU/g increases of *L. monocytogenes* in ricotta to be as follows: 3.0 days for pH 6.2 with 7ºC storage, 3.6 days for pH 6.2 with 6ºC storage, 4.4 days for pH 6.2 with 5ºC storage, 5.6 days for pH 6.2 with 4ºC storage, 6.1 days for pH 5.8 with 4ºC storage, 4.7 days for pH 5.9 with 5ºC storage, 5.6 days for pH 5.5 with 5ºC storage, 6.7 days for pH 5.6 with 4ºC storage, and 67.2 days for pH 5.1 with 4ºC storage. These predictions were shorter than times predicted by Augustin and colleagues for liquid dairy products except in ricotta with pH 5.1 stored at 4ºC, wherein a 2 log CFU/g increase of *L. monocytogenes* was predicted in 52.7 days (Augustin 2005, Tirloni 2019b).

Bolton and colleagues studied *L. monocytogenes* behavior in a Mexican-style cheese-based model system based on salt, pH, and moisture content. The authors produced a soft, directly acidified, renneted fresh cheese made to 6 pH values (5.00, 5.25, 5.50, 5.75, 6.00, and 6.50), 4 moisture contents (42, 50, 55, and 60%), and 4 salt levels (2.0, 4.0, 6.0, and 8.0% w/w). Growth/stasis/death was evaluated at 21 and 42 days storage at 10ºC. Binary logistic regression was used to predict growth/no growth for any of the tested combinations. Ordinal logistic regression was also used to determine odds ratios and probability for growth, stasis, or death. Each model was independently validated with external data. The authors found a concordance rate of 0.7 between predicted and observed growth data, lower than that of several broth studies they referenced in which concordance with predictive models was found to be ≥0.97 (Bolton 1999). Augustin and colleagues additionally found poorness-of-fit in their developed *L. monocytogenes* growth model, in which a 90% coefficient of variation was observed. The authors developed the model based on data from five publications including five fresh soft cheese varieties and seven other cheese varieties and attributed poor fit-to-actual results to be due to both abiotic (heterogeneity of pH, a_w, and lactic acid concentrations) and biotic (competitive

microflora) factors. The authors also noted large variations in optimal growth rates for identical cheeses (Augustin 2005).

Uhlich and colleagues investigated the growth of *L. monocytogenes* on queso blanco slices. The authors inoculated a 5-strain cocktail of *L. monocytogenes* at a level of approximately 2 to 3 log CFU/g onto individual vacuum-packaged slices of commercial queso blanco before storage at 5, 10, 15, 20, and 25ºC for up to 43 days. The authors used a single batch of queso blanco made with pasteurized milk, salt, enzymes, and commercial cheese cultures. The challenged queso blanco had pH 6.80, 48.8% moisture, 2.32% salt, a_w 0.971, and <0.01 g / 100 g lactic acid. The authors noted typical pH values for commercial queso blanco samples range from 5.25 to 5.90, moisture values from 45% to 55%, and salt values from 1.8% to 3%, hence making the tested product more permissive for *L. monocytogenes* growth in terms of pH but within range for other analytical parameters. The authors used DMFit software to fit their primary model, with secondary models developed using TableCurve 2D. The authors found storage at 20 and 25ºC to not significantly differ in terms of growth rate of *L. monocytogenes*. Conversely, growth rates at temperatures of 5, 10, and 15ºC were found to be statistically significantly different from one another as well as from growth rates at 20 and 25ºC. Comparing predicted growth rates based on *L. monocytogenes* strain Scott A in a broth-based model under anaerobic conditions, growth rates in queso blanco were 5 to 7 times lower than broth model predictions (Buchanan and Phillips 1990, Uhlich 2006). None of the tested temperatures prevented *L. monocytogenes* growth over the study period, though the authors found the lag phase to be statistically longer at 5ºC than all other storage temperatures. A lag phase value of 65.3 hours was found for storage temperature 5ºC, versus values of 2.1 to 19.9 hours observed with storage temperatures of 10 to 25ºC. Maximum *L. monocytogenes* population density was

found to range from 7.99 to 8.80 log CFU/g, with no significant differences between the storage temperatures tested. The authors additionally noted that total background APC grew at a similar rate to that of *L. monocytogenes* in the queso blanco tested. Overall, the authors found *L. monocytogenes* to increase ≥6.0 log CFU/g within 17 days at 5ºC, while at 10ºC a level of ≥8.0 log CFU/g was reached within 8 days. Within 35 days storage at 5ºC the authors found the maximum *L. monocytogenes* population density (approximately 8 log CFU/g) to be reached, concerning due to the shelf-life of typical queso blanco being approximately 90 days (Uhlich 2006).

Ostergaard and colleagues developed and validated mathematical models for growth of *L. monocytogenes* and mesophilic LAB starter cultures (*Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*) during chilled storage of cultured cottage cheese with fresh or cultured cream dressing. Overall, the authors found *L. monocytogenes* growth rates between broth and cottage cheese made with fresh cream dressing to be similar, while growth rates in cottage cheese made with cultured cream dressing had a slower growth rate than broth data. The authors used a 4-strain cocktail of *L. monocytogenes* to inoculate cottage cheese samples. The models included temperature, pH, NaCl, lactic acid (naturally occurring), and sorbic acid (as an added preservative) in addition to interaction terms between all variables. LAB starters were predicted to grow on average 16% faster than observed in cheese, while growth of the diacetyl-producing aroma culture (*Lc. lactis* subsp. *lactis* bv. diacetylactis) in the cream dressing was predicted 9% slower than observed in cheese. For fresh and cultured cream dressing cottage cheeses, pH values were 5.18 and 5.39, water-phase NaCl 1.21 and 1.09%, water-phase lactic acid 718 and 1029 ppm, and LAB 5.59 and 6.65 log CFU/g, respectively. The authors found a modeled minimum pH value for *L. monocytogenes* growth to be 4.87, a minimum growth temperature of -

2.01ºC, and MIC values for lactic acid and sorbic acid to be 3.79 mM and 1.90 mM, respectively. Minimum starter and aroma LAB culture populations to prevent *L. monocytogenes* growth were found to be 8.82 and 8.43 log CFU/g, respectively. The authors found *L. monocytogenes* able to grow in cottage cheese with fresh cream dressing at 5, 10, and 15ºC, with maximum *L. monocytogenes* population densities of 6.6, 6.1, and 5.5 log CFU/g, respectively, observed. Concentrations of *L. monocytogenes* in cottage cheese with fresh cream dressing stored at 10 and 15ºC reached a plateau when LAB approached their maximum population levels of 8.6 and 9.0 log CFU/g, respectively. The authors noted *L. monocytogenes* growth rate appeared more dependent on the LAB cultures used for cheese milk fermentation versus on product characteristics of the cheese, as the initial pH of cultured cream cottage cheese was approximately 0.2 units higher than that of fresh cream cottage cheese and a restriction of the maximum population density of *L. monocytogenes* could be achieved with a concomitant increase in numbers of acidifying and flavor-producing cultures during cheese fermentation and over storage (Ostergaard 2014).

Rosshaug and colleagues investigated *L. monocytogenes* growth predictions in a soft blue-white cheese in order to develop a predictive model. The model contained factors temperature, pH, NaCl, and lactic acid. The authors developed the model based on broth data produced in prior studies, generating new data sets in cheese in order to validate the broth model. The authors obtained soft blue-white cheeses manufactured with pasteurized milk directly from a dairy and with an estimated shelf-life of 70 days. The authors found the finished cheese pH to increase from approximately 4.9 to 7.5 by the end of shelf-life while salt remained constant at approximately 1.8%. Lactic acid values were measured as 0.354 g / 100 g cheese at 25 days post-production, 0.126 g / 100 g cheese at 46 days, and 0.010 g / 100 g cheese at 88 days. The authors

found *L. monocytogenes* to increase 3 to 3.5 log CFU/g over the shelf-life of a typical cheese (Rosshaug 2012).

de Araujo and colleagues investigated the growth of *L. monocytogenes* in Coalho cheese, a minimally ripened medium- to high-moisture cheese made with or without starter cultures. The study examined *L. monocytogenes* growth kinetics by the ComBase predictor with varying combinations of temperature, pH, and a_w in commercial Coalho over 14 days storage at 7.5 and 12ºC with initial contamination levels of 3 and 5 log CFU/g. The authors procured 100 samples of Coalho cheese from 10 brands from supermarkets in Brazil. pH and a_w of the samples at the time of collection were measured and used for growth predictions. The authors found at least 25% of samples collected to be above cold storage requirements at the time of collection (≤12ºC per Brazilian legislation). A_w ranged from 0.95 to 0.96 and pH values ranged from 5.59 to 7.00, with most cheeses of pH 6.06 to 7.00 (mean 6.51). The authors found no lag phase and linear growth in all *L. monocytogenes*-inoculated samples. All populations of *L. monocytogenes* grew (>0.5 log CFU/g) by day 14 of storage at either test temperature. ComBase Gr_{max} predictions with temperature, pH, and a_w conditions were generally fail-safe for predicting *L. monocytogenes* growth, however, higher actual Gr_{max} values were found versus ComBase predictions in cheeses stored at 12ºC in most cases. The authors found the challenged cheese to have high levels of *Lactococcus* spp. (7.51 to 8.22 log CFU/g) and *Lactobacillus* spp. (7.33 to 7.95 log CFU/g) at all sampling points and suggested that high levels of these LAB may have contributed to the more modest *L. monocytogenes* growth observed in cheeses versus predicted values based on pH, a_w, and temperature alone. The authors additionally examined growth of *E. coli, S. aureus*, and *Salmonella*, finding Gr_{max} values highest for *L. monocytogenes* and *S. aureus* in the Coalho samples tested. The authors found temperature to be the most influential of all tested growth-

controlling factors in the 4 bacteria studied (de Araujo 2017). Bezerra and colleagues manufactured lab-scale Coalho cheeses using probiotics: (1) *Lc. lactis* + *Lc. lactis* subsp. *cremoris*, (2) *Lb. acidophilus*, (3) *Lb. paracasei*, (4) *Bifidobacterium lactis*, or (5) a mixture of all probiotic strains. Cheeses made with *Lb. acidophilus*, *Lb. paracasei*, or the mixture of all probiotic strains resulted in higher lactose hydrolysis and hence increased organic acid production. While all bacteria studied were shown to increase glucose and galactose levels over 28 days of storage at 10ºC, the *Lb. paracasei* treatment resulted in significantly higher glucose and galactose levels versus the other treatments. This treatment as well as the mixture of all probiotic strains additionally exhibited higher propionic acid production than the other treatments, while the *Bif. lactis* treatment produced higher levels of acetic acid than the other probiotics tested due to its heterofermentative nature. Lactic, formic, citric, propionic, acetic, and pyruvic acids were found in the lab-scale Coalho cheese samples, attributed to metabolism of resident LAB and likely to inhibit pathogen growth, though no challenge study data was generated by Bezerra and colleagues in their model system (Bezerra 2017).

FORMULATION CONTROL OF *L. MONOCYTOGENES*

L. monocytogenes behavior within a cheese varies based on several criteria specific to each variety, including different physico-chemical conditions during manufacture, ripening, and storage as dictated by starter cultures, level and form of acidity, temperature, duration of ripening and storage, moisture, salt, and a_w (Melo 2015). In an interagency risk assessment for listeriosis mitigation from retail delicatessen foods, the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service concluded that inclusion of *L. monocytogenes* growth inhibitors within formulations for RTE products such as cheese, meat, and salads reduced predicted risk of

listeriosis from consuming the foods by 95%. The risk assessors additionally noted delicatessen temperature control, wherein display cases were held ≤5°C / 41°F, to have an impact on mean listeriosis risk, however, of much smaller magnitude (5 to 20% risk reduction) than that achieved with formulation control. While growth inhibitors have little impact on prevalence of *L. monocytogenes* within a food product, they significantly impact levels of the pathogen at the time of consumption and are therefor recommended by USDA for *L. monocytogenes* control in these products (USDA 2013).

CONTROL OF *L. MONOCYTOGENES* VIA ORGANIC ACIDS AND THEIR SALTS

pH is defined as the negative logarithm of the hydrogen ion concentration (i.e. pH = -log[H^+]). At equilibrium, the ratio [H^+] [A^-] / [HA] is a constant, K_a. The pK_a is equal to the pH when the concentration of the dissociated acid and undissociated acid in a solution are equal. In acidic food, the proportion of undissociated acid present increases with the low pH (Mani-Lopez 2012). Strong acids have very low pK_a values, as they are almost entirely dissociated in solution. Organic acids are weak, carboxylic acids that only dissociate partially in aqueous solutions. They have appreciable lipid solubility in their undissociated form, which allows them to diffuse freely through the bacterial plasma membrane into the cytoplasm (ICMSF 1980, Ita 1991). Weak acids have higher pK_a values and are thought to be more inhibitory to *L. monocytogenes* than strong acids at a given pH. When the undissociated acid passes through the plasma membrane into the more alkaline pH of the cytoplasm, it dissociates, releasing a proton and the acid anion and in turn acidifying the cytoplasm. If the cell tries to maintain a constant intracellular pH, it will need to employ a proton translocating ATPase in order to remove protons and thus metabolically burdening the cell while decreasing the energy available to support its growth. If the proton

concentration becomes too high, the pH gradient collapses, leading to cell death. Additionally, the cell accumulates the acid anion, which can disrupt intracellular processes. The antimicrobial potential of a weak acid is therefor determined by its pK_a and by the intrinsic toxicity of the acid anion (Adams 1997). Eklund found the undissociated sorbic acid form to be 10 to 600 times more effective as a bacterial inhibitor than the dissociated acid (Eklund 1983). At pH 6, citric, lactic, acetic, and propionic acids are undissociated 0.006, 0.64, 5.1, and 6.67%, respectively (ICMSF 1980).

Short chain organic acids, including acetic, benzoic, citric, propionic, and sorbic, are most commonly used as food preservatives due to their solubility, taste, and low toxicity. Antimicrobial activity of organic acids generally increases with chain length. Only organic acids that are lipophilic show antimicrobial activity. Undissociated, uncharged weak lipophilic acids (e.g. acetic) are permeable through the cell membrane, while lactic and citric acids are generally not with their passage thought to be carrier-mediated (Ita 1991). Most organic acids are ineffective in inhibiting microorganisms in the pH range 5.5 to 6.8, with the exception of propionic and sorbic acids which show some activity to pH 6.0 and 6.5, respectively. Organic acids are typically ineffective when initial microbial load is high, and many microorganisms use organic acids in metabolism as carbon sources (ICMSF 1980).

Lactic acid is a monocarboxylic acid produced by fermentation (e.g. LAB). Wemmenhove and colleagues found D-lactic acid to be more inhibitory to *L. monocytogenes* than L-lactic acid when studying individual strains in a broth system; however, the authors found the growth of *L. monocytogenes* to be insignificantly different between isomers compared to strain-to-strain variations observed in growth rates (Wemmenhove 2016). Acetic acid is a monocarboxylic acid with a pungent odor and taste, limiting its use in foods. It is highly water

soluble and the principal component of vinegars. Acetic acid is found in pickled products and has GRAS status for miscellaneous and general purpose usage under 21 CFR 184.1005 (Mani-Lopez 2012). The main inhibitory action of acetic acid is to neutralize the electrochemical potential of cell membranes and lower the intracellular pH; lactic acid is thought to work by a similar mechanism (Eifert 1997). Additional mechanisms responsible for lactic acid / lactate inhibition of pathogenic bacteria include a_w lowering, acidification of internal pH, inhibition of ATP formation from proton transfer across bacteria cell membrane, feedback inhibition by lactate anion, the ability of the lactate ion to penetrate the microbial cell membrane, and inhibition of enzymes involved in pyruvate to lactate conversion (Houtsma 1994). Acetic and lactic acids have been found to be more effective in synergistic combinations and under anaerobic conditions. 1 to 2% undissociated acetic acid in food products typically inhibits all microorganisms, with smaller concentrations effective when coupled with refrigeration and high salt or sugar content. 0.1% undissociated acetic acid is inhibitory to most vegetative and sporeforming foodborne pathogens, while molds require 0.3% for inhibition. Only *Acetobacter* spp., certain LAB, and some yeasts and molds are appreciably resistant to acetic acid (ICMSF 1980).

Citric acid is produced naturally by various plants. It is a white powder extracted from citrus fruits including lemons, limes and pineapples and can also be produced via fermentation of glucose. It is a tricarboxylic acid highly soluble in water and primarily insoluble in fat (Jamilah 2008). Citric acid is known to inhibit cells through metal chelation and/or destabilization of the bacterial outer membrane (Mani-Lopez 2012). Burel and colleagues found tribasic citric acid (CA^{3-}) to cause large disruption of the Gram-negative membranes and subsequent inactivation in *Klebsiella aerogenes* and *E. coli* populations, however, the researchers found Gram-positive *S. aureus* able to withstand 10% CA^{3-} with minimal inactivation. The authors concluded that

chelation by CA^{3-} of multivalent ions present in the membrane was likely the observed discrepancy in antimicrobial action between the organisms (Burel 2021).

The pH of cow's milk is typically 6.5 to 6.7. Cow's milk naturally contains 0.05 to 0.08 mM acetate, 9 mM citrate, and <0.4 mM lactate as well as lactose for LAB to convert to lactic acid (Walstra 1984). The pK_a of lactic acid in cheeses is lower than that in water, as Ca^{2+} and Na^+ ions, available to complex with Lac^- ions, are present in cheese in high concentrations, depending on cheese variety. This in turn limits the formation of HLac in cheese by limiting complexation of Lac^- with H^+, thus lowering the pK_a value in the cheese water phase. Conversely, lactic acid in water contains only HLac, H^+, and Lac^- ions and has a pK_a value of 3.86 (Wemmenhove 2019). A lactic acid pK_a value of 3.71 was found in 4-week old Cheddar by Morris and colleagues based on HLac, Lac^-, and H^+ contents (Morris 1988). Citrate can be cometabolized with sugars by several LAB used in cheese production to synthesize diacetyl and with CO_2 as a byproduct, including *Lc. lactis* subsp. *lactis* bv. diacetylactis and *Leuc. mesenteroides*. Trivett and Meyer found 8 citric acid cycle intermediates including citrate to be unacceptable carbon and energy sources for growth of two *L. monocytogenes* strains with or without the addition of an auxiliary carbon source (0.05% glucose). The authors found α-ketoglutarate dehydrogenase and succinate dehydrogenase enzymes to be absent in the studied strains and proposed a split citrate pathway for *L. monocytogenes* (Trivett 1971). The inability of *L. monocytogenes* to utilize citrate as a sole carbon source was additionally found by Friedman and Kautter in a different strain of the pathogen (Friedman 1962).

Organic acids do not fully dissociate in water, and substitution of their donable proton with a monovalent (Na^+, K^+) or multivalent (Ca^{2+}) cation increases their solubility in aqueous solutions. Lactates, acetates, diaceteates, and propionates have been used extensively in meat

products to target bacteria, yeasts, and/or molds (Mani-Lopez 2012). Sodium lactate and sodium diacetate are approved for food use in the U.S., Canada, and Europe. Sodium lactate is the sodium salt of L-lactic acid and available as a 60% aqueous solution (Zuliani 2007). Sodium lactate has been recommended as a flavor enhancer in fresh and cooked meat and poultry when added at levels of 2 to 3% (w/w), with additions of up to 4% found not to alter meat pH (Chen 1992). Salts of organic acids act in three ways for bacterial inhibition: 1) by lowering the a_w of the food, 2) by lowering the cell pH, and 3) by inhibiting enzymes (Houtsma 1994). Because of limitations related to undesirable sensory effects including acid, salty, and metallic flavors, maximum usage rates of 2 to 3% and 0.12% for sodium lactate and sodium diacetate, respectively, in RTE meats are typically utilized for *L. monocytogenes* control (Horita 2018). Potassium and calcium lactate were found to be as effective as sodium lactate in suppressing *L. monocytogenes* growth and a_w in a cooked strained beef meat model system, suggesting the lactate ion to be the inhibitory component of lactate salts (Chen 1992).

COMPARISON OF ORGANIC ACIDS AND THEIR SALTS IN MICROBIOLOGICAL MEDIA

Several authors have compared MIC values of organic acids for *L. monocytogenes* control at pH values relevant to dairy products. Coroller and colleagues documented MIC values of 0.2 to 3.6 mM, 21.5 mM, and 6.4 mM for undissociated citric, acetic, and lactic acids, respectively, in *L. innocua*-inoculated BHIB stored at 30ºC (Coroller 2005), while Le Marc and colleagues found MIC values of 20.3 mM, and 8.0 mM, for undissociated acetic and lactic acids, respectively, in *L. innocua*-inoculated modified BHIB stored at 20ºC (Le Marc 2002). Mejlholm and Dalgaard found MIC values of 2.12 mM, 10.3 mM, and 3.79 mM, for undissociated citric,

acetic, and lactic acids, respectively, in *L. monocytogenes*-inoculated BHIB with pH 6.0 stored at 8ºC (Mejlholm 2009). MIC values of 3.8±0.9 mM, 19.0±6.5 mM, and 5.0±1.5 mM, for undissociated citric, acetic, and lactic acids, respectively, were found by Wemmenhove and colleagues in *L. monocytogenes*-inoculated BHIB with pH values 4.6 to 5.8 stored at 30ºC (Wemmenhove 2016). Additionally, Aryani and colleagues found undissociated lactic acid to have an average MIC of 5.11 mM and a maximum MIC of 6.35 mM at pH 5.50 among 20 *L. monocytogenes* strains assayed in microbiological media (Aryani 2015).

Conner and colleagues investigated behavior of 4 strains of *L. monocytogenes* in TSB with yeast extract (TSBYE) acidified with acetic, citric, hydrochloric, lactic, or propionic acid to pH 4.0 to 6.0 in 0.5-unit increments with incubation at 4 or 30ºC. Minimum inhibitory pH values found were 5.0 for propionic acid, 4.5 for acetic and lactic acids, and 4.0 for citric and hydrochloric acids, representing undissociated acid contents of 0.013 M, 0.044 M, 0.002 M, 0.003 M, and <0.001 M, respectively. Based on total (undissociated and dissociated) acid molarity of the organic acids tested, citric (0.029 M) and propionic (0.031 M) acids had the lowest inhibitory concentrations observed, while acetic acid (0.068 M) had the highest. The authors additionally found lactic and citric acids to have the lowest concentrations of undissociated acid at their inhibitory pH values, while acetic acid had the highest (Conner 1990). This was in agreement with findings from Sorrells and colleagues, who found that on an equal molar basis, citric > lactic > acetic in *L. monocytogenes* inhibition (Sorrells 1989).

Farber and colleagues investigated 4 *L. monocytogenes* strains grown in double-strength BHIB adjusted to various pH values with acetic, lactic, citric, or hydrochloric acid. The authors found acetic acid to be the most effective inhibitor. No growth was observed using acetic acid at pH values <5.6 at 4ºC and <5.0 at 30ºC. HCl was the least effective of the studied acids, with a

minimum pH for growth of 4.3 at 30ºC and 5.0 at 4ºC. In broth adjusted with lactic acid, the authors found a minimum pH for growth of 5.0 to 5.5 at 4ºC and 4.9 to 5.1 at 30ºC. In broth adjusted with citric acid, *L. monocytogenes* growth was observed at pH values of ≥5.3 at 4ºC and ≥4.4 at 30ºC (Farber 1989). Another research group, George and colleagues, found *L. monocytogenes* able to grow at pH values as low as 5.2 at 4ºC with pH adjustment to culture medium with HCl, however, *L. monocytogenes* growth was inhibited at low pH values in the presence of other acidulants including lactic acid (George 1988).

Sorrells and colleagues investigated the effects of hydrochloric, acetic, lactic, malic, and citric acids on *L. monocytogenes* growth in TSB pH-adjusted to pH values 4.4, 4.6, 4.8, 5.0, and 5.2 with storage at 10, 25, and 35ºC for 28 days. The authors observed enhanced growth of *L. monocytogenes* at 25ºC and enhanced *L. monocytogenes* inactivation by organic acids at 35ºC. Antimicrobial activity of the acids fell in decreasing order from acetic > lactic > citric ≈ malic > hydrochloric acid at all incubation times and temperatures at equivalent pH values. Based on equal molar concentrations of organic acids, antimicrobial activity of the acids fell in decreasing order from citric ≈ malic > lactic ≈ acetic > hydrochloric acid at 25ºC and 35ºC and from malic > citric > acetic ≈ lactic > hydrochloric acid at 10ºC. The authors observed maximum pH values where no *L. monocytogenes* growth was observed to be ≤4.4 for malic acid, ≤4.4 for citric acid, 4.4 to 4.6 for lactic acid, ≤4.4 for hydrochloric acid, and 4.8 to 5.0 for acetic acid (Sorrells 1989).

Ita and colleagues investigated the effects of citric, acetic, lactic, and hydrochloric acid on *L. monocytogenes* strain Scott A grown in TSBYE in a fermentation vessel. The authors inoculated the fermentation vessel with 10% (v/v) *L. monocytogenes*. The pH of the media was adjusted from pH 6.5 to pH 6.0, 5.5, 5.0, 4.5, 4.0, or 3.5 by addition of the appropriate acid. pH was held at each point for 4 to 6 h before removing samples for enumeration. At the lower pH

values (≤4.5) an additional sample was transferred and held for 24 h at 37ºC to determine the effect of longer exposure on intracellular pH. In general, intracellular pH values near 5.0 were maintained even at low extracellular pH values for TSBYE adjusted with hydrochloric and lactic acids. Cells treated with acetic acid at an extracellular pH of 4.0 and 3.5 had lower intracellular pH values of 4.84 and 4.65, respectively. Cells treated with citric acid were least able to maintain a pH gradient at low extracellular pH values, and with external pH values of 4.0 and 3.5, intracellular pH values were 4.37 and 4.13, respectively. The authors found acetic acid-treated cells at pH 4.0 and 3.5 held for an additional 24 h to cause much greater reductions in *L. monocytogenes* numbers than the other acids. Lactic acid had a slightly inhibitory effect, while citric and hydrochloric acids were less inhibitory. Acetic acid-treated cells, when held an additional 24 h at 37ºC, were found to decrease the cell count by 4-log with an intracellular pH near 5.0, while the other acids tested showed ≤1-log decreases at the same extracellular pH. Even with an intracellular of pH 3.5 in citric acid-treated cells held for 24 h viable cells were still present in high numbers (7.39 log CFU/ml). The authors concluded that inhibition was caused by a specific effect of protonated acetic acid on metabolic activity of *L. monocytogenes*, rather than the medium pH or the intracellular pH (Ita 1991).

Le Marc and colleagues investigated growth of a single strain of *L. innocua* in the presence of lactic, acetic, and propionic acids at varying temperatures and pH values in modified BHIB. Following autoclaving, half of the BHIB was supplemented with sterile glucose (0.2% w/w) and yeast extract (0.3% w/w). pH adjustments were made using HCl and NaOH. Acetic acid was tested at pH values of 5 to 7.5 (16 to 64 mM), propionic acid at pH values 5.4 to 7.5 (18 to 55 mM), and lactic acid at pH values 4.8 to 7.1 (40 to 138 mM). MIC values for lactic, acetic,

and propionic acids were found to be 8.0, 20.3, and 8.8 mM, respectively, at 20ºC (Le Marc 2002).

Houtsma and colleagues investigated the MIC of sodium lactate for *L. monocytogenes* and spoilage organisms in laboratory media at pH 6.5 and 20ºC. The authors found Gram-positive bacteria to be more sensitive to lactate than Gram-negative bacteria. The authors also found yeasts to be resistant to large amounts of sodium lactate (>10% w/v). In a separate set of experiments, the same research group studied the effect of NaL concentration on the growth of *L. innocua* in yeast extract / peptone broth at pH 5.5, 6.0, 6.5, and 7.0 at 4, 10, 20, and 30ºC. The authors found MIC of NaCl to be significantly higher than MIC of NaL, and that MIC values were not significantly influenced by storage temperature. pH of the medium was shown to influence the MIC of NaL but not of NaCl, and growth inhibition was achieved with lower NaL addition (217 mM) at low pH (5.5) than at high pH (7.0) (1071-1339 mM). No synergistic effect of NaCl and pH on the growth of *L. monocytogenes* was found (Houtsma 1994).

COMPARISON OF ORGANIC ACIDS AND THEIR SALTS IN DAIRY

Glass and colleagues investigated the effect of acid type (citric, malic, or acetic) on the behavior of *L. monocytogenes* in a queso blanco cheese. The authors manufactured queso blanco (pH 5.2) via direct acidification with citric, malic, or acetic acid before inoculating curd with 10^6 CFU/g of a 5-strain cocktail of *L. monocytogenes* prior to hooping, pressing, slicing, and vacuum-sealing. Inoculated cheeses were stored at 4°C for 42 days, with cheeses enumerated for *L. monocytogenes* numbers throughout storage. Average proximate composition for the cheeses was found to be 51 to 53% moisture, 1.9 to 2.0% salt, and pH 5.2 to 5.3. The authors found *L. monocytogenes* numbers decreased 0.7 log CFU/g (from 6.50 to 5.80 log CFU/g) in queso blanco

made with acetic acid, while in cheeses made with malic and citric acids, numbers increased 1.1 and 1.2 log CFU/g (from 6.41 to 7.54 log CFU/g and from 6.52 to 7.67 log CFU/g), respectively, over the course of the study. The authors calculated the percentage of undissociated acetic, malic, and citric acid to be 26.7, 0.65, and 0.13%, respectively, in the tested queso blanco, and speculated that the decreasing percentage of undissociated acid might explain the observed difference in *L. monocytogenes* behavior (Glass 1995).

Silva and colleagues investigated sodium lactate and sodium propionate both in combination with sodium acetate on *L. monocytogenes* growth in the soft cheeses Minas Frescal and Coalho, both produced using rennet and LAB. Cheeses were procured from the supermarket and inoculated with *L. monocytogenes* before treatment via dipping with antimicrobials or sterile water in the case of control cheeses. Included treatments were 2% (w/v) sodium lactate and 0.25% sodium acetate or 2% sodium propionate and 0.25% sodium acetate. Samples were enumerated for *L. monocytogenes* immediately after inoculation and after 7 days storage at 10ºC. pH values for Minas Frescal cheeses at time zero were 6.3, 6.7, and 6.2 in control samples, samples treated with 2% sodium lactate + 0.25% sodium acetate, and samples treated with 2% sodium propionate + 0.25% sodium acetate, respectively, and were reduced to 5.6, 6.2, and 5.9, respectively, following 7 days storage. pH values for Coalho cheese at time zero were 5.0, 5.5, and 5.8 in control samples, samples treated with 2% sodium lactate + 0.25% sodium acetate, and samples treated with 2% sodium propionate + 0.25% sodium acetate, respectively, and changed to 5.3, 5.4, and 5.6, respectively, following 7 days storage. In Minas Frescal cheese, *L. monocytogenes* numbers were reduced from 5.8 to 3.6 log CFU/g at time zero when treated with 2% sodium lactate + 0.25% sodium acetate or 2% sodium propionate + 2% sodium acetate. After 7 days storage at 10ºC, numbers had reached 6.5 log CFU/g in the control Minas Frescal cheese,

while sodium lactate + sodium acetate samples had risen to 5.4 log CFU/g and sodium propionate + sodium acetate to 5.6 log CFU/g. In Coalho cheese, 2% sodium lactate + 0.25% sodium acetate reduced pathogen numbers from 5.4 to 3.7 log CFU/g initially, and after 7 days of 10ºC storage, numbers for control and treatment cheeses were 6.5 and 5.5 log CFU/g, respectively. Coalho cheese treated with 2% sodium propionate + 0.25% sodium acetate *L. monocytogenes* numbers initially and after 7 days storage were found to be 3.6 log CFU/g and 5.7 log CFU/g, respectively. The authors found no significant difference between the two treatment groups in terms of *L. monocytogenes* counts at the beginning and end of storage, however, both treatment groups were found to have significantly lower *L. monocytogenes* counts than control samples (Silva 2012).

Lourenco and colleagues inoculated queso fresco curds (pH 6.0) surface-treated with a sodium lactate (3%)/sodium diacetate (0.22%) mixture with 4 log CFU/g *L. monocytogenes* and stored 3 weeks at 4ºC. The authors observed <1 log CFU/g reduction in final *L. monocytogenes* counts versus control cheese. A combination of sodium lactate (3%)/sodium diacetate (0.22%) and lactic acid (1.2%), however, resulted in a >4 log CFU/g reduction in *L. monocytogenes* final counts versus control queso fresco (Lourenco 2017).

PH THRESHOLD OF *L. MONOCYTOGENES* GROWTH AND SURVIVAL IN CHEESE

Discrepancies between the actual minimum pH value for *L. monocytogenes* growth exist in microbiological media and foodstuffs including cheeses. Irvin found *L. monocytogenes* unable to grow in silage at pH values below 5.5 (Irvin 1969), while a minimum pH of 4.39 for *L. monocytogenes* growth in TSB adjusted with HCl was reported by Sorrells and colleagues (Sorrells 1989). Unclarified cabbage juice adjusted with lactic acid to pH values of ≥5.2

supported immediate *L. monocytogenes* growth at 30ºC, while a pH of 5.0 supported its growth after a 3-day lag phase. No growth of *L. monocytogenes* was observed in cabbage juice with pH values of ≤4.8, with faster inactivation observed with decreasing pH values. Conversely, at 5ºC the cabbage juice was unable to support *L. monocytogenes* growth, with the organism remaining near inoculation levels for 22 days in juices with pH values ≥5.2; a gradual decline of *L. monocytogenes* was observed in samples of pH ≤5.0 (Conner 1986). Among skim milks cultured with *Lc. lactis* subsp. *cremoris* or *Lc. lactis* subsp. *lactis* and inoculated with a single strain of *L. monocytogenes*, pH 4.75 appeared to be completely inhibitory to *L. monocytogenes* growth (Schaak 1988). Ryser and Marth found 4 strains of *L. monocytogenes* unable to grow at 6ºC in cultured or uncultured whey adjusted to pH 5.0 with NaOH or pH 5.2 with lactic acid. In cultured whey with pH 5.4, 2 *L. monocytogenes* strains were unable to grow, while at pH 5.6, growth was observed in all tested strains (Ryser 1988).

In cheese, a pH value of <5.0 was shown to inhibit *L. monocytogenes* growth within Feta by Papageorgiou and Marth (Papageorgiou 1989). Belessi and colleagues observed pH 5.0 to be a threshold allowing prolonged *L. innocua* survival in Feta cheese (moisture 55 to 57%) with storage at 3ºC (Belessi 2008). Govaris and colleagues found Feta cheese of pH 4.55, 53.4% moisture, and 2.14% sodium chloride unable to support *L. monocytogenes* growth, however, survival at 4ºC for 32 days was observed when approximately 4 log CFU/g *L. monocytogenes* was inoculated onto the cheese surface (Govaris 2011). Konteles and colleagues inoculated brined (7% NaCl, w/w) feta cheese with 5 to 6 log CFU/g *L. monocytogenes* and monitored its behavior over 30 days storage at 4°C. Numbers of the organism remained relatively constant, showing no additional growth over the storage period, which may have been due to the high level of LAB found to be 8.5 log CFU/g at the onset of the study and remaining at this high level

over the 30-day study. Analytical values for the affected cheese at study onset were pH 4.58, 56.39% moisture, and 2.83% salt, with little change reported for any of the analytical parameters by the end of study (Konteles 2009). Hariram and colleagues identified pH 5.25 as a threshold for *L. monocytogenes* inhibition in their testing of 8 commercial queso fresco samples at 4 to 7°C (Hariram 2020). In Camembert and Brick cheeses, a pH of >5.75 during ripening allowed substantial growth of *L. monocytogenes* (Ryser 1989). Ryser and Marth found Camembert surfaces and wedges to require minimum pH values of 5.6 to 6.3 and 6.2 to 6.7, respectively, for *L. monocytogenes* growth to occur during ripening of the cheese (Ryser 1987). Genigeorgis and colleagues observed a highly significant effect of pH >5.5 and absence of starter culture to be correlated with *L. monocytogenes* growth across 24 cheese varieties tested (Genigeorgis 1991). Similarly, Jayamanne and Samarajeewa found no growth of *L. monocytogenes* to be observed below pH 5.5 in buffalo curd cheese made using natural starter culture while buffalo milk acidified to pH 5.5 with lactic acid instead of starter culture allowed *L. monocytogenes* growth with ambient storage (Jayamanne 2010). The same threshold (pH >5.5) was observed to delineate cheeses more likely to harbor *Listeria* spp. from those unlikely (Paxson 2008), and other authors have reported *L. monocytogenes* capable of growing only at pH values >5.5 in Galotyri, a traditional Greek soft acid-curd spreadable cheese (Rogga 2005). A pH value of 4.35 in Galotyri cheese was shown to inactivate *L. monocytogenes* in 16 to 27 days, however, a higher pH of 4.53 in Touloumotyri cheese allowed survival of the pathogen for >40 days (Papageorgiou 1998). In Greek whey cheeses Myzithira, Anthotyros, and Manouri with pH values ranging from 6.0 to 6.5, *L. monocytogenes* was shown to grow under refrigerated and ambient conditions; a critical pH of 5.7 was identified for *L. monocytogenes* inhibition in these cheese varieties (Papageorgiou 1996). Within the context of a literature analysis from 114 scientific challenge studies, growth of *L. monocytogenes* introduced either into cheesemilk or onto cheese curd after

fabrication was observed at an average pH of 6.11 (n=413) and inhibition at an average pH of 5.26 (n=690). ≥50% probability of *L. monocytogenes* growth inhibition was estimated in cheeses of pH≤5.64 (95% CI: 5.28, 6.01), and ≥95% probability of *L. monocytogenes* growth inhibition in cheeses of pH≤4.51 (95% CI: 4.11, 4.89) (Engstrom 2012; Appendix 1).

CLEAN-LABEL AND BIOPRESERVATION

Consumer demand for more natural preservatives is increasing as consumer perception of chemically-derived preservatives as chemical or artificial has arisen (Smith-Palmer 2001). Approximately 20% of food products launched from 2015 to 2020 contained a "No Added Preservatives" claim and global clean-label food sales were projected to reach $180 million in 2020 (Kerry 2020). Although no regulatory definition for clean-label has been established, clean-label foods are usually free from artificial colors, flavors, and additives. Consumers additionally expect clean-label foods have simple, easy-to-understand ingredients and for clean-label products to be minimally processed or processed using traditional techniques (Grant 2017). Examples of clean-label ingredients include citrus extracts, fermentates, cultures, vinegar, and lemon juice (Kerry 2020).

Biopreservation, or the use of microorganisms and/or their metabolites to improve the safety of foods, has been used extensively in RTE foods in recent years as a clean-label strategy. Biopreservatives can include LAB and their metabolites, endolysins, bacteriophages, and protective cultures. LAB have major potential for use in biopreservation because they are safe to consume, they dominate the microbiota during storage, and they can effectively inhibit undesirable microorganisms (Holzapfel 1995). Mechanisms of bacterial interference include: 1) competition for nutrients, 2) competition for attachment or adhesion sites, 3) unfavorable

alteration of environment, or combinations of the three. Lactic antagonism mechanisms include production of antibiotics, hydrogen peroxide, diacetyl, bacteriocins, pH depression, and/or nutrient depletion (Said 2019). Principle mechanisms of *L. monocytogenes* inhibition by LAB include the production of organic acid(s) with simultaneous pH decrease in the foods in which they grow and/or the production of ribosomally synthesized proteinaceous inhibitors (bacteriocins) by LAB (Favaro 2015). A primary advantage of using bacteriocin-producing bacterial cultures is that bacteriocin is produced during manufacture versus added as a concentrate and thus potentially considered a food additive requiring labeling (Ross 2000). The use of protective cultures in meats was found to be more acceptable to producers and consumers than the addition of semi-purified bacteriocins (Alves 2006).

Secondary contributors to antilisterial effects include production of hydrogen peroxide, ethanol, carbon dioxide, diacetyl, or reuterin, low redox potential, nutrient depletion and/or competition, and crowding (Adams 1997, Ghrairi 2004, Tirloni 2019). In the presence of oxygen, LAB produce hydrogen peroxide via flavoprotein oxidases. Because LAB are catalase-negative, hydrogen peroxide accumulates and has been shown to be inhibitory against both Gram-negative and Gram-positive foodborne pathogens, while LAB have been shown to be more resistant to the metabolite (Adams 1997). Hydrogen peroxide production depends on the oxygenation of the medium, with more hydrogen peroxide produced at lower temperatures (where solubility of oxygen is higher) and when shaken cultures are used. Hydrogen peroxide accumulation is likely restricted to the surface of semi-solid foods due to permeability restrictions into solid media (Adams 1997). The compound acts alone or in combination with the lactoperoxidase system found in milk, though lactoperoxidase activity is greatly reduced following pasteurization. Dominguez and colleagues found hydrogen peroxide concentrations >0.0495% necessary to

inhibit *L. monocytogenes* growth in fermented milk, as lower concentrations inhibited competing microflora, thus increasing outgrowth of *L. monocytogenes* during fermentation (Dominguez 1987). Diacetyl (2,3-butanedione) is produced from pyruvate by several species of LAB. Little pyruvate is available when LAB are actively growing on a readily fermented carbohydrate because most of the pyruvate is being used to regenerate NAD from NADH. When cells reach stationary phase or when an alternative form of pyruvate (e.g. citrate) is available, diacetyl can accumulate. Gram-negative bacteria are generally more sensitive, however, levels necessary to inhibit yeasts and Gram-negative (200 ppm) or Gram-positive (300 ppm) bacteria are not found in cultured dairy products (Adams 1997).

PROTECTIVE CULTURES

Protective cultures are defined as live microorganisms that are added deliberately to foods to control their bacteriological status without changing their technological and sensory qualities (Said 2019). Starter cultures, on the other hand, are used to deliver beneficial metabolic as well as sensory changes in a food product, generally accompanied by a preservation effect (Holzapfel 1995). Protective cultures should meet the following criteria: 1) have GRAS status, 2) survive and maintain activity during product manufacture and distribution, 3) are able to grow in the food at refrigeration temperature, 4) have no effect on flavor, texture, or intrinsic characteristics, 5) are able to inhibit pathogenic or spoilage organisms in the product, and 6) do not produce harmful substances (Said 2019). Fermenting “protective cultures” should be considered adjunct or multifunctional cultures and fall in-between starter cultures and protective cultures; however, some commercially available protective cultures may be fermentative (Holzapfel 1995). In order for efficient LAB antagonistic activity to stop listerial growth, the

natural or supplemental protective microflora must quickly reach high counts to be favored in microbial competition against *L. monocytogenes* (Tirloni 2019). Bacterial interference usually requires levels of 10^6 CFU/g or higher for effectiveness, a level difficult to achieve and maintain in refrigerated products (Jay 2005).

Very few protective cultures directly targeting *L. monocytogenes* control in cheese are currently commercially available. HOLDBAC™ *Listeria* Dairy is a commercial freeze-dried protective culture known to contain a pediocin AcH/PA-1-producing *Lactobacillus plantarum* strain (Roth 2009). The product claims not to alter the surface ripening flora of cheese nor affect sensory properties, with application at either during dry salting or via directly spraying onto the cheese surface suggested (Vytrasova 2010). Chr. Hansen B-LC-20 is a *Pediococcus acidilactici* protective culture and B-LC 48 a *Lb. curvatus* protective culture, both designed for the control of *L. monocytogenes* in meat products. A third Chr. Hansen protective culture, BS-10, is a *Lc. lactis* subsp. *lactis* culture intended for the control of Gram-positive spoilage organisms in ripened cheeses, including *Clostridium* spp. and *Bacillus* spp. (Gensler 2020). DuPont HOLDBAC™ *Listeria* Dairy and Chr. Hansen BS-10 commercial protective cultures were found by Aljasir and colleagues to effectively inhibit the growth of *L. monocytogenes* and *S. aureus* when the individual cultures were co-cultured in raw milk incubated at elevated temperatures simulating raw milk cheesemaking and ripening (Aljasir 2020). Micocin, a *Carnobacterium maltaromaticum* culture, has been approved by Health Canada for inhibiting *L. monocytogenes* in soft cheeses (Said 2019). LALCULT® Protect *Staphylococcus xylosus* XF01, produced by Lallemand, is a protective culture designed for *L. monocytogenes* control in dairy. Three protective cultures manufactured by SACCO, LPAL, CNBAL, and LRB, are *Lb. plantarum*, *Carnobacterium* spp., and *Lb. rhamnosus* cultures, respectively, with the first two intended for

cheese surface treatment for *L. monocytogenes* inhibition and the third a general inhibitor of yeasts and molds in addition to *L. monocytogenes* (Gensler 2020).

An increased interest in the use of *Enterococcus* spp. as protective cultures in dairy foods has arisen due to their antilisterial activity, attributed to their close phylogenetic vicinity to *Listeria* spp. Concerns over the safety of *Enterococcus* spp. have limited their intentional inclusion in dairy foods, with absence of hemolysin activity for their use as protective cultures stressed as a safety requirement by researchers. Enterococci have been associated with nosocomial infections (e.g. UTIs, catheter-related infections, wound infections), possess multiple antibiotic-resistant genes, and an increased number of vancomycin-resistant strains have been observed in hospitals over recent years. Because of these concerns, enterococci do not yet possess GRAS status, despite their presence in many traditional Mediterranean cheeses (Martin-Platero 2009). Parapouli and colleagues found elimination of *L. monocytogenes* from Galotyri cheese made from ewe's milk to be due to high numbers of enterocin-producing *Enterococcus* spp. present (Parapouli 2016).

NISIN AND OTHER BACTERIOCINS

Bacteriocins are ribosomally synthesized antimicrobial peptides of 3 to 4 kDa molecular weight produced by bacteria (Iseppi 2008). Bacteriocins can be used in 3 ways: 1) as (semi)purified antimicrobials, 2) as bacteriocin-based ingredients from fermentations, or 3) by using bacteriocin-producing starter or protective cultures wherein they are produced *in situ* (Dal Bello 2012, Sobrino-Lopez 2008). The mode of action of bacteriocins can be summarized in the following steps: 1) binding to the cytoplasmic membrane, 2) inserting bacteriocin molecules in the membrane, and 3) forming a pore complex leading to dissipation of the proton motive force

of the target cell (Loessner 2003). Bacteriocins including nisin, lacticin 3147, enterocins A and B, enterocin 416K1, sakacin, and pediocin AcH have all been shown to control *L. monocytogenes* growth (Iseppi 2008). Currently, only nisin and pediocin PA-1/AcH are commercially exploited (Favaro 2015).

Nisin is a bacteriocin produced by strains of *Lc. lactis* subsp. *lactis* and is classified as a Class I, Type A lantibiotic. Nisin was granted GRAS status in 1988 by FDA as an antimicrobial against Gram-positive pathogenic and spoilage organisms and has been approved for use in >48 countries (Ghrairi 2004, von Staszewski 2008). Nisin is currently the only lactococcal bacteriocin that has been granted GRAS status. The U.S. allowable limit of nisin in is 250 μg/g food (Gallo 2007). Nisin binds electrostatically to negatively charged phospholipids, increasing membrane permeability through pore formation and resulting in efflux of essential intracellular components (von Staszewski 2008). Nisin binds with high affinity to the peptidoglycan cell wall precursor lipid II which it uses as a docking molecule for pore-forming, leading to interference with cell wall biosynthesis (Ghrairi 2004). This membrane disruption has been found important in nisin's role of inhibiting spore development into vegetative cells (Gut 2011).

Pediocin PA-1-producing pediococci are limited in their use as cheese starter cultures designed for *L. monocytogenes* control due to their inability to ferment lactose rapidly (Reviriego 2007). Additionally, the production of pediocin PA-1 by *Ped. acidilactii* has been reported to be reduced at pH values exceeding 5.0, commonly encountered in high-moisture cheese varieties (Favaro 2015). Lacticin 3147 is a bacteriocin originally isolated from Irish Kefir grains and used predominantly to inhibit Gram-positive organisms. Lacticin 3147-producing *Lc. lactis* starters have been used for NSLAB as well as *L. monocytogenes* control in dairy products. Researchers have found NSLAB to grow to levels as high as 8 log CFU/g in cheeses depending on ripening

conditions and duration and lacticin 3147 to decrease their levels by at least 100-fold over cheese ripening. Using a lacticin 3147-producing starter in hot-pack cottage cheese versus in Cheddar cheese gives a different mode of action to the starter, as the starter is killed off in processing the first product and antilisterial effects can be attributed to residual lacticin seeded into the cheese during manufacturing (Ross 2000).

Enterocins are the most widely produced non-lactic species of bacteriocin, and some *Enterococcus* spp. strains of dairy origin have shown antilisterial properties while showing no activity against useful dairy LAB starters. Enterocins have additionally been shown to maintain activity in the presence of renneting enzymatic action. Characteristic of enterocins include heat stability, stability over a wide pH range (especially under acidic conditions), and a broad spectrum of activity against Gram-positive pathogens (Giraffa 1995).

BACTERIAL FERMENTATES

Although bacteriocins can be produced by starter or protective cultures *in situ* during cheese production, titers achieved are much lower than in *in vivo* fermentations under optimal conditions, as would be realized during manufacture of commercial bacterial fermentates (Favaro 2015). Bacterial fermentates are industrial-scale fermentation byproducts of GRAS status LAB including *Propionibacterium*, *Lactococcus*, or *Pediococcus*. Fermentates are typically freeze- or spray-dried powdered preparations which can contain the fermenting LAB cells either in an inactive or active state, components of the fermenting organism, culture supernatant, fermentation media, metabolites, and bioactives. An important difference between fermentates and nisin or natamycin preparations is that they are not purified by downstream processing and can be labeled “cultured milk” or “cultured dextrose” in the U.S. depending on the fermentation

substrate used in their manufacture. Some commercial fermentates are blended with dried vinegar to increase antilisterial efficacy (Mathur 2020).

Bacterial fermentates were introduced in the late 1980s and early 1990s with MicroGARD. 30% of cottage cheese produced in the U.S. is estimated to contain MicroGARD, a commercially available bacterial fermentate obtained via fermentation of grade A skim milk or dextrose by *Propionibacterium shermanii* or *Lactococcus*. MicroGARD products contain diacetyl, lactic, propionic, and/or acetic acids and other undefined low molecular-mass (~700 Da) inhibitors (Favaro 2015). MicroGARD have been approved by FDA for use in cottage cheese to control *Listeria* spp. and have been shown to retard growth of fungi by several researchers (Al-Zoreky 1991, Ho 2016, Makhal 2015). Makhal and colleagues found MicroGARD 400 to inhibit the growth of Gram-negative bacteria and fungi, as well as to prevent acidity development and proteolysis, extending the shelf-life of cottage cheese by 2 to 4 weeks when incorporated into the product at a 0.5% level. Increasing usage level of MicroGARD 400 was found to impact cottage cheese sensorially, especially with unfavorable textural issues (Makhal 2015). Al-Zoreky and colleagues found MicroGARD 100 to prolong shelf-life of cottage cheese by inhibiting *Pseudomonas* spp., *Salmonella* spp., *Yersinia* spp., and fungi (Al-Zoreky 1991).

LIMITATIONS OF BACTERIOCINOGENIC CULTURES AND SUGGESTED REMEDIES

Limitations of bacteriocinogenic cultures can include: 1) insufficient level of bacteriocin expression, 2) antagonism of other bacteria towards the producer strain, 3) inadequacy of the producer strain as a starter, 4) low capacity for bacteriocin production within a food system, 5)

safety of the producer strain, 6) interaction between the producer strain and the food matrix, and 7) the effect of physicochemical parameters on the bacteriocin activity (Favaro 2015). Some *Lc. lactis* starter strains are inhibited by nisin due to bacteriocins exhibiting inhibitory activity towards bacteria closely related to their producing strain (Ghrairi 2004). The addition of nisin to cheesemilk of soft, white, fresh cheeses made without starter culture (i.e. directly acidified or rennet-set; e.g. ricotta, panir, queso fresco, or queso blanco) might inhibit *L. monocytogenes*, while cheeses made with starter culture would be unsuitable for nisin use due to inhibition unless nisin-resistant or nisin-producing strains were used (Davies 1997). Using adjunct cultures in addition to a mesophilic starter culture used might cause proteolytic, lipolytic, or lactate-converting activity, potentially changing the pH of the cheese and hence the undissociated lactic acid content, a consideration that may impact growth of *L. monocytogenes* (Wemmenhove 2018). In mold-ripened cheeses (e.g. Camembert), growth of surface mold is accompanied by a considerable increase in cheese pH and subsequent proteolytic activity. In addition to the rise in pH promoting *L. monocytogenes* growth, proteinaceous inhibitory substances such as bacteriocins may be hydrolyzed, leading to resumption of normal *L. monocytogenes* growth (Sulzer 1991). Fast acid producers are commonly used as starter cultures, while slow acid producers are often used as adjunct or protective cultures (Ayad 2004). Typically, bacteriocin-producing cultures produce acid at slower rates and have lower heat resistance and reduced proteolytic activity versus commercial starters (Ryan 1996). Bacteriocinogenic *Lactobacillus* and *Enterococcus* strains generally have poor acidifying capacity in milk. A survey of 250 bacteriocinogenic cultures (mainly *Lactobacillus*, *Enterococcus* and *Streptococcus*) obtained from cheeses found only 8% to exhibit promising lipase and protease production to be used as effective cheese starter cultures and fewer showing sufficient acid production (Favaro 2015). In

addition, nisin-producing lactococci are generally more sensitive to bacteriophage than commercial starters (O'Sullivan 2002).

In a survey of wild *Lc. lactis* strains by Ho and colleagues, only 4 of 14 strains tested which showed antilisterial activity on GM17 agar were found able to produce detectable antilisterial activity when grown in UHT whole milk. The authors speculated that differences in antilisterial activity could be attributed to varying bacteriocin levels when grown in milk versus agar or that the milk components themselves might affect activity, especially as milkfat has been shown to negatively affect the activity of nisin against *L. monocytogenes*, possibly due to adsorption of nisin to milkfat globules (Ho 2018). Furtado and colleagues found a *L. monocytogenes* bacteriocin-producing *Lc. lactis* strain isolated from goat milk to produce bacteriocin at lower levels when cultured in milk versus in de Man Rogosa Sharpe (MRS) broth (Furtado 2015). Sarantinopoulos and colleagues likewise noted low enterocin activity with the use of skimmed milk as a substrate versus maximum activity realized with growth in MRS broth at 37°C and pH 6.5 from an *Ent. faecium* strain isolated from Greek Feta cheese. Additionally, the authors detected no enterocin activity through ripening when utilizing the isolate as an adjunct starter in Feta cheese manufacture. The authors concluded that *in vitro* production of bacteriocin by bacteriocinogenic starter or adjunct cultures does not guarantee *in situ* efficiency (Sarantinopoulos 2002). Liu and colleagues found increasing the inoculum level of a recombinant enterocin A-producing *Lc. lactis* strain capable of producing enterocin A from 2% to 6% in milk or microbiological media did not remedy its poor growth and acid production, leading the authors to use the parental *Lc. lactis* strain as a starter culture in cottage cheese manufacture for *L. monocytogenes* inoculation studies (Liu 2007). Avons and colleagues found lactobacilli of intestinal origin to show only slight growth in milk, and suggested either addition

of a growth factor, cocultivation, or fermentation in a different medium followed by subsequent addition to milk in order to overcome limited growth. The authors found yeast extract supplementation to milk to result in ideal growth and bacteriocin production of a single tested *Lactobacillus* strain (Avons 2004). Previous studies have found MRS or Elliker's broth to be optimal for nisin production (Parente 1992).

Bacteriocin secretion from producing cells increases as cell growth increases, peaking during the late exponential phase of growth. Bacteriocin production has been reported to stabilize or drop sharply during early stationary phase depending on the strain studied (Coelho 2014, Daba 1991, Maisnier-Patin 1992). Typically, good cell growth goes hand-in-hand with high bacteriocin production (Sarantinopoulos 2002). Studying a bacteriocin-producing *Leuc. mesenteroides* strain isolated from Cheddar, Daba and colleagues found no bacteriocin activity in the first 5 h of incubation of the strain in MRS broth at 30ºC, a maximum level of nisin production at 8 h (with corresponding culture pH 5.5 and cell count 8.9 log CFU/ml), and >90% of bacteriocin titers to decrease at 24 h (Daba 1991). Authors have suggested that bacteriocin inactivation may be induced by an enzymatic system produced by Nis^+ *Lc. lactis* strains themselves (Maisnier-Patin 1992). Yezzi and colleagues found bacteriocinogenic *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* grown in pH-controlled 10% reconstituted skim milk to produce 2 to 5 more times nisin than bulk cultures grown in non-pH-controlled reconstituted skim milk, and when the cultures prepared by pH-control were used to manufacture Cheddar, concentrations of nisin increased approximately 20% (Yezzi 1993). A review by Abbasiliasi and colleagues of bacteriocin production by LAB found the effect of aeration to impact bacteriocin production in a strain-dependent manner, with added complexity added for facultatively anaerobic cultures, of which the *Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis* bv.

diacetylactis, and *Lb. plantarum* protective cultures (PC-1, PC-2, and PC-3, respectively) included in Chapter 5 of this dissertation are. Overall, the review found among 14 studies for aeration and/or agitation to result in the same or increased bacteriocin production versus no aeration or agitation in the majority of LAB strains tested (Abbasiliasi 2017). Nisin production by *Lc. lactis* was found to be enhanced with aeration in studies by Amiali and Cabo and associated colleagues while the reverse was found by Hurst (Amiali 1998, Cabo 2001, Hurst 1981). Cretenet and colleagues found organic acid production and final redox values to be the same in a *Lc. lactis* subsp. *cremoris* strain grown under anaerobic or microaerophilic conditions (Cretenet 2014). Conversely, Pedersen and colleagues found a single strain of *Lc. lactis* subsp. *lactis* to acidify to a lesser extent with aerobic versus anaerobic storage (Pedersen 2008). Smetankova and colleagues found no significant differences in production of lactic acid or ethanol by three *Lb. plantarum* strains when grown aerobically or anaerobically; however, the same authors found acetic acid production to differ significantly between the two incubation conditions in a single strain (Smetankova 2012).

LIMITATIONS OF BACTERIOCINS AND SUGGESTED REMEDIES

Qualities of an ideal antimicrobial include: 1) a spectrum of activity directed against harmful flora, 2) physicochemical properties enabling the antimicrobial to resist heat treatments and pH changes encountered in the food industry, and 3) a small size, consistent with rapid diffusion in semi-solid systems; bacteriocins meet some of these requirements (Giraffa 1995). Nisin is bactericidal against *L. monocytogenes*, with its effects enhanced by the addition of NaCl or a reduction in pH (Arques 2015). Samelis and colleaguesnoted the antilisterial activities of nisin and most enterocins to be strongly pH-dependent and limited in cheeses of pH 5.5 to 5.8

(Samelis 2017), while Kykkidou and colleagues also found the action of nisin to be improved at pH 5.5 or below in cheese (Kykkidou 2007). Gallo and colleagues found nisin to be more effective in inhibiting *L. innocua* growth in cheese whey as temperature was increased from 4 to 7ºC and pH lowered from 6.5 to 5.5 (Gallo 2007). Ross and colleagues found a lacticin 3147-producing transconjugate *Lc. lactis* strain able to decrease *L. monocytogenes* by approximately 3 log CFU/g on the surface of mold-ripened cheese (pH ≥6.5), whereas no reduction was observed in the same cheese using a nisin-producing protective culture. The authors attributed their results to nisin being more effective in acidic conditions while lacticin 3147 is effective over a broad pH range (Ross 2000).

The use of nisin extensively in food products is limited due to its adsorption to fat and to the surface of protein globules, limiting its efficacy in high-fat foods such as meats (Favaro 2015). Low solubility, inactivation by endogenous or exogeneous enzymes, inhibition of non-resistant starter cultures, heterogeneous or limited distribution in food products, and flavor alterations have been additionally noted in preventing more widespread use of nisin in meat and dairy products to-date (Alves 2006, Sobrino-Lopez 2008). Increasing milkfat was found to greatly decreases nisin efficacy against *L. monocytogenes*, possibly due to adsorption of nisin to milkfat globules (Ho 2018). Regrowth of *L. monocytogenes* following initial reduction by bacteriocin has been attributed to the emergence of resistant mutants, reported to occur at frequencies of 10^{-6} to 10^{-8}, or to the inactivation of bacteriocin by proteolytic enzymes (Benkerroum 2000). Serial exposure to nisin appears to enhance nisin resistance (Martinez 2005). Additionally, pediocin-resistant mutants of *L. monocytogenes* are reportedly common, and continuous use of pediocin AcH appears not to be suitable as a primary means of food preservation for this reason (Loessner 2003). Starter bacteria and molds isolated from

Camembert cheese have been shown to be responsible for the inactivation of the antilisterial *Carnobacterium* bacteriocin piscicolin (Wan 1997).

Most bacteriocins carry an anionic charge and can bind to phosphate groups of casein (Coelho 2014). Encapsulation of nisin and other bacteriocins has been explored in order to improve their stability and distribution in cheese while preventing their action on cheese starter cultures. Malheiros and colleagues found nisin encapsulated in soy lecithin nanovesicles to inactivate *L. monocytogenes* in milk at low temperatures over 14 days as effectively as free nisin (Malheiros 2010). Other means of improving nisin stability in high-fat, near-neutral pH foods were explored by Ibarra-Sanchez and colleagues, who replaced the nisin variable region with hydrophilic, polar, positively-charged amino acids to increase nisin stability in queso fresco. In a pH 7, 22% milkfat system treated with either Nisaplin (commercial purified nisin A) or its derivatives, the authors detected significantly more residual nisin derivatives versus no detection of residual Nisaplin. While the authors found modified nisin derivatives to have higher MIC values against *L. monocytogenes* strains in a broth system versus Nisaplin, the authors found nisin derivatives to display modest antilisterial enhancement compared with Nisaplin-treated queso fresco samples stored at 4°C (Ibarra-Sanchez 2019).

ANTILISTERIAL ACTIVITY OF PROTECTIVE CULTURES IN SOFT CHEESES

Mendoza-Yepes and colleagues evaluated the ability of Spanish queso fresco (pH 6.5) to support growth of *L. monocytogenes* during storage at 3°C and 7°C. A commercial lactose-negative *Lc. lactis* subsp. diacetylactis starter culture, Fargo 763, was added to cheesemilk at a level of 10%. In cheese made without starter culture, *L. monocytogenes* reached levels of 10^7 CFU/g at 7°C after 10 days, with 1 log growth occurring after 1 week. In the same cheese stored

at 3°C 1 log growth was delayed to approximately 2 weeks, with a final level of approximately 5.5 log CFU/g *L. monocytogenes* observed at the end of the 22-day study. No *L. monocytogenes* growth was observed in cheese made using the starter culture at either 3°C or 7°C over the course of the 22-day study (Mendoza-Yepes 1999).

Vytrasova and colleagues investigated the efficacy of HOLDBAC™ *Listeria* Dairy, a commercial freeze-dried pediocin AcH/PA-1-producing *Lb. plantarum* strain, on *L. innocua* control on the surface of commercial cheese samples including Olomouc brand soft ripened cheese, Loose brand acid curd cheese, and Slovak-style string cheese. The authors surface-inoculated cheese samples with *L. innocua* before treating each with HOLDBAC™ via spraying. Spraying of the HOLDBAC™ product onto cheese was completed in Petri dishes which were subsequently foil wrapped and held at temperatures of 20ºC and 5 to 6ºC for 14 days. In a separate trial, the authors also treated the samples with HOLDBAC™ prior to *L. innocua* inoculation. In control samples, the authors found 3 to 4 log CFU/g increases of *L. innocua* after 14 days at 20ºC, while at 15ºC 1 to 2 log CFU/g increases were found. No analytical data was given for the three cheeses tested. In Loose brand acid curd cheese, the authors found substantial regrowth of *L. innocua* after 5 days, from an initial inoculum level of approximately 4 log CFU/g to approximately 6 log CFU/g when cheese was stored at 20ºC. At 5 to 6ºC, a reduction from approximately 4 log CFU/g to approximately 1.2 log CFU/g in 2 days was recorded, with *L. innocua* numbers remaining around this level for the entirety of the 14-day study (Vytrasova 2010).

Coelho and colleagues selected 8 bacteriocinogenic LAB strains (1 lacticin 481-producing *Lc. lactis* and 7 *Ent. faecalis*) previously isolated from artisanal Azorean Pico cheese for their ability to reduce *L. monocytogenes* in fresh cheese. The authors made fresh cheeses

from pasteurized milk inoculated with 6 log CFU/ml *L. monocytogenes* and each of the LAB strains before storage at 4ºC. All strains were shown to control growth of the organism, and after 7 days, an approximate 4 log reduction in *L. monocytogenes* was observed versus the control. Without a protective culture, *L. monocytogenes* numbers reached approximately 8 log CFU/g after 7 days at 4ºC. Reductions of *L. monocytogenes* were more pronounced using *Ent. faecalis* strains versus *Lc. lactis* strains (3 to 4 log vs. 2 log reductions observed after 7 days) (Coelho 2014).

Benkerroum and colleagues manufactured Moroccan Jben fresh cheese using a bacteriocin-producing *Lc. lactis* subsp. *lactis* strain isolated from raw dairy beverage to control *L. monocytogenes*. The authors fermented cow's milk with the *Lc. lactis* strain (reaching 2.8 x 10^9 CFU/g lactococci by the end of the 30-h fermentation) and contaminated the milk with 10^4 or 10^7 CFU/ml *L. monocytogenes*. A 2.7 log reduction of *L. monocytogenes* was observed in Bac^+ cheese inoculated with 7 log CFU/ml *L. monocytogenes*, while *L. monocytogenes* levels in the Bac^- cheeses remained at the inoculation level. In cheese inoculated with 4 log CFU/ml *L. monocytogenes*, the pathogen was undetectable following 24 h fermentation in Bac^+ cheese, versus increasing approximately 0.5 log in the Bac^- cheese (Benkerroum 2000).

Rodriguez and colleagues evaluated various strains of LAB for their ability to inhibit *L. monocytogenes* strain Ohio in pasteurized milk soft cheese. *L. monocytogenes* was inoculated into the cheesemilk at a level of 10^6 CFU/ml while 1% of a commercial mesophilic starter culture was used in cheese manufacture. Cheesemilks were additionally inoculated individually with 1% of the following cultures: a non-nisin-producing *Lc. lactis*, a pediocin-producing *Ped. acidilacti*, a nisin-producing *Lc. lactis*, a transformant pediocin-producing *Lc. lactis*, or a transformant nisin- and pediocin-producing *Lc. lactis*. Cheeses were vacuum packaged and

ripened for 30 days at 12°C. Cheese pH values after 4 days storage ranged from 4.91 to 5.04 while final pH values ranged from 4.86 to 4.93. *L. monocytogenes* was unable to grow in any of the samples, with day 4 *L. monocytogenes* populations ranging from 5.00 to 6.09 log CFU/g and day 30 *L. monocytogenes* populations ranging from 2.33 to 5.30 log CFU/g. Maximum *L. monocytogenes* populations observed at both sampling times were found in samples containing commercial starter culture only. No moisture nor salt data were given for the tested cheeses, though it was noted that cheeses were brined in 20% brine for 30 minutes before ripening (Rodriguez 2005).

Stecchini and colleagues investigated the behavior of *L. monocytogenes* on commercial Italian mozzarella cheese in the presence and absence of bacteriocin-producing *Lc. lactis* subsp. *lactis* strains. The authors screened 187 LAB strains isolated from raw milk for their antagonistic activity against the *L. monocytogenes* strain Scott A using an agar spot test, with 7 strains found to produce zones of inhibition. The authors inoculated mozzarella with *L. monocytogenes* to a target of 30 CFU/cm^2 on the surface and 3.0 log CFU/ml in the surrounding liquid of the cheese. Inoculated cheese was packaged in bags containing heat-treated neutralized bacteriocin-producing cultures before storage at 5ºC for up to 21 days. Two isolated bacteriocinogenic *Lc. lactis* strains were found to be equally effective at 5°C and 30°C in reducing *L. monocytogenes* numbers to undetectable levels within 24 hours on the surface and surrounding fluid of cheese, however, regrowth of *L. monocytogenes* was observed after 7 days. The authors noted an apparent reduced population of *L. monocytogenes* for 2 to 3 weeks when bacteriocin-producing cultures were employed in comparison to control cheeses without bacteriocin prepared using sterile skim milk in place of culture. The authors observed 1 log *L. monocytogenes* growth from inoculum levels in approximately 1.5 and 2 weeks on the cheese surface and in the fluid treated

with crude bacteriocin, respectively, versus in approximately 0.5 week in both untreated samples. Control cheeses reached a *L. monocytogenes* level of approximately 5.0 log CFU/g within 7 days and a final level of almost 7.0 log CFU/g within 21 days at 5ºC. In cheese treated with bacteriocin-producing culture, a final level of approximately 5.0 log CFU/g *L. monocytogenes* was observed (Stecchini 1995).

McAuliffe and colleagues investigated cottage cheese (pH 5.2) made with a lacticin 3147-producing *Lc. lactis* starter for its ability to support the growth of *L. monocytogenes* over 7 days with storage at 4, 18, and 30°C. pH was noted to remain stable over the course of the study. Initial levels of *L. monocytogenes* (approximately 10^4 CFU/g), which was inoculated into the cream dressing, remained relatively stable over 7 days at 4 and 18°C, dropping to approximately 3.5 log CFU/g, while levels dropped below detection limits (<10 CFU/g) in 3 days at 30°C (McAuliffe 1999). Dal Bello and colleagues found nisin A- and lacticin 481-producing *Lc. lactis* starter cultures to effectively control *L. monocytogenes* populations in cottage cheese (pH 4.70 to 4.80) stored at 4°C, wither higher antilisterial activity with the nisin A-producing strains observed. Conversely, a nisin Z-producing *Lc. lactis* starter was ineffective in controlling the pathogen, which was attributed to its weak acidification ability (cheese pH 5.86) (Dal Bello 2012).

Liu and colleagues investigated the control of *L. monocytogenes* by a recombinant enterocin A-producing *Lc. lactis* adjunct culture in cottage cheese manufacture. The authors found the cell activity of cell-free supernatant from the test strain to be 4-fold less than that of the parent *Ent. faecium* strain, though sufficient enterocin A by the test strain was reported. The test strain was combined with its parent *Lc. lactis* strain, included to compensate for an associated reduction in acid production by the test strain, and inoculated into pasteurized milk at a level of

2% before rennet treatment. *L. monocytogenes* was added after draining curds to a level of 4 to 7 log CFU/g and samples stored at 4ºC for 15 days. Control cottage cheese using only the parent *Lc. lactis* starter was additionally manufactured. pH values for the test and control cheeses were shown to be statistically similar. The presence of the test strain resulted in a dramatic decrease in *L. monocytogenes* numbers in cheese inoculated with 4 log CFU/g, decreasing below detection limits (3 log CFU/g) in 2 days. In control cheese, *L. monocytogenes* was still detected after 10 days storage at 4ºC. With a 5 log CFU/g inoculation level, there was a significantly beneficial difference in using the test strain, with *L. monocytogenes* falling approximately 0.3 log CFU/g until day 15, when a drastic drop to undetectable counts in the test cheese was observed. Likewise in cheeses inoculated with 6 and 7 log CFU/g, a similar statistically significant trend was observed, though less dramatic, between the test and control cheeses (Liu 2007).

Furtado and colleagues incorporated a bacteriocinogenic *Lc. lactis* subsp. *lactis* strain isolated from raw goat milk into fresh Minas-type goat cheese at a level of 10^6 CFU/ml into the cheesemilk before finishing cheese and inoculating curd with 3.91 log CFU/g *L. monocytogenes*. Following 10 days storage of cheese at 8 to 10ºC, the authors found increases in *L. monocytogenes* of +0.24, +0.20, and +2.41 log CFU/g in cheeses made using the bacteriocinogenic strain, a non-bacteriocinogenic *Lc. lactis* strain, and control cheese containing no protective culture, respectively. The authors additionally tested the incorporation of 12.5 mg/kg commercial purified nisin into cheese made without protective culture, finding *L. monocytogenes* to decrease to undetectable limits within 2 days and remain undetectable for the 10-day study. The authors postulated that the low storage temperature coupled with the heterogeneous cheese matrix may have inhibited growth and bacteriocin production of the protective culture, leading the authors to suggest the addition of purified or semi-purified

bacteriocin, as would be realized in the incorporation of bacterial fermentates, to fresh soft cheeses to be a more effective option to control *L. monocytogenes* growth during refrigerated storage (Furtado 2015).

ANTILISTERIAL ACTIVITY OF PROTECTIVE CULTURES IN SURFACE-RIPENED CHEESES

Roth and colleagues used a 3% w/v concentration of HOLDBAC™ *Listeria* Dairy protective culture, a commercial freeze-dried pediocin AcH/PA-1-producing *Lb. plantarum* strain, in smear brine for Raclette-type cheese challenged with *L. innocua* on days 3 and 4 (one trial) or days 7 and 8 (second trial). The authors used a ripening temperature of 11ºC and RH of 95% for 37 or 77 days in the first and second trials, respectively. Cheeses were turned and smeared on a daily basis for 2 weeks and twice a week thereafter. Cheeses were inoculated with *L. innocua* on both sides on 2 successive days, with control cheeses treated with only a commercial surface culture containing *Brevibacterium linens*, *Arthrobacter arilaitensis*, and two yeasts and test cheeses treated with surface culture + HOLDBAC™ *Listeria* Dairy or surface culture in addition to a different protective culture including nisin Z-producing *Lc. lactis*, pediocin PA-1-producing *Ped. acidilactici*, or plantaracin SM71-producing *Lb. plantarum*. Protective cultures were applied 3 times on both sides, once before and twice after *L. innocua* contamination. Trial 1 *L. innocua* inoculation occurred on days 3 and 4, while inoculation occurred on days 7 and 8 in trial 2. Bacterial counts of the commercial smear culture were 7.0 log and 8.7 log CFU/ml in the smear brine in trials 1 and 2, respectively, while yeast counts were 5.0 and 7.0 CFU/ml in trials 1 and 2, respectively. *L. innocua* smears were at levels of 3.0 and 3.7 log CFU/ml in smears from trials 1 and 2, respectively, while protective cultures were

applied at rates of 8% (w/v) or 3% (w/v) for non-HOLDBAC™ and HOLDBAC™ cultures, respectively. Initial starting *L. innocua* populations recovered in trials 1 and 2 were found to be approximately 0.6 and 2.8 log CFU/cm^2, respectively. In the first trial, the authors found *L. innocua* counts to be below detectable limits between days 7 and 22 on control cheese before increasing to 1.3 log CFU/cm^2 on day 37. *L. innocua* was found to be undetectable on cheeses treated with *Ped. acidilactici*, *Lb. plantarum*, or HOLDBAC™ *Listeria* Dairy protective cultures during the entire cheese ripening period, however, the cheese treated with HOLDBAC™ *Listeria* Dairy was found to be positive for *L. innocua* presence on day 37 following enrichment of samples. Conversely, *L. innocua* populations in cheese treated with *Lc. lactis* protective culture were found to be approximately 1 log CFU/cm^2 higher compared to control cheese after 37 days' ripening, with 2.3 log CFU/cm^2 recovered. In the second trial, the authors found *L. innocua* to increase on control cheese to 4.5 log CFU/cm^2 by day 22, remaining stable for the remainder of the study period. Application of *Ped. acidilactici* or *Lb. plantarum* protective cultures caused reductions in *L. innocua* populations to undetectable limits by day 11, while application of HOLDBAC™ *Listeria* Dairy caused a reduction in *L. innocua* counts to 1.2 log CFU/cm^2. However, a rapid increase in *L. innocua* was observed thereafter in the treated samples, with *L. innocua* populations reaching 5.6 log CFU/cm^2 by day 37, which was higher than that observed in control cheese (3.9 log CFU/cm^2) (Roth 2009).

Maisnier-Patin and colleagues manufactured Camembert cheese using milk inoculated with 1, 3, or 5 log CFU/ml *L. monocytogenes* strain V7 and using a nisin-producing starter composed of a pair of isogenic protease-positive and protease-negative strains of *Lc. lactis* subsp. *lactis*. The authors added active cultures of Protease$^+$ and Protease$^-$ strains grown in skim milk to cheese milk to provide an inoculum of 2% (0.4% Prt$^+$ and 1.6% Prt$^-$) for a total of

approximately 7 log CFU/ml. An isogenic Prt^+ and Prt^- strain mixture was included to reduce cheese bitterness. Camembert was manufactured in a pilot plant by adding *L. monocytogenes*, starter, spores of *Penicillium camemberti*, and rennet to pasteurized partially skimmed milk. The authors tested the following combinations: 10^1 CFU/ml *L. monocytogenes*/Nis^+ or Nis^-; 10^3 CFU/ml *L. monocytogenes*/Nis^+ or Nis^-; and 10^5 CFU/ml *L. monocytogenes*/Nis^+ or Nis^-. Nisin concentration, as measured via plate diffusion assay, in curd and cheese paralleled the growth of *Lc. lactis*, with a maximum concentration of 700 IU/g nisin observed in curd after 9 hours fermentation before dropping slowly from 9 to 24 h and dramatically between 24 h and 1 week of aging. The authors found nisin content in the curd to be 2 to 3 times lower than nisin concentration in cheesemilk, which they attributed to potential loss in whey as well as enzymatic inactivation. *L. monocytogenes* in nisin-producing starter cheese remained approximately 2.5 log CFU/g lower throughout aging than in non-nisin-producing starter cheese following an initial average 3.3 log CFU/g reduction. Regrowth of *L. monocytogenes* was more extensive on the surface than in the core of the cheese. The authors found the effectiveness of nisin to be greater with lower ($\leq 10^3$ CFU/ml) *L. monocytogenes* inoculum and recovered no *L. monocytogenes* in either cheese inoculated with 10^1 CFU/ml over storage. The authors postulated that nisin-producing starters could be useful for making safe raw milk cheese or in preventing post-processing low-level contamination (Maisnier-Patin 1992).

Sulzer and Busse investigated *Listeria* growth on Camembert cheese in the presence of *Ent. faecalis, Lb. paracasei*, and nisin-producing *Lc. lactis*. Cheeses were inoculated with a single strain of either *L. monocytogenes* or *L. innocua* into the cheesemilk before manufacture or alternatively contaminated at different stages of ripening by spraying *Listeria* cell suspensions onto the cheese surface. Strains of *Enterococcus, Lactococcus*, and *Lactobacillus* were either

used as starter cultures or added with a commercial starter culture to the cheesemilk. The authors grew strains 24 hours at 30ºC in sterile whole milk before adding at 1% (v/v) to cheesemilk. *Ent. faecalis*, at an initial level of 1.0×10^3 CFU/ml, was used with a commercial starter culture and increased to 10^6 CFU/g cheese at the time of salting before approaching 10^9 CFU/g by the end of 10 days ripening. Development of *Listeria* in *Ent. faecalis*-treated Camembert was found to be the same as in control Camembert. The authors found a single strain of *L. innocua* to be sensitive to a 6-strain cocktail of *Ent. faecalis* when *L. innocua* inoculation occurred 1 hour post-salting; this sensitivity was not replicated when inoculation occurred later (as late as 2 days after salting). Similarly, the authors found using a nisin-producing *Lc. lactis* strain in conjunction with commercial starter or 6 strains of *Lb. paracasei* employed as a sole starter cocktail to have no effect on *L. monocytogenes* growth compared to control cheese when *L. monocytogenes* inoculation occurred 2 days post-brining or later. The authors did observe, however, *L. monocytogenes* to be completely suppressed if nisin-producing *Lc. lactis* was used as the sole starter and *L. monocytogenes* was added to cheese 1 hour post-brining. Conversely, the authors found *Lc. lactis* unable to inhibit *L. monocytogenes* when cheesemilk was inoculated with 10 CFU/ml *L. monocytogenes*. The authors noted that if *Listeria* contamination occurred around the time of brining, *Listeria* growth was insignificant during the first 3 to 6 days of cheese ripening because of the low pH (4.9 to 5.0) on the cheese surface. Conversely, *Listeria* contamination on the surface 3 days post-brining appeared to support *Listeria* growth immediately, as the pH increased from 5.1 to ≥7.0 from the third to fifth day after brining. The authors noted that in either case (with *Listeria* inoculation at 1 hour or 3 days post-brining) *Listeria* reached approximately 4 log CFU/g on the cheese surface within the first 2 weeks and 5 to 6 log CFU/g by 5 to 6 weeks, the main period of consumption for Camembert. The authors reported that

Listeria could be suppressed on Camembert when an inhibitory strain was used as the sole starter culture and when *Listeria* contamination occurred in the early stage of ripening. The authors additionally noted their results to find no effect of protective cultures when *Listeria* was inoculated into the vat cheesemilk versus onto cheese in the early ripening stage. The authors postulated that this observation was due to *Listeria* being allowed considerable multiplication before acidification terminated growth when *Listeria* was inoculated into cheesemilk, versus a small *Listeria* population being exposed to the adverse effects of a low pH in addition to inhibitory substance action within the young cheese (Sulzer 1991).

A lacticin 3147-producing transconjugate *Lc. lactis* was used as a protective culture against *L. monocytogenes* strain Scott A on the surface of a mold-ripened cheese by Ross and colleagues. *L. monocytogenes* was found to be reduced 1000-fold on the surface of the challenged cheese. Conversely, a nisin-producing protective culture caused no reduction in *L. monocytogenes* compared with the control cheese. No pH values for the tested cheese were given, however, the authors noted that pH values on the surface of mold-ripened cheeses can vary from 6.5 to 8.0. The authors speculated that the ineffectiveness of the nisin-producing strain against *L. monocytogenes* was likely due to nisin being more effective in acidic conditions whereas lacticin 3147 is effective over a broad pH range (Ross 2000).

Stauber and colleagues found *Enterococcus* spp. inhibitory to *Listeria* spp. unable to inhibit its growth when sprayed onto Camembert cheese surfaces, despite an increase in *Enterococcus* numbers over ripening (Stauber 1990). Similarly, Picchioni found *Ent. faecium* 7C5 at 10^6 CFU/cm^2 on the surface of Taleggio, an Italian semi-soft smear-ripened cheese, unable to inhibit the growth of *L. innocua* (10 to 100 CFU/cm^2) when sprayed onto cheese at various stages during ripening (Picchioni 1994). Izquierdo and colleagues found *Ent. faecium*

when used as a starter culture in manufacture of a red smear soft Munster cheese to reduce numbers of inoculated *L. monocytogenes* on the cheese surface, while incorporating *Ent. faecium* into the cheese brine resulted in complete inhibition of the pathogen. With no inclusion of *Ent. faecium*, control samples supported 4 log CFU/g growth in the cheese (Izquierdo 2009). Giraffa and colleagues found *L. innocua* to be inhibited by a combination of pH decrease and enterocin production by *Enterococcus* spp. strains in Taleggio, while growth and acidification by the thermophilic starter used in Taleggio manufacture was found to be unaffected by enterocins when the *Enterococcus* spp. strains were used as adjunct cultures (Giraffa 1994).

Ennahar and colleagues investigated the antilisterial activity of a pediocin AcH-producing *Lb. plantarum* strain on a smear surface-ripened soft Muenster cheese. After growing the bacteriocinogenic strain to the late exponential phase, the authors sprayed the cell suspension (approximately 5 log CFU/ml) on the cheese before ripening at 95% RH and 15°C and inoculating with *L. monocytogenes* after 1 week. After addition of *L. monocytogenes*, it was not detected on subsequent sampling days (11, 14, 17, and 21) in 5 trials using the bacteriocinogenic culture. Conversely, in control cheeses, *L. monocytogenes* grew to levels $>10^4$ CFU/g over 21 days. Mean pH of test and control cheeses were not significantly different, nor were fungi or pigmented bacterial populations (Ennahar 1998). The authors stressed a low level of *L. monocytogenes* contamination as being critical in successfully utilizing a pediocin-producing culture in their application, a finding that was similarly reached by O'Sullivan and colleagues using a lacticin 3147-producing *Lc. lactis* culture to control *L. monocytogenes* on surface smear-ripened cheese and Loessner and colleagues using a pediocin AcH-producing *Lb. plantarum* culture to control *L. monocytogenes* on red smear model cheese (Loessner 2003, O'Sullivan 2006).

O'Sullivan and colleagues investigated a lacticin 3147-producing *Lc. lactis* culture for its ability to inhibit *L. monocytogenes* on the surface of smear-ripened cheese. The authors sprayed the test strain onto the surface of cheese either before or after *L. monocytogenes* inoculation to the cheese surface before ripening cheeses at 16°C with 95% RH for 3 weeks. When applied directly after *L. monocytogenes* inoculation, the authors found an immediate 3-log decrease in *L. monocytogenes* numbers with subsequent regrowth after 10 days ripening to a final *L. monocytogenes* population of approximately 3 log CFU/g less than observed in control cheese by the end of ripening. When applied before *L. monocytogenes* inoculation, the test strain was unable to immediately decrease nor control *L. monocytogenes* numbers during ripening, showing no significant difference compared with results in inoculated control cheese. The authors speculated that components of the cheese environment in their application were interacting with lacticin 3147 and making it unavailable upon subsequent *L. monocytogenes* inoculation, noting additionally that a pronounced reduction (from 640 initially to 320 AU/ml) of the bacteriocin was measured 6 days post-application (O'Sullivan 2006).

ANTILISTERIAL ACTIVITY OF PROTECTIVE CULTURES IN HARD AND SEMI-HARD CHEESES

Samelis and colleagues investigated *L. monocytogenes* and *L. innocua* behavior in model Graviera mini cheeses made with an indigenous nisin A-producing *Lc. lactis* subsp. *cremoris* culture isolated from raw milk used as a co-starter culture in combination with a commercial starter. The authors used 2 *L. innocua* strains and 1 avirulent *L. monocytogenes* strain to form a cocktail with starting inoculum of approximately 3 log CFU/g. The commercial strain was a mixture of *Str. thermophilus*, *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *lactis* bv. diacetylactis, and

Leuconostoc spp. and was added into thermized (63°C for 30 s) cheesemilk at a level of approximately 6 log CFU/ml before renneting and curd cooking. Model cheeses were ripened at 18°C and 90% RH for 20 days and stored vacuum-sealed at 4°C for 60 days. pH values decreased from 6.5-6.6 to 5.9 within the first 24 hours irrespective of strains used. pH values further decreased to 5.4 to 5.5 over storage with confluent LAB growth observed. Cheese moisture values were 40.5 to 41.0% at the beginning of ripening and decreased to 31.6 to 32.9% by the end of ripening, while salt level rose to 2.2 to 2.4% by the end of ripening. Concentration of main organic acids (lactic, acetic, citric and butyric) was monitored. Citric acid remained stable, while the others increased during ripening. Lactose and glucose were depleted by day 1 and <5 mg/100 g ethanol and formic and propionic acids were detected. Citric, lactic, and acetic acid levels rose from 655-705, 689-827, and 14-24 mg/100 g cheese on day 1 to 658-777, 920-1,141, and 108-111 mg/100 g cheese on day 60, respectively. The authors found reducing the curd cooking temperature from 48 to 42°C to increase the antilisterial effectiveness of the indigenous protective culture during cheese fermentation. Regardless, none of the cheeses in the study were found able to support *Listeria* growth over the ripening period (Samelis 2017). Giannou and colleagues found no enhanced *L. monocytogenes* inactivation beyond controls in Graviera cheese (pH 5.6) made with an indigenous enterocin-producing *Ent. faecium* culture used as a co-starter culture when cheeses were surface inoculated with *L. monocytogenes* and stored at 4, 12, or 25ºC storage aerobically or in vacuum packages (Giannou 2009).

Buyong and colleagues investigated manufacture of Cheddar with a pediocin-producing *Lc. lactis* subsp. *lactis* starter culture. The authors inoculated pasteurized milk with 10^6 CFU/ml of the pediocin-producing starter or a control isogenic, non-pediocin-producing *Lc. lactis* subsp. *lactis* starter and 10^3 CFU/ml of a 3-strain *L. monocytogenes* cocktail before ripening at 8°C.

Cheddar manufactured with the test strain decreased *L. monocytogenes* to 10^2 CFU/g after 1 week before decreasing to approximately 10 CFU/g following 3 months ripening. Conversely, in Cheddar made with the control strain, *L. monocytogenes* was found to increase to approximately 7 log CFU/g after 2 weeks before gradually decreasing to 3 log CFU/g after 6 months ripening. Pediocin levels decreased from approximately 64,000 AU/g after 1 day to 2,000 AU/g after 6 months, while no pediocin activity was detected in control cheeses (Buyong 1998).

Mills and colleagues investigated the inhibition of *L. innocua* by *Lb. plantarum* in Gouda cheese with 41% moisture, pH 5.2, and 3% salt. The authors manufactured laboratory-scale Gouda using two *Lc. lactis* starter cultures (one commercial cheese starter, one nisin-producing culture) and a *Lb. plantarum* protective culture isolated from soft French artisanal cheese in various combinations. A single strain of *L. innocua* was added to each vat at 3 log CFU/ml before rennet addition. Cheeses were vacuum-packaged and ripened at 12ºC for 4 weeks. None of the cheeses supported *L. innocua* growth, however, more inhibition was realized using *Lb. plantarum* in addition the commercial cheese starter versus the commercial cheese starter alone or in combination with the nisin-producing *Lc. lactis* starter. Additional *L. innocua* inhibition was observed when the three strains were used together in cheese manufacture (Mills 2011).

Arques and colleagues found 7 strains of bacteriocinogenic LAB (including nisin A- and nisin Z-producing *Lc. lactis* subsp. *lactis*; lacticin-481-producing *Lc. lactis* subsp. *cremoris*, TAB 57-producing *Lc. lactis* subsp. *lactis* bv. diacetylactis; TAB 7-producing *Ent. faecium*; and enterocin-I- and enterocin-AS-48-producing *Ent. faecalis*) grown in MRS or milk unable to appreciably control *L. monocytogenes* populations during 60 days raw milk cheese ripening at 12°C when co-inoculated with commercial LAB starter. *L. monocytogenes* populations were 0.29 to 0.97 log CFU/g lower on day 3 of cheese ripening, and 0.00 to 0.68 log CFU/g lower at

the end of ripening in cheeses made with each bacteriocinogenic LAB (Arques 2015). Rodriguez and colleagues observed a lacticin 481-producing *Lc. lactis* subsp. *cremoris* strain used as a single strain starter in semi-hard raw milk cheese to show a decrease of *L. monocytogenes* approximately 2.5 log greater than that observed in cheese made with a commercial starter (Rodriguez 2001). Nunez and colleagues found an enterocin-producing *Ent. faecalis* strain when used as a starter in Manchego cheese manufacture to realize inhibition of *L. monocytogenes* strain Ohio (serotype 4b), however, no inhibition of *L. monocytogenes* strain Scott A (also serotype 4b) was observed (Nunez 1997).

ANTILISTERIAL ACTIVITY OF PROTECTIVE CULTURES IN MICROBIOLOGICAL MEDIA AND MODEL SYSTEMS

Ribeiro and colleagues found 5.9 log CFU/ml of casein-pectin microencapsulated *Lb. paraplantarum* FT-259 co-inoculated with 3.4 log CFU/ml of a single strain of *L. monocytogenes* in Brazilian fresh Minas laboratory model cheese to significantly suppress the maximum level of *L. monocytogenes* when cheeses were stored at 8°C for 21 days to 5.5 log CFU/ml. When free *Lb. paraplantarum* FT-259 was incorporated at a level of 5.2 log CFU/ml, *L. monocytogenes* reached a maximum level of 7.3 log CFU/ml, compared with 8.2 log CFU/ml in the control cheese, levels which were found statistically insignificant from one another. Findings from the researchers suggested microencapsulation may increase the efficacy of *Lb. paraplantarum* protective cultures against *L. monocytogenes* (Ribeiro 2020). However, experiments should be replicated in trials with equal initial levels of free and microencapsulated *Lb. paraplantarum* in future work, as protective culture manufacturer specifications typically call for 6 to 7 log CFU/g usage rates in dairy products (Gensler 2020) and statistical differences in

final *L. monocytogenes* counts could potentially be explained by the low (5.2 log CFU/ml) usage rate of free *Lb. paraplantarum*. Nevertheless, ≥1 log CFU/ml *L. monocytogenes* growth was observed in both treatment and control samples within <5 days, suggesting that the use of *Lb. paraplantarum* FT-259 as a sole means of *L. monocytogenes* growth control was insufficient and would require additional barriers for safety in Brazilian fresh Minas model cheeses (Ribeiro 2020).

Loessner and colleagues investigated the growth of a single *L. monocytogenes* strain in the presence and absence of a pediocin AcH-producing *Lb. plantarum* strain (ALC 01; commercially available from DuPont) on red smear model cheese. The authors used either complex wash-off cultures from commercial red smear cheeses or a commercial defined ripening culture. *L. monocytogenes* was added to the smear brine and applied to model cheeses on day 1, when *Lb. plantarum* counts were found to be approximately 8.7 CFU/cm^2. *L. monocytogenes* inhibition was dependent on contamination level, with inhibition of 1 to 2 log over the ripening period demonstrated versus control cheese when a starting *L. monocytogenes* population of approximately 3.6 CFU/ml brine was applied. With a lower inoculation level of approximately 2.3 CFU/ml brine, more pronounced *L. monocytogenes* inhibition was observed (Loessner 2003).

Reviriego and colleagues found *L. innocua* growth to be controlled by recombinant *Lc. lactis* producers of pediocin PA-1 as well as wild-type Nis$^+$ and Nis$^-$ *Lc. lactis* in a model cheese system incorporating each individually as starter cultures. *L. innocua* reductions of 2.30 to 2.60, 1.65, and 1.49 log CFU/g, respectively, following 28 days storage at 12°C were recorded. Conversely, in control cheeses made without starter culture, *L. innocua* growth could not be controlled, resulting in an increase of 2.57 log CFU/g after 28 days storage (Reviriego 2007).

Mojgani and colleagues investigated the growth of *L. monocytogenes* at 4 and 35ºC in salted (3% NaCl) and unsalted lab-scale cheese samples treated with a purified bacteriocin lactocin RN78 and the *Lb. casei* producer strain. The authors produced cheese using pasteurized cow's milk with 2% commercial starter and rennet. Following cheese manufacture, cheese surface was inoculated via shaking with approximately 4 log CFU/g *L. monocytogenes* and coated with 7 log CFU/g *Lb. casei* or 6400 AU/g lactocin. *L. monocytogenes* counts remained high (5.30 log CFU/g after 90 days versus 5.56 log CFU/g after 3 days) in cheese treated with *Lb. casei* stored at 4ºC, while at 35ºC this cheese showed reduction from 5.88 log CFU/g on day 3 to 2.94 log CFU/g on day 15. In cheese treated with purified lactocin, *L. monocytogenes* reduced from 5.81 log CFU/g to 3.16 log CFU/g on days 3 and 15, respectively, at 4ºC and from 5.81 log CFU/g to 3.08 log CFU/g on days 3 and 15, respectively, at 35ºC. Cheese with 3% NaCl showed reductions of *L. monocytogenes* from 5.40 log CFU/g to 2.99 log CFU/g on days 3 and 15, respectively, at 4ºC and from 5.31 log CFU/g to 3.32 log CFU/g on day 3 and 30, respectively, at 35ºC. In cheese with purified lactocin and 3% NaCl, *L. monocytogenes* numbers reduced from 5.48 log CFU/g to 1.46 log CFU/g on days 3 and 15, respectively, at 4ºC and from 5.42 log CFU/g to 1.26 log CFU/g on days 3 and 15, respectively, in cheese stored at 35ºC. In cheese with *Lb. casei* and purified lactocin, *L. monocytogenes* reduced from 5.09 log CFU/g to 2.45 log CFU/g on days 3 and 15, respectively, at 4ºC and from 5.82 log CFU/g to 1.09 log CFU/g on days 3 and 15, respectively, at 35ºC. In cheese with *Lb. casei* and 3% NaCl, *L. monocytogenes* reduced from 5.86 log CFU/g to 3.65 on days 3 and 15, respectively, at 4ºC and from 5.78 log CFU/g to 1.97 log CFU/g on days 3 and 15, respectively, at 35ºC. *L. monocytogenes* numbers did not change considerably in control cheeses at either storage temperature. The authors found NaCl and purified lactocin to completely inhibit growth of *L.*

monocytogenes (to undetectable limits at 90 days) in cheese stored at 4ºC. pH values on day 3 of storage ranged from 5.54 to 5.94 in cheeses stored at 4ºC and from 5.41 to 5.90 in cheeses stored at 35ºC. Untreated control cheese pH values were 5.59 and 5.57 after 3 days storage at 4 and 35ºC, respectively. The authors found cheese pH to decrease significantly (to pH 4.8 to 5.0) in samples treated with live culture (Mojgani 2010).

Dal Bello and colleagues identified 38 *Lactococcus* strains from fresh or ripened artisanal Italian cheeses showing inhibition against a single strain of *L. monocytogenes*. Inhibitory strains were identified as *Lc. lactis*, *Lc. lactis* subsp. *lactis*, or *Lc. lactis* subsp. *cremoris*. All *Lactococcus* strains showing inhibitory action against *L. monocytogenes* also were found inhibitory to a tested *S. aureus* strain. The authors additionally identified 20 *Enterococcus* strains showing *L. monocytogenes* inhibition and identified as *Ent. faecium*, *Enterococcus* spp., or *Ent. faecalis* (Dal Bello 2010). Nespolo and Brandelli investigated 112 LAB isolates from ewe's milk and cheeses in Brazil. Of 59 strains screened, 21% showed antimicrobial, proteolytic, and lipolytic activities. *Lb. plantarum* and *Lb. rhamnosus* (one strain of each) were tested for bacteriocin-like substance production, with both showing antimicrobial activity against *L. monocytogenes* by disk diffusion assay (Nespolo 2010). Martin-Platero and colleagues investigated 95 *Enterococcus* strains (*Ent. devriesei*, *Ent. faecalis*, and *Ent. malodoratus*) isolated from 3 goat's milk cheeses. The authors found <13% of tested isolates (mostly *Ent. devriesei*) to possess antimicrobial activity against *L. innocua* (Martin-Platero 2009). Ryser and colleagues investigated 105 traditional French cheeses for surface smear microorganisms inhibitory to *L. monocytogenes*. <0.1% of 12,500 colonies screened produced visible zones of inhibition, with isolates found to be inhibitory being *Ent. faecalis*, *Staphylococcus* spp., and coryneforms (Ryser 1984).

Ho and colleagues found 40 of 897 LAB isolates from herbs, fruits, and vegetables to show inhibition zones against *L. monocytogenes* using an MRS agar overlay assay. Isolates inhibitory to *L. monocytogenes* were identified as *Lc. lactis, Lc. raffinolactis, Leuc. mesenteroides, Leuc. pseudomesenteroides, Weissella soli*, and *Weissella viridescens.* 14 isolates with strong activity were identified by 16S rRNA gene sequencing as *Lc. lactis* and all contained the *nisZ* gene cluster encoding for nisin. 4 of 14 strains (2 each from fresh herb and baby spinach) showed significant antilisterial activity when grown in UHT whole milk alone or in combination with an industrial *Lc. lactis* starter culture. The authors found ethanol, a product of mixed acid fermentation, produced in milk containing the wild strains, but not in milk fermented with the industrial starter only. They also found the ethanol levels to be lower in co-cultured milk compared to the wild strain only samples. In whole genome sequencing of 4 wild strains (3 antilisterial and 1 negative control) and 2 industrial *Lc. lactis* strains, the authors found a complete nisin gene cluster containing genes encoding the nisin structural protein, biosynthetic enzymes, transporters, immunity proteins, and regulatory proteins in the 3 wild strains showing antilisterial activity but not in the other strains. Additionally, the *lac* operon was found in both industrial strains but not in the antilisterial wild strains, which contained homologs of genes shown to form alternative lactose transport and metabolism pathways. All wild *Lc. lactis* strains tested were slow acid producers showing a reduction of ≤0.4 pH units after 5 hours in UHT whole milk (Ho 2018).

ANTILISTERIAL ACTIVITY OF PROTECTIVE CULTURES IN OTHER FOODS

Martinez and colleagues tested milk fermented by a nisin-producing *Lc. lactis*, finding nisin-resistant *L. monocytogenes* variants able to survive and multiply while wild-type *L.*

monocytogenes strains were inhibited. The authors used reconstituted skim milk (11%), pasteurized and inoculated at 4% with an overnight culture of a nisin Z-producing *Lc. lactis* strain grown in milk. Inoculated skim milk was then incubated at 32ºC 3 to 4 h until pH 5.8±0.1 was reached, after which milk was inoculated with 6 to 7 log CFU/ml of a single wild-type or respective nisin-resistant derivative *L. monocytogenes* strain. Inoculated samples were incubated 10ºC for 15 days. After 2 days storage, the authors found *L. monocytogenes* numbers between the inoculated milks to be significantly different, with viable counts of the wild-type strain to be approximately 2 log lower for the duration of storage (15 days) than the nisin-resistant strain. The authors replicated the study with an additional *L. monocytogenes* strain and its nisin-resistant mutant, finding similar results. pH value of the inoculated milk dropped from approximately 5.7 to 5.0 within 1 day, pH 4.7 within 2 days, and settled around pH 4.5 by days 6 to 15 (Martinez 2005).

Lb. delbrueckii subsp. *bulgaricus* and *Str. thermophilus*, both used in cheesemaking, were found to be inhibitory to *L. monocytogenes* in skim milk and yogurt by Schaak and Marth, with greater inhibition observed using *Lb. delbrueckii* subsp. *bulgaricus*. The authors additionally found the temperature of incubation and the level of starter addition to affect the level of *L. monocytogenes* inhibition (Schaak 1988b). In a separate study, Schaack and Marth investigated autoclaved skim milk inoculated with 10^3 CFU/ml of *L. monocytogenes* strain V7 and 5.0, 1.0, 0.5, or 0.1% of either *Lc. lactis* subsp. *cremoris* or *Lc. lactis* subsp. *lactis*. Inoculated milks were allowed to ferment for 15 h at 21 or 30ºC before refrigeration at 4ºC. pH 4.75 appeared to be completely inhibitory to *L. monocytogenes* growth among combinations tested and *Lc. lactis* subsp. *lactis* reduced the pH of fermented milks more than *Lc. lactis* subsp. *cremoris*. *L. monocytogenes* and LAB culture inocula were grown in sterile skim milk for 35ºC for 48 h and

12 h at 21ºC, respectively, before co-inoculation. Enumeration for *L. monocytogenes* and LAB as well as direct pH measurement were completed every 3 hours. Growth of *L. monocytogenes* at 21ºC in the presence of lactic culture was minimal, with a final pH of 4.9. Inhibition was greater at 30ºC, with *L. monocytogenes* decreasing at 12 to 15 h incubation and a final pH of 4.43 observed. Decreasing LAB inoculum to 0.5% with 21ºC incubation allowed for *L. monocytogenes* growth. The authors found *Lc. lactis* subsp. *lactis* to be more inhibitory to *L. monocytogenes* than *Lc. lactis* subsp. *cremoris*, however, results from both LAB cultures were similar. With a 1.0% inoculum of *Lc. lactis* subsp. *lactis* and 21ºC incubation, *L. monocytogenes* grew better than when the lactic inoculum was 5.0%. The authors found the population of LAB to grow at the same rate and extent with or without co-inoculation with *L. monocytogenes* (Schaak 1988).

Pitt and colleagues investigated *L. monocytogenes* behavior in pasteurized milk during fermentation with starter and nonstarter LAB. The authors co-inoculated pasteurized milk with approximately 3 to 4 log CFU/ml of a single strain of *L. monocytogenes* and 6 log CFU/ml *Lc. lactis* subsp. *lactis, Lc. lactis* subsp. *cremoris, Lb. plantarum, Lb. bulgaricus*, or *Str. thermophilus* before incubation at 30 or 37ºC for 24 to 72 hours. Freeze-dried and frozen cultures were reactivated by rehydration in 10 ml of sterile 10% (w/v) reconstituted skim milk before incubating at 30 to 37ºC for 48 hours. *L. monocytogenes* and a single lactic culture were co-inoculated into 100-ml screw-cap bottles containing 99 ml pasteurized milk, while controls with only *L. monocytogenes*, only the lactic culture, and only pasteurized milk were additionally tested. After 8 hours incubation at 30 to 37ºC, populations of LAB increased to >8 log CFU/ml in both control and treatment samples, remaining constant throughout the remainder of the experimental period regardless of storage duration and unaffected by the presence of *L.*

monocytogenes or by the reduction in pH of milk to <4.2. No difference in LAB growth was observed between treatment samples and controls. pH values for all treatments and controls were found to be 6.6 to 6.7 at the outset of the study. The authors observed no difference between the pH of treatment samples and that of controls or uninoculated milk samples, and all had final pH values around 4.2. A pH of 5 was reached in 40 hours for *Lc. lactis* subsp. *cremoris* at 30ºC, 18 hours for *Lc. lactis* at 30ºC, 20 hours for *Lb. plantarum* at 30ºC, 12 hours for *Lb. bulgaricus* at 37ºC, and 10 hours for *Str. thermophilus* at 37ºC. With co-inoculation with *Lc. lactis* subsp. *lactis, Lc. lactis* subsp. *cremoris*, and *Lb. plantarum, L. monocytogenes* numbers increased to approximately 7 log CFU/ml after 8 to 16 hours of incubation at 30ºC, but declined steadily afterwards, with 89%, 98%, and 100% inhibition versus controls observed, respectively. *L. monocytogenes* was completely inactivated in the presence of *Lb. plantarum* after 64 hours incubation at 30ºC. When incubated at 37ºC with *Lb. bulgaricus* or *Str. thermophilus, L. monocytogenes* populations increased to approximately 5 and 6 log CFU/ml after 12 and 20 hours, respectively. Thereafter, the pathogen reduced in number, and was inhibited by 93% and 100% compared to controls. *L. monocytogenes* was found to survive the fermentation process by *Str. thermophilus*, however, after 20 hours the pathogen was completely inactivated in the presence of *Lb. bulgaricus*. The authors concluded that *Lb. bulgaricus* and *Lb. plantarum* profoundly inhibited *L. monocytogenes* growth, noting that only the *Lb. plantarum* strain used in the study was known to be bacteriocinogenic, producing a pediocin. The authors speculated that pH alone could not have been responsible for the decline in growth of the pathogen, as populations of the pathogen in treatment samples were considerably less than those in controls at similar hydrogen ion concentrations. They also speculated that it was likely a combined effect of large numbers of competing LAB and low pH that caused antilisterial activity in the treatments.

The authors also cautioned that starter culture alone was insufficient in preventing *L. monocytogenes* outgrowth, as *Str. thermophilus, Lc. lactis* subsp. *lactis*, and *Lc. lactis* subsp. *cremoris* co-inoculated milks, despite decreases in *L. monocytogenes* numbers, allowed for pathogen survival, remaining at high populations in these co-inoculated milks at the end of storage with approximately 6 log CFU/ml at 24 hours for *Str. thermophilus*, 2.5 log CFU/ml at 72 hours for *Lc. lactis* subsp. *cremoris*, and 3.5 log CFU/ml at 72 hours for *Lc. lactis* subsp. *lactis* treated milks (Pitt 2000).

Alves and colleagues found *L. monocytogenes* growth on cooked ham to be significantly inhibited in the presence of Bac^+ and Bac^- *Lb. sakei* isolated from Brazilian fresh pork sausage (2 or 6 log applied via surface inoculation) with 8°C incubation for 10 days. *L. monocytogenes* strains tested were serotypes 4b and 1/2a, tested in separate experiments. Bac^+ and Bac^- LAB strain growth was not influenced by the presence of *L. monocytogenes*, with both LAB strains reaching 8 log CFU/g after 5 days storage. The authors found no additional barrier to listerial growth using the Bac^+ strain versus the Bac^- strain. The authors found bacteriocin production by Bac^+ *Lb. sakei* in ham co-inoculated with *L. monocytogenes* serotype 4b but not 1/2a, attributing the effectiveness of the tested *Lb. sakei* strains to nutrient competition, acid production, and/or production of other antilisterial metabolites (Alves 2006).

ANTILISTERIAL ACTIVITY OF PURIFIED BACTERIOCINS IN CHEESE

Pucci and colleagues found dried pediocin preparation applied to cottage cheese, cream, and cheese sauce to decrease *L. monocytogenes* counts before regrowth (Pucci 1988). Regrowth of *L. monocytogenes* was also observed by Wan and colleagues with treatment of Camembert cheese with piscicolin 126, a bacteriocin produced by *Carnobacterium* spp. (Wan 1997).

Maisnier-Patin and colleague found nisin-producing starter cultures to inactivate *L. monocytogenes* in Camembert cheese, however, regrowth of *L. monocytogenes* was observed as the pH of the cheese increased due to mold growth during ripening (Maisnier-Patin 1992). Conversely, greater success was found by Farias and colleagues, finding an enterocin able to reduce *Listeria* spp. up to 9 log units in goat cheese by the end of ripening (Farias 1999).

Benkerroum and Sandine studied the effect of nisin on *L. monocytogenes* inoculated into sterilized and non-sterilized cottage cheese (pH 4.9 to 5.0). To sterilized cottage cheese, the authors added 2550 IU/g (2.55 mg/g) nisin and 5.54 log CFU/g *L. monocytogenes* before storage for 30 days at 4 or 37ºC. Sterilized cottage cheese samples without nisin stored at 4 and 37ºC supported 1 log growth within 2 days and 1 day, respectively, reaching maximum levels of approximately 7.8 and 9.0 log CFU/g, respectively, over the study period. Samples containing nisin at the 2 test temperatures showed immediate eradication of *L. monocytogenes* with no recovery following enrichment. Additionally, the authors noted that non-sterilized cottage cheese made without nisin spoiled 1 week earlier than those with the antibiotic, as determined via smell and appearance (Benkerroum 1988). In a lower-pH (pH 4.6 to 4.7) commercial low-fat cottage cheese tested by Ferreira and Lund, 4 log CFU/g *L. monocytogenes* was found unable to grow in the cheese with or without the addition of 2000 IU/g (2 mg/g) nisin and incubation at 20ºC. *L. monocytogenes* numbers were reduced to approximately 1.8 log CFU/g in the nisin-treated samples, versus remaining near inoculation levels in control samples following 7 days storage (Ferreira 1996). In commercial low-fat cottage cheese (pH 4.3, 0.5% salt) inoculated with approximately 6 log CFU/g *L. monocytogenes*, the addition of 2 mg/g Nisaplin contributed to a greater log-kill of the pathogen versus in control cheese (log-kill of 3.5 vs. 2.8 log CFU/g, respectively) following 3 days storage at 20ºC (Collins 2011).

Morgan and colleagues found lacticin 3147 to inactivate *L. monocytogenes* in cottage cheese. The authors inoculated commercial cottage cheese with *L. monocytogenes* at a level of 4 log CFU/ml in the presence of 10% (w/v) of a lacticin 3147 bacteriocin powder. Holding the cheese at 30°C for 120 minutes realized a 85% reduction of *L. monocytogenes* versus the control cheese, which maintained the 10^4 CFU/ml inoculum level. No analytical values were given for the commercial cheese used (Morgan 2001b). Davies and colleagues found 2.5 mg/L Nisaplin, when added to cheesemilk, to inhibit growth of *L. monocytogenes* in a ricotta-type cheese (pH 5.9; directly acidified with acetic acid) for ≥8 weeks' storage at 6 to 8ºC. Conversely, in cheese manufactured without Nisaplin, growth of *L. monocytogenes* was observed within 1 to 2 weeks. Inclusion of Nisaplin produced an immediate circa 0.8 log listericidal effect in the studied cheeses (Davies 1997). Al-Holy and colleagues found a combination of mild heating and nisin treatment (1000 or 1500 IU/ml cheesemilk) to totally eliminate *L. innocua* from brined white cheeses stored at 4 and 10ºC (Al-Holy 2012). Ibarra-Sanchez and colleagues found a combined treatment of nisin and PlyP100 (an endolysin) to dramatically reduce *L. monocytogenes* in a model queso fresco system during refrigerated storage (Ibarra-Sanchez 2019).

Samelis and colleagues investigated Anthotyros, a traditional Greek fresh whey cheese, in its ability to support the anaerobic growth of *L. monocytogenes* at 4ºC for up to 45 days when treated with purified nisin. The authors prepared Anthotyros from whey (pH 6.5 to 6.7) left over from Feta cheese manufacturing and subjected it either to natural acidification (pH 5.3, adjusted to 6.2 with 10% NaOH) or direct acidification with 10% citric acid. The authors included treatments with Nisaplin at 100 or 500 IU/g to the whey prior to cheesemaking or to the finished cheese at a level of 500 IU/g as well as a control nisin-free sample. Cheeses were inoculated with approximately 4 log CFU/g of a single strain of *L. monocytogenes* before being vacuum sealed

and stored at 4ºC for up to 45 days. An immediate 2.0 to 2.2 log CFU/g reduction of *L. monocytogenes* was observed in samples with 500 IU/g nisin added to the whey, a notably greater reduction than that realized from the same level of nisin added to the finished cheese wherein a 0.8 to 0.9 log CFU/g reduction was observed. Levels of *L. monocytogenes* remained notably lower in the 500 IU/g nisin into whey samples throughout the study, remaining below inoculation levels for 45 days in cheese made with whey directly acidified with citric acid and remaining below inoculation levels for 35 days before reaching an approximately 5 log CFU/g level by the end of storage in naturally acidified whey cheese. Anthotyros made with naturally acidified whey supported immediate growth of *L. monocytogenes* (to ≥1 log CFU/g above the inoculum level) within 3 days in the control sample and within 8 days in the post-manufacture nisin-treated sample. In Anthotyros made with whey acidified using citric acid, growth of *L. monocytogenes* occurred within 3 days in the control cheese and within 16 days in the post-manufacture nisin-treated sample. Initial pH values of the challenged cheeses manufactured with naturally acidified whey were found to be 6.88, 7.14, 7.12, and 6.89 for control cheese, cheese treated with 100 IU/g nisin into the whey, cheese treated with 500 IU/g nisin into the whey, and cheese treated with 500 IU/g nisin onto the cheese surface post-manufacture, respectively. Initial pH values of the challenged cheeses manufactured using whey acidified using citric acid were found to be 7.08, 7.08, 7.11, and 7.01 for control cheese, cheese treated with 100 IU/g nisin into the whey, cheese treated with 500 IU/g nisin into the whey, and cheese treated with 500 IU/g nisin onto the cheese surface post-manufacture, respectively. Final pH values ranged from 6.34 to 6.91, with reductions in pH value apparent in all samples included in the study. The authors noted that results from their work varied from those of Davies and colleagues, who found complete inhibition (to <2 log CFU/g) of *L. monocytogenes* up to 55 and 70 days with the use of

100 IU/g nisin added into the cheesemilk heated with or without direct acidification by acetic acid in making ricotta-type cheese with storage at 6 to 8ºC. The latter study, however, included 500 mg/L of potassium sorbate into the milk, which may have increased the antilisterial effectiveness of nisin and/or acetic acid (Davies 1997, Samelis 2003).

Iseppi and colleagues investigated an enterocin produced by *Ent. casseliflavus* for its protection against *L. monocytogenes* when incorporated into an organic–inorganic hybrid coating applied to polyethylene film in direct contact with inoculated fresh cheeses stored at room temperature and under refrigeration. Bacterial growth curves using enterocin-treated packaging mimicked control films except for an initial approximately 1-log decrease in *L. monocytogenes* counts followed by subsequent regrowth. The authors additionally found spoilage of fresh soft cheese stored 28 days at 4ºC to be significantly inhibited using the enterocin-treated film versus controls (Iseppi 2008).

ANTILISTERIAL ACTIVITY OF PURIFIED BACTERIOCINS IN OTHER FOODS AND MICROBIOLOGICAL MEDIA

Ghrairi and colleagues found a MIC of nisin Z to be 420 ng/ml against *L. ivanovii* and a MIC of a novel enterocin produced by *Ent. faecalis* to be 677 ng/ml in a broth system (Ghrairi 2004). Schillinger and colleagues found 3000 IU/ml nisin necessary to cause a significant reduction in *L. monocytogenes* in tofu, however, the authors noted that rapid regrowth *of L. monocytogenes* was observed in samples stored at 10ºC (Schillinger 2001). Samelis and colleagues found nisin with lactic or acetic acid to show an antagonistic effect on the initial reduction of *L. monocytogenes* in sliced pork bologna samples, additionally finding growth of *L. monocytogenes* to commence earlier in bolognas immersed first in acids then nisin versus the

reverse (Samelis 2005). Alves and colleagues found the use of Chrisin (a commercial purified nisin; applied at 10^2 IU/g ham) unable to suppress *L. monocytogenes* growth beyond control samples when serotype 4b and 1/2a *L. monocytogenes* strains were inoculated individually via surface inoculation before storage of samples at 8°C for 10 days (Alves 2006).

Martinez and colleagues tested 4 *L. innocua* and 7 *L. monocytogenes* strains isolated from artisanal cheeses commercialized in northern Spain for their susceptibility to pediocin PA-1, enterocin AS-48, nisin, and plantaricin C. The authors found the most active bacteriocin against *Listeria* spp. to be pediocin PA-1 followed by enterocin AS-48 and nisin. MIC for plantaricin C was found to be much higher than the other bacteriocins studied. *L. monocytogenes* MIC values of 0.469 to 0.938 μM for nisin, 0.109 to 0.055 μM for enterocin AS-48, 0.007 to >0.215 μM for pediocin PA-1, and 17.5 to 70 uM for plantaricin C were observed. The authors found no large differences in nisin MIC values between strains but found *L. innocua* strains to be equally or less susceptible to nisin than *L. monocytogenes* strains. The authors noted that strains more resistant to one bacteriocin were not necessarily more resistant to other bacteriocins. The authors additionally found that serial exposure to nisin appeared to enhance nisin resistance (Martinez 2005).

ANTILISTERIAL ACTIVITY OF COMMERCIAL FERMENTATES

Glass and colleagues investigated the effect of 0.6% Alta^{TM} 2341, a commercial fermentate containing corn syrup solids, hydrolyzed yeast, and hydrolyzed vegetable protein, and organic acid type (citric, malic, or acetic) on the behavior of *L. monocytogenes* in queso blanco. The authors prepared the cheese via direct acidification to a final pH of 5.2 with and without the addition of 0.6% Alta^{TM}. Average proximate composition for the cheeses was found to be 51 to

53% moisture, 1.9 to 2.0% salt, and pH 5.2 to 5.3. The cheese was inoculated to a level of 10^6 CFU/g *L. monocytogenes* before storage at 4 or 20ºC. The authors observed an immediate 0.5 to 0.7 log decrease in *L. monocytogenes* in cheeses made with all acid types and 0.6% Alta™. Time-to-1-log growth was delayed approximately 7 days at 4ºC and 1 day at 20ºC for samples containing 0.6% Alta™ and malic or citric acid, respectively, versus in samples containing no fermentate. Samples formulated with acetic acid were unable to support *L. monocytogenes* growth at 4ºC, however, final populations were reduced by approximately 0.7 log CFU/g following 42 days' storage in samples containing 0.6% Alta™ and acetic acid versus samples containing acetic acid only. The authors additionally compared the effects of acetic acid alone or in combination with 0.6 or 2.5% Alta™ against 10^2 and 10^6 CFU/g *L. monocytogenes*. The incorporation of 2.5% Alta™ into cheese manufactured with acetic acid and with a lower starting inoculum reduced *L. monocytogenes* counts to undetectable levels following 21 days storage at 4ºC, though the pathogen could still be recovered via enrichment. Though the antimicrobial component of Alta™ was unknown to the authors, they noted that the ingredient showed similar antilisterial activity to pediocin AcH (Glass 1995).

von Staszewski and Jagus tested MicroGARD and nisin, in combination or separately, in a liquid cheese whey system inoculated with a single strain of *L. innocua* and stored at 7, 12, 20, or 25ºC. The liquid cheese whey system was prepared from 34% whey protein concentrate to solids levels of 0.8 or 8% and pH adjusted to 5.5 with HCl. The authors used a 40% solution of MicroGARD 300, developed to target Gram-positive organisms specifically, in sterile water before pH-adjusting to 5.5 with HCl. The authors prepared a stock solution of 10^5 IU/ml Nisaplin in sterile water pH adjusted to 2.0 and stored at -20°C to ensure bacteriocin solubility. When added to whey samples, neither MicroGARD nor nisin preparations were found to affect pH of

the whey system. 5% MicroGARD 300 with 50, 100, or 200 IU/ml nisin samples were prepared in addition to whey samples treated with only 5% MicroGARD 300 or 50, 100, or 200 IU/ml nisin. The authors found 100 and 200 IU/ml nisin when applied alone to show an immediate reduction in *L. innocua* by 5.3 and 6.7 log CFU/ml, respectively, which was subsequently followed by regrowth of *L. innocua*. Regrowth was restored almost immediately in samples stored at 20 and 25ºC, while growth was restored after 50 hours in samples treated with 50 or 100 IU/ml nisin and stored at 7ºC. The authors found combinations of MicroGARD and nisin to be less effective in immediately reducing *L. innocua* counts, however, by the end of storage, counts were 2 to 3 log CFU/ml lower than treatments containing single ingredients with an exception of 100 IU/ml nisin-treated whey stored at 7ºC which was found more effective than combination treatments. The authors found treatment of whey with 5% MicroGARD 300 to give similar results to untreated whey samples at all temperatures (von Staszewski 2008).

Zuckerman and Avraham found nisin and MicroGARD to have greater efficacy against *L. monocytogenes* when combined versus MicroGARD alone in fresh chilled salmon stored at 6°C, and additionally found MicroGARD alone in poultry unable to inhibit growth of *L. monocytogenes*. However, treatment of salmon with nisin in addition to MicroGARD resulted in lower efficacy against *L. monocytogenes* than in salmon treated with nisin alone (Zuckerman 2002). This finding was similar to those of von Staszewski and Jagus, who found a liquid cheese whey system to have better performance against *L. monocytogenes* with 100 IU/ml nisin alone versus in combination with 5% MicroGARD 300 in samples stored at 7°C. However, their work found combinations of the two ingredients to show better antilisterial efficacy versus either ingredient alone in whey samples stored at 3 higher test temperatures (12, 20, and 25°C) (von Staszewski 2008).

BIOPRESERVATIVE EFFECTS ON SPOILAGE

An additional consideration for identifying appropriate biopreservation strategies may be due not to product safety but product spoilage. LAB may produce antifungals including proteinaceous compounds, phenyllactic acid and cyclic dipeptides, hydroxylated fatty acids, bacteriocin-like substances and other low or medium MW compounds (Voulgari 2010). Settanni and colleagues found that in making Tosèla cheese, a traditional high-moisture cheese made with pasteurized milk, calf rennet, and without a starter culture, with incorporating a mixed culture of *Lb. paracasei* and *Str. macedonicus* a higher number of *Lactobacillus* (7.90 log CFU/g versus 4.86 log CFU/g in control cheese) and *Streptococcus* (6.10 log CFU/g versus 4.89 log CFU/g in control cheese) and a lower number of coliforms (3.10 log CFU/g versus 4.93 log CFU/g in control cheese) and *Staphylococcus* (2.78 log CFU/g versus 5.00 log CFU/g in control cheese) could be realized. The incorporated *Lb. paracasei* strain was additionally found to produce bacteriocin with antilisterial activity in microbiological media (Settanni 2011).

Voulgari and colleagues investigated the antifungal activity of 81 NSLAB isolates against molds and yeasts developed on the hard surface of cheese. Molds used were *Penicillium* spp. and yeasts *Debaryomyces hansenii* and *Saccharomyces cerevisiae*. The authors screened 10 *Leuc. mesenteroides* from goat's milk Teleme cheese, 17 *Lb. paracasei* subsp. *paracasei* and 15 *Lb. delbrueckii* subsp. *bulgaricus* from various cheeses, and 31 from Feta, including 11 facultative heterofermentative *Lactobacillus* and 20 obligate heterofermentative *Lactobacillus*. The authors found all isolates from Feta cheese to exhibit antifungal properties in addition to zones of inhibition for *L. innocua* and *L. monocytogenes* in agar disk assays (Voulgari 2010).

Campos and colleagues investigated Minas frescal cheeses made with either 0.1% or 0.5% mesophilic starter or via direct acidification with lactic acid. The group found elevated

levels of coliforms, yeasts, and molds in the directly acidified cheeses versus those made with lactic starter (Campos 2000). Kykkidou and colleagues investigated inclusion of nisin in Galotyri Greek soft acid-curd cheese (approximate pH 4.0 and 75% moisture) stored aerobically at 4ºC for 42 days, monitoring spoilage organisms. The authors found *Lactobacillus, Lactococcus*, and yeasts to predominate in the samples, and found 50 IU/g nisin to extend the shelf-life of the cheese by 7 days and 150 IU/g nisin by 21 days based on sensory analysis as well as a critical limit on yeast of 5 log CFU/g. Galotyri included in the study was made from ewe's milk and commercial starter culture, with initial counts of 8.0 log CFU/g *Lactobacillus* spp., 8.5 log CFU/g *Lactococcus* spp., <1.0 log CFU/g *Enterobacter* spp., and <2.0 log CFU/g *Pseudomonas* spp., *Enterococcus* spp., *S. aureus*, and yeasts measured in the product. The authors found the most pronounced effect of nisin to be on yeast inhibition in the Galotyri studied (Kykkidou 2007).

CONCLUSIONS

Cheese is a diverse food group, with several intrinsic and extrinsic factors dictating pathogen behavior. Regulatory issues surrounding cheeses are complex and continue to be examined in order to ensure best practices for consumer safety and product quality. High-risk cheese varieties, including raw milk cheeses and high-moisture, low-acid fresh cheeses, continue to increase in popularity in the U.S., making pathogen control in both groups imperative. A large body of published works on pathogen control measures in cheese exists and should direct future research in the area of cheese safety.

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CHAPTER 2:

Heat-induced inactivation of microorganisms in milk and dairy products

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ABSTRACT

It is well understood that pasteurization of milk allows its safe consumption in terms of foodborne illness, while failure in adequate heat treatment has resulted in both product recalls and also foodborne disease outbreaks. Aspects of different heat treatments within the dairy industry that affect relevant microorganisms, with an emphasis on bacteria, will be discussed in this review. This includes a description of D- and Z-values as measures of heat resistance, the factors that affect D-values, such as different dairy matrices, a discussion of some of the mechanisms associated with heat resistance in bacteria important for dairy products, case studies on different products illustrating different types of heating effects on microorganisms present, and recommendations for the most appropriate experimental design for understanding how heat affects microorganisms.

Key words

heat inactivation, mechanisms, pasteurization, milk, dairy, pathogens

INTRODUCTION – MILK AND HEAT TREATMENT

Foods obtained from animal sources provide humans with a balance of nutrients which are not readily available from plants (Murphy 2003), and evidence in ancient pottery shards has indicated that humans have been preserving and possibly even processing dairy products since the 6th millennium BC (Salque 2012). Bovine milk is an example of a nutrient-rich animal-sourced food, containing lipids, proteins (casein), carbohydrates (lactose), amino acids, vitamins and minerals (calcium) and has overall dietetic benefits from a human perspective (Haug 2007, Steijns 2008). However, due to its nutritional properties, milk is also a growth matrix for a variety of spoilage and potentially pathogenic microorganisms.

The idea that heating milk would improve its storage quality was recognised even before Pasteur showed that heating would inactivate the bacteria present in wine (Holsinger 1997). Coupled with this was the discovery by Robert Koch of the bacterial cause of tuberculosis, and by 1886, 85% of Medical Officers of Health in the UK believed that tuberculosis was spread by consumption of raw milk and undercooked meat (Atkins 1999, Savage 1912). In the 1890s, bovine tuberculosis was also recognised as a serious human and animal health concern in New Zealand (Bryder 1991, Ford 2013). The uptake of pasteurization as a technology to treat milk was rapid in Europe where Germany, followed by Denmark and Sweden, was the first to install commercial pasteurizers in the 1880s (Holsinger 1997). By contrast, this technology was slow to be accepted in New Zealand, where only 20 of the 345 factories used pasteurization by 1909 (Ford 2013). A similar controversial view of the technology was held in the USA where the first commercial-scale pasteurizer was only installed in New York City in 1907 (Boor 2017). This was despite the study of Smith who in 1899 showed that *Mycobacterium tuberculosis* was inactivated in milk heated at 60°C for 15 minutes (Smith 1899, Holsinger 1997).

Today, it is well understood that pasteurization of milk and dairy products keep consumers safe from foodborne illness (Costard 2017), while failure in adequate heat treatment has resulted in both product recalls, and also foodborne disease outbreaks (see Table 2-1 for some examples).

OVERVIEW OF TYPES OF HEATING IN DAIRY MANUFACTURING PROCESSES

During the manufacture of dairy products, milk is subjected to a variety of different heating processes. A summary of the most relevant heating types for microbiological inactivation can be found in the factsheet produced by the International Dairy Federation (Deeth 2018). The objective of thermal processing is to increase the keeping quality by inactivating microorganisms. Disadvantages of thermal processing include organoleptic degradation (browning, cooked flavour), decreased nutritional value (loss of nutrients) and technological problems (poor solubility of milk powder). For the purposes of this review, six main types of heat treatments are highlighted (Table 2-2). These include:

Pasteurization. High temperature short time (HTST) pasteurization involves heating the milk to at least 72°C for 15 s or low-temperature long time (LTLT) to 63°C for 30 min. The technical aspects of pasteurization and its effect on the microbiological and nutritional aspects of milk are well documented in IDF Bulletin 496/2019 (IDF 2019). Higher fat (>10%), higher solids (>18%) or added sugar products typically have a higher viscosity and lower heat transfer coefficients, and so may require higher temperatures or longer holding times.

Batch pasteurization. In the dairy industry there are numerous different batch pasteurization temperatures and times that are used. Generally, the ingredients are standardized or recombined into a batch tank and heat treated to kill at least any vegetative pathogens that may be present. Two examples are: 1) Milk used for starter culture and yogurt manufacture receives a significant heat treatment of 90-95°C. The objective of the heat treatment is to destroy bacteriophages, vegetative bacteria, and some though not all spores, eliminate inhibitory substances, denature some protein, and expel dissolved oxygen. 2) Processed cheese batch ingredients usually receive a process equivalent to, or greater than, pasteurization to kill vegetative cells, and there is a wide range of process parameters used to achieve this.

Thermization. This process occurs between 57 – 68°C for 5 s up to 30 minutes. Thermization targets vegetative psychrotrophs (that produce heat-resistant lipases and proteinases) and is sometimes used in the manufacture of cheese (an example is described below in the *Listeria* case study; see Table 2-4 for thermization definitions relevant to raw milk cheesemaking), or to extend the keeping quality of milk during chilled storage prior to further processing.

Extended shelf-life processing (ESL) or Ultra pasteurization. Milk that is to be stored for extended periods at refrigeration prior to use can also be treated with temperatures and times higher than pasteurization (125 – 140°C for 1 – 10s).

Ultra high temperature (UHT). Milk that is stored for long periods of time (6 – 12 months) at ambient temperature prior to use is UHT-heat treated at 135- 150°C/1-10 s, with the

aim of achieving a 9-log reduction in the thermophilic spore count. When this 9-log reduction is achieved at a particular time and temperature combination, it is sometimes referred to as the bacteriological index (or B*). At this time/temperature combination, the B* has a value of 1 (Kessler 1981). There are two main types of UHT systems, direct and indirect. In the direct system the product comes into direct contact with the heating medium. The product is rapidly heated and cooled and considered to have a better flavor profile than indirectly heated product. In the indirect system the heating medium does not contact the product and heat is transferred through a heat exchanger. Indirect heating is usually more severe than the direct process (Tetra Pak 2005).

In-container sterilization. Cans filled with evaporated/condensed milk are sealed and autoclaved, which can either operate continuously or in batches. The cans are kept in motion during sterilisation, to distribute the heat more quickly and more evenly through the cans. Any protein precipitated during the heat treatment is uniformly distributed throughout the milk. The milk reaches the sterilisation temperature of 110-120°C for 15-20 minutes, after which the milk is cooled to storage temperature. The heat treatment is intense and results in light brown colouration because of Maillard reactions (Tetra Pak 2003).

For commercially sterile products with an extended ambient shelf-life, the food safety concern is inactivation of spores of pathogens i.e. *Clostridium botulinum*. Because milk is a low-acid food (pH > 4.5), the food safety aim is to achieve 12 decimal reductions for *C. botulinum* (this is the same criterion for other canned/sterile foods). The minimum botulinum cook (time/temperature combination) will produce a product that is microbiologically safe (i.e. *C. botulinum* spores are inactivated), but not necessarily sterile (i.e. some more heat resistant spores

may still be present, and this is referred to as 'commercially sterile'). These more heat-resistant spores, which may cause spoilage, are for example *Geobacillus stearothermophilus* and *Bacillus sporothermodurans* (Lewis 2003). Therefore, the heat treatments in dairy processes that control these more heat-resistant spores will also control *C. botulinum* spores. Note that *C. botulinum* is rarely associated with raw milk or pasteurized dairy products (Doyle 2015).

IMPORTANCE OF UNDERSTANDING D-VALUES AND Z-VALUES

Historically, the thermal death point was a concept used to describe the heat sensitivity of bacteria and has been defined as the length of time needed to completely inactivate a suspension of an organism (including prokaryotic and eukaryotic cells) at a single constant temperature (Tischer 1954). For example, Park showed that the thermal death point for the tubercle bacilli in milk was 1 minute at 68°C (Park 1927). However, the thermal death point has largely fallen out of use as it depends on the size of the population present, type of organisms, and gives the impression of an instant kill at one point in time at a set temperature. Calculation of the D-value is more useful as it provides information on the heat resistance of bacteria (Juffs 2007).

What are D- and Z-values? Since the inactivation of different microorganisms by heat varies, a simple measure of how resistant they are to heating processes is needed. Hence the use of D-values (decimal reduction time - DRT). The D-value is defined as the time taken to reduce the population by 1 log CFU or 90%. D-values for bacteria are dependent on the type of heating matrix (e.g. liquid milk versus concentrated milk products), the testing method used (e.g. capillary tube-based versus pilot scale pasteurizer), the type and strain of bacterium tested and the temperature applied. The change in the microbial number from before heating to after heating

is considered to be linear, which allows the D-value to be easily calculated from the slope, m, of the linear regression line of the plot of the log survivors against time at a specific treatment temperature (Berk 2009).

The equation is:

$$D = 1/(u)$$

where u is the rate of decline of the population given by the absolute value of slope m.

Early work by Bigelow and Esty demonstrated this linear relationship between the logarithm of the DRT and the temperature and is referred to as the classical thermal death model (Bigelow 1921, Bigelow 1920). However, there are occasions where “shoulders” or “tailing” of survivors occurs (Ross 1998). “Shoulders” are considered to be a lag in inactivation response by the cells being tested, while “tails” represent sub-populations that are reportedly more resistant to the temperature applied than the general population of cells being tested (Ross 1998). In the case of “shoulders” or “tailing”, the D-value is calculated from the resulting curve using models such as Weibull (Huertas 2015). Table 2-3 shows the D-values of various bacteria of importance in dairy manufacturing and in various dairy matrices or their derivatives.

The Z-value is used to define the temperature that would be needed to reduce the D-value by a factor of 10 and is useful for predicting how resistant bacteria may be to heat treatment. For example, if the D-value for a bacterium in milk at 65°C is 10 s, and its Z-value is 7ºC, then it can be predicted that at 72°C the D-value is 1 s. So for this bacterium, a treatment of 15 s at 72°C (standard pasteurization), would result in a 15 log reduction in the population of that particular bacterium. Using a real dairy example, the D-value of *L. monocytogenes* strain 1151 in butter treated at 68°C was calculated as 11.3 s, with a Z-value of 6.71°C (Casadei 1998). At 72.7°C

(pasteurization), the D-value was 1.5 s (i.e. pasteurization of butter would result in 10 log reductions of this strain of *L. monocytogenes* when carried out at the standard 15 s).

Some factors reportedly affecting the D-values of microorganisms in the dairy context. There are a number of factors that influence the heat resistance of microorganisms. One factor is the fat content of the dairy matrix. A recent study has found that oil helps to protect bacteria from thermal inactivation in two ways: i) desiccation of cells (i.e. a low water activity), and ii) by protecting the cells by creating a moisture barrier which prevents water vapor from rehydrating the cells, thus preventing the inactivation process (Yang 2020). That study was carried out in peanut oil, but may also explain some of the phenomena seen in dairy matrices with higher fat contents. For example, higher exposure times to pasteurization-like temperatures were required to inactivate Hepatitis A virus in cream compared to skim or homogenized milks (Bidawid 2000). For *Cronobacter* spp. in reconstituted infant formula, higher fat whole milk formula resulted in a higher D-value compared to the D-value for low fat or skim milk counterparts at 58°C under laboratory experimental conditions (Osaili 2009) (Table 2-3). However, for bacteria, an increased fat content may not always result in a higher D-value. For example, Casadei and colleagues showed that *L. monocytogenes* strain 1151 had a higher D-value in butter (11.30 s) compared to double cream (7.86 s) at 68°C (Table 2-3) which was expected due to the higher fat content of butter. However, for a different strain, Scott A, there was a higher D-value in double cream (9.46 s) compared to butter (6.45 s), seemingly indicating strain-specific behavior. Similarly, *E. coli* ATCC 9637 was shown to have a higher D-value at 76°C when treated in milk compared to 40% cream (Table 2-3) (Read 1961).

Recent studies have also shown that minerals contained within milk also increase or decrease the heat resistance of different bacteria. Calcium and magnesium were shown to increase the heat resistance of *Salmonella* Seftenburg 775W at 63°C by stabilizing the cell envelope (Mañas 2001), while higher levels of phosphate were shown to decrease the heat resistance of *Geobacillus* spores to heat at 110°C (Kumar 2019).

Desiccation or a change in osmotic tolerance (e.g. higher total percentage solids) are well known factors that increase the heat resistance of some pathogens in dairy products, in particular for *Salmonella*. In liquid milk, *Salmonella* is easily inactivated by pasteurization (72°C for 15s). Work carried out by Pearce et al. (2012) who tested 32 different serotypes of *Salmonella*, showed that a > 6.9 log reduction was achieved at 61.5°C for 15 s for the most heat resistant strain tested (*S*. Typhumurium NZRM 4220). However, when milk is concentrated, the D-values at 57°C for *Salmonella* increased from 1.4 min at 10% TS to 26.6 min at 51% TS (Table 3). In addition, it has been known for many decades that *Salmonella* are reduced during the spray drying process of milk (~4.5 – 6 log reduction in powder with 20% TS and 3% moisture) (Miller 1972). A more recent study has confirmed that historical work, and shown that drying of milk from a water activity of 0.9 to a water activity of 0.25 resulted in a 4 log reduction in *Salmonella* (Lang 2017). However, studies have shown that desiccated *Salmonella* are more heat resistant than their non-desiccated counterparts. Gruzdev and colleagues showed that desiccated cells of *S*. Typhimurium, grown from laboratory medium, were only reduced by 3 log when heated for 1 hour at 100°C (dry heat), compared to non-desiccated cells that were completely inactivated within 10 min (~ 6 log reduction) (Gruzdev 2011). Similarly in dairy media, a study by Sekhon and colleagues showed that a 6 – 7 log reduction was achieved in reconstituted skim milk at 65°C for 2 minutes for strains of *S*. Enteritidis, *S*. Montevideo, *S*. Newport and *S*. Typhimurium

versus a 4 – 5 log reduction when these same strains were treated in skim milk powder at 80°C (Sekhon 2020).

The strain type also influences how bacteria respond to heat treatments, with some strains naturally more tolerant than others. *Cronobacter* spp. strain 607 was found to be more resistant to heating in the same reconstituted infant formula at 58°C (D value of 8.8 – 10.4 min) compared to strain 51329 (~ 0.51 min) (Edelson-Mammel 2004). *L. monocytogenes* strains belonging to serogroup 1 were found to be more resistant than serogroup 4 strains when heated at 72°C in semi-skimmed sterilized milk (Lemaire 1989). Similarly, a study on thermization of raw milk for cheese manufacture using a pilot-plant pasteurizer showed that some strains of *E. coli* were inherently more resistant to heating than others, with *E. coli* O16:H21 (FAM21846) exhibiting a D-value of 3.3 s at 65°C compared to *E. coli* O68:H14 (FAM21805), which had a D-value of 93.4 s at 65°C (Peng 2013).

The initial preparation of the culture for testing also affects the resulting D-value. Elevating the growth temperature of *L. monocytogenes* strain Scott A from 37°C to 48°C resulted in a higher subsequent D-value in UHT milk (Fedio, 1989), while coupling 43°C incubation with anaerobic storage resulted in a higher D-value for *L. monocytogenes* strain F5069 in sterile whole milk than did 37°C aerobic storage (Knabel 1990). *L. monocytogenes* cells in the stationary phase of growth were additionally found to show increased thermal resistance versus those in the log phase (Jørgensen 1999, Lou 1996).

The heating methodology used in determining D-values also impacts the resultant values. For liquid products, best-case results are obtained by laboratory-scale pasteurization with flow, while worst-case results are obtained with a large sample volume in a water bath (van Lieverloo 2011). Presence of air in liquid samples should be considered, as *L. innocua* cells were found via

scanning electron microscopy to be trapped within large air pockets and recoverable via enrichment and confirmation in inoculated ice cream mix following heating at 69°C for 30 min (Neha 2019). Donnelly and colleagues found that test tube methodology overestimated the thermal resistance of *L. monocytogenes* in reconstituted non-fat dry milk compared to values derived using sealed glass tubes (Donnelly 1987). This finding was substantiated by Sorqvist, who found *Yersinia enterocolitica* D-values at 60°C in physiological saline to be 8 to 29 times greater using 9-mm test tubes than when using capillary tubes (Sorqvist 1989). Caution must additionally be exercised in applying batch system kinetics to those of a continuous flow system. As evidenced by results of Fairchild and others, significantly different *L. innocua* Z-values were obtained when raw skim milk was heated in sealed capillary tubes, as compared to values obtained with a laboratory-scale pasteurizer (Fairchild 1994). Nevertheless, van Lieverloo and colleagues found insignificant difference in *L. monocytogenes* D-values obtained using four different heating methodologies (lab-scale pasteurizer, large volume pre-heated menstruum, submerged capillary tubes, or submerged vials); their analysis of 735 published D-values obtained from dairy and non-dairy liquid foodstuffs and microbiological media demonstrated a high overall R^2 value of 0.88 (van Lieverloo 2011).

As part of the inherent tolerance to heat, sporulation of bacteria must be included. Spores from spore-forming bacteria are naturally more heat resistant because of the nature of the bacterial spore (Table 2-3). The proposed mechanisms of heat resistance for the various microorganisms are discussed in more detail below.

MECHANISMS OF HEAT INACTIVATION OR RESISTANCE FOR DIFFERENT MICROORGANISMS OF DAIRY RELEVANCE

Russell has previously discussed the different mechanisms for heat inactivation of bacteria (Russell 2003). While these mechanisms can be applied generally to heat inactivated bacteria in dairy processing, the possible mechanisms associated with dairy relevant microorganisms are considered.

Important foodborne pathogens. An explanation for why *Salmonella* species in milk powder may tend to have a higher heat resistance in the desiccated state may be found in the ribosome (McDonough 1968). It has been suggested that ribosomal degradation is important for inactivation of *Salmonella* at higher treatment temperatures, and that a low water activity may inhibit this degradation by stabilizing the ribosomes, perhaps by magnesium ions (Aljarallah 2007, Tolker-Nielsen 1996). The ribosome hypothesis is also supported by recent work indicating that the glass transition temperature (Tg) in *S. enterica* serovars increased as the water activity decreased (maximum 57.5°C at a water activity of 0.87, compared to maximum 83.3°C at a water activity of 0.43) (Lee 2020). The glass transition temperature is the temperature at which polymers containing 30 – 50 carbon chains begin to move from a rigid state, in which they act as glass, to a flexible state (Shrivastava 2018). The higher Tg also corresponded to more cell survival at 60°C for 10 min. It is logical that molecules in a rigid state at a lower water activity would be less vulnerable to physical or chemical interventions than molecules in a more fluid state. Thus, it seems that the observed heat resistance of *Salmonella* in a desiccated form is not due to any inherent physiology of the bacterial cell itself but has more to do with the environment. This is supported by work showing that *Salmonella*, originally isolated from

milk powder, lose their heat resistance when heat tested after re-growth in laboratory medium and subsequent re-inoculation into liquid milk (Read 1968).

Listeria monocytogenes is an important pathogen in dairy manufacturing and continues to result in disease outbreaks and dairy product recalls (Bourdichon 2019). Unlike *Salmonella*, ribosomal damage does not appear to be a major cause of cell death in *Listeria* species when heated between 57 and 63°C (Skåra 2011). For *Listeria monocytogenes*, heat treatment appears to result in cell membrane damage and cell wall thinning (Bermúdez-Aguirre 2011, Somolinos 2010), which is supported by studies showing a down regulation in genes that govern cell division and cell wall synthesis when *L. monocytogenes* are heat shocked (van der Veen 2007). The *sigB* gene, together with a cascade of stress response mechanisms, play a role in heat tolerance (Somolinos 2010). Several heat-shock genes are also activated during heat treatment, as well as the SOS response (a DNA repair mechanism in its broadest sense) (van der Veen 2007). The alternative sigma factor σB is activated in *Listeria* species as a general response to various types of stress, such as acid, oxidative stress and ethanol stresses (Chaturongakul 2006). These environmental stresses, which could occur in dairy manufacturing environments through acid, peroxide or ethanol sanitizer use, for example, are also known to confer a cross-protection to *Listeria* when subsequently treated with mild heat (e.g. 60°C) (Lou 1996). A further tolerance mechanism may be the presence of plasmids. Researchers in Finland have recently found a strain of *L. monocytogenes* reportedly tolerant to heating at 55°C which is conferred by the presence of a plasmid carrying a gene for the ATP-dependent protease ClpL (Pöntinen 2017). However, that isolate was found in a meat manufacturing plant, and was not dairy-associated, but it does demonstrate strain-dependent resistance mechanisms that can be found in different matrices.

Cronobacter spp. are important opportunistic pathogens, and internationally, it is recognised that all infants (<12 months of age) are the population of concern for foodborne infections with *Cronobacter* spp. (CAC 2008). In terms of dairy manufacturing, *Cronobacter* spp. are able to survive the drying process used to manufacture infant powder products (more resistant to drying than even *Salmonella*) (Lang 2017). For *Cronobacter* spp. in broth culture, heat treatment (48°C) damages cell membranes, and associated cell leakage of proteins, enzymes and nucleic acid occurs (Chang 2009). However, similar to *Salmonella* spp., *Cronobacter* spp. in the desiccated form in milk powder are resistant to dry heating (Jaradat 2014). It has been speculated that *Cronobacter* spp. in this dried state in milk powder undergo a decrease in their respiratory ability during heat inactivation, contributing to this heat tolerance (Lang 2018). Another mechanism based on a unique thermotolerant genomic island has been found in thermotolerant *Cronobacter* spp. (Gajdosova 2011, Orieskova 2013). The thermotolerant effect is coded for by a gene on a genomic island of other stress-response genes in strains of thermotolerant *Cronobacter* spp. strains (37 – 54°C) (Orieskova 2013). A 20-fold increase in the transcription of the thermotolerance genes occurred in such *Cronobacter* spp. cells when the heat treatment was increased from 37 to 54°C (Orieskova 2013). However, as for other pathogenic bacteria, there is no evidence that such thermotolerance affects the inactivation of *Cronobacter* spp. during commercial pasteurization processes (e.g. *C. sakazakii* was reduced by > 6.7 log CFU at 67.5°C in a pasteurizer operating under validated turbulent flow) (Pearce 2012).

Potential spoilage bacteria. The heat resistance of spores of spore-forming mesophilic and thermophilic bacteria is a well-established phenomenon (Davies 1975), but the heat resistance (D-values) of the spores from these groups varies (Table 2-3). In general terms,

membrane permeability changes, protein or enzyme damage, and DNA damage are all considered to be mechanisms of heat inactivation of bacterial spores (Russell 2003). In addition, dry heat is also less effective than wet heat against bacterial spores due to the dehydration of the spore core (Setlow 2006). However, a few recent discoveries may help to shed light on the heat inactivation of bacterial spores in the dairy context.

A mobile genetic element (transposon) Tn1546-like carrying an operon called *spoVA*^{2mob} has recently been found in strains of *B. subtilis* and has been linked to high heat resistance of the spores produced by the strains tested (Berendsen 2016). However, the two dairy isolates tested in that study, *B. amyloliquefaciens* B425 isolated from sterilized milk and *B. licheniformis* B4092 isolated from buttermilk powder, did not appear to contain the transposon.

For thermophilic *Geobacillus* spp., a recent study using scanning electron microscopy visualised damage to the inner spore core. Heat treatment (120°C for 10 min) caused membrane permeability and release of intracellular components (Rozali 2017). This correlates with studies showing that heating of *Geobacillus* spp. spores at 121°C for 30 min released dipicolinic acid (Reyes 2019). The presence of phosphate has also been shown to accelerate the loss of cations from dairy-relevant *Geobacillus* spp. spores, resulting in increased heat sensitivity (Kumar 2019).

Streptococcus thermophilus is a thermoduric bacterium used as a yogurt culture in dairy manufacturing, but can also be an important spoilage bacterium. *Str. thermophilus* exhibits a complex array of responses to heat including up-regulation of several heat shock genes, signal transduction genes, cell wall genes, iron homeostasis, ABC transporters and restriction-modification system when treated at 50°C (Li 2011). Interestingly, the heat shock gene *ClpL* also plays important role in the physiology of *Str. thermophilus* at high temperature, similar to other

Gram-positive bacteria, like the heat tolerant *Listeria* carrying the plasmid for this gene (Varcamonti 2006). Recently, *ClpL* in a heat-tolerant *L. monocytogenes* strain was found to share 98% nucleotide sequence identity with *ClpL2* of *Lactobacillus rhamnosus*, with researchers suggesting the heat shock gene may have been acquired through horizontal gene transfer (Pöntinen 2017).

From a probiotics perspective, where maintaining the viability of the cells is important, ribosomal and cytoplasmic membrane damage play key roles. The ribosome and cytoplasmic membranes of *Lactobacillus bulgaricus* were shown to be damaged when heated at 65°C in skimmed milk (Teixeira 1997). Similarly, Ananta and colleagues showed that spray drying caused disintegration of the cell membranes in *Lactobacillus rhamnosus* GG (Ananta 2005). Hence, the need for molecules that protect and stabilise the cell membrane during heating (Agudelo 2017, Chávez 2007, Lapsiri 2013, Liu 2017).

CASE STUDY EXAMPLES

Inactivation of L. monocytogenes in cheese. The Centers for Disease Control and Prevention has reported that unpasteurized milk dairy products cause 840 times more illnesses and 45 times more hospitalizations than pasteurized milk dairy products in the U.S. (Costard 2017). A review of 1993 to 2006 U.S. foodborne disease outbreaks associated with raw milk products found cheese to be the causative agent in 27 of those outbreaks (Langer 2012). With the exception of Feta cheese (in which the pH is reduced to 4.4 and curd cooked to 48°C during manufacture) and other cheeses involving curd-cooking steps (e.g. Swiss or pasta filata styles), the safety of raw milk cheeses is primarily dictated by the microbiological quality of the milk itself and not the ability of the cheesemaking process to inactivate pathogens (Condron 2009,

Donnelly 2018). While raw milk cheeses may be technically in compliance with FDA regulations mandating a minimum of 60 days' aging at ≥2°C, this aging period has been found to be insufficient in eliminating foodborne pathogens from certain cheese varieties (D'Amico 2010, Reitsma 1996, Ryser 1987, Schlesser 2006). Milk thermization has been used as a means of reducing bacterial counts in raw cheesemilk while still meeting labelling requirements for raw milk cheese designation in the U.S. (Johnson 1990) and has been recently recommended by Canadian regulators to increase the microbial safety of Gouda or Gouda-like cheeses (Boyd 2021). Among > 900 U.S. artisan, farmstead, and specialty cheese producers surveyed in 2018, 50% used raw (no heat treatment) and 17% used thermized (some heat treatment but below legal pasteurization requirements) milk in cheesemaking, up from 32% and 6%, respectively, as reported in 2016 (ACS 2016, ACS 2018). Several working definitions of thermization exist (Table 2-4), but no established definition in U.S. regulations has been declared.

L. monocytogenes and Shiga toxin-producing *Escherichia coli* (STEC) have been identified as especially high-risk pathogens in raw milk cheeses due to the severity of illness associated with each as well as their ability to survive or grow during the aging process of certain cheeses, even when initially present in milk at very low levels (Condron 2009, Donnelly 2018, Johnson 1990). Montel and colleagues found STEC to be more resistant to naturally occurring lactic acid bacteria (LAB) in unpasteurized milk compared to *L. monocytogenes*, *Salmonella*, and *Staphylocccus aureus* (Montel 2014) while Pereira and colleagues found *L. innocua* and non-Shiga toxin-producing *E. coli* to be the most difficult contaminants to control by co-inoculation with LAB isolated from raw milk cheeses when tested in a raw milk model cheese (Pereira 2009). *L. monocytogenes* has been shown to be more heat-tolerant than most other non-sporeforming pathogens (Doyle 2001); however, as STEC strains are less heat-tolerant than *L.*

monocytogenes, thermization treatments to reduce *L. monocytogenes* should sufficiently reduce STEC at equal or higher rates (Betts 2000, Fernandes 2009, Sorqvist 2003, van Asselt 2006).

A significant body of research on the thermal reduction of *L. monocytogenes* in liquid dairy and other food products exists, with predictive models constructed from meta-analyses by several authors (Mackey 1989, Sorqvist 2003, van Asselt 2006, van Lieverloo 2011, van Lieverloo 2013). However, differences in methods limit the usefulness of these models to appropriately facilitate a definition of thermization conditions necessary for the thermal reduction of *L. monocytogenes* in cheesemilk. Differences which affect the results between models include incorporation of reconstituted and/or skim milks (Mackey 1989, van Lieverloo 2013), inclusion of high fat or high solids dairy products (e.g. butter, cream, ice cream mix) (van Asselt 2006), pooling of milk D-values with other foods and microbiological media (Sorqvist 2003, van Asselt 2006, van Lieverloo 2011), and inclusion of temperatures higher than HTST pasteurization (Mackey 1989, Sorqvist 2003, van Asselt 2006, van Lieverloo 2011, van Lieverloo 2013). Predicted *L. monocytogenes* Z-values of 5.7°C to 7.0°C and D-values at 60°C of 87 to 140 s were recorded in those published predictive models. Comparatively, a predicted *L. monocytogenes* Z-value of 6.1°C and D-value at 60°C of 130 s were found by a meta-analysis of 162 whole milks inoculated with *L. monocytogenes* and heated to temperatures 55°C to 71.7°C (Figure 2-1).

Fewer studies investigating STEC in liquid dairy and other products exist in the scientific literature, with an additional limitation for cheese thermization application due to the inclusion of non-STEC general *E. coli* D-values in two existing published meta-analyses (Sorqvist 2003, van Asselt 2006). Predictive models published by Sorqvist and van Asselt predicted *E. coli* Z-values of 6.0°C and 10.6°C, and D-values at 60°C of 39 and 113 s, respectively. Comparatively, a

predicted STEC Z-value of 4.5°C and D-value at 60°C of 115 s were found in a meta-analysis of 25 whole milks inoculated with STEC and heated to temperatures 55°C to 65°C (S. Engstrom, unpublished results).

Case study example – extended shelf-life milk. Extended shelf-life (ESL) milk can be produced by thermal processes, or non-thermal processes such as microfiltration or bactofugation. Only thermally processed ESL is considered in this review. ESL heat treatment is undertaken with the aims of extending the shelf-life of the milk (beyond pasteurized milk) and maintaining optimum flavor. In effect, the amount of chemical change to the milk constituents should be minimal. There are no international standards for the heat treatment, but generally temperatures of between 125 and 130°C for 2 to 6 s are used, with the aim of achieving a 6-log reduction in the thermophilic spore count. The method of heat treatment can be direct, by contact with dry steam, or indirect, involving heat exchangers. ESL milk can be packaged aseptically, in which case bacteria surviving the heating process are a consideration, or under clean (but not aseptic) conditions, in which bacteria entering the milk post-processing are a further consideration. The shelf-life of ESL milk can be 30 to 60 days, depending on the rate of post-heat treatment contamination. For further details see Deeth (2017).

The production process of ESL milk will inactivate all vegetative cells, but spore-forming bacteria can survive the heat treatment process. Thus, the quality of the raw milk used is a consideration. If protease producing bacteria in raw milk (for example, species of *Pseudomonas*) get the opportunity to grow before processing, the proteases produced can be heat-stable, surviving the heat treatment and causing spoilage in the processed milk. Production of ESL milk is a balance between microbiological quality, organoleptic acceptability and chemical damage.

The higher the temperature and the longer the time the safer the milk is microbiologically, but the more chemical injury there is and the less organoleptically acceptable the milk is.

The measurement of chemical change in ESL milk is best measured by the degree of denaturation of the whey protein β-lactoglobulin. The volatile sulphur compounds formed during degradation of β-lactoglobulin contribute to the 'cooked' flavour of heat-treated milk. Approximately 50% of the native β-lactoglobulin should remain in ESL milk; otherwise the flavor may be affected.

Some spore-forming bacteria can survive ESL milk processing and some spore-forming and non-sporeforming bacteria can enter the ESL milk after the heat treatment, from filling equipment and packaging (Mugadza 2019) if aseptic filling is not used. At farm level, spores can enter raw milk from the surface of cow's teats, bedding, fodder, pasture or milking equipment, among other sources (Gleeson 2013).

The diversity of bacteria in ESL milk has been studied (Mayr 2004, Mugadza 2017, Mugadza 2018; for review see Deeth 2017). When spoilage occurred (in 191 samples), Mayr and colleagues found that 76 samples contained aerobic spore-formers while 31 contained Gram-negative bacteria. The contamination included species of *Rhodococcus*, *Anguinibacter*, *Arthrobacter*, *Microbacterium*, *Enterococcus*, *Staphylococcus* and *Micrococcus*. The presence of non-spore-forming bacteria in the ESL milk indicates that there was post-heat treatment contamination. Mugadza and Buys also found evidence of post-heat treatment contamination by *Bacillus cereus* strains, as well as evidence of survival of the heat treatment (Mugadza 2017).

In ESL milk, psychrotrophic spore formers, such as *Bacillus* and *Clostridia* spp., are a concern. These are bacteria that can survive the heating process and can then grow in the milk, even if it is stored at refrigeration temperatures. Processing can activate the spores leading to

germination and growth at low temperatures. Growth can be greater in cases of temperature abuse.

In general, strains of *Bacillus* species will cause spoilage rather than safety issues. Some strains of *B. cereus*, *B. licheniformis* and other *Bacillus* species can produce heat-stable toxins (Salkinoja-Salonen 1999, Tirloni 2020) leading to potential safety issues. *B. cereus* can produce emetic or diarrhoeal toxins, from growth in the milk or after ingestion, respectively. The spores of some strains of *B. cereus* are heat resistant and therefore *B. cereus* are of particular concern with ESL milk. A wide range of D- and Z-values, as measures of this heat resistance, have been reported for spores of *B. cereus* strains (for review see Deeth 2017), which are difficult to compare because of the different temperatures used. For example, D-values at 100°C from 3 to 36 s have been reported, presumably depending on the strain tested, but it could also depend on the method used. This range of temperatures doesn't include particularly heat resistant strains of *B. cereus*, such as that which was reported to have a D-value at 129.4°C of 14.4 s (Bradshaw 1975). The range of Z-values reported is also variable (for review see Deeth 2017), with values from 6.7 to 13.8°C reported. All of this shows that there is a wide range of heat resistance of spores among different strains of *B. cereus*. Similar variation has also been reported for spores of other species (Janstova 2001).

The *B. cereus* group of bacteria (*B. cereus sensu lato*) consists of 6 closely related species that are difficult to differentiate, even with 16S sequencing. Except for *B. anthracis*, which is not associated with milk, only strains of the *true B. cereus* species (*B. cereus sensu stricto*) are potentially pathogenic to humans. Many authors don't differentiate between *B. cereus sensu stricto* and *B. cereus sensu lato*, making it difficult to analyse the literature with regard to

pathogenicity of *B. cereus* strains that have been isolated from milk. Refrigerated storage of ESL milk will, in general, slow the growth and toxin production of *B. cereus* strains.

Sulphite reducing clostridia (SRC) are a group of bacteria consisting of a number of difficult-to-differentiate species, grouped by their ability to reduce sulphite. They are prevalent in the farm environment, but most dairy associated species are not pathogenic to humans as only a few strains carry toxin genes (MPI 2014).

For clostridia species, *C. botulinum* strains of Groups I and II are the main concerns for human health. *C. botulinum* is rarely associated with pasteurized dairy products, and anaerobic conditions are needed for its growth. On the occasion it does occur, Group I strains are most common (Doyle 2015). The minimum temperature for growth of strains of *C. botulinum* Group I is about 12°C, and under anaerobic conditions. Therefore, any surviving *C. botulinum* Group I strains in ESL milk are unlikely to grow and produce toxin if the heat-treated milk is not subjected to temperature abuse, and anaerobic conditions. *C. botulinum* Group II strains are psychrotrophs with the ability to grow and produce neurotoxin at temperatures as low as 3°C, but the spores are only moderately heat-resistant and, if present, will probably be inactivated during the heating process (MPI 2020).

Paenibacillus species, which were formerly part of the *Bacillus* genus, have emerged recently as a spoilage concern in dairy products. They can survive the heat treatment of ESL milk and they have a growth range from about 5 - 55°C, meaning that they can be psychotropic, mesophilic or thermophilic. They are mainly an issue with spoilage of ESL, particularly if there is temperature abuse.

Case study example – UHT milk. The UHT process involves a pre-heating stage, the high-heat stage, cooling, homogenising and aseptic packaging. The aim of such a process is to inactivate bacteria (and spores) so that the milk can be stored at ambient temperature. UHT milk is not a sterile product (it is referred to as commercially sterile) as some spores can survive the heat treatment and cause spoilage or be of a public health concern, particularly if there is temperature abuse, such as storage at ambient temperature of above 40°C. The heat-treated milk is packaged aseptically so the main concern is bacterial spore survival of the heating process, rather than post-processing contamination.

At the processing temperatures used, all vegetative bacterial cells will be inactivated. Additionally, many bacterial spores will be inactivated and those that survive are referred to as high-heat resistant spores. There is no standard method for the isolation of high-heat resistant spores but they can be isolated by heating the sample to 100°C for 30 min and incubating the agar plates aerobically or anaerobically at 30°C for mesophilic or 55°C for thermophilic spores (ISO 2009, Wehr 2004).

Spores surviving UHT heat treatment can be a quality or a safety concern. From a quality perspective, species like *B. sporothermodurans*, first isolated from UHT milk (Pettersson 1996), and *G. stearothermophilus* are a concern. These spores generally grow to low numbers in milk and growth is temperature dependent, but some strains can exhibit proteolytic activity, thus causing spoilage (Pinto 2018). Occasionally other *Bacillus* species causing spoilage have also been isolated from UHT milk, such as *Paenibacillus lactis* (Scheldeman 2004).

From a safety perspective, strains of *B. cereus* are a safety concern (as discussed for ESL milk). However, *B. cereus* spores are not known to survive UHT heating, and spoilage of UHT product from *B. cereus* growth is associated with post-heat re-contamination. The high-heat

resistance of some strains of *B. licheniformis* spores has been reported to be conferred by an operon called spoVA which is carried on Transposon (Tn) 1546, which was found on strains of *B. amyloliquefaciens* and *B. licheniformis*. When the operon was introduced into a *B. subtilis* strain, the ability to produce high-heat resistant spores was conferred (Berendsen 2016). In most instances, *B. licheniformis* is associated with spoilage of dairy products, and although some strains have the capability to produce a heat-stable toxin, lichenysin A (100°C for 20 minutes) (Mikkola 2000, Salkinoja-Salonen 1999), foodborne outbreaks are rare. In addition, although *B. licheniformis* toxin gene presence has been studied in isolates from milk powder (Li 2019), gene expression and pathogenicity in dairy products has not been studied.

METHODS FOR ENUMERATION OF HEATED MICROORGANISMS

Experimental design - Why is it important? Internationally, food-safety management approaches are moving toward science- and risk-based approaches. It follows that experimental studies should be performed in a way that reflects or is demonstrably relevant to the thermal treatment processes and technologies that are used commercially in the international dairy industry. Furthermore, the availability of better tools to take into account processing conditions, more advanced software, and better mathematical models are an aid in modelling inactivation kinetics of microorganisms (Smelt 2014). The implementation of such recommendations (described above) would contribute to an improved comparability of published results and to a better reliability of the mathematical quantitative risk assessment models in the future (Condron 2015).

For any alternative milk treatment processes, in order to make informed risk management decisions on their appropriateness for health protection, it is essential that they be compared

against pasteurization as the reference point. Simulation of commercial pasteurization conditions should allow key pathogen inactivation parameters to be derived for qualitative risk assessment (QRA) models. However, even today, different researchers use different techniques, a situation that makes it difficult, if not impossible, to compare the results, and leads to disagreement that cannot be resolved easily. Examples of differences include: heating in open vs. closed vials, laboratory vs. industrial setup, laboratory vs. wild strains, strains isolated from the environment vs. from infected animals, accounting or not for non-linear death kinetics, etc. Although many data on heat resistance in buffer systems are available for modelling, data in real foods are comparatively scarce (Smelt 2014). The big challenge of predictive microbiology is to design experiments in model systems that give reliable information for real food situations. D-values can vary by a factor 10 in different food matrices, but it is not always known whether that is due to interlaboratory variation or due to differences in food matrices (Smelt 2014).

Condron and colleagues outline key considerations that underpin developing a harmonized protocol for the study of the heat resistance of pathogens, which highlights the need for all experimental data to come from a carefully designed protocol, with understanding and knowledge gained through commercial experience and/or epidemiological data (Condron 2015). Regarding the selection of micro-organisms that should be used in a challenge trial, raw food products often naturally contain a variety of microorganisms and data collection should be focused on pathogens relevant for that food, in this case milk and dairy products; furthermore, as the sensory quality of food is influenced by the metabolic activities of spoilage organisms, thus limiting the food's shelf-life, an inactivation process, in this case a thermal process, may be applied to control not just those organisms of food safety significance, but also relevant organisms capable of growth and spoilage (Ross 2011). Where one specific microbial species is

added to a chosen matrix under conditions relevant to commercial operations, technological parameters such as pH, water activity, temperature, fat content, etc. must be considered and robustly documented.

Culture selection and preparation. Inactivation studies would ideally employ the key organism of concern with the greatest resistance to the treatment process - *Mycobacterium bovis* in the case of milk pasteurization – *Geobacillus stearothermophilus* and *Clostridium botulinum* in the case of a UHT treatment. However, this is not always possible in a food production environment. In such a situation surrogates are sometimes used. However, such surrogates would have to have robustly demonstrated a high level of similarity in heat resistance with the specific organism of interest. For example, an attenuated strain of *E. coli* O157:H42, demonstrated to have similar resistance to the most heat resistant virulent O157 strains screened was selected for heat inactivation trials in milk (Pearce 2012).

Pearce and colleagues also recommended using single strain isolates rather than using a cocktail when evaluating inactivation kinetics, as a mixture of several different heat tolerances would distort the survival curves, and also different strains may respond differently to recovery conditions, potentially requiring growth media suited to the recovery of more than one strain (i.e. may not be the optimum growth medium for particular strains tested) (Pearce 2012). However, in the absence of certainty that the most heat resistant strain has been isolated, a mixture or "cocktail" of strains may on the other hand increase confidence that the upper limit of heat resistance is established (Condron 2015).

While a given laboratory heating method will not necessarily predict actual commercial-type HTST pasteurization results, some methods, if well controlled and adequately reproducible,

can be useful for comparing relative heat resistances between strains. As an example of the importance of strain selection for sensitivity to the applied stress (i.e. heat treatment), Pearce and colleagues reported that pathogens *S. aureus* and *E. coli* showed a 5 log, or greater, spread of relative heat resistances during screening tests (Pearce 2012). This wide spread of resistance illustrated the danger of using results solely derived from a single strain, without adequate strain screening, in order to determine an inactivation rate representative of that species.

The inactivation medium of raw vs sterilized milk and relevance to culture preparation and recovery. When considering the heat inactivation medium, i.e. the milk, it is well known that changes in milk fat and protein contents are major elements in the seasonal compositional variation of milk (Auldist 1998) that can influence heat inactivation in other systems (Keller 2008, Ma 2007). To eliminate this possible variable, standardization of the milk substrate fat and protein levels to relevant levels across experiments would be required.

The growth conditions for preparation of the inoculum for challenge are known to affect the tolerance of organisms to subsequent stress. These particularly include the effect of temperature, pH, a_w, spore/cell age, stage of growth and availability of nutrients on the composition of the cell and its metabolic activity. Cells in the exponential growth phase are typically less resistant to a range of stresses than stationary phase cells. Consequently, it may be appropriate to select conditions for growth that mimic the likely physiological state of the cells in the milk (or dairy product) prior to processing. For example, cold shock, which occurs when stationary phase bacteria are chilled before being heat challenged, thus not allowing toxic molecules to form; with *L. monocytogenes*, thermotolerance can be reduced by chilling to 0°C (Bayles 2000). This increase in heat sensitivity results from reactive oxygen species formed

during the metabolism of growing cells. These toxic products can damage injured cells, reducing the number that are able to form colonies after heating. While catalase (McCleery 1995) and reduced oxygen levels (George 1998) can enhance the recovery of cells injured by cold shock, to minimize the effect of any cold shock and to maximize recovery of the heat treatment survivors, inocula should not be held on ice, but instead used as soon as harvested (Pearce 2012).

Furthermore, when exposed to a stress, many bacteria instigate a series of responses that make them generally more resistant to a range of stresses (Ait-Ouazzou 2012, Condron 2015, Henge-Aronis 2004). Further to this point, when considering the state of the heat inactivation medium and relevance to microbiological enumeration and recovery from the milk, at one extreme this milk could be sterile, or at the other extreme unpasteurized (raw) and containing 'natural' levels of other microbial contaminants, and possibly active natural antimicrobial systems. Should non-sterile product be used, e.g. raw milk, the inherent background contamination levels naturally present can create difficulties in enumeration of the test organism because selective media may be required to eliminate these organisms to enable enumeration of the challenge organisms. As selective media often contain antibiotics or other selective agents that can prevent the growth of stressed and/or injured surviving cells, this can run the risk of leading to overestimation of the lethality of the treatment. For this reason, some investigators (e.g. Pearce 2012) have preferred to use heat treated milk (e.g. UHT) that has very low levels of microbial contaminants. The use of different methods may result in poor repeatability of inactivation determinations.

Regarding inoculum density - ideally, the density of the inoculum used in the challenge study should reflect levels that would be expected to occur naturally. However, when validating a process lethality step such as a heat treatment, this would mean that very high inoculum levels

are required, for example 10^6 to 10^7 CFU/g of products, to be able to measure the extent of reduction in challenge organisms. Alternatively, lower cell densities may be used and enumerated if large sample volumes can be processed, e.g. using most probable number (MPN) methods (Duquet 1987) or cell concentration methods such as filtration. To minimize the effect of the suspending medium on the properties of the heating medium (milk) and also to minimize time for heat transfer, inocula should be suspended in the smallest practical volume of medium that allows even distribution of the organism throughout the heating medium.

Regarding clumping of culture organisms and intracellular pathogens - some organisms normally grow in close association, resulting in aggregates of cells rather than individual cells. This may be exacerbated in broth media, particularly for organisms that form pellicles. Clumping can lead to underestimation of cell numbers by plate count methods. Clumping during heating leads also to the tailing of survivor curves, leading to the wrong interpretation that there is a more heat-resistant spore fraction (Condron 2015, Klijn 2001). Clumping has also been suggested to provide protection from heat to cells on the inside of the clump, although several studies have demonstrated that the effect is negligible for HTST processes (Cerf 2007, Davey 1990, Hastings 2001).

Scale-up & other practical considerations for commercial process validation and verification. Regarding the challenge trial heat treatment design - testing commercial systems directly generally involves a high volume of product and challenge microorganisms, in addition to associated practical issues such as the need to decontaminate the plant between challenge trials and the problems of deliberately introducing high levels of challenge organisms into factory environments. Consequently, some researchers have opted for inferred pasteurization efficiency

experiments, as determined by extrapolation of the results of studies conducted in simpler, or smaller, experimental systems. Examples of systems used for basic data collection include test-tubes, vials, capillary tubes, and submerged coils, although it is well-known that systems that are not totally immersed in the heating medium can cause artefacts (Cerf 2007).

Regarding the thermal treatment apparatus, this will depend on the type of determination being undertaken, viz. whether to establish basic pathogen thermal inactivation data or to validate a commercial scale process. In the case of verification of a commercial heat inactivation process (e.g. milk pasteurization), the evaluation of the heat treatment (i.e. pasteurization) performance would ideally be undertaken by direct challenge of the equipment with naturally contaminated milk. However, as naturally contaminated product would not be expected to have a consistent level of pathogens, it is more practical to test systems against relatively high levels of challenge organisms. While commercial processes can be partially verified by testing for the absence of the challenge strain after the process, or by demonstration of the achievement of the required reduction, the knowledge gained is limited in its utility as it cannot be readily extrapolated to other conditions (e.g. microbial levels, variations in milk composition etc.) or processes (e.g. minor deviations in temperature or time conditions). Such an approach simply shows the absence of survivor(s) in the treated and tested volume. It gives no indication of the probability of survivor presence in larger volumes as those of continuous flow plants. Consequently, there is little certainty that the process would be reliable under all realistic pathogen loads and operating conditions. Also, such an approach cannot be used to optimize a process or to develop alternative processes. This requires a more fundamental and detailed knowledge of the effects of time and temperature on the kinetics of inactivation, i.e. the change in microbial numbers as a function of heating time, and survival of the survivor organism, under

conditions that encompass the full range of likely conditions, including the least lethal combination of processing and product formulation, with a perspective of relevance for the organisms of concern.

Piyasena and colleagues demonstrated the importance of using commercial-type conditions with turbulent flow in pasteurization experiments, and they pointed out that batch processes cannot be readily extrapolated to continuous HTST pasteurization as they do not take into account shear force and other physical stress (Piyasena 1998). A kinetic study of the heat inactivation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) further emphasized this importance and provides valuable insights into the methodology required to obtain inactivation data under commercial-type conditions in a turbulent-flow pasteurizer (Pearce 2001). In 2012, Pearce and colleagues further developed application of these protocols with a view to provide robust kinetic data collected from a standardized, repeatable, practical, safe, and cost-effective protocol for modelling the heat inactivation of various non-spore-forming pathogens from raw milk on a commercial-type scale (Pearce 2012). The extrapolation of results from simpler, or smaller, systems (e.g. laboratory-scale pasteurizers and pilot-scale pasteurizers) to full scale commercial pasteurizers is clearly not straightforward, because of the combination of potential physiological changes (i.e. induction of stress responses during warming) in cells experiencing dynamic temperature processes compared to exposure to static temperature and, more particularly, because of the differences in the time–temperature profile in simple systems compared with e.g. fluid milk passing through modern commercial pasteurizers. In addition, turbulent flow, associated with increased Reynold's numbers (Re), provides for more efficient residence times and heat transfer. It is essential that data derived from one (experimental) pasteurization system can be compared to data from others. While the “residence time” in the

holding tube at the nominal pasteurization temperature is the main process step governing the extent of inactivation, other stages before and after this can also contribute to pathogen inactivation. This requires, for example, that inactivation during “come-up” and “cool-down” is determined or can be calculated by full characterization of the time-temperature profiles experienced by cells in the experimental system. Pasteurization processes are designed to ensure that the “fastest moving particle” (FMP), i.e. that particle which has the minimum residence time, still is exposed to the required time/temperature conditions to achieve the required thermal inactivation. Laminar flow results in shorter minimum residence time than does turbulent flow. As flow becomes more turbulent the residence time of the FMP more closely approaches the average of the residence time distribution (RTD) and the movement of milk through the pipe may ultimately approximate “plug” flow, i.e. when all particles effectively take the same time to pass through the tube. The flow characteristics can be deduced from calculation of the Re-value which is a function of the average flow speed through the tube, tube diameter, fluid viscosity and fluid density. A higher Re indicates more turbulent flow. Thus, each of these characteristics should be reported when inactivation data derived from flow-through systems are presented.

For complex systems, with different geometries, temperature ranges and times, the combined effect can be estimated as the sum of the effects of the component steps. This approach considers the warming, heating and cooling processes as several individual simpler processes with different residence times (Kiesner 2004). When validating a process, steps should also be taken to verify that the process is operating as planned, e.g. to ensure the integrity of the system so that cross-contamination of raw to pasteurized product does not confound the results.

Thermal inactivation models – application, future benefit, modelling for Quantitative Risk Assessment (QRA). As previously discussed for D- and Z-values, thermal inactivation of pathogens is accepted as an exponential process, i.e. in a given time interval, and at a given temperature, a constant proportion of the surviving cells will be killed. D- and Z-values are readily derived from appropriate experimental data and are convenient because they can be easily combined, mathematically, with time-temperature data describing pasteurization processes to estimate expected pathogen reduction in commercial pasteurizers.

The classical thermal death model works well in situations where the temperature increase and decrease times are very short relative to the hold time. It is well known, however, that data describing thermal inactivation of microbial populations often do not form a straight line when plotted as described for the classical thermal death model. Besides "shoulders" and "tailing", other deviations from exponential decline have also been described. Theoretical reasons for these variations are well described in the literature (Cerf 1977, McClure 2004, Stumbo 1973).

Pearce and colleagues described an alternative pragmatic approach, when the increase and decrease times are appreciable (Pearce 2012). Traditional D- and Z-value determinations on a laboratory scale are derived from plots of survival versus time over a range of temperatures. However, if these determinations were to be scaled up to pilot-scale turbulent flow pasteurization, each time point would require changing the holding-tube with associated sterilization before and after. As this approach is impractical, Pearce and colleagues pioneered an approach whereby they developed kinetic data for a pilot-scale pasteurizer operating under commercial conditions of turbulent flow and used survival data at different temperatures to derive the required parameters from the appropriate mathematical model (Pearce 2001).

Most mathematical models require assumptions about the nature of the process that they describe and, when fitted to the data, will provide an equation that represents the best interpretation of the data given the assumptions or hypotheses inherent in the model. As such, reporting of raw results, as opposed to derived results such as D- and Z-values or other fitted model parameters, is important because it can reveal reproducible behaviour that the fitted model "ignores". Apart from the benefits of data summary and revealing patterns of behavior, fitting data to a model can also quantify variability in the data, which can be used to determine confidence intervals for the predictions of the model or the inherent variability in the process and thereby to specify operating limits that consistently provide the required level of safety.

While available models can account for complex inactivation kinetics, greater complexity could be caused by use of a mixture of strains, as advocated above, and experimenters need to be aware that the interpretation of theoretical models for thermal inactivation may not be valid, or may need to be modified, when multiple strains are used in experiments. Thus, design of experiments for process verification can be based on reasonable expectations of performance based on inactivation data and models that are integrated with mathematical descriptions of time-temperature conditions in the pasteurizer. More importantly, good inactivation data and models combined with detailed process models provide a rigorous framework for identifying reasons for unexpected process failures and a rational basis for modification of the process to overcome them. As identified by Hastings and colleagues, pasteurization process design and verification will require the combined expertise and perspectives of engineers and microbiologists (Condron 2015, Hastings 2001).

Future technologies. Food processing using non-thermal processes such as high-pressure processing (HPP), cold plasma, membrane filtration, pulsed electric fields (PEF), irradiation or a combination of any of these through a hurdle approach represents a change from the traditional heat processes that are relatively well characterized. The need for novel or alternative processing technologies in the food industry is a direct result of consumer demand for fresh, high quality and healthy products that are also safe. Validating and verifying a novel process is a relatively straight-forward process if the product has been made for years by a known process, such as milk pasteurisation or UHT treatment, and is considered very safe. However, in many of these cases, one immediately thinks about the time and temperature of a heat-treatment step. Nevertheless, the drive for fresher, less-processed foods has resulted in many products without a recognized kill step. Consequently, how to verify and validate any process, including some of these non-traditional processes, requires consideration of microbial reduction requirements and the conditions necessary to achieve this. The key microorganism(s) of concern for the new process/product must be established from the food safety and spoilage perspectives. Alternative process technologies, for example HPP or PEF, bring about different microbial kill mechanisms that can influence the type of organism likely to be of concern and be most resilient to that alternative process (examples – *B. amyloliquefaciens* for HPP, *Geobacillus* spp. for UHT). As the microbial kill mechanism(s) are not necessarily directly comparable to heat treatment (Barbosa-Canovas 2008) establishing microbial equivalence to heat treatment is not a straightforward process. To robustly validate an alternative method still requires identification of the food safety objective (FSO) required to confidently achieve an appropriate level of protection (ALOP) and adherence to the elements described in this paper.

CONCLUSIONS

Pasteurization of milk was a step change technology that allowed nutritious dairy products to be safely consumed by mankind. However, research has shown that different microorganisms respond in different ways to heat depending on a variety of factors. Evaluating the risks of pathogens and microorganisms that compromise quality in heat treated dairy products needs a clear understanding of the dairy matrix, type of heat used, genetics of the microorganism present and methodology used to collect data on D- and Z-values. Future technologies would need to meet the same inactivation abilities as current heating knowledge. Despite the developments of alternative technologies for destroying microorganisms, such as HPP and PEF technology, thermal treatments remain the preferred option for most processes in the dairy industry. Heat treatment of milk is a proven reliable technology for the production of microbiologically safe, quality dairy products.

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Table 2-1. Examples of cases where improper heat treatment has led directly to foodborne illness or recalls of dairy products.

Dairy category	Product details	Date	Country	Foodborne illness or recall
Liquid dairy	Milk	1986	USA	Foodborne illness (*Campylobacter)*
	Milk	1992	USA	Foodborne illness (*Campylobacter)*
	Milk	1997	UK	Foodborne illness (*Cryptosporidium*)
	Milk & Cream	2013	UK	Recall
	Milk	2015	USA	Recall
	Chocolate milk	2015	USA	Recall
	Various milk and cream products	2016	USA	Recall
	Double cream	2017	USA	Recall
	Milk	2017	USA	Recall
	Milk	2017	USA	Recall
	Milk	2018	USA	Recall
	Chocolate milk	2018	USA	Recall
	Chocolate milk	2018	USA	Recall
	Strawberry milk	2018	USA	Recall
	Milk, chocolate milk and ice cream	2020	USA	Recall
Cheese	Cheese	1986	USA	Foodborne illness (*Salmonella)*
	Mascarpone cream cheese	1996	Italy	Foodborne illness (*Clostridium botulinum*)
	Fresh cheese curd	1998	USA	Foodborne illness ***(E. coli*** O157 H7)
	Fresh cheese ("queso fresco")	2003	Spain	Foodborne illness *(Streptococcus equi subsp. zooepidemicus)*
	Morra bocconcini cheese	2004	Canada	Recall and Foodborne illness *(L. monocytogenes)*
	Cheese	2010	Australia	Recall
	Latin-style soft cheese	2015	USA	Recall and Foodborne illness *(L. monocytogenes)*
	Cottage cheese	2016	USA	Recall
	Cheese	2019	USA	Recall
	Cheese ("Margie cheese")	2018	USA	Recall
Ice cream	Ice cream and milk	2016	USA	Recall
	Ice cream	2016	USA	Recall
Yogurt	Yogurt	2014	USA	Recall
	Yogurt	2016	USA	Recall

Table 2-2. Examples of common types of heat inactivation in dairy manufacturing processes (adapted from Deeth 2018).

Process	Temperature (°C)	Time	Examples of applications	Microorganism inactivated
Pasteurization				
LTLT milk	63	30 min	Regulatory requirement for pasteurization, Pasteurized milk, cream, cheese, powders, recombined milks	Non-spore-forming pathogens, psychrotrophic spoilage bacteria e.g. Gram-negative *Pseudomonas* and Enterobacteriaceae (not spores or thermoduric bacteria e.g. *Streptococcus, Enterococcus*)
HTST milk	72 -75	15 – 20 s		
HTST cream	>80	1-5 s		
Batch pasteurization				
Starter/yogurt milk	90 - 95	15-30	Starter manufacture / Yoghurt manufacture	All non-spore-forming bacteria and some psychrotrophic & mesophilic spores (depending on the specific heat treatment)
Cheese milk	70 - >95	4 – 15 min	Process cheese	
Thermization	57 – 68	5 s – 30 min	Further processing & manufacture of some cheese	Some lipase & proteinase producing vegetative cells of spoilage psychrotrophs (some pathogens may remain viable)
ESL or Ultra-pasteurized milk	125 – 140	1 – 10 s	Extended shelf life refrigerated milk	All non-spore-forming bacteria and most psychrotrophic and mesophilic spores.
UHT	135 - 150	1 - 10 s	Ambient drinking milk with long shelf life (6-9 months)	All non-spore-forming bacteria and all spores except highly heat-resistant spores of *G. sterothermophilus* and *B. sporothermodurans*); produces 'commercially sterile product'
In container sterilization	110 - 120	10 – 20 min	Evaporated/condensed milk, drinking milk with long shelf life. Nutritional dairy beverages	All bacteriophages, non-spore-forming and many spores except highly heat-resistant ones

Table 2-3. Examples of D-values of bacteria important in dairy manufacturing, and in various dairy matrices and dairy derivatives.

Bacterium	Inactivation temperature (°C)	D-value (min)	Type of medium	Reference
Non spore forming bacteria				
E. coli	62.8 - O104:H7	2.8	TSB with 51% milk solids	Dega 1972
	64 - ATCC 9637	0.385	Ice-cream mix	Desmarchelier 2003
	66.5 - O104:H7	0.968	Non-dairy medium containing 51% milk solids	Desmarchelier 2003
	76 - ATCC 9637	0.00195	Milk	Read 1961
	76 - ATCC 9637	0.00265	Chocolate milk	Read 1961
	76 - ATCC 9637	0.00147	Ice cream mix	Read 1961
	76 - ATCC 9637	0.00093	40% cream	Read 1961
S. aureus	70	0.1	Milk	ICMSF 1996
	75	0.02	Milk	ICMSF 1996
L. monocytogenes	68 - strain 1151	0.19	Butter	Casadei 1998
	68 - strain 1151	0.15	Half cream	Casadei 1998
	68 - strain 1151	0.13	Double cream	Casadei 1998
	68	0.116	Reconstituted skim or UF whole milk (27% TS)	Szlachta 2010
	72	0.015 – 0.045	Milk	Sutherland 1997
Salmonella	57	1.4	TSB (10% milk solids)	Dega 1972
	57	26.6	TSB (51% milk solids)	Dega 1972
	60	< 0.2	Milk	D'Aoust 1987
	63	1.2	Whole milk	Mañas 2001
	63	1.1	Skim milk	Mañas 2001
	63	1.3	Whey ultrafiltrate	Mañas 2001
	73 - 74	0.035	Retentates (4X conc. skim or whole milk)	Kornacki 1993
	85	0.07	Galacto-ologosaccharide syrup (74% total solids)	Bang 2017
Cronobacter spp.	58 - *C. sakazakii* DPC 6529	0.55	Reconstituted infant formula	Huertas 2015
	58	0.68	Reconstituted infant formula - whole milk	Osaili 2009
	58	0.62	Reconstituted infant formula - low fat milk	Osaili 2009

	58	0.51	Reconstituted infant formula - skim milk	Osaili 2009
	60	1.1 – 4.4	Reconstituted infant formula	Edelson-Mammel 2004 Iversen 2004, Nazarowec-White 2003
	70	0.07	Reconstituted infant formula	Edelson-Mammel 2004
	85	0.08	Galacto-ologosaccharide syrup (74% total solids)	Bang 2017
Spore forming bacteria				
Bacillus cereus	90	1 - 13	UHT milk (2% fat)	Stoeckel 2013
	95	2.6 – 2.9	Micellar casein concentrate (1.5 – 14.3 % protein)	Stoeckel 2014
	95	2.6	Whole milk	Stoeckel 2014
	95	0.91 - 0.16	Cream	Mazas 1999
	95	6.3	Infant formula	ICMSF 1996
	100	1.09 - 0.27	Skim milk	Mazas 1999
	100	1.03 - 0.31	Conc skim and whole	Mazas 1999
	100	2.7 – 3.1	Skim milk medium	Mikolajcik 1970
	110	0.34 – 0.65	Infant formula (10 – 50% total solids)	Stoeckel 2013
B. licheniformis	100	4 – 8	General	Brown, 2000
	100	20 – 103	From raw milk, in Ringer's solution	Scheldeman 2006
	100	2.8 – 4.1	Skim milk medium	Mikolajcik 1970
	140	0.006	Milk powder	Hill 2004
B. subtilis	95	2 – 6	Milk	Janstova 2001
	121	~ 0.5	General	Brown 2000
B. pumilus	95	~ 4	Milk	Janstova 2001
	100	0.875	Skim milk medium	Mikolajcik 1970
B. coagulans	95	4 – 8	Milk	Janstova 2001
	100	1.97	Skim milk medium	Mikolajcik 1970
B. sporothermodurans	125	2 - 13	Raw milk	Burgess 2010
	140	0.06 – 0.13	UHT milk	Huemer 1998
	140	~ 0.083	UHT milk	Scheldeman 2006
Anoxybacillus	140	~3.6	Milk powder	Hill 2004
Geobacillus spp.	140	0.06 – 1	Milk powder	Eijlander 2019, Hill 2004
	140	0.02	UHT milk	Huemer 1998

Table 2-4. Cheesemilk thermization definitions and predicted decreases in *Listeria monocytogenes* based on literature model given in Figure 2-1.

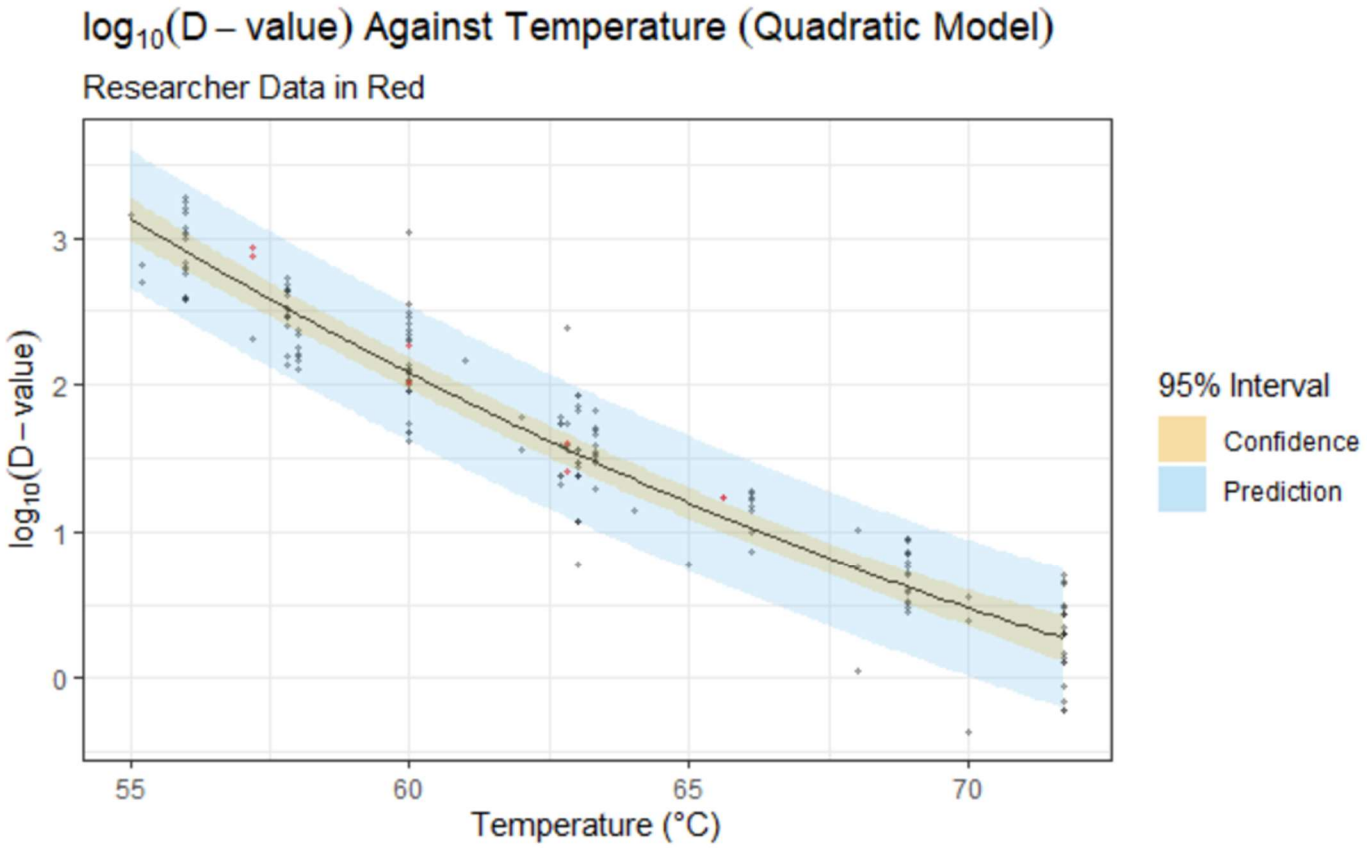
Heat Treatment	Duration	Notes	Predicted *L. monocytogenes* Decrease (log CFU/ml)	Reference(s)
55°C	2-16 s	"Much of the aged raw milk cheese produced in the US is subjected to some form of heat treatment, generally thermization. This treatment generally consists of heat treatment at 55°C for a period ranging from 2 to 16 sec."	0.0 to 0.0	Donnelly 2004
57°C	30 min	Swiss alpine-style specific; suggested to be equivalent to thermization at 60°C for 5 minutes or 65°C for 15 sec	3.6	Jakob 2015
57-68°C	5 s-30 min	Noted for psychrotroph control as well as destruction of some non-sporeforming pathogens; "Some pathogens may remain viable"	0.0 to >8.0*	Deeth 2018
57-68°C	10-20 s	Noted for psychrotroph control; "Not sufficient to reduce significantly the population of vegetative cells of the more heat resistance bacterial pathogens but creates a suitable environment for the multiplication of selected starter cultures" (Condron 2009)	0.0 to 3.6	Eugster 2019, Rukke 2011, Stepaniak 2002
57-68°C	≤15 s	Specification for certain Swiss PDO cheeses; *L. monocytogenes* reductions of <0.1 log, 0.2 log, 0.7 log, and 2.0 log for 15 s treatment at temperatures 57, 62, 65, and 68°C, respectively, expected (Eugster 2019, Sorqvist 2003)	≤0.0 to ≤2.7	Eugster 2019
57-68°C	≥15 s	"At the lower end of the temperature range for thermization, there is little if any destruction of *L. monocytogenes*" (Condron 2009)	≥0.0 to ≥2.7	EU Directive 92/46/EEC 2014
57-68°C	15-20 s	Noted for psychrotroph control; *L. monocytogenes* noted to be able to survive	0.0 to 3.6	Fernandes 2009
57-68°C	15-30 s	Noted for psychrotroph control	0.0 to 5.4	Panthi 2017
60-62.8°C	15-20 s	"Used to kill most bacteria found in milk; does not kill all pathogenic bacteria, spores or most non-starter lactic acid bacteria"	0.1 to 0.5	Johnson 2002
60-68.5°C	≤15 s	Noted for psychrotroph control	≤0.1 to ≤3.1	Johnson 1988
60°C	5 min	Swiss alpine-style specific; suggested to be equivalent to thermization at 57°C for 30 minutes or 65°C for 15 sec	2.5	Jakob 2015
61°C	15 s	Blue specific; allows for survival of some yeasts	0.2	Dines Cantor 2017

61-62°C	15 s	Blue specific	0.2 to 0.3	Cantor 2004
≥62°C	≥15 s	Australian thermization requirement with ≥90 days aging at ≥2°C; "Could inactivate up to 3 log of pathogenic *E. coli*" (with 2 log attributed to thermization, 1 log to aging); "*L. monocytogenes* likely to survive the combined treatment in some cheeses, e.g., Camembert"	≥0.3	Condron 2009
≥62°C	≥16 s	Canadian thermization guideline with ≥60 days aging at ≥2°C and cheese pH ≤5.5 and a_w ≤0.95 required at the end of the manufacturing process	≥0.3	Government of Canada 1996
62-65°C	10-15 min	For selective pressure for natural milk starter cultures in Italian traditional cheese manufacture	>8.0	Parente 2004
62-67°C	20-90 s	Industrial thermization conditions (France)	0.4 to >8.0	Levieux 2007
62-68°C	15 s	"Practiced widely" for thermization	0.3 to 2.7	Rukke 2011
63°C	10-15 s	Noted for psychrotroph control; milk may be subsequently pasteurized before cheesemaking	0.3 to 0.4	McSweeney 2007
63°C	15 s	Camembert, Brie, and Limburger specific	0.4	Farkye 2002
~63°C	15 s	Noted for psychrotroph control	0.4	Chambers 2002
63°C	15 min	For selective pressure of natural milk starter cultures in Traditional Specialty Guaranteed Mozzarella	>8.0	Parente 2017
63-65°C	15 s	"Typical" thermization	0.4 to 1.0	Panthi 2017
63-65°C	15-20 s	Noted for psychrotroph control with enzymes unaffected; "Likely to result in 2-5 log reduction of *E. coli*" (Condron 2009)	0.4 to 1.3	Fox 1993, IDF 1981, Johnson 1991
63-65°C	16 s	Cheddar specific	0.5 to 1.0	Hirvi 1998
64.4°C	16 s	Proposed as a guideline for minimum thermization	0.8	Bishop 2001, Johnson 1990
64.4°C	17.5 s	"Can achieve at least a five-log reduction of *E. coli* O157:H7"	0.9	Boyd 2021
64.6-65°C	15.5 s	Shown to eliminate coliforms, reduce APC by 1.71 log CFU/ml	0.8 to 1.0	Johnson 1991
65°C	15 s	Noted for psychrotroph control; milk may be subsequently pasteurized before cheesemaking; suggested to be equivalent to thermization at 57°C for 30 minutes or 60°C for 5 minutes (Jakob 2015); recommended minimum thermization for control of STEC in mature cheese (Eugster 2019)	1.0	Dusterhoft 2017, Eugster 2019, Fox 2004, Fox 2017, Jakob 2015, Martin 2011

65°C	16-18 s	"Will destroy pathogenic organisms that threaten cheese safety"	1.0 to 1.1	Wendorff 2007
65°C	20 s	Upper limit for thermization; >50% of alkaline phosphatase remains active	1.3	Panthi 2017
65-66°C	16-18 s	"Lethal for virtually all pathogenic microorganisms present in milk that pose major threats to cheese safety"	1.0 to 1.6	Marcos 2004
65-70°C	15-20 s	Noted for cheesemilk (general)	1.0 to 6.6	Fernandes 2009
65-70°C	16-20 s	Typical thermization for raw milk cheeses	1.0 to 6.6	Johnson 2001
66°C	10-15 s	Noted for psychrotroph control	0.9 to 1.4	Van der Berg 2004
66°C	15 s	Noted for psychrotroph control	1.4	Bennett 2004, Legg 2017
66.7°C	16 s	Cheddar specific	1.9	D'Aoust 1985
67°C	20 s	For reduction of amino acid decarboxylase-positive bacteria	2.6	Martin 2011
67-70°C	20 s	Cheddar specific	2.6 to 6.6	Johnson 2001
67.7-70°C	≤15 s	Swiss specific	≤2.4 to ≤5.0	Johnson 1988

*For predictions giving >8.0 log CFU/ml reduction, ">8.0" value assigned.

Figure 2-1: Log D-value versus heating temperature for *Listeria monocytogenes*-inoculated fluid whole bovine milk samples in the scientific literature. For relevance to thermization of raw milk intended for cheesemaking, dataset omits non-*monocytogenes* *Listeria* species, reconstituted milks, and temperatures <55°C or ≥72°C. Dataset includes whole (standardized or raw) milks at all pasteurization levels with all methods of culture preparation, inoculation, and heating. (Bradshaw 1985, Bradshaw 1987, Bradshaw 1991, Bunning 1986, Bunning 1992, Crawford 1989, Donnelly 1986, Farber 1992, Fedio 1989, Holsinger 1992, Kamau 1990, Kenney 2004, Knabel 1990, Pearce 2012, Rowan 1998, Szlachta 2010, van der Veen 2009).



CHAPTER 3:

Development and validation of D-values for *Listeria monocytogenes* and Shiga toxin-producing *Escherichia coli* in milk to reduce pathogen risks in unpasteurized milk cheeses

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INTERPRETIVE SUMMARY

Thermization has been proposed to reduce the risk of pathogens in raw cheesemilk, however, the temperature / time combinations needed to enhance safety have not been well characterized. In the current study, process lethality data for *Listeria monocytogenes* and Shiga toxin-producing *Escherichia coli* (including O157:H7 and six other serotypes) were generated and validated in non-homogenized, pasteurized whole milk heated to thermization temperatures. These data can be used to increase the safety of raw milk artisanal cheeses to reduce populations of pathogens to levels where they will not be infectious to consumers.

ABSTRACT

Several US cheeses can be legally produced using raw milk, but certain varieties have been shown to support growth of *Listeria monocytogenes* or survival of Shiga toxin-producing *Escherichia coli* (STEC) beyond 60-day aging requirements. Thermization has been proposed to reduce the risk of pathogens in raw cheesemilk, however, the temperature / time combinations needed to enhance safety have not been well characterized. The objective of this research was to determine and validate D-values of *L. monocytogenes* and STEC at thermization temperatures 65.6, 62.8, and 60.0°C; a D-value at 57.2°C was additionally determined for *L. monocytogenes* only. Non-homogenized, pasteurized whole milk samples (1 ml) were inoculated with 8-log CFU/ml *L. monocytogenes* or STEC (5- or 7-strain mixture), vacuum-sealed in moisture-impermeable pouches and heated via water bath submersion. Duplicate samples were removed from heating at appropriate intervals and immediately cooled in an ice bath. Survivors were enumerated on Modified Oxford or Sorbitol MacConkey overlaid with Tryptic Soy agar to aid in the recovery of heat-injured cells. Duplicate trials were conducted, and survival data were used to calculate thermal inactivation rates. $D_{65.6°C}$, $D_{62.8°C}$, and $D_{60.0°C}$-values ($\mu \pm SD$; in s) of 17.1 ± 0.3 and 7.2 ± 0.7, 33.8 ± 9.8 and 16.9 ± 1.3, and 146.6 ± 59.5 and 60.0 ± 7.4 were found for *L. monocytogenes* and STEC, respectively, and a $D_{57.2°C}$-value of 825.2 ± 46.9 s was determined for *L. monocytogenes*. Predicted times to 3-log reduction were validated in triplicate trials for each test temperature using 100 ml milk inoculated with 3-4 log CFU/ml of pathogen cocktails, finding fail-safety of generated D-values. Generated D-values were additionally compared to published values from 21 scientific studies investigating *L. monocytogenes* and STEC in whole milk heated to thermization temperatures (55.0 to 71.7°C). These data can be used to create

flexible thermal processes for artisanal raw milk cheese to reduce *L. monocytogenes* and STEC populations to levels where they are not infectious to consumers.

Key words: *Listeria monocytogenes*, Shiga toxin-producing *Escherichia coli*, cheese, raw milk, thermization

INTRODUCTION

Artisanal cheese sales in the US totaled $4.42 billion in 2016, a 23.4% increase since 2012, with projected 21% growth expected by 2021, driven in part by an increasing demand for unpasteurized or raw milk cheeses (Specialty Food Association 2016. Most US raw milk cheese consumers are undeterred by potential health issues related to consumption of raw milk cheeses and purchase these products for their perceived flavor complexity or beliefs that they are more naturally or traditionally produced than pasteurized milk cheeses (Licitra 2019). Though only a small portion of the US population consumes unpasteurized milk cheeses, estimated at 1.6% in 2017 by Costard and colleagues, the authors noted that a doubling in consumption of these products could increase outbreak-related illnesses by 96% (Costard 2017). *Listeria monocytogenes*, Shiga toxin-producing *Escherichia coli* (STEC; including O157:H7), *Salmonella* spp., and *Staphylococcus aureus* have been identified as primary pathogens of concern in raw milk natural cheeses (Donnelly 2018, Fernandes 2009, Panthi 2017). *L. monocytogenes* and STEC have been classified as especially high-risk to raw milk cheeses due to the severity of associated illnesses (Condron 2009, Donnelly 2018), their ability to grow (Ryser 1987, Sanaa 2004) or survive (D'Amico 2010, Reitsma 1996, Schlesser 2006) in certain raw milk cheeses even when initially present at low levels in the cheesemilk, and their increased resistance to starter cultures and native microbiota found in raw milk cheese compared to other vegetative pathogens (Montel 2014, Pereira 2009).

While milk pasteurization is known to eliminate vegetative pathogens, current US regulations allow for 47 cheeses varieties classified by Code of Federal Regulations (CFR) Title 21 Part 133 to be legally manufactured using raw milk, including those falling under 21 CFR 133.182, "Soft ripened cheeses." This category includes cheeses with moisture values <50% that

are not otherwise defined in 21 CFR 133 (e.g. surface-mold-ripened cheeses such as Camembert and Brie) given that they are aged a minimum of 60 days at ≥2°C (Donnelly 2018). Soft ripened cheeses carry an estimated 50- to 160-fold greater risk for listeriosis per serving when made with unpasteurized milk (Jackson 2018) and disproportionately account for a high number of listeriosis outbreaks implicating dairy foods in the US (CDC 2017, Langer 2012). STEC, on the other hand, accounted for only 11% of US unpasteurized cheese outbreaks from 1998-2011 (Gould 2014), however, were attributed to 6 of 8 unpasteurized milk cheese outbreaks from 1983-2018 in the UK (Donnelly 2018). STEC are especially hazardous to raw milk cheeses ripened for long periods due to their low infectious dose (<100 CFU) (Donnelly 2018, Farrokh 2013) and ability to survive in acidic environments such as those found in aged cheeses (Maher 2001, Ramsaran 1998, Reitsma 1996).

Except for cheeses involving extensive acidification or curd-cooking in manufacture (e.g. feta, pasta filata, or Swiss-style cheeses), the safety of unpasteurized milk cheeses is primarily dictated by the microbiological quality of the milk itself and not the ability of the cheesemaking process to inactivate pathogens (Condron 2009, Donnelly 2018). Milk thermization has been proposed as a strategy to make safer unpasteurized milk cheeses while still meeting labeling requirements for raw milk cheese designation (Johnson 1990). Following three North American STEC outbreaks in raw milk Gouda and Gouda-like cheeses aged ≥60 days including a 2018 outbreak of *E. coli* O121 in raw milk Gouda-like cheese in British Columbia, Canadian regulators recommended thermization of milk prior to the production of Gouda or Gouda-like cheeses to increase their microbial safety (Boyd 2021). Several working definitions of thermization exist (see Table 2-4) with no established definition in US regulations currently declared, though a general microbial reduction of 3-4 log CFU/ml is expected (CAC 2004) and a

positive phosphatase test for the treated cheesemilk is observed (Eugster 2019). Among >900 US artisan, farmstead, and specialty cheese producers surveyed in 2018, 50% were using raw and 17% thermized cheesemilk (ACS 2018), representing substantial increases from 2016 survey numbers (32% and 6%, respectively) (ACS 2016). A joint FDA / Health Canada risk assessment concluded that a 3-log decrease in *L. monocytogenes* in raw milk before cheesemaking would reduce the mean risk of listeriosis approximately 7.2- to 10-fold lower than baseline estimates for soft ripened cheese made from raw milk (FDA 2015). STEC are less heat resistant than *L. monocytogenes* and therefore thermization treatment sufficient to reduce *L. monocytogenes* should sufficiently reduce STEC at equal or higher rates (Fernandes 2009, Sorqvist 2003, van Asselt 2006). STEC serovars O26, O45, O103, O111, O121, O145, and O157 have been implicated in prior foodborne outbreaks, with currently no published reports suggesting non-O157 STEC to have higher heat tolerance than O157 strains (King 2014). To characterize the reduction of bacteria due to thermization, the D-value, or the time at a particular temperature needed to reduce cell numbers by one log cycle, is used (Peng 2013).

The objective of the current study was to generate and validate D-values for *L. monocytogenes* and STEC in order to establish process lethality guidelines for whole cheesemilk thermization. Predicted times to 3-log decrease based on generated D-values were subsequently validated using methodology simulating vat heat treatment and compared with values published in the existing scientific literature.

MATERIALS AND METHODS

Inoculum preparation. *L. monocytogenes* strains LM 108 (hard salami isolate, serotype 1/2b), LM 301 (cheddar cheese isolate, serotype 1/2a), LM 310 (goat's milk cheese isolate

associated with illness, serotype 4b), R2-500 (soft Hispanic-style cheese isolate, 4b), and R2-501 (clinical isolate associated with soft Hispanic-style cheese, 4b) and STEC strains O111:H8-strain 00-3142 (clinical isolate), O103:H2–strain 01-3002 (clinical isolate), O121:H9-strain 01-3434 (clinical isolate), O45:H2-strain 01-3510 (clinical isolate), O145:NM-strain 99-3311 (clinical isolate), O26:H11-strain H30 (clinical isolate), and O157:H7 strain FRIK47 (ATCC 43895) (clinical isolate) were grown individually in 10 ml Trypticase Soy broth (TSB; BBL, BD, Sparks, MD) at 37°C for 18-22 h. For each strain, 0.2 ml aliquots of overnight culture were spread-plated on Trypticase Soy agar (TSA; BBL, BD) and incubated 37°C for 20-24 h. Cells were harvested via scraping plate surfaces with a sterile inoculating loop and suspending lawns from each individual strain in 4.5 ml 0.1% buffered peptone water (BPW; pH 7.2) to achieve approximately 10 log CFU/ml. Equivalent populations of each isolate were combined to provide ~10 log CFU/ml of a 5-strain and 7-strain mixture of *L. monocytogenes* and STEC, respectively. Strain purities were verified by streaking on modified Oxford agar (MOX; *Listeria* selective agar base, Difco, BD, Sparks, MD) or Sorbitol MacConkey agar (SMAC; BBL, BD) for *L. monocytogenes* and STEC, respectively, as well as on TSA. Strain and cocktail populations were verified by plating on MOX and SMAC for *L. monocytogenes* and STEC, respectively.

Sample inoculation. 50 ml of non-homogenized, pasteurized whole milk (SuperNatural Organic Whole Milk, Kalona Organics, Kalona, IA) was hand-shaken to distribute cream layer and subsequently pipetted into a sterile 50-ml centrifuge tube (Falcon, BD Biosciences, Sparks, MD) before batch inoculation at a 1.0% (v/v) level with *L. monocytogenes* or STEC strain cocktail to provide approximately 8 log CFU/ml milk. Commercial pasteurized whole milk was used in place of raw whole milk to ensure consistency of background microflora and fat content

between trials and was supported by findings of other authors noting equivalent or increased recovery of heat-injured *L. monocytogenes* in sterilized and pasteurized whole milks compared to raw milk (Bunning 1988, Crawford 1989, Mathew 2002). Additionally, non-homogenized whole milk was used to emulate typical milk used for cheesemaking. Following vortexing, 1-ml aliquots of inoculated milk were pipetted into water- and gas-impermeable pouches (3-mil high barrier EVOH pouches, Deli 1 material, oxygen transmission rate of 2.3 cm^3/cm^2 for 24 h at 23°C, water transmission rate of 7.8 g/m^2 for 24 h at 37.8°C, and 90% RH; WinPak, Winnipeg, Manitoba, Canada) and vacuum-packaged (Multivac AGW, Sepp Haggemuller KG, Wolfertschewenden, Germany). Pouches were flattened to a uniform thickness prior to heating.

Heating of pouched samples. Sample pouches were attached to a fabricated sampling rack in order to provide even distribution of samples bags within a waterbath and to allow for simultaneous and efficient immersion. The sampling rack was submerged in a circulating waterbath (Magniwhirl Constant Temp Bath, Blue M Electric Company, Blue Island, IL) heated to 1 of 4 target temperatures (57.2, 60.0, 62.8, or 65.6°C) with samples removed at pre-determined time intervals. Sample temperature was monitored with a digital thermocouple (Fisher Scientific Traceable Thermometer and type K probe, Thermo Fisher Scientific, Waltham, MA) calibrated against a factory-calibrated mercury-filled thermometer (FisherBrand, factory calibrated to meet the requirements of ISO/EC Guide 25, ANSI/NCSL 2540-1-1994, ISO 9000/QS 9000 Series of Quality Standards, and MIL STD 45662A) and inserted through a rubber septum (TRU-FLATE chembond round patches, Plews & Edelmann, Dixon, IL) into a vacuum-sealed pouch containing 1 ml of uninoculated milk. The time needed for the sample to reach the target treatment temperature (come-up time) was recorded for each trial and averaged

10 sec. Time 0 samples were not removed from waterbath until samples reached the specified target temperature. At each sampling point, duplicate inoculated samples were removed and immediately submerged in an ice bath for a minimum of 2 min to reach ≤ 4°C. Chilled sample pouches were removed from the ice bath, dried, and sanitized with 70% ethanol before opening. 9.0 ml BPW was added to each pouch (1:10 dilution) before stomaching (Neutec Masticator, Neutec Group, Inc., Farmingdale, NY) samples for 30 s. Trials for each temperature / pathogen combination were conducted in duplicate.

Heating of flasked validation samples. D-values generated from heating milk in vacuum-sealed bags in a waterbath were subsequently validated by heating of milk in sterilized flasks. 100 ml samples of non-homogenized, pasteurized whole milk (Kalona brand) were pipetted into sterilized 250-ml Büchner flasks containing a stirbar. The flasks were prewarmed in a waterbath prior to testing. The flasks were individually immersed in a stainless steel pan waterbath over a magnetic hotplate stirrer (Corning, Model PC-620D), with water reaching >2 inches above the milk surface, and heated with constant stirring to target temperatures. Once the target temperature of the milk was reached and stabilized, milk was inoculated with a target of 3-4 log CFU/ml *L. monocytogenes* or STEC (1% v/v), with samples removed at pre-determined time intervals. Sample temperature was monitored with a factory-calibrated mercury-filled thermometer (FisherBrand), which remained suspended in the milk throughout the heating period. Immediately following inoculation as well as at each sampling point, a 2-ml sample was pipetted from the flask into a 15-ml centrifuge tube (Falcon, BD Biosciences) fully submerged in an ice bath. Immediately following sampling, each sample was shaken vigorously in the ice bath for 10 s to cool the sample to ≤ 4°C. Chilled milk sample tubes were removed from the ice bath,

dried, and sanitized with 70% ethanol before opening. Samples were serially diluted as well as directly plated to reduce the detectable level of *L. monocytogenes* and STEC. Trials for each temperature / pathogen combination were conducted in triplicate.

Enumeration and data handling. Samples were serially diluted with BPW and surface plated on MOX and SMAC agars overlayed with TSA for *L. monocytogenes* and STEC, respectively, in order to aid recovery of heat-injured cells (Kang 2000). Uninoculated samples of tested milk were surface plated on TSA on the day of testing to observe background counts. Plates were incubated for either 24 h (STEC) or 48 h (*L. monocytogenes* and uninoculated samples) at 37°C after which colonies were counted. Log CFU/ml versus heating time were plotted for each pathogen/temperature/trial combination. The come-up time for each trial was not included in the respective survival curve, although additional lethality may have occurred during the time it took for samples to reach their target temperature (average 10 s). Individual survival curves for each pathogen/temperature/trial were fitted using linear regression, with at least 5 sampling points included for each curve. D-values were estimated as the average of the absolute inverse of the slopes of the regression line for each pathogen/temperature/trial combination. Survival curves were additionally fitted using seven other relevant models (e.g. Weibull, biphasic) in the GlnaFiT add-on for Excel (Microsoft Excel 2016, Microsoft, Redmond, WA, Geeraerd 2005), however, D-values calculated from linear regression of each survivor curve were found to have highest R^2 values and were thus reported. The z-value, or temperature increase needed for a 1-log reduction of the D-value, of *L. monocytogenes* and STEC were determined over the test temperatures 60.0°C to 65.6°C in order to compare relative heat resistance between the two pathogens. Z-values were determined by graphing log D-value versus

temperature for each pathogen (Figure 3-1 and 3-2 for *L. monocytogenes* and STEC, respectively), with z-values equaling the absolute inverse of the slope of each linear regression line.

Literature analysis. Thermal lethality data from 18 studies investigating *L. monocytogenes* and 3 studies investigating STEC in whole milk samples heated to thermization temperatures (55°C to 71.7°C) were compiled. The thermization temperature range was based on a range found in a compilation of 40 published definitions of thermization specific to cheesemaking (Table 2-4). Datasets included bovine whole milk samples only with any level of homogenization and/or pasteurization/sterilization and heated by any methodology. A total of 162 and 25 D-values for *L. monocytogenes* (Table 3-S1) and STEC (Table 3-S2), respectively, were included. Log D-values versus heat treatment temperature were graphed, with individual thermal destruction curves constructed for *L. monocytogenes* (Figure 3-1) and STEC (Figure 3-2).

Statistical analysis of published L. monocytogenes D-values. Published *L. monocytogenes* D-values (n=162) and standard deviations (n=96 of 162) compiled from literature analysis were fitted in order to find a model that most accurately predicted log D-value from test temperature while minimizing D-value underprediction. Linear, piecewise, and quadratic model shapes with Ordinary Least Squares, Inverse-Variance Weighted Least Squares, Study Effect, and Inverse-Variance Weighted + Study Effects models were separately constructed and evaluated. Univariate outlier detection for numeric variables was carried out via visual inspection of boxplots and multivariate outlier detection using Mahalanobis Distance in

the performance R package (R Core Team, Vienna, Austria) with a threshold of 0.05. To assess the predictive accuracy of the three candidate model shapes, repeated stratified 6-fold cross-validation was performed on the full data set. Generated D-values from the current study (8 observations, 2 for each test temperature) were used to validate models in each fold of each repetition. See Appendix 2 for additional information regarding statistical analysis.

RESULTS AND DISCUSSION

This study investigated thermization (i.e. sub-pasteurization) treatments necessary for the reduction of *L. monocytogenes* and STEC in non-homogenized whole milk to levels where they would not be infectious to consumers if applied to raw milk cheesemaking. Following generation of D- and z-values for each pathogen, predicted times to 3-log decrease were validated in a larger volume of inoculated milk with lower inoculum to ensure fail-safety of the generated D-values. Due to the availability of a significant body of published data on *L. monocytogenes* inactivation in whole milk samples heated to thermization temperatures, compiled lethality data for *L. monocytogenes* were additionally modeled, finding non-linearity of fit over thermization temperatures of 55°C to 71.7°C (Figure 3-1).

Evaluation and validation of D-values. Survival curves for *L. monocytogenes* and STEC in whole milk revealed a linear decrease in populations across all test temperatures and trials, with R^2 values ranging from 0.84 to 0.99 for individual pathogen, temperature, and trial combinations tested (Figures 3-3 to 3-6). As expected, D-values for STEC were shorter than those for *L. monocytogenes* at each test temperature (Tables 3-1 and 3-2); z-value for both pathogens in the whole milk matrix was 6.1°C over the temperatures tested for both pathogens

(60.0°C to 65.6°C). As calculated z-values are dependent on the temperature range included in their determination (van Doornmalen 2009), z-values should be interpreted with caution and used solely as a reference (Peng 2013). To this point, a higher z-value was found for the least thermotolerant strain tested among 4 STEC and 5 generic *E. coli* dairy isolates heated in raw milk to thermization temperatures by Peng and colleagues (Peng 2013).

In the validation experiments, *L. monocytogenes* populations decreased from ~3.5-log CFU/ml to undetectable levels (<0.48 log CFU/ml) in 15-26 s, 60-90 s, 180-210 s, and 1200-1800 s at test temperatures of 65.6, 62.8, 60.0, and 57.2°C, respectively, while >3.5-log reduction of STEC to undetectable levels were observed in 5-10 s, 12-25 s, 94 s, and 189 s at the same test temperatures (Table 3-2). The observed lethality in the validation experiments was faster than that predicted based on the D-value experimental data. This finding suggests fail-safety of the D-values generated in the current study when heating larger volumes of non-homogenized whole milk, as would occur during typical batch thermization treatments. However, to ensure sufficient lethality, particularly when using high-temperature, short-time pasteurization (HTST) equipment, establishing a thermal process based on the more conservative D- and z-values (Table 3-1) will deliver a greater margin of safety compared to using the values from the validation study alone (Table 3-2).

Review of published studies confirm the relative rate of inactivation for the two pathogen types, even when using different food matrices and methodologies. In a screening of 32 STEC strains isolated from raw milk cheeses and cattle feces, Peng and colleagues found that none of the tested strains survived heating at 55°C for 15 min in microbiological media (Peng 2012). In contrast Pöntinen and colleagues reported 0.0 to 1.4 log reductions in two *L. monocytogenes* serotype 1/2c strains when TSB was heated at 55°C for 40 minutes (Pöntinen 2017). Ercolini and

colleagues reported a 1.1 log CFU/g reduction of *E. coli* O157:H7 in Grana cheese curd cooked at 55°C for 20 min, while no reduction of *L. monocytogenes* was observed following the same treatment (Ercolini 2005). Arocha and colleagues reported heating cottage cheese curd at 57°C for 1.5 h to reduce *E. coli* O157:H7 populations from >7.5 log CFU/g to less than detectable limits (Arocha 1992). Skandamis and colleagues reported survival of a 10-strain *L. monocytogenes* cocktail exposed to mild acid (min pH 5.04, adjusted with lactic acid) or salt stress (3.5% NaCl) followed by subsequent heating at 57°C for 2 h in TSB. The authors reported linear decreases from ~8 log CFU/ml *L. monocytogenes* within the first hour of heating, however, less lethality was observed in the second hour, with final *L. monocytogenes* levels found to be >1.3 CFU/ml, regardless of initial stresses applied (Skandamis 2009). These data suggest temperatures in the lower range recommended for cheesemilk thermization (e.g., 57°C) are sub-lethal to *L. monocytogenes* but lethal to STEC.

The z-value, or temperature increase required to obtain a decimal reduction of D-value, can additionally be used to compare heat resistance between microorganisms when evaluated over a uniform temperature range (van Doornmalen 2009). At the temperatures tested in the current study (60.0°C to 65.6°C) to generate D-values, both *L. monocytogenes* and STEC returned the same z-value (6.1°C). *L. monocytogenes* z-values of 5.7°C to 7.0°C were reported in predictive models for the pathogen constructed from data in microbiological media and a variety of foodstuffs including dairy (Mackey 1989, Sorqvist, 2003, van Asselt et al., 2006, van Lieverloo 2011, van Lieverloo 2013). Though no predictive models specifically for STEC have been published to date, generic *E. coli* z-values of 6.0°C and 10.6°C were suggested by Sorqvist and van Asselt, respectively, from their meta-analyses in liquid foods and media (Sorqvist, 2003, van Asselt 2006).

Lastly, an additional single trial of D-value determination for *L. monocytogenes* at 57.2°C was conducted in pasteurized homogenized whole milk to evaluate whether the homogenization status of the milk used had an impact on D-value. For homogenized milk $D_{57.2°C}$ was ~683 s (11.4 min; data not shown) compared to ~825 s (13.75 min) for the non-homogenized milk; however, given the scatter of data along the regression line, no significant difference was found between survival curves in the two milk types tested (P = 0.49). Among compiled thermization recommendations specific to cheesemaking, the maximum suggested milk treatment times were 30 min, suggesting the time required to reduce *L. monocytogenes* by 3-log may be longer than practical at this lower treatment temperature. In agreement with our findings, regulators have suggested milk thermization at temperatures ≤58°C to be inadequate or less effective than higher thermization temperatures (CAC 1999).

Comparison with modeling of published D-values. Among the modeling approaches considered for fitting *L. monocytogenes* published D-values in whole milk samples, a mixed-effect model with a study-level random effect yielded the best fit for the data, with quadratic modeling performing best in repeated 6-fold cross validation and yielding the smallest prediction errors and the lowest rate of under-prediction. Quadratic fitting of the dataset returned the lowest mean squared prediction error (MSPE) both in-sample (MSPE = 0.047) and out-of-sample (MSPE = 0.053). The difference in accuracy between in-sample and out-of-sample prediction was not found to be significant (mean difference = -0.006, 95% confidence interval: -0.042–0.021). The quadratic model additionally resulted in a lower Akaike information criterion than linear fitting (20.62 vs. 32.93), an indication of better model fit (Appendix 2). The final model was found to underpredict three of 162 observations (Figure 3-1), a rate of 1.9%, though two of

these observations were found to be statistical outliers in preliminary analyses. These outlying D-values were observed in sterile and UHT milks by Knabel, and Fedio, respectively (Fedio 1989, Knabel 1990). Elevated *L. monocytogenes* inoculum growth temperatures of 43°C and 48°C were used by the two authors, which has been shown to alter lipid and protein biosynthesis, membrane composition, and subsequently increase *L. monocytogenes* thermal resistance in liquid dairy products (Doyle 2001, Pagan 1997). Experimental conditions including growth of test bacterial strains, inoculation procedures, details on milk samples used, and methodology for heating and enumeration are given in Tables 3-S1 and 3-S2 for literature datasets for *L. monocytogenes* and STEC, respectively.

Curvature of compiled *L. monocytogenes* log D-value versus temperature plots in milk samples heated to temperatures 52°C to 75°C was also observed by Mackey and Bratchell (Mackey 1989). The authors attributed curvature to differences in heating methodology, especially between the use of sealed tubes and slug-flow heat exchanger, which they found to result in significantly different z-values of 6.1°C or 7.4°C, respectively (Mackey 1989). Conversely, van Lieverloo and colleagues found no significant effect of heating methodologies in a model for *L. monocytogenes* inactivation in raw milk constructed from published D-values even between best-case (lab-scale pasteurizer with flow) and worst-case (large sample volume heated in waterbath) methodologies (van Lieverloo 2011). To our knowledge, other authors to-date have not compiled strictly STEC log D-values in milk samples, however, researchers have reported generic *E. coli* thermal inactivation models based on compiled D-values in microbiological media and foodstuffs that included milk and dairy datasets (Sorqvist 2003, van Asselt 2006). Due to the limited published data available on STEC in whole milk at thermization

temperatures, a relevant predictive model beyond linear fitting for STEC inactivation could not be realized in the current study (Figure 3-2).

CONCLUSIONS

As the popularity of artisanal raw milk cheeses continues to grow in the US, control measures to enhance their safety must be scientifically evaluated and optimized. Though hygienic controls can help to reduce microbial populations in raw cheesemilk, heat treatment is the most important process used for eliminating vegetative bacterial pathogens from the finished product. Thermization can retain some beneficial properties of raw cheesemilk while improving safety. This study aimed to identify thermization conditions necessary for 3-log reductions of *L. monocytogenes* and STEC in whole milk and can be used to create flexible thermal processes to improve the safety of certain artisanal raw milk cheeses. Risk assessors still agree that even with the addition of thermization, raw milk cheeses, particularly soft ripened varieties, still present a higher risk to consumers than those made from pasteurized milk. Strict hygienic controls must still be observed at the farm and plant level to ensure high quality milk and subsequent cheese.

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Table 3-1. Generated D- and z-values for *Listeria monocytogenes* and Shiga toxin-producing *Escherichia coli* in non-homogenized, pasteurized, whole milk. Values represent average ± SD of duplicate trials for each pathogen and temperature combination.

Pathogen	D-value (s) for Temperature				z-value
	57.2°C	60.0°C	62.8°C	65.6°C	
L. monocytogenes	825.2 ± 46.9	146.6 ± 59.5	33.8 ± 9.8	17.1 ± 0.3	6.1°C
STEC	N/A	60.0 ± 7.4	16.9 ± 1.3	7.2 ± 0.7	6.1°C

Table 3-2. Time (in min:s) and temperature combinations necessary for 3-log reduction of *Listeria monocytogenes* and Shiga toxin-producing *Escherichia coli* in non-homogenized, pasteurized, whole milk based on generated D-values (duplicate trials) and time to >3 log killed based on validation in flasks (triplicate trials; time to undetectable levels <0.48-log CFU/ml, from starting average 4.12 + 0.29 log CFU/ml of STEC or 3.57+0.55 log CFU/ml *L. monocytogenes*; rounded up to nearest 5 second interval).

Temperature (°C)	*L. monocytogenes*		STEC	
	Predicted time to 3-log decrease	*Time to >3- log kill in flask validation*	*Predicted time to 3-log decrease*	*Time to >3- log kill in flask validation*
65.6	0:51	0:30	0:21	0:10
62.8	1:39	1:30	0:51	0:25
60.0	7:21	3:00	3:00	1:35
57.2	41:16	30:00	Not tested	3:10

Table 3-S1. Published *Listeria monocytogenes* D-values in whole milks heated to thermization temperatures (55°C to 71.7°C).

Menstruum	Strain	Temperature (°C)	D-value (s)	Comments	Reference
Raw whole milk; antibiotic-free, SPC <1000 CFU/ml	Scott A	57.8	289.6 ± 28.4	• Scott A found to be more heat resistant than strains Murray B, V7, and V37 in sterile whole UHT milk in preliminary study • Heating completed in a waterbath in sealed 13 X 100-mm borosilicate glass tubes • Starting inoculum 10^5 CFU/ml Strain grown in TSBYE 37°C 24 h	Bradshaw 1985
		63.3	19.9 ± 6.0		
		66.1	7.3 ± 1.8		
		68.9	3.0 ± 0.8		
		71.7	0.9 ± 0.1		
Sterile (121°C 20 min) whole milk	Scott A	57.8	255.6	• Heating completed in a waterbath in sealed 13 X 100-mm borosilicate glass tubes • Starting inoculum 10^5 CFU/ml • Strain grown in TSBYE 37°C 24 h	Bradshaw 1987
		63.3	34.9		
		66.1	9.9		
		68.9	3.2		
		71.7	2.0		
Commercially sterile whole milk	Scott A	57.8	298.0		
		66.1	13.9		
		68.9	5.8		
		71.7	2.0		
		71.7	2.7		
		71.7	2.7		
Raw whole milk	Scott A	57.8	289.9		
	BS-9	57.8	435.6		

Farm bulk tank raw milk		63.3	38.7	• Heating completed in a waterbath in sealed 13 X 100-mm borosilicate glass tubes • Starting inoculum 10^5 CFU/ml • Strain grown in TSBYE 37°C 24 h	Bradshaw 1991
		68.9	3.3		
		71.7	2.2		
	Scott A	57.8	330.0		
		63.3	31.0		
		68.9	4.0		
		71.7	2.0		
	SE-31	57.8	528.6		
		63.3	46.1		
		68.9	2.8		
		71.7	1.5		
Sterile (121°C 20 min) bulk tank milk	BS-9	52.2	2848.3		
		57.8	409.0		
		63.3	68.0		
		68.9	9.1		
	Scott A	52.2	1704.8		
		57.8	290.2		
		63.3	50.6		
		68.9	7.3		
	SE-31	57.8	440.5		
		63.3	49.6		
		68.9	6.2		
		71.7	4.4		
Raw milk (1.5 ml) with freely suspended *L. monocytogenes*	Scott A	57.8	445.0 ± 102.8	• Heating completed in a waterbath in sealed borosilate tubes for 1.5 ml test volume and in slug flow heat exchanger for 4 L test volume • Starting inocula 6.9 x 10^3 to 6.0 x 10^5 CFU/ml for freely suspended bacteria and 1.2 x 10^4 to 9 x 10^5 CFU/ml for intracellular bacteria (inside murine macrophages via *in vitro* procedure) • Strain grown in TSBYE 37°C 24 h	Bunning 1986
		63.3	33.4 ± 9.9		
		68.9	7.2 ± 2.0		
Raw milk (4 L) with freely suspended *L. monocytogenes*	Scott A	63.3	29.7 ± 0.2		
		66.1	15.1 ± 4.2		
		68.9	5.3 ± 0.3		
		71.7	1.3 ± 0.1		
Raw milk (1.5 ml) with intracellular *L. monocytogenes*	Scott A	57.8	490.1 ± 64.7		
		63.3	33.3 ± 8.5		
		68.9	7.0 ± 4.4		
	F5069	57.8	331 ± 99.3		Bunning 1988

Sterile whole milk (80-100 ml) with freely suspended *L. monocytogenes*		62.8	38.3 ± 7.3	• Heating completed in a waterbath in sealed borosilate tubes for 80-100 ml test volume and in slug flow heat exchanger for 2 L test volume • Starting inocula 10^6 to 10^8 CFU/ml for freely suspended bacteria and 5.0 x 10^5 to 8.5 x 10^6 CFU/ml for intracellular bacteria (inside bovine phagocytes via *in vitro* procedure) • Strain grown in TSBYE 37°C 18 h	
		66.1	16.9 ± 1.8		
		68.9	8.6 ± 0.4		
Sterile whole milk (80-100 ml) with intracellular *L. monocytogenes*	F5069	57.8	429.8 ± 32.7		
		62.8	55.2 ± 22.1		
		66.1	16.7 ± 5.4		
		68.9	3.9 ± 0.5		
Sterile whole milk (2 L) with freely suspended *L. monocytogenes*	F5069	66.1	19.1 ± 8.4		
		68.9	5.1 ± 0.6		
		71.7	3.1 ± 1.1		
Sterile whole milk (2 L) with intracellular *L. monocytogenes*	F5069	66.1	18.4 ± 1.1		
		68.9	9.1 ± 0.7		
		71.7	5.0 ± 0.4		
Sterile whole milk with non heat-shocked *L. monocytogenes*	F5069	71.7	3.0 ± 1.0	• Heating completed in slug flow heat exchanger • Strain grown in TSBYE 35°C	Bunning 1992
Sterile whole milk with heat-shocked *L. monocytogenes*	F5069	71.7	4.6 ± 0.5		
Sterile whole milk (FDA enrichment)	F5069	71.7	1.4 ± 0.3	• Heating completed in slug flow heat exchanger • Starting inoculum 10^7 CFU/ml • Strain grown in TSBYE	Crawford 1989
Sterile whole milk (NSB enrichment)	F5069	71.7	2.0 ± 0.5		
Sterile whole milk (USDA enrichment)	F5069	71.7	0.6 ± 0.2		

Sterile whole milk (cold enrichment)	F5069	71.7	0.6 ± 0.1		
Sterile whole milk (plated on LPM)	F5069	71.7	0.7 ± 0.2		
Sterile whole milk (plated on MMA)	F5069	71.7	1.3 ± 0.4		
Sterile whole milk (plated on TSYEA)	F5069	71.7	2.7 ± 0.8		
Whole milk	F5069	55.0	1440.0	• Heating completed in in a waterbath in sealed 2-ml glass reaction vials (1.5 ml test volume) • Starting inoculum 10^8 CFU/ml • Strain grown in sterile whole milk unless otherwise indicated	Donnelly 1986
Whole milk	F5027	62.7	21.0		
Whole milk	ATCC 19111	62.7	39.0		
Whole milk	ATCC 19113	62.7	24.0		
Whole milk	ATCC 19115	62.7	24.0		
Whole milk	F5069	62.7	60.0		

Whole milk	F5069	65.0	6.0		
Whole milk; *L. monocytogenes* grown in 11% NFDM	F5069	62.7	54.0		
Whole milk; *L. monocytogenes* grown in skim milk	F5069	62.7	54.0		
Sterile whole milk	V7 and F6861	58.0	130.2	• Heating completed in a waterbath in 2 ml glass chromatographic vials, covered and crimped • Starting inoculum 10^7 CFU/ml • Strains grown in TSBYE 30°C 24 h • Acid shock (pH 6.8 to pH 4.0) of cultures completed using 10 N HCl	Farber 1992
Sterile whole milk	V7 and F6861	58.0	147.0		
Sterile whole milk	V7 and F6861	58.0	165.0		
Sterile whole milk; immediate acid shock of *L. monocytogenes*	V7 and F6861	58.0	234.0		
Sterile whole milk; 4 h acid shock of *L. monocytogenes*	V7 and F6861	58.0	159.6		

Sterile whole milk; 24 h acid shock of *L. monocytogenes*	V7 and F6861	58.0	180.0		
UHT milk, LPM agar used	Scott A	60.0	208.3	• Heating completed in 2 L Erlenmeyer flask containing 300 ml test volume • Starting inoculum 8.5 log CFU/ml • Strains grown in TSBYE 37°C 24 h • Inoculated milks held at 48°C for 1 h as pre-heating treatment where noted	Fedio 1989
UHT milk preheated to 48°C 1 h, LPM agar used	Scott A	60.0	312.5		
UHT milk, TSAYE agar used	Scott A	60.0	357.4		
UHT milk preheated to 48°C 1 h, TSAYE agar used	Scott A	60.0	1111.1		
Homogenized milk	Scott A	57.2	203.4	• Heating completed in a waterbath in 9-ml screw cap vials containing 3 ml test volume • Starting inoculum 10^9 CFU/ml • Strain grown in BHIB 37°C 18 h • Heating completed in a waterbath in glass ampules with 2 ml test volume • Starting inoculum 5-6 log CFU/ml • Strain grown in TSBYE 35°C 18-20 h	Holsinger 1992
		60.0	123.0		
Raw milk	Scott A	55.2	492.0 ± 66.0		Kamau 1990
		57.8	138.0 ± 6.0		
Raw milk with 0.6 mM H_2O_2	Scott A	55.2	666.0 ± 54.0		
		57.8	156.0 ± 30.0		
Whole milk, BHI agar used	302	60.0	199.8		Kenney 2004

Whole milk, MOX agar used	302	60.0	127.8	• Heating completed in a waterbath in sealed capillary tubes (1 mm x 90 mm) with 50 µl test volume • Starting inoculum 8.7 to 9.3 log CFU/ml • Strain grown in BHIB 37°C 48 h	
Sterile whole milk; *L. monocytogenes* 37°C aerobic growth and plating	F5069	62.8	36.0	• Heating completed in a waterbath in sealed Pyrex TDT tubes (1 mm x 90 mm) • Starting inoculum 10^6 CFU/ml • Strain grown in TSBYE	Knabel 1990
Sterile whole milk; *L. monocytogenes* 43°C anaerobic growth and plating	F5069	62.8	243.0		
Standardized whole UHT milk (non-homogenized)	NZRM4237	61.0 62.0 63.0 64.0	146.0 ± 34.0 61.0 ± 8.0 28.0 ± 5.0 14.0 ± 3.0	• Heating completed in pilot-plant scale pasteurizer with 120 L test volume • Starting inoculum 10^7 CFU/ml Strain grown in TSB	Pearce 2012
Pasteurized whole milk; *L. monocytogenes* grown at 37°C, plated on LSA	NCTC 9863, smooth S-type	56.0 60.0 63.0	384.0 ± 12.0 42.0 ± 6.0 6.0 ± 6.0	• Heating completed in a waterbath in 28-ml screw-cap dilution bottles with 10 ml test volume • Parent smooth S-type cultures were grown in tyndallized whole milk at 42.8°C 24 h prior to heat treatment at 60-63°C for 3-7 min to obtain rough R-type cultures • Starting inoculum 1.5 x 10^8 to 3 x 10^8 CFU/ml	Rowan 1998
Pasteurized whole milk; *L. monocytogenes* grown at 42.8°C, plated on LSA	NCTC 9863, smooth S-type	56.0 60.0 63.0	990.0 ± 18.0 108.0 ± 12.0 24.0 ± 6.0		
Pasteurized whole milk; *L. monocytogenes*	NCTC 9863, smooth S-type	56.0 60.0	576.0 ± 12.0 90.0	• Strains grown in TSBYE at 30°C before regrowth in sterilized whole milk at 37°C or 42.8°C prior to heating trials	

grown at 37°C, plated on TSYEA		63.0	24.0 ± 6.0
Pasteurized whole milk; *L. monocytogenes* grown at 42.8°C, plated on TSYEA	NCTC 9863, smooth S-type	56.0	1470.0 ± 42.0
		60.0	222.0 ± 12.0
		63.0	66.0 ± 6.0
Pasteurized whole milk; *L. monocytogenes* grown at 37°C, plated on LSA	NCTC 11994, smooth S-type	56.0	384.0 ± 12.0
		60.0	48.0 ± 6.0
		63.0	12.0 ± 6.0
Pasteurized whole milk; *L. monocytogenes* grown at 42.8°C, plated on LSA	NCTC 11994, smooth S-type	56.0	1068.0 ± 18.0
		60.0	120.0 ± 6.0
		63.0	30.0 ± 6.0
Pasteurized whole milk; *L. monocytogenes* grown at 37°C, plated on TSYEA	NCTC 11994, smooth S-type	56.0	606.0 ± 12.0
		60.0	90.0 ± 12.0
		63.0	24.0 ± 6.0
Pasteurized whole milk; *L. monocytogenes* grown at 42.8°C, plated on TSYEA	NCTC 11994, smooth S-type	56.0	1590.0 ± 48.0
		60.0	234.0 ± 6.0
		63.0	72.0
Pasteurized whole milk; *L. monocytogenes* grown at 37°C, plated on LSA	NCTC 9863, rough R-type	56.0	390.0 12.0
		60.0	48.0 ± 6.0
		63.0	12.0

Pasteurized whole milk; *L. monocytogenes* grown at 42.8°C, plated on LSA	NCTC 9863, rough R-type	56.0	1086.0 ± 6.0
		60.0	126.0 ± 6.0
		63.0	36.0
Pasteurized whole milk; *L. monocytogenes* grown at 37°C, plated on TSYEA	NCTC 9863, rough R-type	56.0	630.0 ± 6.0
		60.0	102.0 ± 6.0
		63.0	24.0
Pasteurized whole milk; *L. monocytogenes* grown at 42.8°C, plated on TSYEA	NCTC 9863, rough R-type	56.0	1746.0 ± 18.0
		60.0	264.0 ± 12.0
		63.0	84.0 ± 6.0
Pasteurized whole milk; *L. monocytogenes* grown at 37°C, plated on LSA	NCTC 11994, rough R-type	56.0	396.0 ± 6.0
		60.0	54.0 ± 6.0
		63.0	12.0
Pasteurized whole milk; *L. monocytogenes* grown at 42.8°C, plated on LSA	NCTC 11994, rough R-type	56.0	1170.0 ± 12.0
		60.0	138.0 ± 12.0
		63.0	36.0 ± 6.0
Pasteurized whole milk; *L. monocytogenes* grown at 37°C, plated on TSYEA	NCTC 11994, rough R-type	56.0	684.0 ± 12.0
		60.0	108.0 ± 6.0
		63.0	30.0 ± 6.0
		56.0	1890.0 ± 48.0

Pasteurized whole milk; *L. monocytogenes* grown at 42.8°C, plated on TSYEA	NCTC 9863, rough R-type	60.0	288.0		
		63.0	84.0 ± 6.0		
Whole milk	Scott A	58.0	219.6 ± 16.8	• Heating completed in a waterbath in sealed capillary tubes with 0.1 ml test volume • Starting inoculum 10^8 CFU/ml • Strain grown in BHIB 37°C 24 h	Szlachta 2010
		60.0	91.2 ± 3.6		
		62.0	36.6 ± 0.6		
UHT whole milk	1E	68.0	10.3 ± 0.3	• Heating completed in small-scale continuous flow heater ("microheater") • Starting inoculum 10^8 CFU/ml • Strains grown first in BHIB at 30°C overnight, then resuspended in whole milk and grown at 7°C for 7 days	Van der Veen 2009
		70.0	3.5 ± 0.2		
UHT whole milk	NV8	68.0	5.8 ± 0.1		
		70.0	2.5 ± 0.0		
UHT whole milk	Scott A	68.0	1.1 ± 0.0		
		70.0	0.4 ± 0.0		

Table 3-S2. Published Shiga toxin-producing *Escherichia coli* D-values in whole milks heated to thermization temperatures (55°C to 71.7°C).

Menstruum	Strain	Temperature (°C)	D-value (s)	Comments	Reference
UHT whole milk; plated on TSAYE	*E. coli* O157:H7 NCTC	55.0	2500.0	• Heating completed in a Standsted Food Lab 9000 high-pressure isostat in 30-ml low-density polyethylene bottles with 30-ml test volume • Starting inoculum 10^9 CFU/ml • Strain grown in TSBYE at 37°C for 16 h	Patterson 1998
		60.0	769.2		
UHT whole milk; plated on TSAYE + 0.5% NaCl	*E. coli* O157:H7 NCTC	55.0	1111.1		
		60.0	192.3		
Raw milk	*E. coli* K133 (serotype O113:H4)	60.0	48.4 ± 22.0	• Heating completed in pilot-plant scale pasteurizer with 30 L test volume • Strains grown in TSB at 37°C for 24 h	Peng 2013
		62.5	49.7 ± 19.9		
		65.0	4.6 ± 0.9		
Raw milk	*E. coli* N09-1208 (serotype O26:H11)	60.0	49.8 ± 16.4		
		62.5	32.1 ± 0.3		
		65.0	3.4 ± 0.6		
Raw milk	*E. coli* K331/4 (serotype O91:H21)	60.0	71.7 ± 26.8		
		62.5	23.6 ± 12.4		
		65.0	3.2 ± 0.4		
Raw milk	*E. coli* K356 (serotype O2:H21)	60.0	80.5 ± 26.0		
		62.5	47.9 ± 7.7		
		65.0	3.0 ± 0.2		
Whole milk	*E. coli* O111:B4	55.0	450.0	• Heating completed in waterbath in 5-ml sealed glass ampules with 2 ml test volume • Starting inoculum 10^5 CFU/ml	Singh 1980
		55.0	393.6		
		60.0	75.0		
Whole milk	*E. coli* O127:B8	55.0	2025.0		
		55.0	1996.8		
		60.0	495.0		
		60.0	496.8		
		63.0	120.0		
		63.0	115.8		

Figure 3-1. Literature (○) and study (●) log D-values for *Listeria monocytogenes* in inoculated whole milks versus test temperature. Literature values were compiled for whole bovine milks heated to thermization temperatures (55°C to 71.7°C) and based on data given in Table 3-S1. Fitted thermal destruction curve with the equation log D-value = $0.0034x^2 - 0.5958x + 25.7639$ (—; $R^2 = 0.94$) shown with 95% confidence (- -) and prediction (**...**) intervals. Literature D-values underpredicted by thermal destruction curve (×) are additionally displayed.

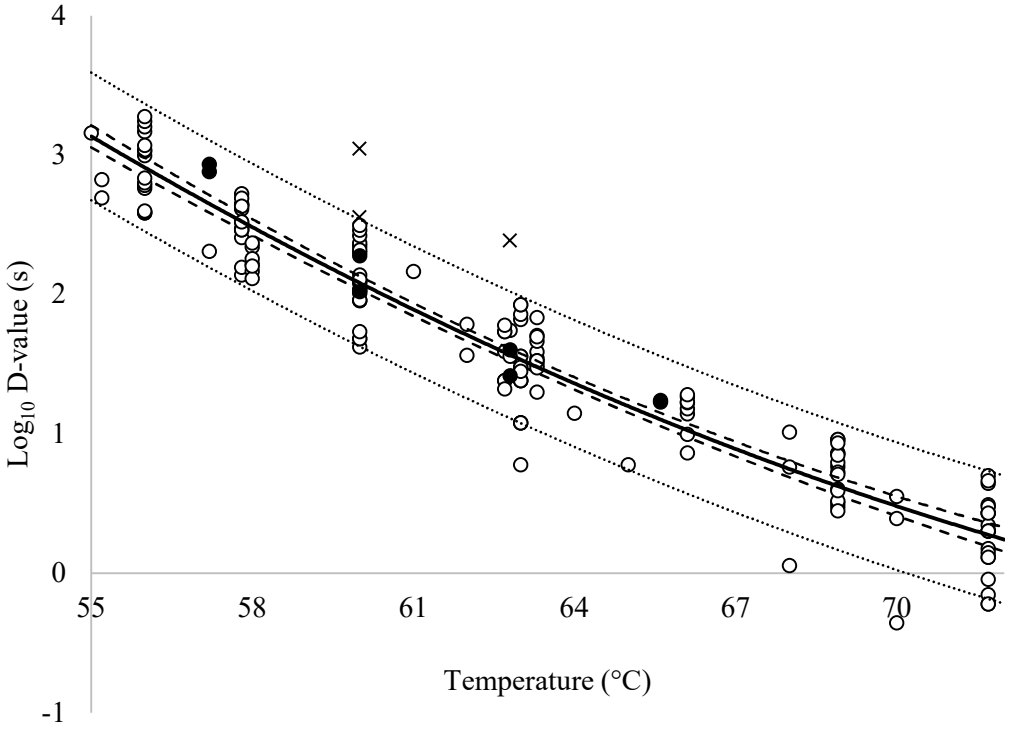


Figure 3-2. Literature (○) and study (●) log D-values for Shiga toxin-producing *Escherichia coli* in inoculated whole milks versus test temperature. Literature values were compiled for whole bovine milks heated to thermization temperatures (55°C to 71.7°C) and based on data given in Table 3-S2. Fitted thermal destruction curve with the equation log D-value = –0.2224x + 15.3830 (—; $R^2 = 0.79$) shown with 95% confidence (- -) and prediction (**...**) intervals.

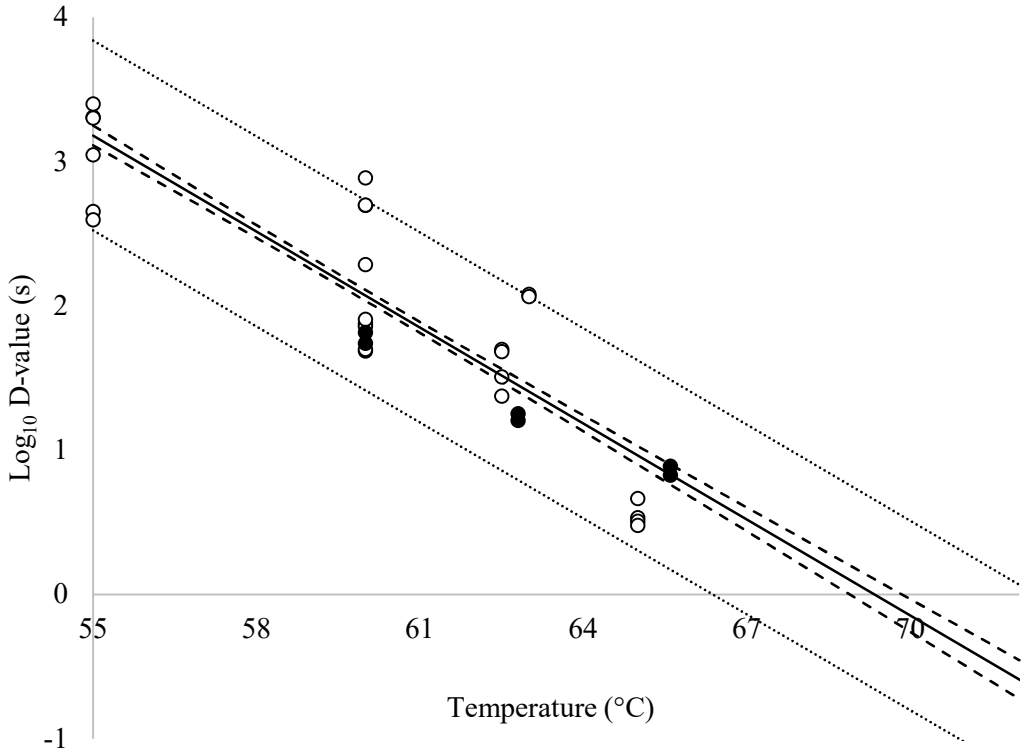


Figure 3-3. Log CFU/ml *Listeria monocytogenes* (○ / ···) or Shiga toxin-producing *Escherichia coli* (STEC; ● / —) versus time in non-homogenized, pasteurized, whole milk heated at 65.6°C. Pooled results from duplicate trials are shown, with data points representing the mean population of duplicate samples and error bars for the standard deviation for each timepoint. Linear regression for survival curves from single *L. monocytogenes* (R^2 values 0.91 and 0.94 for trials 1 and 2, respectively) and STEC (R^2 values 0.92 and 0.90 for trials 1 and 2, respectively) trials were used for D-value calculations.

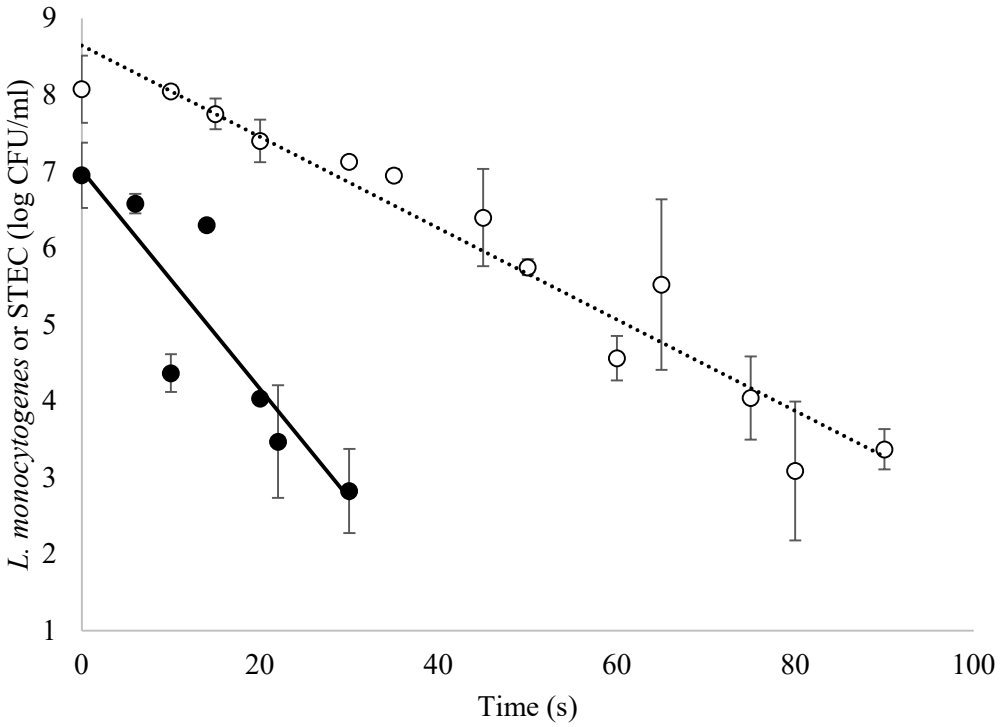


Figure 3-4. Log CFU/ml *Listeria monocytogenes* (○ / ···) or Shiga toxin-producing *Escherichia coli* (STEC; ● / —) versus time in non-homogenized, pasteurized, whole milk heated at 62.8°C. Pooled results from duplicate trials are shown, with data points representing the mean population of duplicate samples and error bars for the standard deviation for each timepoint. Linear regression for survival curves from single *L. monocytogenes* (R^2 values 0.87 and 0.89 for trials 1 and 2, respectively) and STEC (R^2 values 0.95 and 0.88 for trials 1 and 2, respectively) trials were used for D-value calculation.

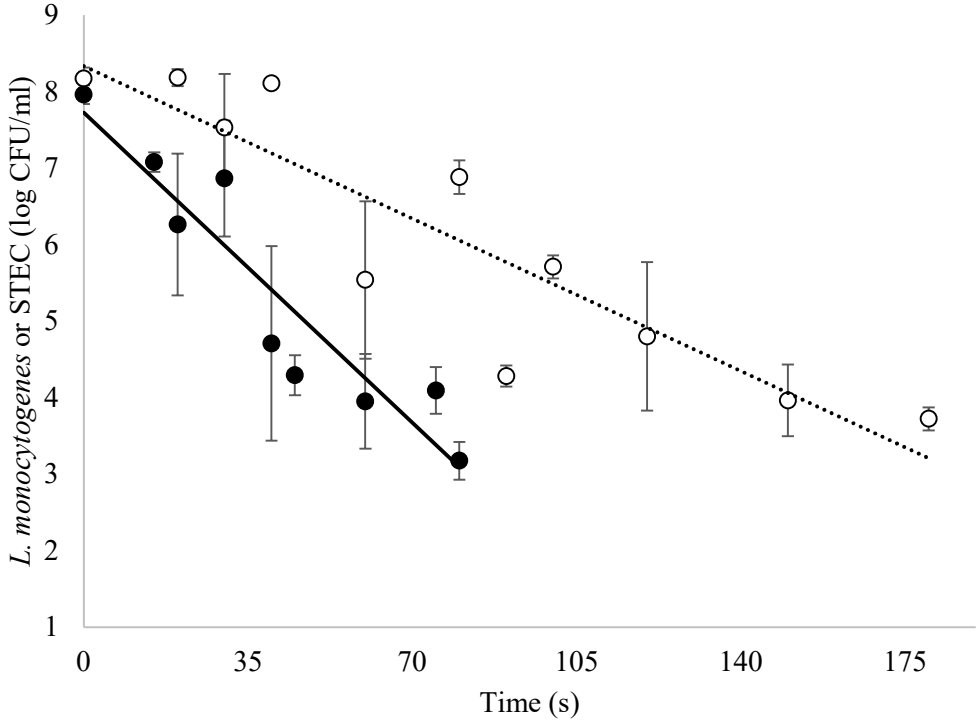


Figure 3-5. Log CFU/ml *Listeria monocytogenes* (○ / ···) or Shiga toxin-producing *Escherichia coli* (STEC; ● / —) versus time in non-homogenized, pasteurized, whole milk heated at 60.0°C. Pooled results from duplicate trials are shown, with data points representing the mean population of duplicate samples and error bars for the standard deviation for each timepoint. Linear regression for survival curves from single *L. monocytogenes* (R^2 values 0.84 and 0.90 for trials 1 and 2, respectively) and STEC (R^2 values 0.94 and 0.99 for trials 1 and 2, respectively) trials were used for D-value calculation.

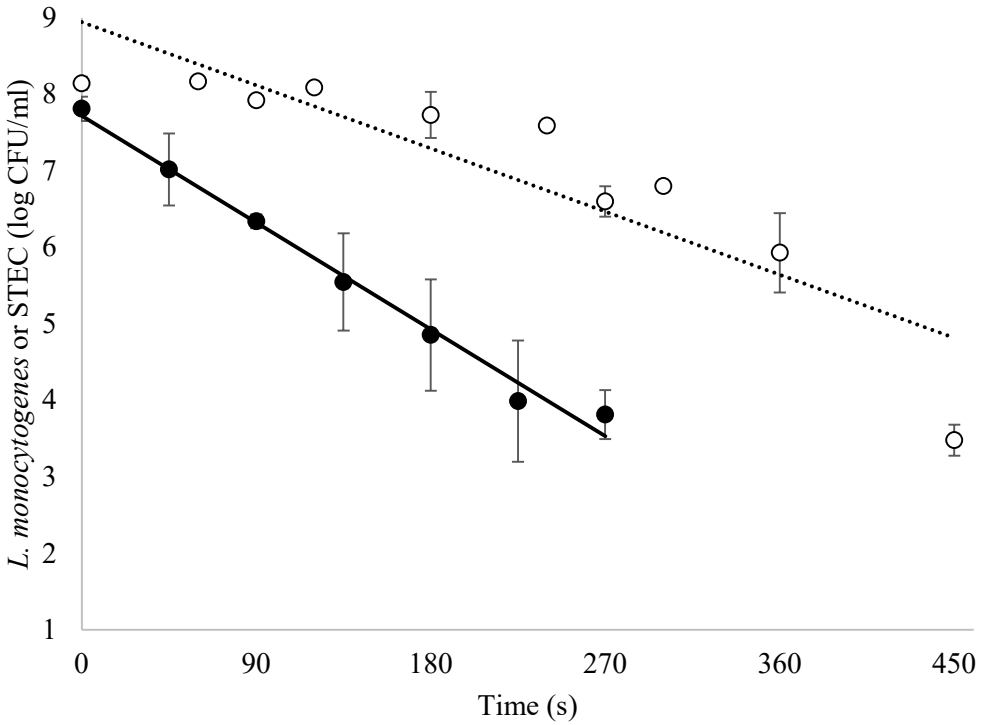
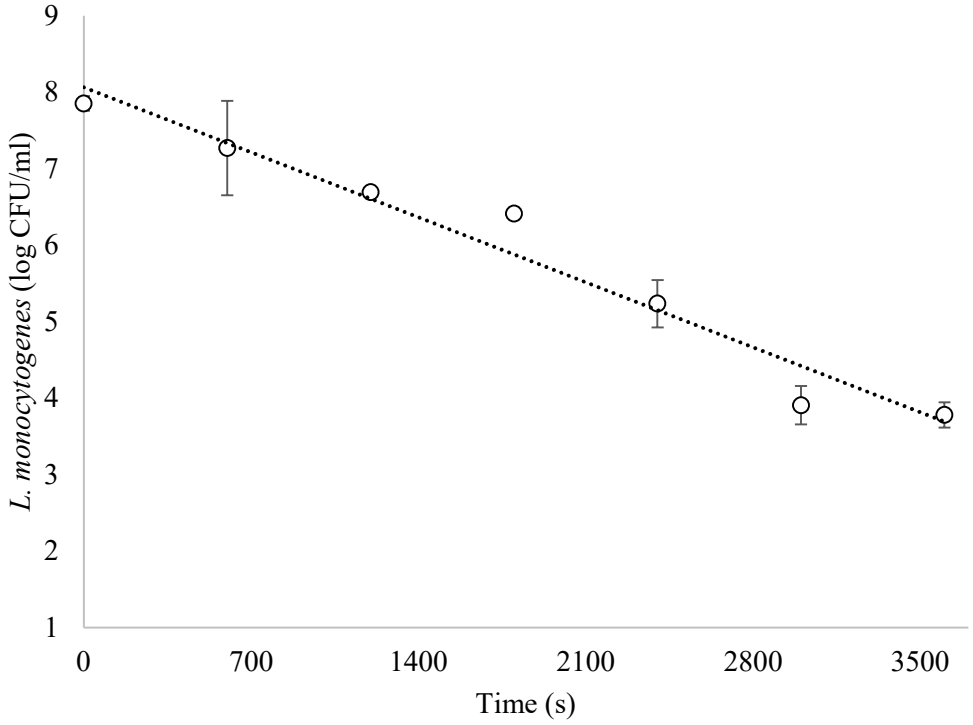


Figure 3-6. Log CFU/ml *Listeria monocytogenes* (○ / ···) versus time in non-homogenized, pasteurized, whole milk heated at 57.2°C. Pooled results from duplicate trials are shown, with data points representing the mean population of duplicate samples and error bars for the standard deviation for each timepoint. Linear regression for survival curves from single *L. monocytogenes* (R^2 values 0.96 and 0.93 for trials 1 and 2, respectively) trials were used for D-value calculation.



CHAPTER 4:

Growth of *Listeria monocytogenes* in a model high-moisture cheese on the basis of pH, moisture, and acid type

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ABSTRACT

High-moisture, low-acid cheeses have been shown to support *Listeria monocytogenes* growth during refrigerated storage. Prior studies have suggested that organic acids vary in their antilisterial activity and that cheeses of lower pH delay growth longer than those of higher pH; however, no standard pH value for *Listeria* control in cheese exists. The objective of this research was to create a predictive model to include the effects of acid type, pH, and moisture on the growth of *L. monocytogenes* in a model cheese system. Cream, micellar casein, water, lactose, salt, and acid (citric, lactic, acetic, or propionic) were combined in 32 formulations targeting 4 pH values (5.25, 5.50, 5.75, and 6.00) and 2 moisture levels (50 and 56%). Each was inoculated with 3-log CFU/g *L. monocytogenes* (5-strain mixture) after which 25-g samples were vacuum-sealed and stored 8 weeks at 4ºC. Triplicate samples were enumerated on Modified Oxford agar weekly in duplicate trials. Model cheeses formulated with acetic and propionic acids inhibited growth (i.e. no observed rise in *L. monocytogenes* over 8 weeks) at pH ≤5.75, while those formulated with lactic acid inhibited growth at pH 5.25 only. All model cheeses formulated with citric acid supported growth. Resulting growth curves were fitted for lag phase and growth rate before constructing models for each. pH and acid type were found to significantly affect both growth parameters ($p < 0.05$), while moisture (50-56%) was not statistically significant in either model ($p \geq 0.05$). The effects of acetic and propionic acid were not significantly different. In contrast, model cheeses made with citric acid had significantly shorter lag phases than the other acids tested, but growth rates after lag were statistically similar to model cheeses made with lactic acid. These data suggest propionic ~ acetic > lactic > citric acids in antilisterial activity within the model cheese system developed and can be used in formulating safe high-moisture cheeses.

HIGHLIGHTS

- pH and organic acid significantly affect *L. monocytogenes* growth in model cheese
- Moisture (50-56%) did not significantly affect *L. monocytogenes* growth
- Propionic ~ acetic > lactic > citric acid in antilisterial activity in model cheese

INTRODUCTION

Annual economic burden of *L. monocytogenes* has been estimated at $2.8 billion (Hoffmann 2015), with an estimated $773 million implicating raw and pasteurized dairy products (Batz 2012). *L. monocytogenes* is of particular concern to the dairy industry due to its ability to grow to high levels in fresh cheeses, defined as those with moisture values ≥50% and including fresh soft (e.g. queso fresco, queso blanco, and fresh mozzarella) and soft unripened (e.g. cottage, ricotta, and quark) varieties, among others (FDA 2003). Risk assessments suggest a >80% reduction in the number of listeriosis cases could be realized by eliminating high dose exposures to *L. monocytogenes* (≥1,000 CFU/g) (FDA 2003, ILSI 2005). Following these assessments, efforts have focused on adjusting formulations with the intent of preventing proliferation throughout product shelf-life.

Growth limits for *L. monocytogenes* include a wide range of pH values (4.4-9.4), salt levels (up to 10%), water activities (a_w; ≥0.92) and temperatures (-0.4 to 45°C) when grown under ideal conditions in microbiological media (FDA 2011). Queso fresco, the most produced and most consumed Hispanic-style cheese in the U.S. (Gonzáles-Córdova 2016), has a moisture content of 46-57%, salt content of 1-3%, and average pH >6.1 (Van Hekken 2003). Refrigerated shelf-life from time of manufacture to consumption is 1-45 days in fresh soft cheeses (FDA 2003), though shelf-lives of up to 60 days have been reported (Leggett 2012). Refrigeration is generally the only post-manufacturing hurdle to *L. monocytogenes* growth in several fresh soft cheese varieties (ILSI 2005); however, many studies (Cataldo 2007, Davies 1997,Genigeorgis 1991, Glass 1995, Leggett 2012, Lourenco 2017, Uhlich 2006) have shown *L. monocytogenes* capable of growing ≥1 log CFU/g in soft fresh cheeses within 1-2 week's storage at refrigeration temperatures when not formulated for its control.

Several soft cheeses are manufactured via the direct acidification of pasteurized milk in the absence of starter culture and with or without rennet (Kosikowski 1997). Typical acidulants used to manufacture these cheeses include citric, acetic, lactic, tartaric, phosphoric acids, acid whey concentrates, and fruit juices, among others (Guinee 1993, Koskiwoski 1997), with commonly used sources of citric, acetic, and lactic acids added in the form of lemon juice, vinegar, and various starter cultures, respectively. It is widely accepted that *L. monocytogenes* inhibition via organic acids is attributed to the undissociated form of the relevant acid. Studies have shown that the pK_a value—the pH at which 50% of total acid is undissociated—of organic acids dictates behavior of *L. monocytogenes* in a broth media system, with acids of higher pK_a values imparting more inhibition than those of lower pK_a values (Ahamad 1989, Ita 1991, Le Marc 2002, Sorrells 1989, Wemmenhove 2016). However, studies directly comparing organic acids against each other in refrigerated foods remain sparse (Barmpalia 2004, Dubal 2004, Samelis 2001), particularly those that compare organic acids in fresh soft cheeses (Glass 1995).

Recent work has explored methods to predict *L. monocytogenes* behavior in cheese (De Araujo 2017, Ostergaard 2014, Ostergaard 2015, Uhlich 2006) and model cheese systems (Bolton 1999, Schvartzman 2011) based on physical parameters (e.g. pH, moisture, salt, and/or a_w) and storage temperature, with the goal of identifying limits for its control. Additionally, existing models include lactic acid starter cultures in cheese formulation (De Araujo 2017, Ostergaard 2014, Ostergaard 2015, Uhlich 2006), which have repeatedly been shown to confer additional *L. monocytogenes* protection beyond that of formulation (Alves 2006, Naldini 2009, Pingitore 2012). Beyond this concern, a number of researchers have found notable variation between cheese varieties in allowing for *L. monocytogenes* growth (Augustin 2005, Genigeorgis 1991) and existing models built from broth system data (e.g. ComBase) have been shown by

other authors to underestimate observed growth levels of *L. monocytogenes* in cheeses with very high (0.98-0.99) a_w values, such as cottage cheese (a_w ~0.98) (Schvartzman 2011). Unfortunately, no published model has identified parameters for *L. monocytogenes* control in fresh soft cheese during extended refrigerated shelf-life (e.g. 8 weeks at 4°C). Therefore, a knowledge gap exists in safely formulating fresh soft cheeses using only acidulating organic acid(s) and refrigeration for listerial control. Manufacturers of soft fresh cheese varieties would benefit from a model that could predict growth or inhibition boundaries based on the acidulant used as well as the cheese pH in validating and adjusting their cheese formulations.

The objective of the current study was to evaluate the effects of four acid types (citric, lactic, acetic, and propionic) on *L. monocytogenes* behavior in a model cheese system designed to mimic a directly acidified fresh soft cheese at varying pH and moisture levels. Subsequently, a statistical model predicting refrigerated growth of the pathogen based on model cheese parameters was constructed.

MATERIALS AND METHODS

Model cheese manufacture. Thirty-two formulations were identified using a full-factorial design targeting four acid types used (citric, lactic, acetic, and propionic), four pH values (5.25, 5.50, 5.75, and 6.00), and two moisture levels (50% and 56%) (Table 4-1). Combinations of model cheese formulations were designed to represent a system similar to fresh soft cheese varieties (e.g. queso fresco, ricotta, or fresh mozzarella). All treatments were replicated in duplicate trials.

Formulations included pasteurized cream (Select Heavy Whipping Cream, 36% milkfat, Kemp's, Minneapolis, MN), sterile deionized water, micellar casein (CasPro™ 8500, Milk

Specialties Global, Eden Prairie, MN), lactose (NF Lactose Monohydrate, Foremost Farms, Baraboo, WI), sodium chloride (Fisher, Waltham, MA), and one of four organic acids—citric (monohydrate, Fisher; diluted to 50% acid strength in DI water), lactic (85%, Millipore, Burlington, MA), acetic (Fisher; diluted to 85% strength), or propionic (Fisher; diluted to 85% strength). Ingredient levels were adjusted as needed to meet model criteria, with organic acids added at levels to reach target pH values. Model cheese pH before acid addition was found to be ~6.1-6.2.

All ingredients were combined in a sterilized stand mixer bowl (KitchenAid model KV25G0X, Whirlpool Co., Benton Harbor, MI) with a batch size of ~1,500 g (Table 4-S1). Ingredients were mixed for 2 minutes until homogeneous. 700 g was transferred into a sterilized beaker for uninoculated samples and 50 g into a gas-impermeable pouch (3-mil high barrier EVOH pouches, Deli 1 material, oxygen transmission rate of 2.3 cm^3/cm^2 for 24 h at 23°C, water transmission rate of 7.8 g/m^2 for 24 h at 37.8°C, and 90% relative humidity; WinPak, Winnipeg, Manitoba, Canada) and vacuum-packaged (Multivac AGW, Sepp Haggemuller KG, Wolfertschewenden, Germany) for proximate analysis. The remaining 750 g portion was used for inoculated samples.

Sample proximate analysis. Moisture (5 h, 100°C vacuum oven method, AOAC 926.08), pH (direct measurement, Accumet Basic pH meter and Orion 8104 combination electrode, Thermo Fisher Scientific, Waltham, MA, AOAC 981.12), NaCl (measured as percentage of Cl^-, $AgNO_3$ potentiometric titration; Mettler DL22 food and beverage analyzer, Columbus, OH, AOAC 983.14), and water activity (Decagon AquaLab 4TE Water Activity Meter, Pullman, WA) were measured in triplicate uninoculated samples for each treatment (AOAC 2000).

Samples were stored vacuum-sealed at 4°C, with analyses conducted within 72 hours of model cheese manufacture.

Inoculum preparation. *L. monocytogenes* strains LM 108 (hard salami isolate, serotype 1/2b), LM 301 (cheddar cheese isolate, serotype 1/2a), LM 310 (goat's milk cheese isolate associated with illness, serotype 4b), R2-500 (soft Hispanic-style cheese isolate, 4b), and R2-501 (clinical isolate associated with soft Hispanic-style cheese, 4b) were grown individually in 10 ml Trypticase soy broth (TSB; BBL, BD, Sparks, MD) at 37°C for 18-22 h. Cells were harvested via centrifugation (2,500 × *g* for 20 min) and suspended in 4.5 ml 0.1% buffered peptone water (BPW; pH 7.2) after which equivalent populations of each isolate were combined in BPW in a five-strain cocktail. Strain purities were verified by streaking on modified Oxford agar (MOX; *Listeria* selective agar base, Difco, BD, Sparks, MD) and Trypticase soy agar (BBL, BD). Strain and cocktail populations were verified by plating on MOX.

Inoculation and storage. Model cheeses were batch inoculated at a 0.5% (v/w) level with *L. monocytogenes* directly into mixer bowls in which model cheeses were made to provide approximately 3 log CFU/g cheese. To distribute inoculum, cheeses were mixed at high speed for 2 min. Thereafter, 25-g inoculated samples were placed into gas-impermeable pouches and vacuum-packaged. Uninoculated samples of 25-g were additionally placed into pouches and vacuum-packaged. All samples were stored at 4 ± 0.5°C for 8 weeks.

Microbiological enumeration and pH/appearance monitoring. Triplicate inoculated samples and duplicate uninoculated samples were assayed for *L. monocytogenes* and pH,

respectively, at time zero and weeks 1-8. Additionally, at time zero and weeks 4 and 8 duplicate uninoculated samples were assayed for aerobic plate count (APC), lactic acid bacteria (LAB), and yeasts/mold (YM). 25-g samples were enumerated following addition of 50 ml BPW to each package and homogenizing with a stomacher for 30 sec (Neutec Masticator, Neutec Group, Inc., Farmingdale, NY). Serial dilutions of inoculated samples were spread plated on MOX (35°C, 48 h), while uninoculated samples were pour plated for APC (Plate Count agar, 35°C, 48 h, BBL, BD) and LAB (De Man, Rogosa and Sharpe agar, 35°C, 48 h anaerobic storage, BBL, BD) and spread plated for YM (Potato Dextrose agar, 21°C, 120 h, BBL, BD). Changes in direct pH and odor/appearance were monitored weekly.

Development of predictive model. Individual growth curves of *L. monocytogenes* in model cheeses were modeled as a function of time using the Combase DMFit (Institute of Food Research, Norwich, U.K.) Excel add-on (version 2007, Microsoft Corporation, Seattle, WA) based on Baranyi models (Baranyi 2006). Data points (n=9, each representing the average of triplicate *L. monocytogenes* samples enumerated at time points 0-8 weeks) from individual treatments in each trial were analyzed separately to obtain growth curves, with a total of 64 *L. monocytogenes* growth curves generated. DMFit software was used to generate the growth rate (in log CFU/g per week) and lag phase (in weeks) for each fitted curve. In treatments with a decline in *L. monocytogenes* levels over the 8-week study period (and hence a fitted negative growth rate value), growth rate was assigned as 0 log CFU/g per week and lag phase assigned as 8 weeks. The time endpoint of 8 weeks was considered right-censored as no observed data past this time was used. Individual curves were analyzed using JMP statistical analysis software (JMP 13, SAS, Cary, NC). Independent, continuous factors were target pH (5.25-6.00) and target

moisture (50-56%), while independent, fixed factors were acid type (citric, lactic, acetic, or propionic) and trial (1 or 2). Dependent variables were growth rate and lag phase. The full model containing linear and quadratic terms for all independent factors and a single pairwise factor interaction term (target pH*target moisture) served as the initial model. The model was revised by removing statistically insignificant terms (those with p-values >0.05).

RESULTS

Composition of model cheeses. The primary objective of this project was to identify *L. monocytogenes* growth and inhibition limits in high-moisture cheeses stored at 4°C under differing combinations of acid type, pH, and moisture in a representative model cheese system. Proximate analysis results are reported in Table 4-S2. Total acid and undissociated acid levels were calculated for each treatment based on sample pH and pK_a values of the tested acids (3.13 for citric, 3.86 for lactic, 4.76 for acetic, and 4.87 for propionic) using the Henderson-Hasselbalch equation [$pH = pK_a + \log([A^-]/[HA])$].

Uninoculated sample analysis. APC, LAB, and YM counts as well as pH stayed relatively stable throughout the 8-week study in uninoculated samples stored vacuum-packaged at 4°C (data not shown). Average change in pH for all formulations over 8 weeks was found to be +0.04, ranging from -0.13 to +0.21 pH units within a single formulation and trial (data not shown). Time 0 APC, LAB, and YM counts were 2.14 ± 0.73, 1.60 ± 0.51, and 1.63 ± 0.61 log CFU/g and increased to 2.66 ± 1.43, 2.55 ± 1.46, and 2.68 ± 1.49 log CFU/g, respectively, by the 8-week sampling point (data not shown). Changes in native microflora of the samples did not

noticeably correspond to acid type, target cheese pH, or target cheese moisture. No obvious changes in odor or appearance were found in any of the samples tested.

L. monocytogenes *behavior.* Thirty-two formulations were inoculated and tested through 8 weeks' storage at 4°C. Starting inoculum level was 3.49 ± 0.25 log CFU/g. Eighteen treatments supported *L. monocytogenes* growth (defined as ≥1 log CFU/g increase from time zero *L. monocytogenes* count). Based on lag phase and growth rate models, predicted times-to-growth ranged from 0.7 to >8.0 weeks (Table 4-2).

All treatments formulated with citric acid supported growth of the pathogen, with 1-log growth predicted at 0.7 to 5.1 weeks. Lower pH (5.25 and 5.50) citric acid formulated samples showed a slight delay of *L. monocytogenes* growth from that of higher pH (5.75 and 6.00) samples (Figure 4-1).

Treatments formulated with lactic acid to pH values ≥5.50 supported growth of *L. monocytogenes* over the study period. Predicted times-to-growth ranged from 0.8 to 6.9 weeks. Samples formulated to pH 5.50 showed times-to-growth of 2 and 4 weeks in 56% and 50% moisture samples, respectively; however, the models found no statistical difference between moisture levels ($p>0.05$) and predicted 4.5 weeks time-to-growth for both formulations. Samples formulated with lactic acid to target pH 5.25 inhibited *L. monocytogenes* growth for 8 weeks at 4°C, regardless of moisture level (Figure 4-2).

In contrast, none of the samples formulated to pH 5.75 and below with acetic (Figure 4-3) or propionic acid (Figure 4-4) supported growth through the 8 week storage period. Samples formulated to pH 6.00 with acetic acid realized growth in 1.5-2.5 and 2.0-4.5 weeks in samples formulated to target 56% and 50% moisture, respectively, compared with model predictions of

5.4 weeks for each. Samples formulated to pH 6.00 with propionic acid realized growth in 1.5-3.0 and 3.5-5.5 weeks in samples formulated to target 56% and 50% moisture, respectively, compared with model predictions of 5.5 weeks for each.

Calculated undissociated acid ranges in which *L. monocytogenes* growth was observed in model cheeses were 0.01-0.21 mM, 0.10-1.94 mM, 0.67-0.72 mM, and 0.97-1.03 mM for citric, lactic, acetic, and propionic acids, respectively. Minimum undissociated acid levels inhibiting *L. monocytogenes* growth over the 8-week study period were 4.87, 4.08, and 4.67 mM in cheeses formulated with lactic, acetic, and propionic acids, respectively, while 0.21 mM undissociated citric acid formulated into pH-5.25 targeted model cheese was found insufficient to prevent listerial growth.

Modeling microbial growth. R^2 values for DMFit growth curves (i.e. for those treatments in which growth rate and lag phase were not censored to 0 log CFU/g per week and 8 weeks, respectively) ranged from 0.83 to 1.00 and averaged 0.95. Growth rate values ranged from 0.00 to 2.57 log CFU/g per week, while lag phase values ranged from 0.00 to 8.00 weeks (Table 4-2).

All growth curves generated were inputted into JMP statistical software and analyzed using a Standard Least Squares model. Full models containing linear terms for all independent factors (acid type, target pH, target moisture, and trial) and a single pairwise factor interaction term (target pH*target moisture) served as the initial models for growth rate and lag phase. Models were reduced by removing statistically insignificant terms (those with p-values > 0.05), including target moisture ($p = 0.5185$ for the full growth rate model, $p = 0.6855$ for the full lag phase model), target pH*target moisture ($p = 0.2575$ for the full growth rate model, $p = 0.9277$ for the full lag phase model), and trial ($p = 0.8553$ for the full growth rate model, $p = 0.8178$ for

the full lag phase model). The reduced growth rate model contained terms acid type ($p < 0.0001$ for citric, lactic, and acetic acids) and target pH ($p < 0.0001$), and the reduced lag phase model contained terms acid type ($p < 0.0001$, $p = 0.0066$, and < 0.0001 for citric, lactic, and acetic acids, respectively) and target pH ($p < 0.0001$) (2 models constructed, Table 4-3). Growth rate could be estimated by the equation Growth Rate (in log CFU/g per week) = –5.58 + 1.09(pH) – 0.36(acetic) + 0.47(citric) + 0.35(lactic) and lag phase by the equation Lag Phase (in weeks) = 41.89 – 6.79(pH) + 2.62 (acetic) – 3.69(citric) – 1.33(lactic). For both models, the use of an organic acid (acetic, citric, or lactic) directs the assignment of 1 to its multiplier and 0 to the multipliers of the other organic acids. R^2 values for reduced growth rate and lag phase models were 0.73 and 0.71, respectively. Although there were observable differences in time-to-growth between cheeses of varying moisture levels, moisture was statistically insignificant ($p > 0.05$) across all acid types and pH values tested.

Tukey's Honest Significant Differences (HSD) analysis of the reduced growth rate model identified two distinct, significantly different ($p < 0.05$) groups containing citric and lactic acids (group I) and acetic and propionic acids (group II). Tukey's HSD analysis of the reduced lag phase model identified three distinct groups: citric (group I), lactic (group II), and acetic and propionic acid (group III). Predicted growth rate, lag phase, and *L. monocytogenes* time-to-growth (1-log CFU/g) for model cheese formulations of pH 5.25 to 6.00 in 0.05 pH unit increments are listed in Table 4-4 and model parameter significant effects given in Table 4-5.

DISCUSSION

It is well documented that organic acids have varying degrees of listerial inhibition. Minimum inhibitory concentration (MIC) values of undissociated citric, lactic, acetic, and

propionic acids within a pH range of 5.2 to 5.6 were found to be 3.8, 5.0, 19.0 and 11.0 mM, respectively, for *L. monocytogenes* strains grown in a broth system at 30°C (Wemmenhove 2016). Undissociated citric, lactic, acetic, and propionic acid concentrations in which *L. monocytogenes* growth was observed in model cheeses in our study were ≤0.21 mM, ≤1.94 mM, ≤0.72 mM, and ≤1.03 mM, respectively, all below the MIC values identified (Wemmenhove 2016).

L. monocytogenes inoculation studies in fresh soft cheese varieties have shown variable efficacy of organic acids. Davies and others manufactured and inoculated ricotta (pH 5.9, manufactured with vinegar) with 10^2-10^3 CFU/g *L. monocytogenes* and monitored the pathogen's growth with storage at 6-8°C. The group found *L. monocytogenes* had increased >1 log CFU/g after 2 weeks and continued to grow to a final level of ca. 7 log CFU/g) (Davies 1997). Comparatively, our model estimated 6.5 weeks' time-to-growth in pH 5.9 fresh soft cheese made with acetic acid; however, the typical moisture content for ricotta (~70%) and elevated storage temperature fall outside our model parameters.

Within the context of a literature model constructed from 114 scientific challenge studies of all cheese varieties stored under a variety of temperatures and conditions, growth of *L. monocytogenes* was observed at an average pH of 6.11 (n=413), while inhibition was observed at an average pH of 5.26 (n=690). Growth probabilities for pH 5.25, 5.5, 5.75, and 6.0 model cheeses of 56% moisture were calculated to be 55%, 70%, 82%, and 90%, respectively (Engstrom 2012). Coincidentally, several cheese varieties, especially within categories outside of fresh soft, employ the use of lactic acid-producing starter cultures, and agreement between the literature and fresh soft cheese models upon inhibition near pH 5.25 reflects this.

Genigeorgis and colleagues evaluated the ability of 24 varieties of store-bought cheeses to support growth of *L. monocytogenes* at 4°C, finding fresh soft varieties able to support growth including queso fresco (pH 6.5), queso ranchero (pH 6.2), queso panella (pH 6.2-6.7), ricotta (pH 5.9-6.1), and cottage cheese (pH 4.9-5.1; moisture approx. 80%) and those unable to support *L. monocytogenes* growth including cream cheese (pH 4.8) and feta (pH 4.2-4.3) (Genigeorgis 1991). Ricotta was found to be the most supportive cheese of *L. monocytogenes* growth among those tested, with its low water-phase-salt (<0.7%), relatively high pH (5.9-6.1), and low level of competitive microflora (<2.0 log CFU/g) attributed to this designation. All three ricotta samples challenged by the authors contained vinegar, suggesting reduced efficacy of acetic acid at high pH values, a finding confirmed in our study. The authors challenged five commercial cottage cheeses, with a single cottage cheese containing acetic acid (pH 5.0, 1.16% brine, 5.00 CFU/g APC at day zero, no starter culture) found to inhibit growth; the other 4 cottage cheeses (all made without the use of acetic acid and with pH values of 4.9-5.1, water-phase-salt 1.00-1.14%, 3.00-6.41 CFU/g APC at day zero, and with or without starter culture) were found to support growth at 4°C (Genigeorgis 1991). Genigeorgis and colleagues found day zero APC for directly acidified retail soft cheeses, all of which supported *L. monocytogenes* growth at 4°C, to range from <2.00 to 7.87 log CFU/g, compared with day zero APC of 2.14 ± 0.73 log CFU/g observed in the current study. Though our data fall in the lower end of this range, the authors noted that APC do not consistently dictate the growth of *L. monocytogenes* in cheeses made without starter cultures (Genigeorgis 1991).

Despite significant research on *L. monocytogenes* behavior in a variety of cheeses, accurately predicting the pathogen's growth in the commodity remains elusive. Schvartzman and colleagues found a critical a_w value of ~0.965 in highly contaminated (~3 log CFU/ml) semisoft

laboratory scale model cheeses made using pasteurized milk, skim milk powder, sodium chloride, and rennet, with pH adjustments using lactic acid or NaOH to pH 5.6-6.5 and a_w 0.938-0.996 at two contamination levels (~1 and ~3 log CFU/ml) (Schvartzman 2011). The authors validated their resulting growth model with model cheeses formulated to pH values of 5.84-6.44 and a_w values of 0.959-0.982. When growth was observed, increase in *L. monocytogenes* was found to be a_w and contamination level dependent, with no discernable effect from pH; the authors did note, however, that the relatively narrow range of pH's tested (5.6-6.5) might account for the limited effect of pH on *L. monocytogenes* growth observed in their model. In comparing actual data to ComBase predictions, the authors found ComBase to have a poor fit ($R^2 = 0.37$) versus observed data, while the fit with the authors' own ordinary logistic regression model had a good fit ($R^2 = 0.94$). The authors noted that ComBase underestimated the observed amount of growth in 70% of cases at a_w values of 0.98-0.99 while the model overestimated cases where a_w values were ~0.96-0.97; overall, ComBase correctly predicted growth initiation (≥0.5 log CFU/g) in 41% of cases in which growth was actually observed (Schvartzman 2011).

This study investigated the factors of pH, acid type, and moisture on *L. monocytogenes* behavior at 4°C in a model system meant to mimic fresh soft cheese. Predictive models developed for growth rate and lag phase confirmed that antilisterial activity of the studied organic acids falls in the descending order of propionic ~ acetic > lactic > citric acid in high-moisture cheeses, and suggest that modifying pH or acidulant significantly influences *L. monocytogenes* growth. These models can be used in estimating *L. monocytogenes* boundary conditions for growth within fresh soft cheeses.

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Table 4-1. Target ranges for three factors in design of experiment.

Factor	**Level 1**	**Level 2**	**Level 3**	**Level 4**
Acid Type	Citric	Lactic	Acetic	Propionic
pH	5.25	5.50	5.75	6.00
% Moisture	50	56		

Table 4-2. Modeled lag phase, growth rate, and predicted time to 1-log growth of *Listeria monocytogenes* compared with observed time to 1-log growth in model cheeses of 50-56% moisture stored at 4°C for 8 weeks.

Formulation				Lag Phase (weeks)*	Growth Rate (log CFU/g per week)*	Time to 1-log Growth (weeks)	
Acid Type	pH	Moisture (%)	NaCl (%)			Predicted	Observed**
Citric	5.25	50	1.25	0.0 0.0	0.9 0.8	5.13	1.5 1.5
Citric	5.25	56	1.25	0.0 0.0	0.6 0.7	5.13	2.5 2.0
Citric	5.50	50	1.25	0.0 0.0	1.1 1.1	3.10	1.5 1.0
Citric	5.50	56	1.25	0.0 0.0	0.7 1.3	3.10	1.5 1.0
Citric	5.75	50	1.25	0.0 0.0	1.2 1.0	0.85	0.5 1.0
Citric	5.75	56	1.25	0.0 0.0	1.3 1.3	0.85	1.0 0.5
Citric	6.00	50	1.25	0.0 0.0	1.0 1.1	0.70	0.5 0.5
Citric	6.00	56	1.25	0.0 0.0	0.9 1.7	0.70	1.0 0.5
Lactic	5.25	50	1.25	8.0 8.0	0.0 0.0	6.92	>8.0 >8.0
Lactic	5.25	56	1.25	8.0 8.0	0.0 0.0	6.92	>8.0 >8.0
Lactic	5.50	50	1.25	1.6 3.2	0.4 1.1	4.51	4.0 4.0
Lactic	5.50	56	1.25	0.0 0.0	0.7 0.6	4.51	2.0 2.0
Lactic	5.75	50	1.25	0.0 0.0	1.2 0.8	2.47	1.0 1.5
Lactic	5.75	56	1.25	0.4 0.0	1.5 1.0	2.47	1.0 1.0
Lactic	6.00	50	1.25	0.0	1.4	0.76	1.0

				0.0	1.6		1.0
Lactic	6.00	56	1.25	0.5	2.6	0.76	1.0
				0.0	1.6		0.5
Acetic	5.25	50	1.25	8.0	0.0	>8.0	>8.0
				8.0	0.0		>8.0
Acetic	5.25	56	1.25	8.0	0.0	>8.0	>8.0
				8.0	0.0		>8.0
Acetic	5.50	50	1.25	8.0	0.0	>8.0	>8.0
				8.0	0.0		>8.0
Acetic	5.50	56	1.25	8.0	0.0	>8.0	>8.0
				8.0	0.0		>8.0
Acetic	5.75	50	1.25	8.0	0.0	>8.0	>8.0
				8.0	0.0		>8.0
Acetic	5.75	56	1.25	8.0	0.0	>8.0	>8.0
				8.0	0.0		>8.0
Acetic	6.00	50	1.25	0.0	0.6	5.41	2.0
				4.9	1.3		4.5
Acetic	6.00	56	1.25	0.0	0.6	5.41	1.5
				0.0	0.6		2.5
Propionic	5.25	50	1.25	8.0	0.0	>8.0	>8.0
				8.0	0.0		>8.0
Propionic	5.25	56	1.25	8.0	0.0	>8.0	>8.0
				8.0	0.0		>8.0
Propionic	5.50	50	1.25	8.0	0.0	>8.0	>8.0
				8.0	0.0		>8.0
Propionic	5.50	56	1.25	8.0	0.0	>8.0	>8.0
				8.0	0.0		>8.0
Propionic	5.75	50	1.25	8.0	0.0	>8.0	>8.0
				8.0	0.0		>8.0
Propionic	5.75	56	1.25	8.0	0.0	>8.0	>8.0
				8.0	0.0		>8.0
Propionic	6.00	50	1.25	0.0	0.4	5.47	3.5
				0.0	0.2		5.5
Propionic	6.00	56	1.25	0.0	0.5	5.47	3.0
				0.0	0.5		1.5

*Lag Phase and Growth Rate determined using DMFit modeling of growth curves from duplicate trials.
**Point of 1-log CFU/g increase from time 0 count in duplicate trials; rounded to nearest half or full integer.

Table 4-3. Models for lag phase and growth rate of *Listeria monocytogenes* at 4°C in high-moisture model cheeses of pH 5.25-6.00, 50-56% moisture, and 1.25% salt.

	Lag Phase (weeks)		Growth Rate (log CFU/g per week)	
Term	**Coefficient**	**P-value**	**Coefficient**	**P-value**
Intercept	41.89	<0.0001	-5.58	<0.0001
pH	-6.79	<0.0001	1.09	<0.0001
Acid Type [Citric]	-3.69	<0.0001	0.47	<0.0001
Acid Type [Lactic]	-1.33	0.0066	0.35	<0.0001
Acid Type [Acetic]	2.62	<0.0001	-0.36	<0.0001

Table 4-4. Predicted *Listeria monocytogenes* lag phase, growth rate, and time to 1-log growth in model cheeses of 50-56% moisture stored at 4°C based on cheese pH and acid type used. Predicted lag phase and growth rate were based on primary and secondary models using DMFit and JMP statistical programs.

	Acid Type											
	Citric			Lactic			Acetic			Propionic		
Cheese pH	Lag Phase (weeks)	Growth Rate (log CFU/g per week)	Time to 1-log Growth (weeks)	Lag Phase (weeks)	Growth Rate (log CFU/g per week)	Time to 1-log Growth (weeks)	Lag Phase (weeks)	Growth Rate (log CFU/g per week)	Time to 1-log Growth (weeks)	Lag Phase (weeks)	Growth Rate (log CFU/g per week)	Time to 1-log Growth (weeks)
5.25	2.5	0.6	4.2	4.9	0.5	6.9	>8.0*	0.0**	>8.0	>8.0*	0.0**	>8.0
5.30	2.2	0.7	3.7	4.6	0.6	6.4	>8.0*	0.0**	>8.0	>8.0*	0.0**	>8.0
5.35	1.9	0.7	3.2	4.2	0.6	5.9	>8.0*	0.0**	>8.0	8.0	0.0**	>8.0
5.40	1.5	0.8	2.8	3.9	0.7	5.4	7.8	0.0**	>8.0	7.6	0.0**	>8.0
5.45	1.2	0.8	2.4	3.5	0.7	4.9	7.5	0.0	>8.0	7.3	0.0**	>8.0
5.50	0.9	0.9	2.0	3.2	0.8	4.5	7.2	0.1	>8.0	6.9	0.0**	>8.0
5.55	0.5	0.9	1.6	2.9	0.8	4.1	6.8	0.1	>8.0	6.6	0.0	>8.0
5.60	0.2	1.0	1.2	2.5	0.9	3.7	6.5	0.2	>8.0	6.3	0.1	>8.0
5.65	0.1	1.1	1.0	2.2	0.9	3.3	6.1	0.2	>8.0	5.9	0.1	>8.0
5.70	0.0*	1.1	0.9	1.9	1.0	2.9	5.8	0.3	>8.0	5.6	0.2	>8.0
5.75	0.0*	1.2	0.9	1.5	1.0	2.5	5.5	0.3	>8.0	5.2	0.2	>8.0
5.80	0.0*	1.2	0.8	1.2	1.1	2.1	5.1	0.4	7.7	4.9	0.3	>8.0
5.85	0.0*	1.3	0.8	0.8	1.2	1.7	4.8	0.4	7.0	4.6	0.4	7.4
5.90	0.0*	1.3	0.8	0.5	1.2	1.3	4.4	0.5	6.5	4.2	0.4	6.7
5.95	0.0*	1.4	0.8	0.2	1.3	0.9	4.1	0.6	5.9	3.9	0.5	6.0
6.00	0.0*	1.4	0.7	0.0*	1.3	0.8	3.8	0.6	5.4	3.6	0.5	5.5

*Predicted lag phase was <0.0 or ≥8.1 weeks; therefore, censored values of 0.0 or >8.0 were assigned.
**Predicted growth rate was <0.0; therefore, a censored value of 0.0 was assigned.

Table 4-5. Model parameter significant effects (P < 0.05) for lag phase and growth rate of *Listeria monocytogenes* in model cheeses of pH 5.25-6.0 and 50-56% moisture stored at 4ºC.

	Lag Phase (weeks)		Growth Rate (log CFU/g per week)	
Variable	**ChiSquare**	**Prob > ChiSq**	**ChiSquare**	**Prob > ChiSq**
Target pH	48.30	<0.0001	55.61	<0.0001
Acid type	31.30	<0.0001	33.33	<0.0001

Table 4-S1. Model cheese ingredient formulations.

Acid	Target pH	Target Moisture (%)	Micellar Casein (%)	Cream (%)	Sterile Water (%)	Lactose (%)	NaCl (%)	Acid (%)			
								Citric (50% strength)	Lactic (85% strength)	Acetic (85% strength)	Propionic (85% strength)
Citric	5.25	50	20.60	64.45	10.24	2.40	1.15	1.15	0	0	0
Citric	5.25	56	18.88	53.66	22.75	2.40	1.16	1.15	0	0	0
Citric	5.50	50	21.14	64.45	10.15	2.40	1.14	0.71	0	0	0
Citric	5.50	56	19.37	53.66	22.71	2.40	1.15	0.71	0	0	0
Citric	5.75	50	21.14	64.45	10.48	2.40	1.14	0.39	0	0	0
Citric	5.75	56	19.37	53.66	23.03	2.40	1.15	0.39	0	0	0
Citric	6.00	50	21.14	64.45	10.73	2.40	1.14	0.14	0	0	0
Citric	6.00	56	19.71	53.66	22.93	2.40	1.15	0.14	0	0	0
Lactic	5.25	50	20.49	64.15	10.73	2.40	1.13	0	1.10	0	0
Lactic	5.25	56	19.06	53.66	22.73	2.40	1.15	0	1.00	0	0
Lactic	5.50	50	20.80	64.45	10.57	2.40	1.13	0	0.65	0	0
Lactic	5.50	56	19.37	53.66	22.81	2.40	1.15	0	0.61	0	0
Lactic	5.75	50	20.80	64.45	10.84	2.40	1.13	0	0.38	0	0
Lactic	5.75	56	19.37	53.66	23.04	2.40	1.15	0	0.38	0	0
Lactic	6.00	50	20.80	64.45	11.08	2.40	1.13	0	0.14	0	0
Lactic	6.00	56	19.37	53.66	23.28	2.40	1.15	0	0.14	0	0
Acetic	5.25	50	21.28	64.45	10.07	2.40	1.13	0	0	0.67	0
Acetic	5.25	56	19.37	53.66	22.75	2.40	1.15	0	0	0.67	0
Acetic	5.50	50	21.28	64.45	10.33	2.40	1.13	0	0	0.41	0
Acetic	5.50	56	19.05	53.66	23.34	2.40	1.15	0	0	0.41	0
Acetic	5.75	50	21.28	64.45	10.48	2.40	1.13	0	0	0.26	0
Acetic	5.75	56	19.05	53.66	23.49	2.40	1.15	0	0	0.26	0
Acetic	6.00	50	21.28	64.45	10.65	2.40	1.13	0	0	0.08	0
Acetic	6.00	56	19.05	53.66	23.66	2.40	1.15	0	0	0.08	0
Propionic	5.25	50	20.62	64.45	9.28	2.40	1.13	0	0	0	2.11
Propionic	5.25	56	18.88	53.66	21.79	2.40	1.15	0	0	0	2.11
Propionic	5.50	50	20.80	64.45	9.72	2.40	1.13	0	0	0	1.50
Propionic	5.50	56	19.37	53.66	21.92	2.40	1.15	0	0	0	1.50
Propionic	5.75	50	20.80	64.45	10.34	2.40	1.13	0	0	0	0.88

Propionic	5.75	56	19.37	53.66	22.54	2.40	1.15	0	0	0	0.88
Propionic	6.00	50	20.80	64.45	10.92	2.40	1.13	0	0	0	0.30
Propionic	6.00	56	19.37	53.66	23.12	2.40	1.15	0	0	0	0.30

Table 4-S2. Physicochemical analysis of model cheese formulations.

Formulation #	Acid	pH	Moisture (%)	NaCl (%)	a_w	Total Acid (mM)	*Calculated Undissociated Acid (mM)
1	Citric	5.29±0.05	50.84±0.20	1.25±0.01	0.974±0.006	29.98±0.00	0.21±0.02
2	Citric	5.30±0.08	56.47±0.01	1.23±0.00	0.977±0.007	29.88±0.00	0.20±0.04
3	Citric	5.52±0.03	50.70±0.29	1.23±0.04	0.975±0.006	18.59±0.00	0.08±0.00
4	Citric	5.52±0.03	56.49±0.16	1.26±0.02	0.978±0.008	18.45±0.00	0.07±0.00
5	Citric	5.71±0.02	50.52±0.29	1.23±0.04	0.977±0.005	10.24±0.12	0.03±0.00
6	Citric	5.69±0.04	56.37±0.36	1.23±0.00	0.979±0.007	10.22±0.14	0.03±0.00
7	Citric	5.99±0.02	51.06±0.28	1.24±0.00	0.979±0.007	3.65±0.04	0.01±0.00
8	Citric	6.00±0.04	56.23±0.46	1.22±0.00	0.979±0.004	3.70±0.01	0.01±0.00
9	Lactic	5.09±0.11	49.73±0.43	1.23±0.02	0.959±0.009	151.63±38.77	8.88±4.18
10	Lactic	5.27±0.04	55.61±0.18	1.25±0.01	0.970±0.002	129.21±22.97	4.87±0.49
11	Lactic	5.45±0.05	50.04±0.22	1.23±0.00	0.968±0.003	76.26±4.00	1.94±0.32
12	Lactic	5.51±0.06	55.63±0.07	1.25±0.01	0.973±0.002	68.02±1.30	1.52±0.25
13	Lactic	5.75±0.06	50.33±0.09	1.24±0.00	0.970±0.005	41.82±1.56	0.54±0.10
14	Lactic	5.72±0.03	56.07±0.37	1.25±0.01	0.973±0.003	42.15±1.09	0.57±0.05
15	Lactic	6.02±0.04	50.39±0.10	1.26±0.02	0.973±0.001	13.52±3.22	0.10±0.03
16	Lactic	6.01±0.03	56.00±0.09	1.24±0.01	0.976±0.001	14.63±1.66	0.10±0.02
17	Acetic	5.24±0.01	50.23±0.48	1.25±0.02	0.963±0.007	99.09±0.00	24.86±0.30
18	Acetic	5.24±0.01	56.07±0.78	1.25±0.01	0.971±0.001	99.09±0.00	24.65±0.60
19	Acetic	5.48±0.04	50.16±0.59	1.25±0.02	0.968±0.008	60.61±0.00	9.71±0.80
20	Acetic	5.50±0.04	56.03±0.11	1.24±0.01	0.972±0.005	60.61±0.00	9.43±0.65
21	Acetic	5.69±0.02	50.26±0.43	1.23±0.01	0.973±0.004	38.38±0.14	4.08±0.16
22	Acetic	5.69±0.04	56.48±0.04	1.24±0.00	0.973±0.006	38.38±0.14	4.08±0.28
23	Acetic	5.97±0.04	49.67±0.97	1.25±0.02	0.969±0.002	12.41±0.00	0.72±0.07
24	Acetic	6.01±0.02	56.61±0.22	1.24±0.01	0.976±0.008	12.41±0.00	0.67±0.03
25	Propionic	5.30±0.03	51.04±0.49	1.21±0.01	0.972±0.006	117.70±6.16	31.86±0.16
26	Propionic	5.30±0.01	56.95±0.06	1.23±0.00	0.977±0.001	117.70±6.16	32.14±1.30

27	Propionic	5.49±0.04	50.32±0.49	1.20±0.01	0.971±0.004	80.65±0.00	15.62±1.23
28	Propionic	5.49±0.02	56.05±0.27	1.24±0.00	0.977±0.003	80.65±0.00	15.75±0.62
29	Propionic	5.75±0.04	50.35±0.06	1.24±0.03	0.973±0.005	40.10±0.00	4.68±0.40
30	Propionic	5.75±0.03	56.28±0.09	1.23±0.00	0.979±0.004	40.10±0.00	4.67±0.27
31	Propionic	6.00±0.01	50.60±0.14	1.23±0.01	0.972±0.002	14.82±0.00	1.03±0.02
32	Propionic	6.03±0.02	56.12±1.05	1.23±0.01	0.979±0.004	14.82±0.00	0.97±0.04

*Calculated undissociated acid values were determined via the Henderson-Hasselbalch equation [$pH = pK_a + \log([A^-]/[HA])$]. pK_a values used for citric, lactic, acetic, and propionic acids were 3.13, 3.86, 4.76, and 4.87, respectively.

FIGURE LEGENDS

Figure 4-1. *Listeria monocytogenes* growth at 4°C in model cheeses formulated with citric acid to pH 5.25 and 50% (○) or 56% (●) moisture, pH 5.50 and 50% (∆) or 56% (▲) moisture, pH 5.75 and 50% (□) or 56% (■) moisture, and pH 6.00 and 50% (◇) or 56% (◆) moisture.

Figure 4-2. *Listeria monocytogenes* growth at 4°C in model cheeses formulated with lactic acid to pH 5.25 and 50% (○) or 56% (●) moisture, pH 5.50 and 50% (∆) or 56% (▲) moisture, pH 5.75 and 50% (□) or 56% (■) moisture, and pH 6.00 and 50% (◇) or 56% (◆) moisture.

Figure 4-3. *Listeria monocytogenes* growth at 4°C in model cheeses formulated with acetic acid to pH 5.25 and 50% (○) or 56% (●) moisture, pH 5.50 and 50% (∆) or 56% (▲) moisture, pH 5.75 and 50% (□) or 56% (■) moisture, and pH 6.00 and 50% (◇) or 56% (◆) moisture.

Figure 4-4. *Listeria monocytogenes* growth at 4°C in model cheeses formulated with propionic acid to pH 5.25 and 50% (○) or 56% (●) moisture, pH 5.50 and 50% (∆) or 56% (▲) moisture, pH 5.75 and 50% (□) or 56% (■) moisture, and pH 6.00 and 50% (◇) or 56% (◆) moisture.

Figure 4-1

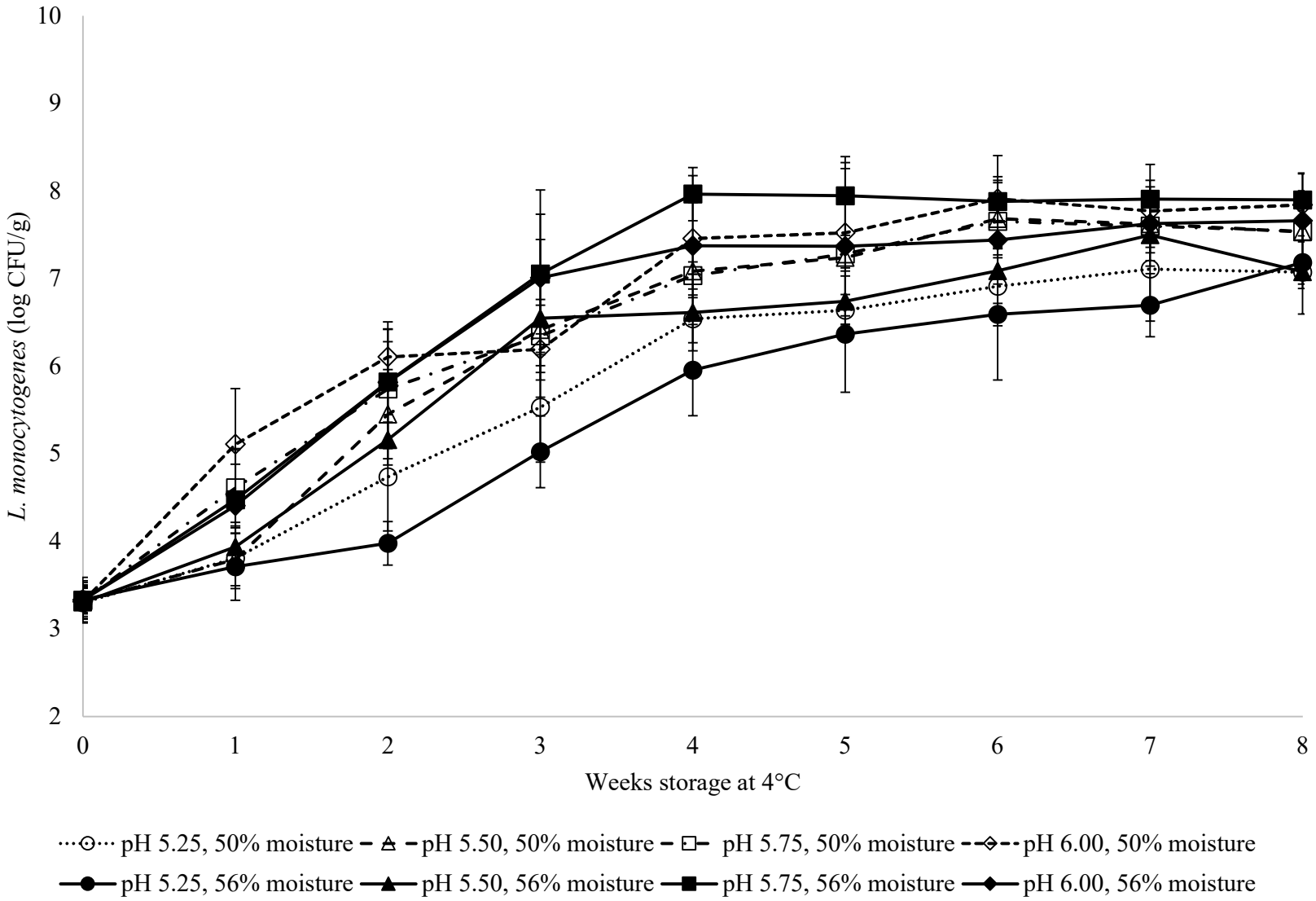

10
9
8
7
6
5
4
3
2
L. monocytogenes (log CFU/g)
0
1
2
3
4
5
6
7
8
Weeks storage at 4°C
pH 5.25, 50% moisture
pH 5.50, 50% moisture
pH 5.75, 50% moisture
pH 6.00, 50% moisture
pH 5.25, 56% moisture
pH 5.50, 56% moisture
pH 5.75, 56% moisture
pH 6.00, 56% moisture

Figure 4-2

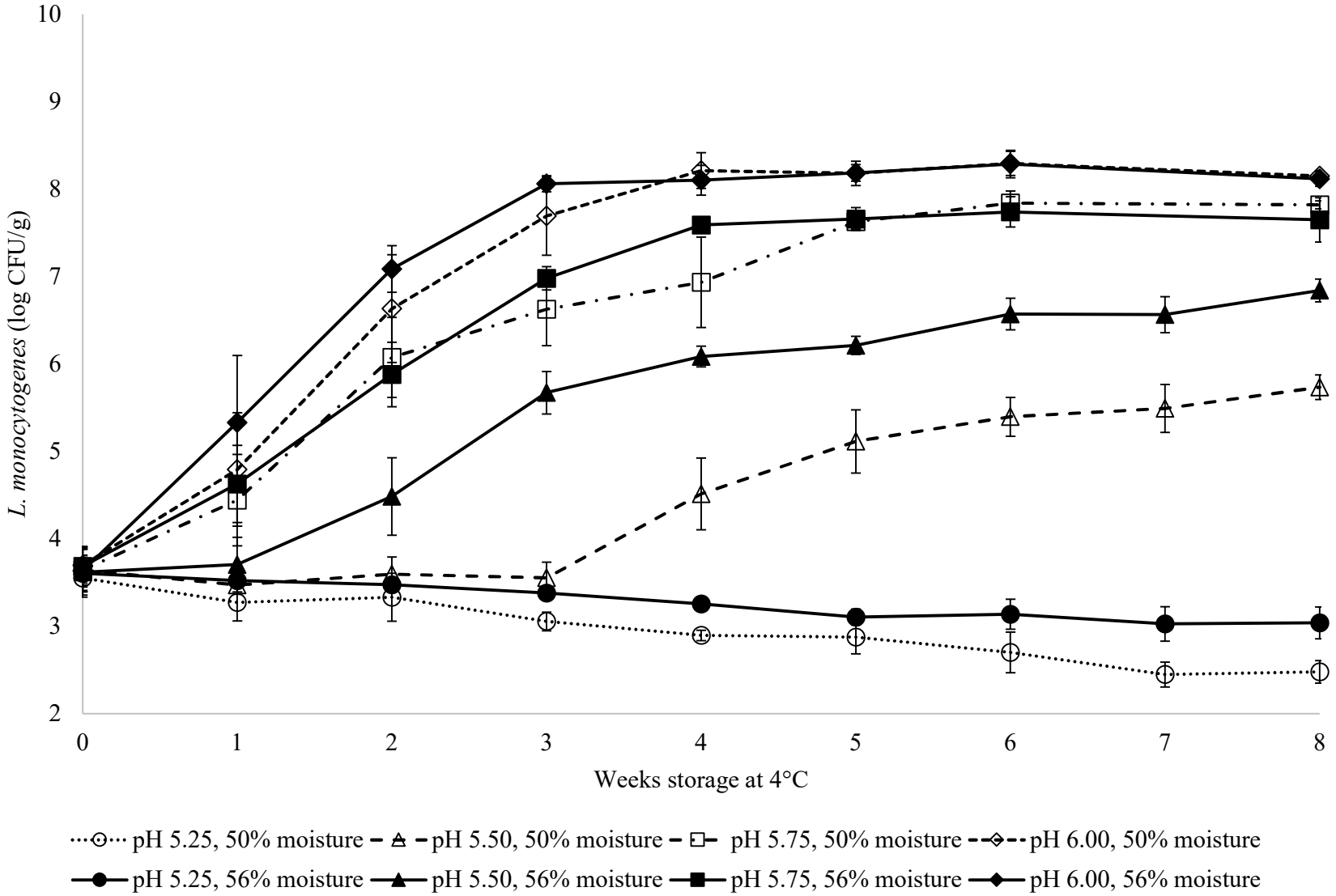

10
9
8
7
6
5
4
3
2
L. monocytogenes (log CFU/g)
0
1
2
3
4
5
6
7
8
Weeks storage at 4°C
pH 5.25, 50% moisture
pH 5.50, 50% moisture
pH 5.75, 50% moisture
pH 6.00, 50% moisture
pH 5.25, 56% moisture
pH 5.50, 56% moisture
pH 5.75, 56% moisture
pH 6.00, 56% moisture

Figure 4-3

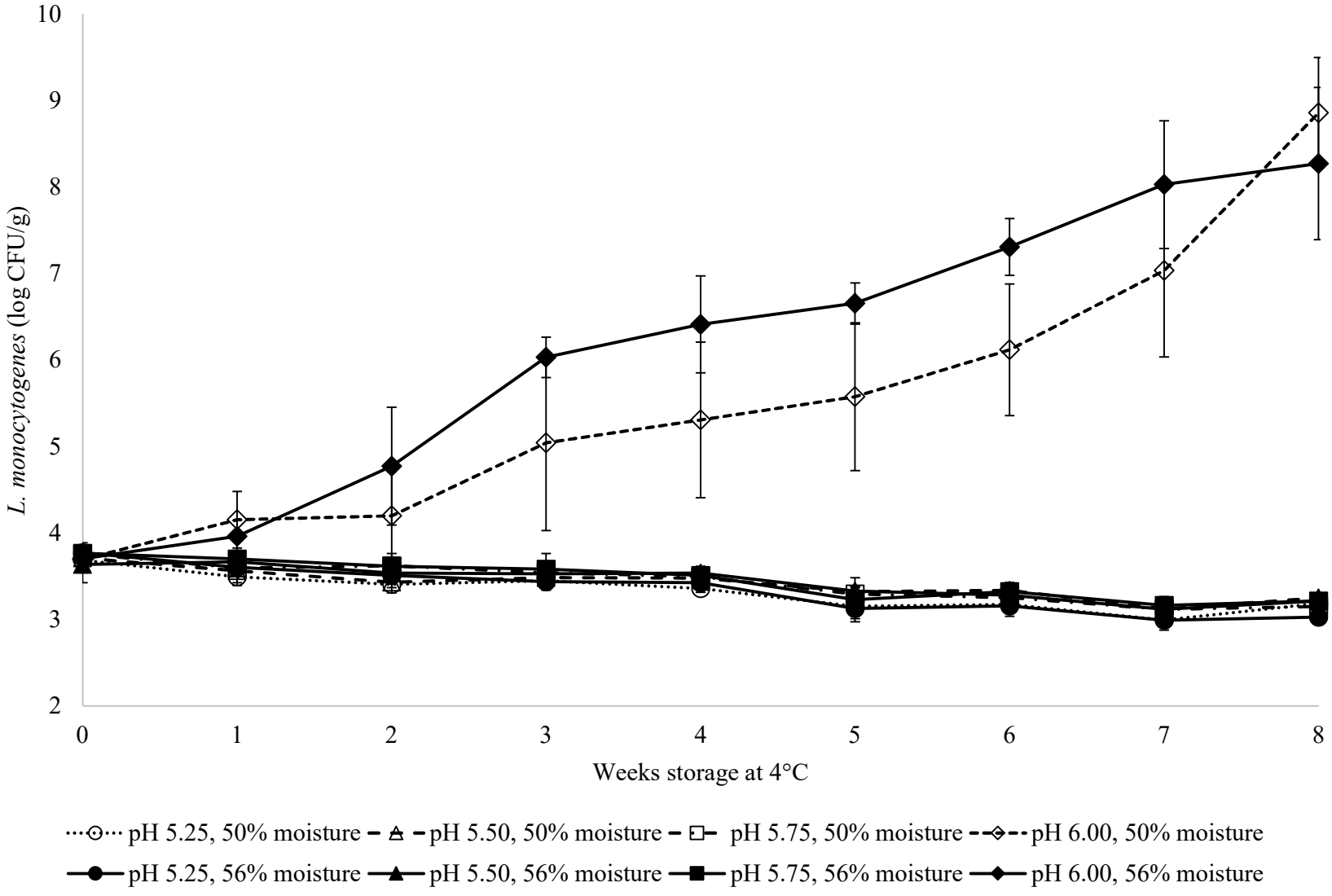
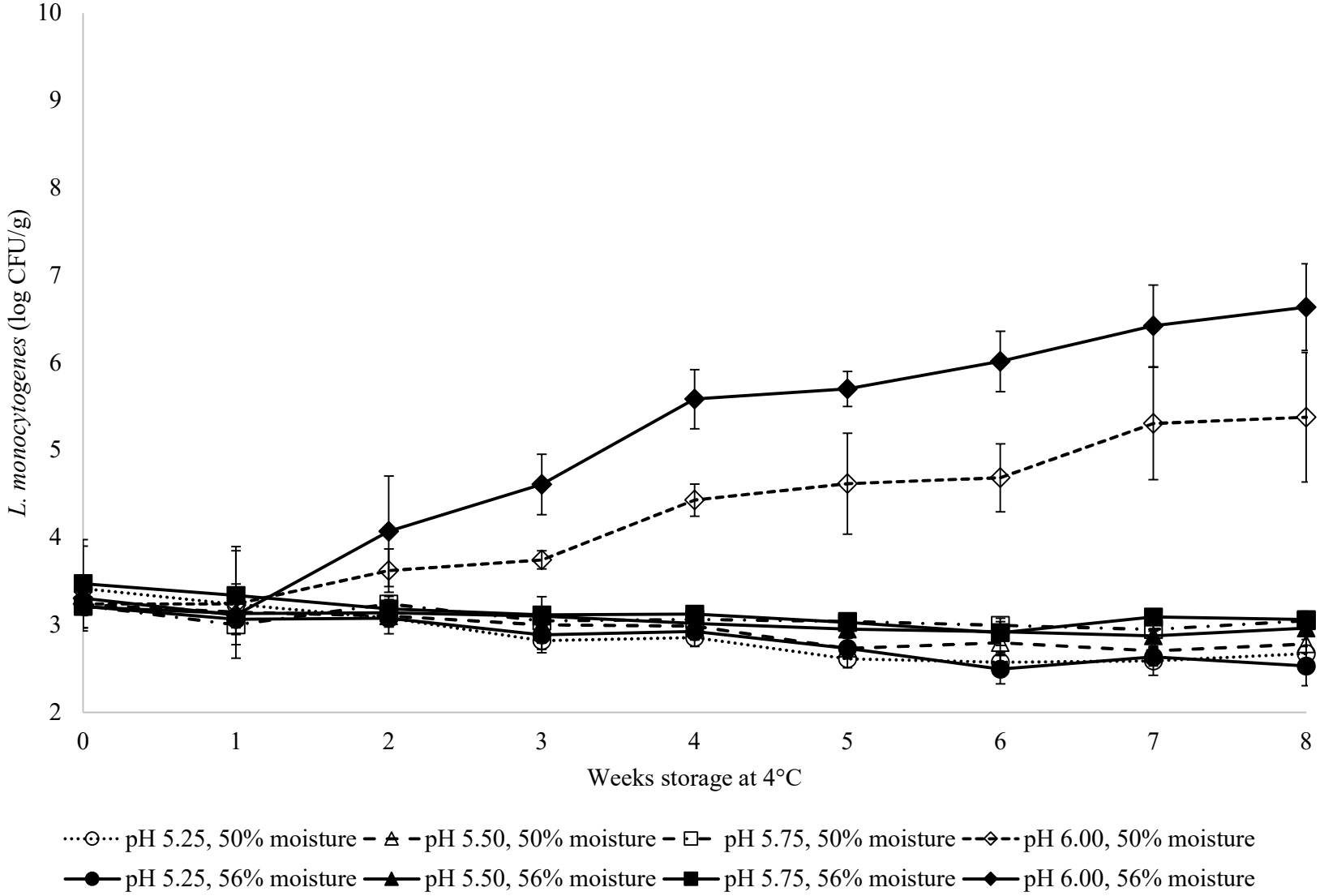

10
9
8
7
6
5
4
3
2
L. monocytogenes (log CFU/g)
0
1
2
3
4
5
6
7
8
Weeks storage at 4°C
pH 5.25, 50% moisture
pH 5.50, 50% moisture
pH 5.75, 50% moisture
pH 6.00, 50% moisture
pH 5.25, 56% moisture
pH 5.50, 56% moisture
pH 5.75, 56% moisture
pH 6.00, 56% moisture

Figure 4-4


10
9
8
7
6
5
4
3
2
L. monocytogenes (log CFU/g)
0
1
2
3
4
5
6
7
8
Weeks storage at 4°C
pH 5.25, 50% moisture
pH 5.50, 50% moisture
pH 5.75, 50% moisture
pH 6.00, 50% moisture
pH 5.25, 56% moisture
pH 5.50, 56% moisture
pH 5.75, 56% moisture
pH 6.00, 56% moisture

CHAPTER 5:

Effect of commercial protective cultures and bacterial fermentates on *Listeria monocytogenes* growth in a refrigerated high-moisture model cheese

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ABSTRACT

Biopreservatives are clean-label ingredients used to control pathogenic and spoilage microorganisms in ready-to-eat foods including cheese. In a first set of experiments, the efficacies of six commercial biopreservatives in controlling *L. monocytogenes* growth at 4°C were tested in a high-moisture model cheese (pH 6.00, 56% moisture, 1.25% salt) made of cream, micellar casein, water, salt, lactose, lactic acid, and a single protective culture (PC-1, PC-2, or PC-3, 10^6 CFU/g target) or bacterial fermentate (CM-1 or CM-2 [cultured milk] or CSV-1 [cultured sugar-vinegar blend], 0.5% or 1.0% level). Cheeses were inoculated with 3-log CFU/g *L. monocytogenes* (5-strain cocktail), after which 25-g samples were vacuum-sealed and stored at 4°C for 8 weeks. *L. monocytogenes* populations from triplicate samples were enumerated weekly on Modified Oxford agar in duplicate trials. *L. monocytogenes* growth (≥1-log increase) was observed in approximately 1 week in control cheese and those formulated with 10^6 CFU/g PC-1 or PC-2. Growth was delayed to 2.5 weeks in model cheeses formulated with 10^6 CFU/g PC-3 or 0.5% CM-2 and to 3 weeks with 0.5% CM-1 or CSV-1. Growth was further delayed to 6.5-7.5 weeks in model cheeses formulated with 1.0% CM-1 or CM-2, while formulation with 1.0% CSV-1 inhibited *L. monocytogenes* growth for 8 weeks. In a second set of experiments, the combined effect of pH and 0.5% CSV-1 on *L. monocytogenes* inhibition was investigated. Incorporation of 0.5% CSV-1 delayed *L. monocytogenes* growth to 3, 6, and >10 weeks in cheeses of pH 6.00, 5.75, and 5.50, respectively, versus growth observed in 1, 1, and 3.5 weeks in control cheeses. These data suggest that certain fermentates have greater antilisterial activity than protective cultures in directly acidified cheeses with direct biopreservative incorporation and refrigerated storage. Further research is needed to optimize conditions to prevent listerial growth utilizing protective cultures in fresh, soft cheeses.

HIGHLIGHTS

- PC-1 and PC-2 did not inhibit *L. monocytogenes* growth under refrigeration
- PC-3 was more effective when incorporated directly versus as propagated cells
- Bacterial fermentates delayed *L. monocytogenes* growth ≥1.5 weeks in model cheese
- Cultured sugar-vinegar blend was more inhibitory than cultured milks
- Reducing pH further delayed growth in model cheese with 0.5% cultured sugar-vinegar

INTRODUCTION

Consumer preference for free-from-synthetic preservatives and easy-to-understand ingredients drove clean-label foods to become a global $165-billion-dollar industry in 2015 with projections to reach $180 billion in 2020 (Food Insider Journal 2017). Biopreservation, or the use of microorganisms or their metabolites to improve the safety of foods (Holzapfel 1995, Stiles 1996), is an attractive option for *Listeria monocytogenes* control in clean-label foods. Protective cultures and bacterial fermentates represent two classes of biopreservatives commonly used in refrigerated foods such as meats (Alves 2006, Castellano 2006, McDonnell 2013, Melero 2013) and cheeses (Mendoza-Yepes 1999, Naldini 2009, Pingitore 2012, Vytrasova 2010). In fact, a single product line of bacterial fermentate products (MicroGARD^{TM}) is added to an estimated 30% of US-produced cottage cheeses to inhibit spoilage or pathogenic bacteria throughout product shelf-life (Favaro 2015).

Protective cultures and bacterial fermentate biopreservatives are primarily sourced from lactic acid bacteria due to their GRAS (Generally Recognized As Safe) status and natural predominance in several foods (Castellano 2006, Melero 2013, Stiles 1996). Mechanisms for pathogen inhibition by protective cultures are theorized to include displacement/exclusion on food or food-contact surfaces, competition for nutrients and/or oxygen, their ability to produce bacteriocins active against particular pathogens, and/or their ability to generate other inhibitory compounds such as organic acids (Melero 2013, Said 2019). Qualities of an ideal protective culture include GRAS status, bacteriocin production, acidification ability, and capability to survive and grow or metabolize in the food product (Favaro 2015). Protective cultures differ from starter cultures in that they are non-fermenting and have little influence on sensory characteristics, whereas starter cultures are involved in fermentation processes and change the

taste, flavor, and texture of foods; adjunct or multifunctional cultures can deliver functions in the middle of the two groups (Said 2019, Thun 2005).

Commercial bacterial fermentates are produced via fermentation of milk or sugars by bacteria such as *Propionibacteria shermanii*, *Lactobacillus plantarum*, or specific lactococci (Favaro 2015, Vytrasova 2010). Products of these fermentations include organic acids (lactic, propionic, or acetic), diacetyl, hydrogen peroxide, carbon dioxide, alcohols, fatty acids, acetaldehyde, reuterin, bacteriocins, and/or undefined low molecular weight inhibitors (considered to be bacteriocin-like inhibitory substances) (Favaro 2015, Thun 2005). These metabolites, in addition to other thus far unidentified effects, are believed to be responsible for the pathogen inhibition by these commercial ingredients (Favaro 2015, Vytrasova 2010).

Fresh, soft cheeses made via direct acidification of pasteurized milk present unique challenges for *L. monocytogenes* control, as their physiochemical properties (e.g. 46-57% moisture, 1-3% salt, pH >6.1 for queso fresco) may fall within growth parameters for *L. monocytogenes* (FDA 2011, van Hekken 2003). Additionally, starter cultures, which are absent in many fresh, soft cheeses, have been shown to confer additional antilisterial protection beyond that of product formulation (Alves 2006, Naldini 2009, Pingitore 2012). While several studies have investigated protective cultures or bacterial fermentates for their efficacy against spoilage organisms in dairy products (Al-Zoreky 1991, Buehler 2018, Cheong 2014, Delavenne 2013, Lacanin 2017, Salih 1990), few studies have investigated their use against *L. monocytogenes* in fresh, soft cheeses (Glass 1995, McAuliffe 1999, Mendoza-Yepes 1999*)*, with fewer still directly comparing commercially available biopreservatives for their respective antilisterial effectiveness (Aljasir 202, Gensler 2020).

The objective of the current study was to evaluate three commercial protective cultures and three commercial bacterial fermentates on *L. monocytogenes* behavior in a model system designed to mimic a directly acidified fresh, soft cheese (pH 6.00, 56% moisture, 1.25% salt, and made with lactic acid) when stored under refrigeration (4°C). The study additionally assessed whether pH reduction coupled with incorporation of 0.5% bacterial fermentate CSV-1 in the tested model cheese system could further inhibit *L. monocytogenes* growth at 4°C.

MATERIALS AND METHODS

Inoculum preparation. *L. monocytogenes* strains LM 108 (hard salami isolate, serotype 1/2b), LM 301 (cheddar cheese isolate, serotype 1/2a), LM 310 (goat's milk cheese isolate associated with illness, serotype 4b), R2-500 (soft Hispanic-style cheese isolate, 4b), and R2-501 (clinical isolate associated with soft Hispanic-style cheese, 4b) were grown individually in 10 ml Trypticase soy broth (TSB; BBL, BD, Sparks, MD) at 37°C for 18-22 h. Cells were harvested via centrifugation (2,500 × *g* for 20 min) and suspended in 4.5 ml 0.1% buffered peptone water (BPW; pH 7.2) after which equivalent populations of each isolate were combined in BPW in a five-strain cocktail. Strain purities were verified by streaking on modified Oxford agar (MOX; *Listeria* selective agar base, Difco, BD, Sparks, MD) and Trypticase soy agar (BBL, BD). Serial dilutions (in BPW) of strain and cocktail populations were verified by spread-plating on MOX (35°C, 48 h).

Protective culture preparation. Protective cultures were enumerated via diluting in BPW and pour-plating with de Man, Rogosa, Sharpe agar (MRS; BBL, BD) with anaerobic incubation at 30°C for 48 h in order to determine necessary grams of each culture needed to achieve

targeted level (10^6 CFU/g) in model cheese formulations. A targeted protective culture level of 10^6 CFU/g was chosen based PC-1, PC-2, and PC-3 manufacturer recommended usage levels necessary for *L. monocytogenes* growth inhibition. Counts for PC-1, PC-2, and PC-3 were found to be 11.33±0.04, 10.82±0.16, and 7.71±0.86 log CFU/g, respectively. In duplicate trials, protective cultures were added as received from the supplier at 0.01% to 4.35% (w/w) to standardize to the targeted level of 10^6 CFU/g. In a separate experiment, each single-strain protective culture was individually grown in 10 ml MRS broth (BBL, BD) at 30°C for 18-22 h after which cells were harvested via centrifugation (2,500 × *g* for 20 min) and suspended in 4.5 ml 0.1% BPW. Diluted protective culture strains were incorporated into model cheeses at the time of manufacture at a level of 0.25% (v/w) to deliver 10^6 CFU/g. This second methodology of protective culture incorporation was tested in order to control for the large variation in CFU level between the included protective cultures as well as to test whether 10^6 CFU/g propagated cells of each single-strain culture would affect *L. monocytogenes* growth. Though this application was not the intended use for any of the protective cultures tested, results by Loessner found 4.5 log CFU/cm^2 of a twice-washed aerobically-propagated pediocin AcH-producing *Lb. plantarum* cell pellet to inhibit *L. monocytogenes* growth on the surface of soft smear cheese stored at 4°C, leading us to test a similar methodology in our own soft model cheese system (Loessner 2003). Aerobic incubation of propagated protective cultures was exercised in our study due to the facultatively anaerobic status of each culture.

Model cheese manufacture. Six commercial biopreservatives were selected for evaluation (Table 5-1). In a first set of experiments, three protective cultures (PC-1, PC-2, and PC-3, target 10^6 CFU/g) and three bacterial fermentates (CM-1 or CM-2 [cultured milk] or CSV-

1 [cultured sugar-vinegar blend], 0.5% or 1.0% level) were individually tested in a high-moisture model cheese system (pH 6.00, 56% moisture, 1.25% salt, made with lactic acid) meant to mimic directly acidified high-moisture cheese (e.g. queso fresco or ricotta). This model cheese was previously shown to permit *L. monocytogenes* >1 log growth at 4°C in 1 week (Engstrom 2020). Based on results from the first set of experiments, a second set of experiments was completed to test a single bacterial fermentate (CSV-1, 0.5% level) in model cheeses (56% moisture, 1.25% salt, made with lactic acid) at different pH values (5.25, 5.50, 5.75, or 6.00). All treatments were replicated in duplicate trials.

Model cheeses were manufactured following a protocol by Engstrom et al. (2020). Briefly, model cheese formulations were prepared with pasteurized cream (Select Heavy Whipping Cream, 36% milkfat, Kemp's, Minneapolis, MN), sterile deionized water, micellar casein (CasPro™ 8500, Milk Specialties Global, Eden Prairie, MN), lactose (NF Lactose Monohydrate, Foremost Farms, Baraboo, WI), sodium chloride (Fisher, Waltham, MA), lactic acid (85%, Millipore, Burlington, MA), and either a single commercial protective culture (targeting 10^6 CFU/g) or commercial bacterial fermentate (target levels of 0.5% and 1.0%). Commercial protective cultures (PC-1, PC-2, and PC-3) or commercial fermentates (CM-1, CM-2, and CSV-1) were added to liquid ingredients before addition of dry ingredients to ensure complete dispersion in the model cheese system. Ingredient levels were adjusted as needed to meet analytical targets (56% moisture, 1.25% salt, pH adjustment with lactic acid). Control model cheeses without protective culture or fermentate were included in each trial.

All ingredients were combined in a sterilized stand mixer bowl (KitchenAid model KV25G0X, Whirlpool Co., Benton Harbor, MI) with a batch size of ~1,500 g and mixed for 2 minutes until homogeneous. 700 g was transferred into a sterilized beaker for uninoculated

samples and 50 g for proximate analysis transferred into a gas-impermeable pouch (3-mil high barrier EVOH pouches, Deli 1 material, oxygen transmission rate of 2.3 cm^3/cm^2 for 24 h at 23°C, water transmission rate of 7.8 g/m^2 for 24 h at 37.8°C, and 90% relative humidity; WinPak, Winnipeg, Manitoba, Canada) and vacuum-packaged (Multivac AGW, Sepp Haggemuller KG, Wolfertschewenden, Germany). Samples were stored at 4°C, with proximate analyses conducted within 72 hours of model cheese manufacture. The remaining 750 g portion was used for inoculated samples.

Inoculation and storage. Model cheeses were batch inoculated (0.5% v/w liquid inoculum) in mixer bowls with *L. monocytogenes* to deliver approximately 3-log CFU/g cheese. For model cheeses co-inoculated with 0.25% (v/w) propagated protective culture, *L. monocytogenes* inoculum volume was adjusted to 0.25% (v/w; total 0.5% v/w liquid addition). To distribute inoculum, cheeses were mixed at high speed for 2 min. Thereafter, 25-g inoculated portions or uninoculated samples were placed into gas-impermeable pouches and vacuum-packaged. All samples were stored at 4 ± 0.5°C for 8-10 weeks.

Sample proximate analysis. Moisture (5 h, 100°C vacuum oven method, AOAC 926.08), pH (direct measurement, Accumet Basic pH meter and Orion 8104 combination electrode, Thermo Fisher Scientific, Waltham, MA, AOAC 981.12), NaCl (measured as percentage of Cl^-, $AgNO_3$ potentiometric titration; Mettler DL22 food and beverage analyzer, Columbus, OH, AOAC 983.14), and water activity (a_w; Decagon AquaLab 4TE Water Activity Meter, Pullman, WA) were measured in triplicate uninoculated samples for each treatment (AOAC 2000).

Microbiological enumeration and pH/appearance monitoring. Triplicate inoculated samples and duplicate uninoculated samples were assayed for *L. monocytogenes* and pH, respectively, at time zero and weekly thereafter. Additionally, at time zero and weeks 4 and 8, duplicate uninoculated samples were assayed for aerobic plate count (APC), lactic acid bacteria (LAB), and yeasts/mold (YM); duplicate uninoculated samples formulated with protective cultures were additionally assayed for LAB at all weekly sampling points to monitor protective culture populations. 25-g samples were enumerated following addition of 50 ml BPW to each package and homogenizing with a stomacher for 30 sec (Neutec Masticator, Neutec Group, Inc., Farmingdale, NY). Serial dilutions of inoculated samples were spread plated on MOX (35°C, 48 h), while uninoculated samples were pour plated for APC (Plate Count agar, 35°C, 48 h, BBL, BD) and LAB (MRS agar, 30°C, 48 h anaerobic storage, BBL, BD) and spread plated for YM (Potato Dextrose agar, 21°C, 120 h, BBL, BD). Changes in direct pH and odor/appearance were monitored weekly.

Statistical analysis. Individual growth curves of *L. monocytogenes* in model cheeses formulated with protective cultures were modeled as a function of time using the Combase DMFit (Institute of Food Research, Norwich, U.K.) Excel add-on (version 2007, Microsoft Corporation, Seattle, WA) based on Baranyi models (Baranyi 2006*)*. Data points (each representing the average of triplicate *L. monocytogenes* samples enumerated at time points 0-8 weeks) from individual treatments in each trial were analyzed separately to obtain growth curves, and DMFit software was used to generate the growth rate (in log CFU/g per week) and lag phase (in weeks) for each fitted curve. Individual curves were analyzed using JMP statistical analysis software (JMP 13, SAS, Cary, NC). Independent, fixed factors were culture (PC-1, PC-2, PC-3,

or none), methodology (direct ingredient incorporation or washed propagated cells incorporation), and trial, and dependent factors were growth rate and lag phase.

RESULTS AND DISCUSSION

The objective of the first set of experiments was to evaluate three commercial protective cultures (PC-1, PC-2, and PC-3, target 10^6 CFU/g) and three commercial bacterial fermentates (CM-1, CM-2, and CSV-1, 0.5% or 1.0% level) for their ability to inhibit *L. monocytogenes* growth in a model cheese designed to mimic a directly acidified soft cheese (pH 6.00, 56% moisture, 1.25% salt, and made with lactic acid) stored at 4°C for 8 weeks. Based on the inhibition observed using fermentate CSV-1 at a 1.0% level, a second set of experiments was undertaken to determine the effect of pH (pH 5.25, 5.50, 5.75, and 6.00) on the inhibition of *L. monocytogenes* in model cheese formulated with 0.5% CSV-1 and stored at 4°C for 10 weeks. Proximate analysis results for the first and second sets of experiments are reported in Tables 5-2 and 5-3, respectively. Average starting populations of *L. monocytogenes* were 3.20 ± 0.17 log CFU/g and 3.34 ± 0.19 log CFU/g in the first and second sets of experiments, respectively. Calculated starting undissociated lactic acid contents were 0.09, 0.61, 1.65, and 4.35 mM, respectively, in model cheeses of target pH 6.00, 5.75, 5.50, and 5.25 based on a pK_a of lactic acid of 3.86.

Uninoculated sample analysis. Addition of protective cultures had no impact on model cheese pH, and no pH adjustment was necessary in model cheeses formulated with protective cultures. Over 8 weeks' storage at 4°C, the pH decreased modestly to minimum values of 5.6 and 5.7 in model cheeses formulated with target 10^6 CFU/g protective cultures PC-1 and PC-3,

respectively, regardless of application method (direct ingredient incorporation or propagated cells incorporation; data not shown). In comparison, no pH change was observed in model cheese formulated with target 10^6 CFU/g PC-2 over the study period, an expected observation based on the inability of PC-2 to ferment lactose. Production of organic acids and consequent lowering of cheese pH are known inhibitory qualities of certain LAB cultures against *L. monocytogenes* (Favaro 2015, Melero 2013). However, ingredient specifications for protective cultures PC-1 and PC-3 noted weak/slow acidification ability of these products and PC-2 was reported to have no acid production capability (Table 5-1). Levels of protective cultures PC-1, PC-2, and PC-3 changed from initial populations of 6.18±0.24, 5.66±0.20, and 5.88±0.66 to 7.20±0.88, 5.42±1.39, and 6.38±1.50 log CFU/g following 8 weeks' storage at 4°C. Application method (direct ingredient incorporation or incorporation of propagated cells) did not significantly impact protective culture populations over the course study period ($P>0.05$). YM counts additionally remained at acceptable levels (≤ 2.83 log CFU/g) over the study period in model cheeses formulated with protective cultures.

Addition of fermentates caused a change in pH of up to ±0.1 in duplicate trials incorporating 0.5% or 1.0% of CM-1, CM-2, or CSV-1 in the first set of experiments. pH adjustment with 10N NaOH or HCl did not impact *L. monocytogenes* behavior between trials ($P>0.05$) and therefore no pH adjustment after fermentate addition was made in the second set of experiments testing 0.5% CSV-1 in model cheeses with varying pH values. In samples formulated with fermentates CM-1, CM-2, and CSV-1 and stored vacuum-packaged at 4°C, APC, LAB, and YM counts increased over the study from 2.09, 1.61, and 1.48 at 0 weeks to 2.94, 2.44, and 2.40 log CFU/g at 8 weeks, for APC, LAB, and YM, respectively. pH values of

model cheeses formulated with fermentates CM-1, CM-2, and CSV-1 remained relatively stable throughout the 8-week study, with final pH values ranging from 6.0 to 6.1.

L. monocytogenes *behavior in samples formulated with protective cultures.* Protective cultures PC-1, PC-2, and PC-3, incorporated as direct ingredients at a target level of 10^6 CFU/g into model cheese before *L. monocytogenes* inoculation and 4°C storage, permitted growth of *L. monocytogenes* (defined as ≥1 log CFU/g increase from time zero) in approximately 1.0 to 2.5 weeks in duplicate trials (Figure 5-1). Because PC-3 is freeze-dried and packaged at ~8 log CFU/g compared to ~11 log CFU/g for frozen concentrated cultures PC-1 and PC-2, the ingredient required a substantially higher weight of culture to be added to model cheese to achieve the desired cell count (10^6 CFU/g). The greater amount of added material contributed to visual and textural differences in the model cheese compared with cheeses containing PC-1 or PC-2. Gensler and others recorded similar observations when incorporating a freeze-dried *Lb. plantarum* protective culture into milk samples compared with other commercial protective cultures tested (Gensler 2020). To control for the difference in cell counts between the commercial protective cultures tested, a second methodology was tested in a single trial wherein each protective culture was grown to stationary phase individually in MRS broth before incorporation of 10^6 CFU/g propagated cells into model cheese. 10^6 CFU/g propagated cells of protective cultures PC-1, PC-2, and PC-3 permitted growth of *L. monocytogenes* in approximately 1 week (Figure 5-S1).

No significant differences ($P>0.05$) in *L. monocytogenes* lag phase or growth rate were observed between cheese formulations containing directly incorporated PC-1 or PC-2 and the control cheese. Cheeses formulated with directly incorporated PC-3 were observed to have a

slower *L. monocytogenes* growth rate (P=0.03) but statistically similar lag phase (P>0.05) to control cheeses and cheeses containing directly incorporated PC-1 or PC-2. Maximum *L. monocytogenes* populations reached in samples formulated with protective cultures PC-1, PC-2, and PC-3 incorporated as direct ingredients were 1.90, 0.99, and 3.11 log CFU/g lower, respectively, than in control samples (Figure 5-1). Suppression of maximum *L. monocytogenes* populations may be credited to competitive inhibition by the protective cultures, a phenomenon which has been well documented in other *Listeria* challenge studies (Alves 2006, Nilsson 1999, Ostergaard 2014, Pitt 2000). For listerial growth to be delayed via competitive inhibition, however, protective cultures must quickly reach high counts and be metabolically active (Tirloni 2019).

The manufacturers of PC-1 and PC-3 noted that their products were capable of bacteriocin production; no mention of bacteriocin-producing capability was given by the manufacturer of PC-2. As bacteriocin production is a growth-related process, bacteriocin activity of LAB is typically highest at the end of the exponential growth phase (Sarantinopoulos 2002). No measurement of bacteriocin (either presence/absence or quantification) was conducted in the current study, however, given that the protective cultures did not grow substantially in model cheeses and storage temperature was below their optimal temperature for metabolism, production of bacteriocin would have been unlikely over the course of the study. In the event that *in situ* bacteriocin production during cheese manufacture with LAB cultures is realized, Favaro and colleagues noted that titers are significantly lower than those achieved during *in vitro* fermentation under optimal conditions, such as those utilized in the production of bacterial fermentates (Favaro 2015). A single trial with freshly propagated cells that removed metabolites during the inoculum preparation suggests the PC-3 efficacy may been less due to the competitive

inhibition or production of antimicrobial compounds *in situ*, and more likely to carry-over of metabolites, as no significant differences (P>0.05) in *L. monocytogenes* lag phase or growth rate was found between the control cheese and cheese containing PC-1, PC-2, or PC-3 in this application (Figure 5-S1). The high degree of variability for the direct addition of protective cultures (Figure 5-1) may reflect inconsistent concentrations of the metabolites from lot-to-lot.

Studies have found mixed results in the ability of protective cultures to control *L. monocytogenes* in soft cheeses. A 1999 study found a lactose-negative *Lc. lactis* subsp. diacetylactis starter culture capable of inhibiting *L. monocytogenes* growth in queso fresco (pH 6.5) for 22 days at 3 or 7ºC when co-inoculated into cheesemilk (Mendoza-Yepes 1999). A 2010 study found an adjunct *Lb. plantarum* strain sprayed onto the surface of commercial soft cheeses caused an immediate ~0.5 to 4.0 log CFU/g reduction in *L. innocua* followed by subsequent regrowth with storage at 20ºC or continued inhibition for 14 days with 5ºC storage (Vytrasova 2010). Furtado and others found that bacteriocin-producing- and non-bacteriocin-producing *Lc. lactis* subsp. *lactis* starter strains demonstrated similar abilities to prevent *L. monocytogenes* growth in fresh cheese during 10 days of storage at 8 to 10°C (0.24 and 0.20 log CFU/g increases, respectively, vs. 2.41 log CFU/g for a control cheese without a protective culture) (Furtado 2015). The authors additionally tested the incorporation of commercial purified nisin (a bacteriocin produced by *Lc. lactis*) into cheese made without protective culture and found that *L. monocytogenes* decreased to undetectable limits within 2 days and remained undetectable for the 10-day study. The authors postulated that the low storage temperature coupled with the heterogeneous cheese matrix may have inhibited growth and bacteriocin production of the protective culture (Furtado 2015).

L. monocytogenes *behavior in samples formulated with bacterial fermentates.* All treatments formulated with 0.5% fermentates CM-1, CM-2, or CSV-1 and pH 6.00 delayed growth of *L. monocytogenes* slightly but did not prevent growth over 8 weeks' storage at 4°C. Times-to-growth varied from approximately 2.5 to 3 weeks among test samples, compared with approximately 1 week observed in the control sample (Figure 5-2). When the level of fermentate was increased to 1.0%, times-to-growth were further delayed to approximately 5.5 to >8 weeks (Figure 5-3). An immediate 0.32 and 0.43 log CFU/g reduction in *L. monocytogenes* populations was observed in model cheeses formulated with 0.5% and 1.0% CM-2, respectively. This phenomenon may be due to the presence of bacteriocin in CM-2, a noted mechanism of antibacterial action by the ingredient supplier; however, the presence of bacteriocin was not confirmed in this study. Initial decreases in *L. monocytogenes* populations followed by subsequent regrowth have been well documented in food challenge studies testing bacteriocins in a variety of products (Dal Bello 2012, Maisnier-Patin 1992, O'Sullivan 2006, Pucci 1988, Schillinger 2001, Stecchini 1995, von Staszewski 2008, Wan 1997*).*

The low-acid high-moisture cheese varieties targeted in this study (e.g. fresh mozzarella, queso fresco, or ricotta) typically have pH values near 5.7-6.1. Although decreasing cheese pH to 5.25 is effective, it may be too low for several cheese varieties practically and/or sensorially. The first set of experiments demonstrated that CSV-1 was the most effective of the three fermentates, and that model cheese formulated with 1.0% CSV-1 inhibited *L. monocytogenes* growth for the duration of the initial 8-week study (Figure 5-3). Therefore, this fermentate was further tested in a second set of experiments to evaluate its ability to delay *L. monocytogenes* growth in model cheeses of pH 5.25, 5.50, 5.75, and 6.00 when incorporated into model cheese at a 0.5% level (Figure 5-4) to determine if pH reduction would improve efficacy of the product. In the second

set of experiments, control model cheeses without CSV-1 and pH ≥5.50 supported growth of *L. monocytogenes* over 10 weeks' storage at 4°C, whereas no growth was observed in the pH 5.25 control cheese. The addition of 0.5% CSV-1 delayed listerial growth by an additional two weeks in cheese with pH 5.75 and 6.00 but did not prevent growth beyond 3 weeks. In contrast, no pathogen growth (<0.5 log increase) for 10 weeks was observed in cheese supplemented with 0.5% CSV-1 when pH was pH 5.50 or 5.25. Incorporation of 0.5% CSV-1 extended times-to-growth from approximately 1 to 3 weeks, 1 to 6 weeks, and 3.5 to >10 weeks, in control and test model cheeses of pH 6.00, 5.75, and 5.50, respectively. Although the combination of pH 5.75 and 0.5% CSV-1 did not prevent growth, it significantly delayed growth ($P<0.05$) compared to the same pH without fermentate. While manufacturers will need additional validation work for their cheeses, these data provide guidance for formulation adjustments to prevent listerial growth and demonstrate the limitations as well as benefits of fermentates to inhibit growth of *L. monocytogenes* in high moisture cheeses.

McDonnell and colleagues found vinegar-containing biopreservatives to inhibit *L. monocytogenes* growth more effectively in turkey slurries stored at 4°C for 4 weeks than those not containing vinegar (McDonnell 2013). Acetic acid, the principal component of vinegar, was shown to be more effective than citric and lactic acids and as effective as propionic acid in controlling *L. monocytogenes* growth during 8 weeks' storage at 4°C when formulated into the model cheese system at varying pH values and moisture levels (Engstrom 2020).

This study evaluated three commercial protective cultures and three commercial bacterial fermentates for their ability to control *L. monocytogenes* growth at 4°C in a model fresh, soft cheese system. Data revealed that fermentates delayed growth for 1.5 to >6.5 weeks beyond that observed in control cheeses depending on ingredient, usage level, and cheese pH. A cultured

sugar-vinegar blend fermentate was found to be more inhibitory than cultured milk fermentates, likely due to the presence of acetic acid. In contrast, two of three protective cultures tested (PC-1 and PC-2) were unable to suppress *L. monocytogenes* growth in model directly acidified high moisture cheese at refrigeration conditions, likely due to storage temperature being below that for optimal metabolism. A third protective culture tested (PC-3) was found to have a slower rate of *L. monocytogenes* growth, however, similar lag phase to cheeses containing PC-1 or PC-2 or control cheese when added to model cheese as a direct ingredient in duplicate trials. Though not inhibitory against most LAB, the low (≤4°C) storage temperature of many fresh, soft cheeses limits the growth and subsequent metabolite production of many protective cultures. Utilizing these or other protective cultures under different conditions (e.g. at a higher incubation temperature, using them in different products or formats, or lowering the pH value of the product) might confer protection against *L. monocytogenes* or other microorganisms.

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Table 5-1. Commercial biopreservatives tested in model cheeses of pH 6.00, 56% moisture, and 1.25% salt, made with lactic acid.

Ingredient	Labeling	Format	Details from Manufacturer	Addition Level(s)
Protective Culture PC-1	"Culture" (*Lc. lactis* subsp. *lactis*)	Frozen pellets	Nisin-producing O-culture targeting *L. monocytogenes* and sporeformers in unripened and ripened cheeses. Shown to effectively inhibit *Clostridia* spp. in Gouda with 8°C ripening and 4°C storage following 20-h fermentation at 22°C. Recommended to be used with an acidifying starter culture. 0.002-0.004% (w/v) recommended usage in cheesemilk.	10^6 CFU/g
Protective Culture PC-2	"Culture" (*Lc. lactis* subsp. *lactis* bv. diacetylactis)	Frozen concentrate	O-culture for direct addition into cooled cottage cheese cream dressing targeting psychrotrophic bacteria with no elevated incubation ≥4.4°C required for effectiveness. 0.018% (w/v) recommended usage in cottage cheese cream dressing.	10^6 CFU/g

Protective Culture PC-3	"Culture" (*Lb. plantarum*)	Powder	Weakly acidifying and aroma-producing culture targeting *Listeria* spp. on cheese. Shown to effectively inhibit *L. innocua* growth on three soft cheese varieties stored at 5-6°C. 2.86% (w/v) recommended usage in solution for cheese surface application. Recommended to be applied at time of dry salting or onto cheeses the day after exit from salt bath.	10^6 CFU/g
Fermentate CM-1	"Cultured milk"	Powder	Skim milk fermented by a combination of LAB and subsequently pasteurized. For spoilage, psychrotroph, yeast, and mold control in dairy products including fresh cheeses. 0.1-1.0% (w/w) recommended usage.	0.5%, 1.0%
Fermentate CM-2	"Cultured milk powder"	Powder	Skim milk fermented by a combination of LAB and subsequently pasteurized. For spoilage, pathogen, yeast, and mold control in dairy products including cottage and fresh cheeses. 0.5-1.0% (w/w) recommended usage in cheese.	0.5%, 1.0%
Fermentate CSV-1	"Cultured sugar, vinegar"	Powder	Cane sugar fermented by a combination of LAB and subsequently pasteurized. For *L. monocytogenes* and *Clostridia* spp. control in RTE uncured meats. 1.5-2.5% (w/w) recommended usage in meat.	0.5%, 1.0%

Table 5-2. Physicochemical analysis of model cheeses made with lactic acid (targeting pH 6.00, 56% moisture, and 1.25% salt) in uninoculated samples at time 0. No significant differences were found between sample pH, moisture, NaCl, or a_w values ($P>0.05$).

Formulation	pH	Moisture (%)	NaCl (%)	A_w	*Protective Culture (log CFU/g)
Control	6.03±0.06	56.04±0.37	1.23±0.01	0.979±0.003	N/A
PC-1, 10^6 CFU/g	6.02±0.04	55.81±0.30	1.21±0.03	0.979±0.008	6.18±0.24
PC-2, 10^6 CFU/g	6.03±0.06	55.77±0.32	1.23±0.03	0.978±0.005	5.66±0.20
PC-3, 10^6 CFU/g	5.99±0.11	55.84±0.18	1.23±0.04	0.978±0.003	5.88±0.66
CM-1, 0.5% level	6.02±0.04	55.85±0.08	1.26±0.01	0.979±0.003	N/A
CM-1, 1.0% level	6.03±0.06	56.16±0.30	1.28±0.02	0.978±0.001	N/A
CM-2, 0.5% level	5.99±0.06	55.90±0.08	1.26±0.02	0.978±0.002	N/A
CM-2, 1.0% level	5.96±0.08	56.10±0.13	1.30±0.01	0.977±0.001	N/A
CSV-1, 0.5% level	6.01±0.01	56.57±0.08	1.23±0.03	0.977±0.004	N/A
CSV-1, 1.0% level	6.02±0.09	56.37±0.14	1.26±0.06	0.976±0.001	N/A

*Protective cultures were enumerated with de Man Rogosa Sharpe (MRS) agar incubated anaerobically at 30°C for 48 hours.

Table 5-3. Physicochemical analysis of model cheeses made with lactic acid and incorporating 0% or 0.5% CSV-1 (targeting pH 5.25 to 6.00, 56% moisture, and 1.25% salt) in uninoculated samples at time 0. No significant differences were found between sample moisture, NaCl, or a_w values (P>0.05).

Formulation	pH	Moisture (%)	NaCl (%)	A_w
pH 5.25	5.30±0.01	55.63±0.27	1.23±0.08	0.978±0.003
pH 5.25, 0.5% CSV-1	5.35±0.02	56.08±1.29	1.25±0.02	0.977±0.001
pH 5.50	5.53±0.03	55.50±0.19	1.28±0.03	0.982±0.002
pH 5.50, 0.5% CSV-1	5.58±0.02	55.93±0.06	1.27±0.02	0.980±0.003
pH 5.75	5.75±0.02	56.14±0.59	1.26±0.04	0.983±0.001
pH 5.75, 0.5% CSV-1	5.80±0.01	56.06±0.24	1.27±0.01	0.981±0.001
pH 6.00	6.02±0.02	56.59±0.20	1.24±0.03	0.985±0.001
pH 6.00, 0.5% CSV-1	6.05±0.03	56.45±0.19	1.28±0.03	0.978±0.003

FIGURE LEGENDS

Figure 5-1. *Listeria monocytogenes* growth at 4°C in model cheeses formulated with lactic acid (pH 6.00, 56% moisture, 1.25% salt) and containing 10^6 CFU/g commercial protective culture PC-1 (Δ), PC-2 (□), PC-3 (◇), or Control (●) containing no protective culture. Commercial protective cultures were directly incorporated into model cheese at 0.01 ± 0.00%, 0.01 ± 0.00%, or 2.42 ± 2.74% (w/w) for PC-1, PC-2, and PC-3, respectively.

Figure 5-2. *Listeria monocytogenes* growth at 4°C in model cheeses formulated with lactic acid (pH 6.00, 56% moisture, 1.25% salt) and containing 0.5% (w/w) commercial bacterial fermentate CM-1 (□), CM-2 (◇), CSV-1 (Δ), or Control (●) containing no fermentate.

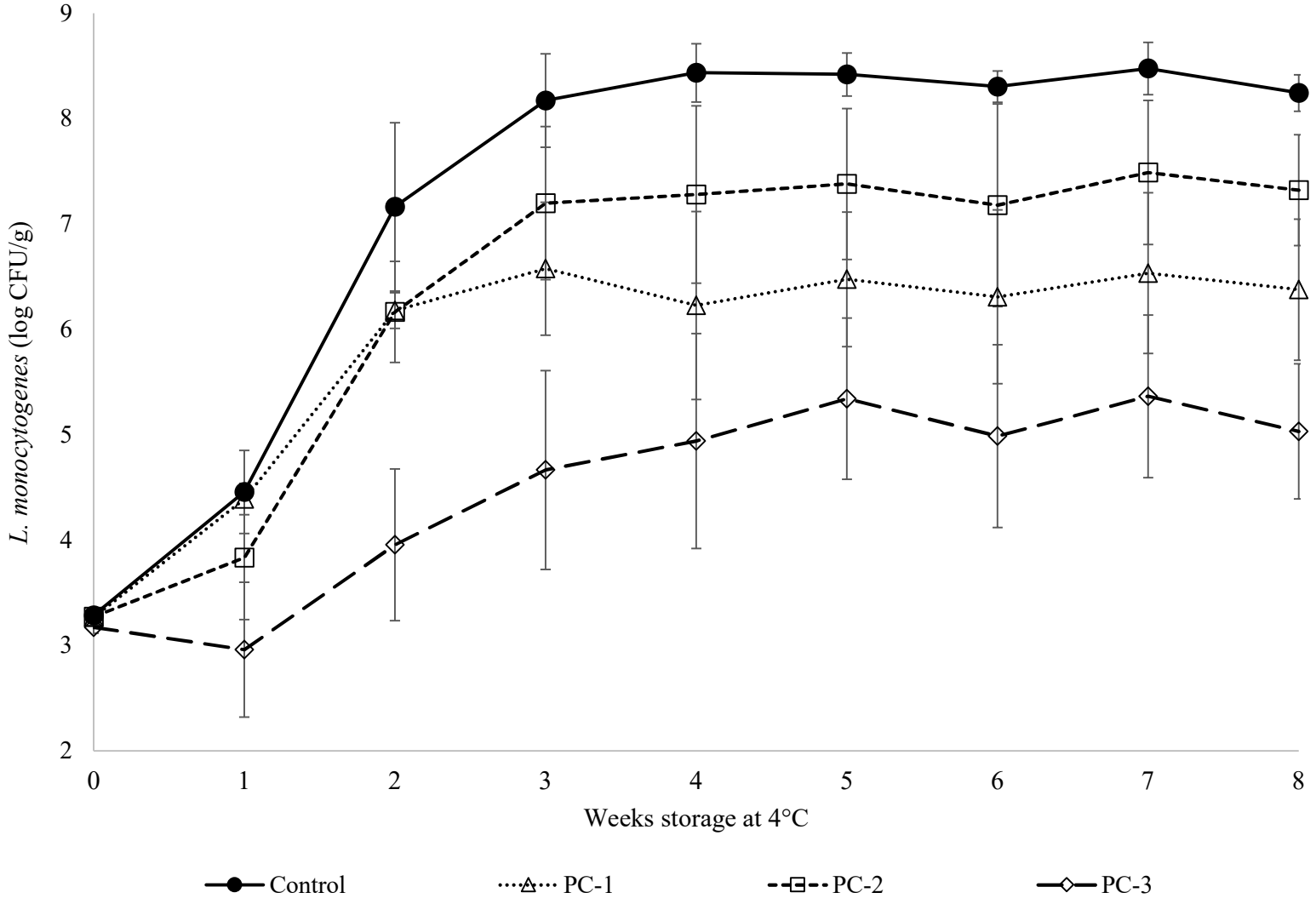
Figure 5-3. *Listeria monocytogenes* growth at 4°C in model cheeses formulated with lactic acid (pH 6.00, 56% moisture, 1.25% salt) and containing 1.0% (w/w) commercial bacterial fermentate CM-1 (□), CM-2 (◇), CSV-1 (Δ), or Control (●) containing no fermentate.

Figure 5-4. *Listeria monocytogenes* growth at 4°C in model cheeses formulated with lactic acid (56% moisture, 1.25% salt) with [pH 5.25 (◇), pH 5.50 (□), pH 5.75 (Δ), or pH 6.00 (○)] or without [pH 5.25 (◆), pH 5.50 (■), pH 5.75 (▲), or pH 6.00 (●)] 0.5% (w/w) CSV-1 commercial bacterial fermentate.

Figure 5-S1. *L. monocytogenes* growth at 4°C in model cheeses formulated with lactic acid (pH 6.00, 56% moisture, 1.25% salt) and containing 10^6 CFU/g commercial protective culture PC-1 (Δ), PC-2 (□), PC-3 (◇), or Control (●) containing no protective culture

(data from single trial shown). Commercial protective cultures were grown in MRS broth at 30°C for 18-22 h after which cells were harvested via centrifugation and suspended in 4.5 ml 0.1% BPW before incorporation into model cheese.

Figure 5-1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L. monocytogenes (log CFU/g)
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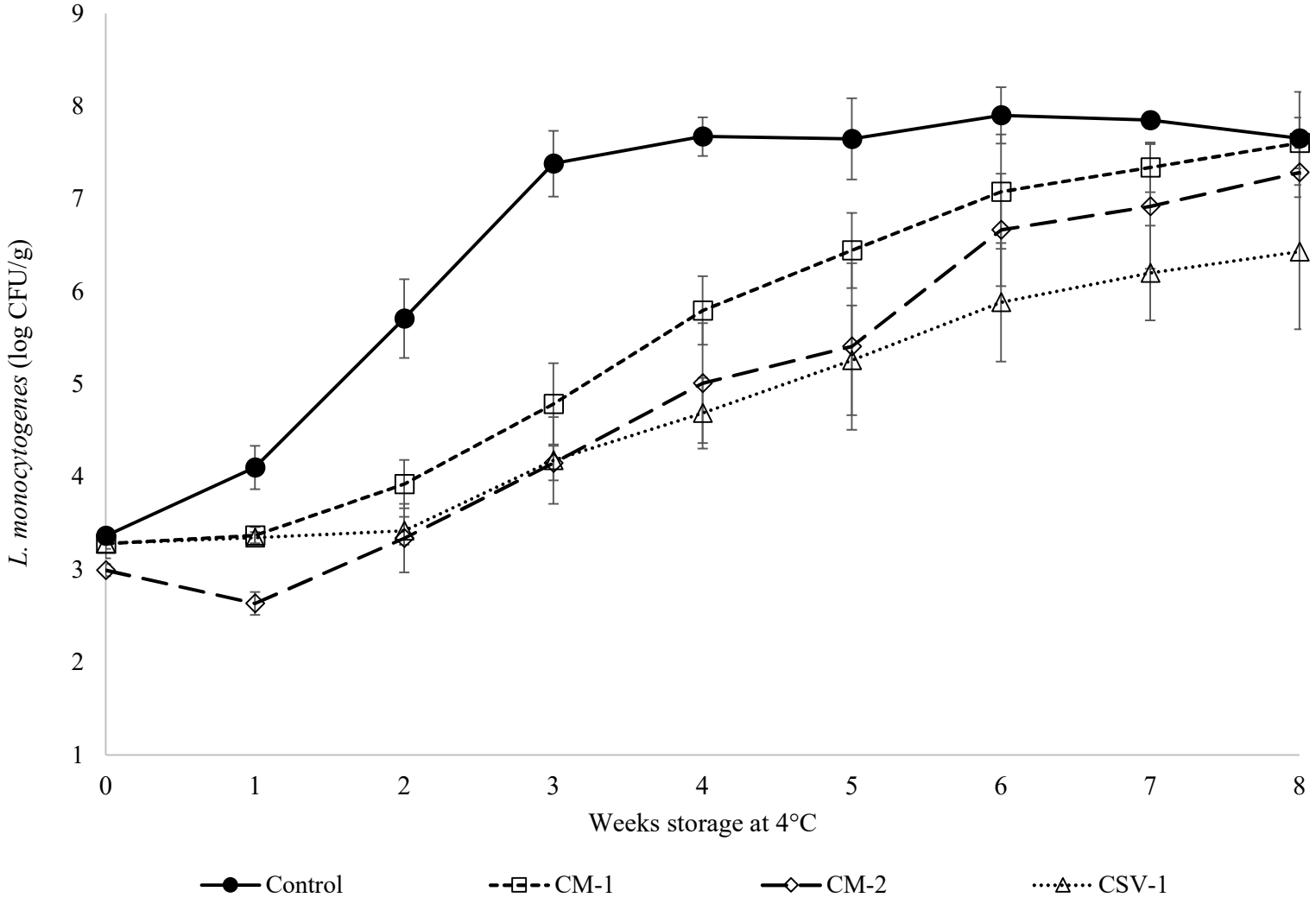
8
Weeks storage at 4°C
Control
PC-1
PC-2
PC-3

Figure 5-2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L. monocytogenes (log CFU/g)
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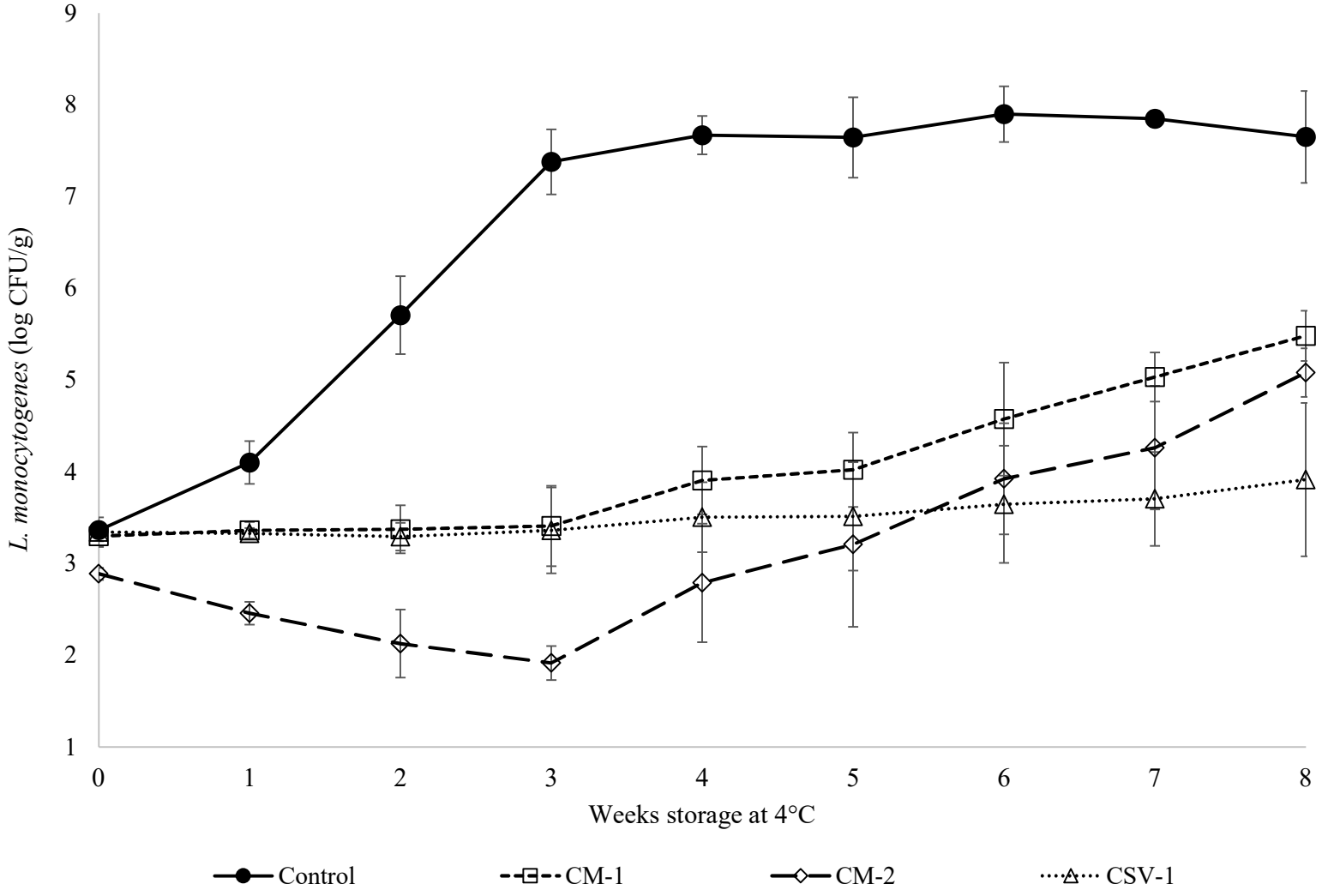
Weeks storage at 4°C
Control
CM-1
CM-2
CSV-1

Figure 5-3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Weeks storage at 4°C
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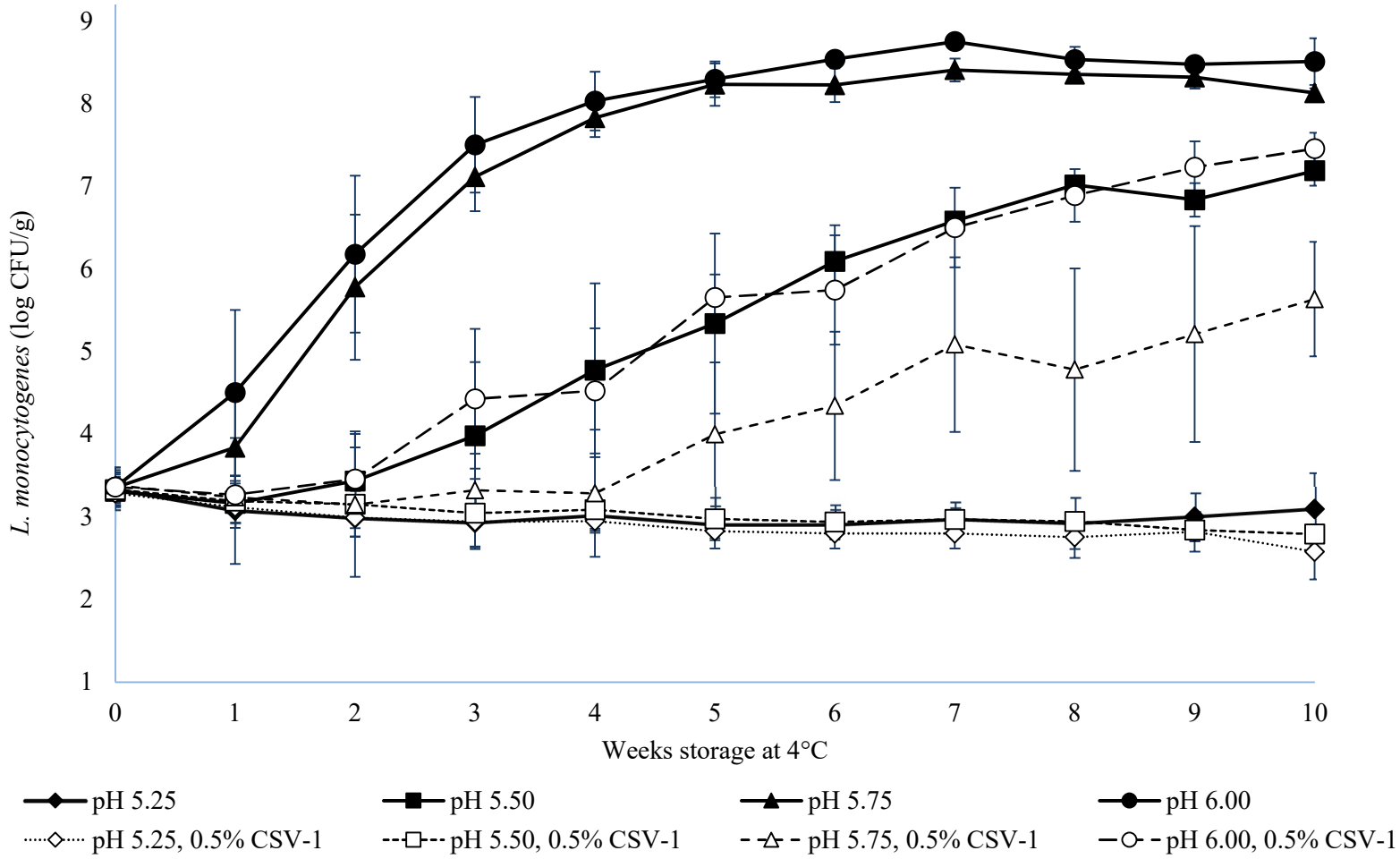
CM-1
CM-2
CSV-1

Figure 5-4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L. monocytogenes (log CFU/g)
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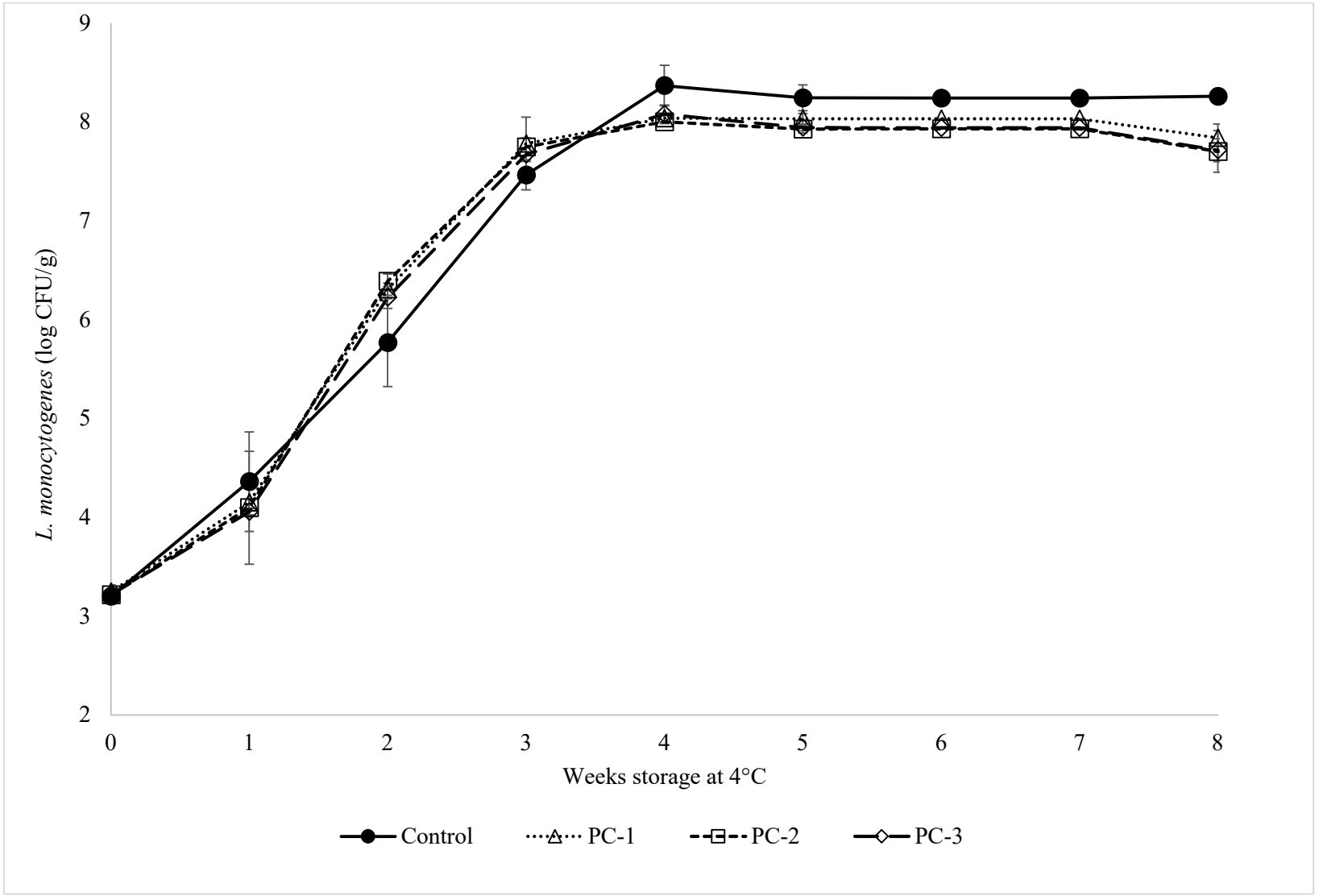
Weeks storage at 4°C
pH 5.25
pH 5.50
pH 5.75
pH 6.00
pH 5.25, 0.5% CSV-1
pH 5.50, 0.5% CSV-1
pH 5.75, 0.5% CSV-1
pH 6.00, 0.5% CSV-1

Figure 5-S1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L. monocytogenes (log CFU/g)
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Weeks storage at 4°C
Control
PC-1
PC-2
PC-3

CHAPTER 4:

Conclusions and Future Work

This research project aimed to develop strategies for high-risk cheese manufacturers, namely those producing unpasteurized milk cheeses or high-moisture, low-acid cheeses. In order to improve the safety of both cheese groups, pathogen inactivation and/or growth inhibition need to be addressed. Chapter 3 focuses on inactivation of 2 pathogens of concern to raw milk cheese manufacturers, *L. monocytogenes* and STEC, while Chapters 4 and 5 focus on *L. monocytogenes* growth control in high-moisture, low-acid cheeses.

The aim of Chapter 3 was to determine thermization time / temperature combinations necessary to improve the safety of raw cheesemilk before subsequent unpasteurized milk cheese manufacture. Estimated and validated times to 3-log reduction (based on generated D-values) for *L. monocytogenes* and STEC in whole milk were found to be 7:21 and 3:00, 1:39 and 0:51, and 0:51 and 0:21 (min:s), for test temperatures 60.0, 62.8, and 65.6°C, respectively. Results from this work intend to be used in development of an application (modeled after USDA FSIS Appendix A for heating meat/poultry) estimating log-kill for each pathogen based on inputs time or temperature for thermization.

A lower test temperature, 57.2°C, was used to generate a D-value for *L. monocytogenes* only. While this temperature was found inadequate in reducing *L. monocytogenes* sufficiently within a practical amount of time for thermization (41:16 required for 3-log decrease), 57.2°C may be an appropriate thermization temperature in long-aged hard cheeses with sufficient hurdles (e.g. high curd cooking temperature, low moisture, and/or high acidity). Generating and validating D-values for STEC at this lower temperature could therefor be a future research direction. Enrichment from thermized milk and/or subsequent cheesemaking and aging with inoculated then thermized milk would likely need to be undertaken for this work.

A novel cheese system was used for *L. monocytogenes* challenge studies in Chapters 4 and 5. Further work may include optimization of this model cheese system for other applications or screening of addition antimicrobials within the system. Validation work beyond literature comparisons should additionally be completed to ensure results from the model system can be directly translated to antimicrobial effects within high-moisture cheese varieties.

The aim of Chapter 4 was to determine combinations of pH, organic acid, and moisture which inhibit *L. monocytogenes* in model high-moisture cheeses of varying pH (5.25-6.00), moisture (50-56%), and organic acid (citric, lactic, acetic, propionic). pH and organic acid were found to significantly affect *L. monocytogenes* growth rate and lag-phase duration at 4°C, while moisture was found to insignificantly affect these parameters. Antilisterial effectiveness of organic acids fell in decreasing order of pK_a values, with propionic ≈ acetic > lactic > citric. Predicted times to 1-log increase of *L. monocytogenes* in pH 5.5 high-moisture cheeses based on the developed models for growth rate and lag phase were 2.0, 4.5, >8.0, and >8.0 weeks, respectively, for high-moisture cheeses pH-adjusted with citric, lactic, acetic, or propionic acids. R^2 values for both models could be improved upon by expanding datasets to include other formulations, especially higher moisture and pH values; this may lead to moisture becoming a significant variable in either model, as the test range was limited (50-56%) in the current study.

In Chapter 5, bacterial fermentates (2 cultured milks [CM-1 and CM-2] and 1 cultured sugar-vinegar blend [CSV-1]) were found to be more efficacious in controlling *L. monocytogenes* at refrigeration than protective cultures tested (2 *Lc. lactis* subsp. *lactis* and 1 *Lb. plantarum* strain). All tested bacterial fermentates delayed *L. monocytogenes* growth ≥1.5 weeks in permissive model cheese (pH 6.00, 56% moisture, made with lactic acid) when formulated at 0.5% (w/w), with CSV-1 found to be more inhibitory than CM-1 or CM-2. Reducing pH further

delayed growth in model cheese with 0.5% CSV. CSV products are used extensively in formulating for control of *L. monocytogenes* growth in RTE meat products. Blending vinegar with cultured milk or cultured sugar fermentates would likely lead to improved antilisterial effects and should be explored in the future, though this work would need to be coupled with sensory analysis to develop solutions suitable to fresh cheese flavors. While a single *Lc. lactis* strain tested and the *Lb. plantarum* protective culture tested have both been found by other authors to effectively control *L. monocytogenes* in cheeses at temperatures ≤7°C, our results suggested utilizing the protective cultures as a sole means for listerial control in fresh soft cheeses would lead to inadequate prevention of *L. monocytogenes* growth if formulated at a level of 10^6 CFU/g with 4°C storage. Further exploration into protective culture applications appropriate for fresh soft cheese varieties could be a future area of development. Additionally, though *L. monocytogenes* control was the principle aim of Chapters 4 and 5, further examination into spoilage control by organic acids and/or biopreservatives could be investigated.

Analysis of existing *L. monocytogenes* cheese challenge studies (Appendix 1) revealed some interesting large-scale conclusions about cheeses that could be used to dictate future research in determining which factors effectively predict whether cheeses are at risk of supporting *L. monocytogenes* growth. Consideration should be given to updating the dataset given in Appendix 1, as it was completed in 2012 and further research on *L. monocytogenes* growth in cheeses has been published since this time. Future variables to look at might be whether single strains or cocktails were used and starting inoculum level.

APPENDIX 1. GENERALIZED LINEAR MODEL OF PUBLISHED *L. MONOCYTOGENES* GROWTH IN CHEESE CHALLENGE STUDIES

Within the context of a literature analysis conducted from 100 scientific challenge studies published of all cheese varieties (including data from 457 "fresh soft," 131 "soft unripened," 271 "soft ripened," 95 "semi-soft," 113 "hard," and 36 "process" experimental cheeses) stored under a variety of temperatures and conditions, growth of *L. monocytogenes* introduced either into cheesemilk or onto cheese curd after fabrication was observed at an average pH of 6.11, while inhibition was observed at an average pH of 5.26 (Figure A1-1). This literature analysis included 1103 cheeses, with 413 showing growth (defined as any observed increase in *L. monocytogenes* in cheese curd over storage period) and 690 cheeses showing no growth. All data were fitted to a binary logistic regression model using SAS v. 9.2. The PROC LOGISTIC command of SAS was used to model the binary dependent variable "Growth" based on binary independent categorical variables "Pre/Post-Processing Inoculation," "Presence/Absence of Starter Culture," "Pasteurized/Raw Milk," "Bovine/Other Milk," and continuous independent variables "pH," "Percent Water-Phase Salt," and "Storage Temperature." 95% Confidence Intervals for *L. monocytogenes* "Growth" probabilities were computed for the continuous independent variable "pH" using the SAS command PROC PROBIT with dosage level (d) = logistic INVERSECL. Statistical analysis revealed that "pH," "Percent Water-Phase Salt," "Presence/Absence of Starter Culture," "Pasteurized/Raw Milk," "Bovine/Other Milk," and "Storage Temperature" all significantly impacted *L. monocytogenes* growth ($P < 0.01$), while "Pre/Post-Processing Inoculation" (i.e. *L. monocytogenes* inoculated into cheesemilk or onto cheese curd), was found insignificant in growth estimation of the pathogen ($P = 0.33$). A generalized linear model for *L. monocytogenes* growth was constructed:

log odds Growth = -12.30 + 2.61(pH) - 0.70(bovine=0/other milk=1) + 0.57(pasteurized=0/raw milk=1) - 0.46(percent water-phase salt) + 0.39(absence=0/presence of starter culture=1) - 0.05(storage temperature, °C)

where values >0 predicted ≥50% risk of growth of *L. monocytogenes* in a given cheese. ≥50% probability of *L. monocytogenes* growth inhibition was estimated in cheese with pH≤5.64 (95% CI: 5.28, 6.01), and ≥95% probability of *L. monocytogenes* growth inhibition in cheese with pH≤4.51 (95% CI: 4.11, 4.89)

Figure A1-1. pH versus percent water-phase salt of cheeses challenged with *Listeria monocytogenes* and showing growth or no growth of the pathogen. Dataset given in Table A1-1.

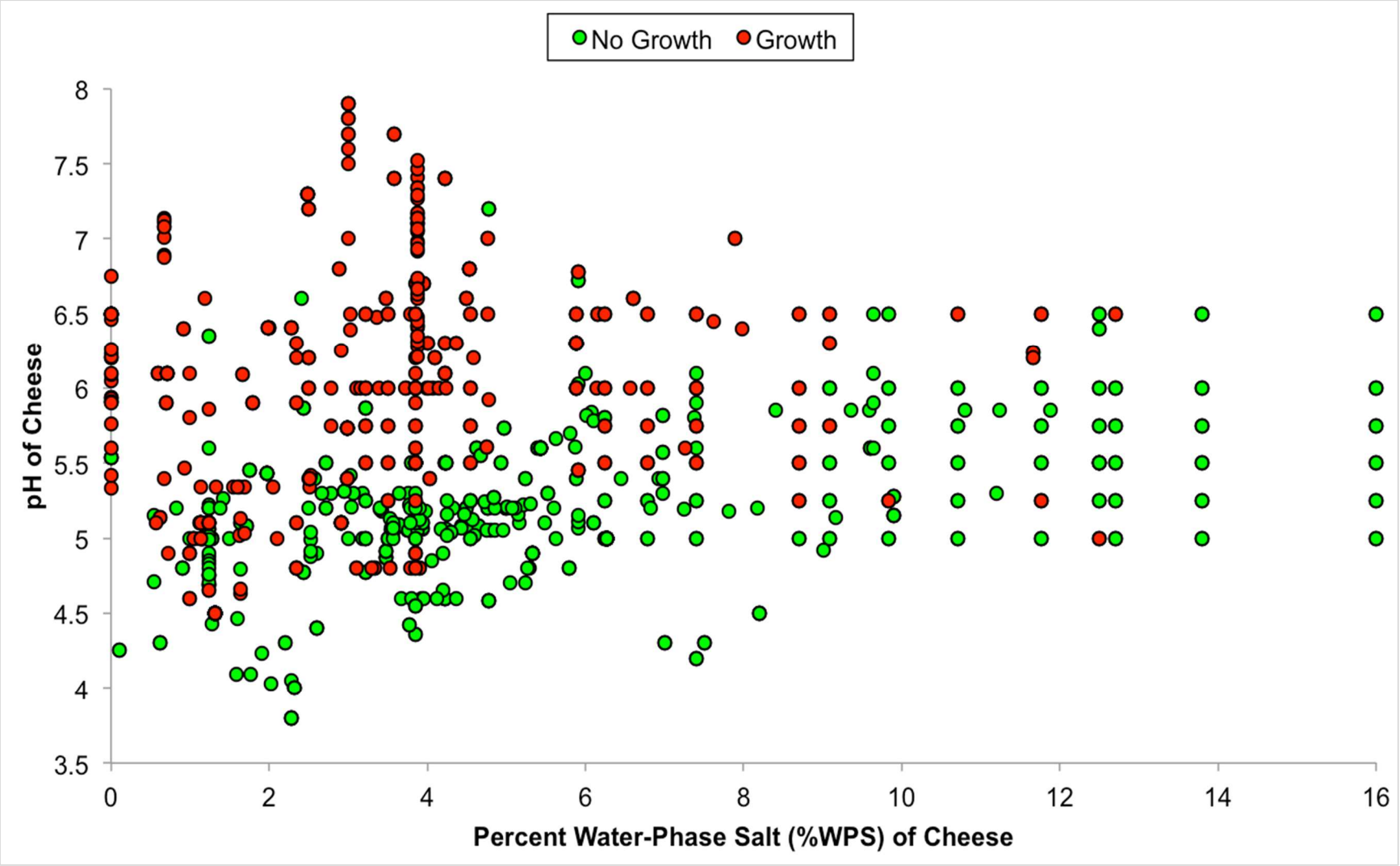

Table A1-1. Literature derived *Listeria monocytogenes* cheese challenge study data used in developing multiple linear regression model. Primary reference citations given in: Engstrom, S.K. 2012. Evaluating the risk of *Listeria monocytogenes* and *Salmonella* spp. growth on semi-soft and hard cheeses stored without refrigeration. M.S. Thesis, University of Wisconsin-Madison.

[1]Binary denotation for pre-processing or post-contamination of cheese, where 0 = "pre-processing" (i.e. inoculation into cheesemilk) and 1 = "post-processing" (ie. inoculation onto cheese sample).
[2]Binary denotation for pasteurized or raw milk cheese, where 0 = "pasteurized" and 1 = "raw" or "thermized" milk cheese.
[3]FDA 2003 Quantitative Risk Assessment cheese category, where 1 = fresh soft, 2 = soft unripened, 3 = soft ripened, 4 = semi-soft, 5 = hard, and 6 = process.
[4]Starting pH value of cheese before storage.
[5]Moisture content of cheese (% moisture).
[6]%WPS = Percent water-phase salt of cheese.
[7]Binary denotation for species of milk used, where 0 = "bovine" and 1 = "other species and/or species blend" milk used.
[8]Binary denotation for presence/absence of starter culture, where 0 = "no starter culture used," and 1 = "starter culture used."
[9]Storage temperature (in °C) of cheese.
[10]Binary denotation for growth of *L. monocytogenes* during cheese storage, where 0 = "no growth," and 1 = "growth." "Growth" was defined as any observed increase in CFU/g cheese during storage.

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Abdalla	1993	0	0	Gibna Bayda white pickled cheese	3	6.45	5.15	62.44	7.62	0	0	4	65	1
Abdalla	1993	0	0	Gibna Bayda white pickled cheese	3	6.40	5.55	63.97	7.98	0	1	4	65	1
Akturkoglu	1999	0	0	Beyaz Peynir (Turkish white cheese) w/ starter	3	6.30	6.00	60.00	9.09	0	1	4	60	1
Akturkoglu	1999	0	0	Beyaz Peynir (Turkish white cheese) w/o starter	3	6.30	6.00	60.00	9.09	0	0	4	60	1
Al-Holy	2012	0	0	White brined cheese, unripened (L. innocua)	2	6.24	6.10	46.20	11.66	0	0	4	12	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Al-Holy	2012	0	0	White brined cheese, unripened (L. innocua)	2	6.20	6.10	46.20	11.66	0	0	4	12	1
Al-Holy	2012	0	0	White brined cheese, unripened (L. innocua)	2	6.24	6.10	46.20	11.66	0	0	10	12	1
Al-Holy	2012	0	0	White brined cheese, unripened (L. innocua)	2	6.20	6.10	46.20	11.66	0	0	10	12	1
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	4	300	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	4	170	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	4	120	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	4	280	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	4	235	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	4	130	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	4	260	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	4	180	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	4	120	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	12	180	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	12	125	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	12	70	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	12	170	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	12	75	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	12	75	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	12	150	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	12	95	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	12	60	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	22	45	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	22	35	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	22	25	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	22	55	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	22	32	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	22	21	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	22	49	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	22	24	0
Angelidis	2010	1	0	Grated process cheese	6	5.00	2.80	41.90	6.26	0	0	22	20	0
Arici	1999	0	0	Turkish white cheese made with cow's milk	3	4.70	3.48	62.92	5.24	0	1	4	133	0
Arici	1999	0	0	Turkish white cheese made with cow's milk	3	4.70	3.35	63.10	5.04	0	1	4	133	0
Arici	1999	0	0	Turkish white cheese made with sheep's milk	3	4.80	3.30	59.04	5.29	1	1	4	133	0
Arici	1999	0	0	Turkish white cheese made with sheep's milk	3	4.80	3.30	59.30	5.27	1	1	4	133	0
Arques	2005	0	1	Raw milk semi-hard cheese	4	5.07	2.00	54.00	3.57	0	1	12	57	0
Arques	2005	0	1	Raw milk semi-hard cheese	4	5.08	2.00	54.00	3.57	0	1	12	57	0
Arques	2005	0	1	Raw milk semi-hard cheese	4	5.08	2.00	54.00	3.57	0	1	12	57	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Arques	2005	0	1	Raw milk semi-hard cheese	4	5.04	2.00	54.00	3.57	0	1	12	57	0
Arques	2005	0	1	Raw milk semi-hard cheese	4	5.09	2.00	54.00	3.57	0	1	12	57	0
Arques	2005	0	1	Raw milk semi-hard cheese	4	5.00	2.00	54.00	3.57	0	1	12	57	0
Arques	2005	0	1	Raw milk semi-hard cheese	4	5.10	2.00	54.00	3.57	0	1	12	57	0
Arques	2005	0	1	Raw milk semi-hard cheese	4	5.07	2.00	54.00	3.57	0	1	12	57	0
Bachmann	1994	0	1	Emmentaler	5	5.26	0.51	35.40	1.42	0	1	11	1	0
Bachmann	1994	0	1	Tilsiter	4	5.21	1.23	39.30	3.03	0	1	11	90	0
Back	1993	1	0	Blue Lymeswold	3	5.50	2.00	50.00	3.85	0	1	3	27	1
Back	1993	1	0	Blue Lymeswold	3	5.50	2.00	50.00	3.85	0	1	6	27	1
Back	1993	1	0	Blue Lymeswold	3	5.50	2.00	50.00	3.85	0	1	6	27	1
Back	1993	1	0	Blue Lymeswold	3	5.50	2.00	50.00	3.85	0	1	10	20	1
Back	1993	1	0	Blue Lymeswold	3	5.50	2.00	50.00	3.85	0	1	10	20	1
Back	1993	1	0	Blue Lymeswold	3	5.50	2.00	50.00	3.85	0	1	3	27	0
Back	1993	1	0	Blue Stilton	5	5.40	2.10	38.00	5.24	0	1	5	14	0
Back	1993	1	0	Blue Stilton	5	5.40	2.10	38.00	5.24	0	1	10	14	0
Back	1993	1	0	Brie with Garlic	3	5.10	1.50	50.00	2.91	0	1	3	15	1
Back	1993	1	0	Brie with Garlic	3	5.10	1.50	50.00	2.91	0	1	3	15	1
Back	1993	1	0	Brie with Garlic	3	5.10	1.50	50.00	2.91	0	1	6	15	1
Back	1993	1	0	Brie with Garlic	3	5.10	1.50	50.00	2.91	0	1	6	15	1
Back	1993	1	0	Cambazola	3	5.10	1.29	53.70	2.35	0	1	5	18	1
Back	1993	1	0	Cambazola	3	5.10	1.29	53.70	2.35	0	1	10	14	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Back	1993	0	0	Camembert (center)	3	4.80	1.29	53.70	2.35	0	1	15	26	1
Back	1993	0	0	Camembert (center)	3	4.80	1.29	53.70	2.35	0	1	3	26	0
Back	1993	0	0	Camembert (center)	3	4.80	1.29	53.70	2.35	0	1	6	26	0
Back	1993	0	0	Camembert (center)	3	4.80	1.29	53.70	2.35	0	1	10	26	0
Back	1993	0	0	Camembert (surface)	3	5.90	1.29	53.70	2.35	0	1	3	26	1
Back	1993	0	0	Camembert (surface)	3	5.90	1.29	53.70	2.35	0	1	6	26	1
Back	1993	0	0	Camembert (surface)	3	6.20	1.29	53.70	2.35	0	1	10	26	1
Back	1993	0	0	Camembert (surface)	3	6.30	1.29	53.70	2.35	0	1	15	26	1
Back	1993	1	0	Chaume	3	5.40	2.50	40.00	5.88	0	1	3	15	0
Back	1993	1	0	Chaume	3	5.40	2.50	40.00	5.88	0	1	6	15	0
Back	1993	1	0	English Brie	3	5.10	0.63	50.00	1.24	0	1	6	24	1
Back	1993	1	0	English Brie	3	5.10	0.63	50.00	1.24	0	1	6	24	1
Back	1993	1	0	English Brie	3	5.10	0.63	50.00	1.24	0	1	10	24	1
Back	1993	1	0	English Brie	3	5.10	0.63	50.00	1.24	0	1	10	24	1
Back	1993	1	0	English Brie	3	5.10	0.63	50.00	1.24	0	1	3	24	0
Back	1993	1	0	English Brie	3	5.10	0.63	50.00	1.24	0	1	3	24	0
Back	1993	1	0	French Brie	3	5.10	0.63	50.00	1.24	0	1	5	18	1
Back	1993	1	0	French Camembert	3	5.40	1.29	50.00	2.52	0	1	3	15	1
Back	1993	1	0	French Camembert	3	5.40	1.29	50.00	2.52	0	1	3	15	1
Back	1993	1	0	French Camembert	3	5.40	1.29	50.00	2.52	0	1	6	15	1
Back	1993	1	0	French Camembert	3	5.40	1.29	50.00	2.52	0	1	6	8	1
Back	1993	1	0	Full-fat soft cheese with garlic and herbs	3	5.10	1.50	50.00	2.91	0	1	3	15	0
Back	1993	1	0	Full-fat soft cheese with garlic and herbs	3	5.10	1.50	50.00	2.91	0	1	3	15	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Back	1993	1	0	Full-fat soft cheese with garlic and herbs	3	5.10	1.50	50.00	2.91	0	1	6	15	0
Back	1993	1	0	Full-fat soft cheese with garlic and herbs	3	5.10	1.50	50.00	2.91	0	1	6	15	0
Back	1993	1	0	Mycella	3	5.40	3.50	47.00	6.93	0	1	5	18	0
Back	1993	1	0	White Lymeswold	3	5.50	2.00	50.00	3.85	0	1	6	23	1
Back	1993	1	0	White Lymeswold	3	5.50	2.00	50.00	3.85	0	1	6	23	1
Back	1993	1	0	White Lymeswold	3	5.50	2.00	50.00	3.85	0	1	10	15	1
Back	1993	1	0	White Lymeswold	3	5.50	2.00	50.00	3.85	0	1	10	23	1
Back	1993	1	0	White Lymeswold	3	5.50	2.00	50.00	3.85	0	1	3	23	0
Back	1993	1	0	White Lymeswold	3	5.50	2.00	50.00	3.85	0	1	3	23	0
Back	1993	1	0	White Stilton	3	5.40	2.10	50.00	4.03	0	1	5	11	1
Back	1993	1	0	White Stilton	3	5.40	2.10	50.00	4.03	0	1	10	11	1
Belessi	2008	1	0	Feta	3	4.50	5.00	56.00	8.20	0	1	3	25	0
Belessi	2008	1	0	Feta	3	4.50	5.00	56.00	8.20	0	1	5	18	0
Belessi	2008	1	0	Feta	3	4.50	5.00	56.00	8.20	0	1	10	17	0
Belessi	2008	1	0	Feta	3	4.50	5.00	56.00	8.20	0	1	15	10	0
Bello	2011	0	0	Cottage	2	5.86	1.00	80.00	1.23	0	1	4	7	1
Bello	2011	0	0	Cottage	2	4.65	1.00	80.00	1.23	0	1	4	7	1
Bello	2011	0	0	Cottage	2	4.80	1.00	80.00	1.23	0	1	4	7	0
Bello	2011	0	0	Cottage	2	4.70	1.00	80.00	1.23	0	1	4	7	0
Bello	2011	0	0	Cottage	2	4.76	1.00	80.00	1.23	0	1	4	7	0
Benech	2002	0	0	Cheddar	5	5.20	2.00	37.11	5.11	0	1	7	180	0
Benech	2002	0	0	Cheddar	5	5.20	2.00	37.46	5.07	0	1	7	180	0
Benech	2002	0	0	Cheddar	5	5.20	2.00	39.19	4.86	0	1	7	180	0
Bolton	1999	1	0	Mexican-style	1	5.00	6.00	42.00	12.50	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.25	4.00	42.00	8.70	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.25	2.00	50.00	3.85	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.25	2.00	55.00	3.51	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.25	6.00	55.00	9.84	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.25	8.00	60.00	11.76	0	0	10	42	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Bolton	1999	1	0	Mexican-style	1	5.50	2.00	42.00	4.55	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.50	4.00	42.00	8.70	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.50	2.00	50.00	3.85	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.50	4.00	50.00	7.41	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.50	2.00	55.00	3.51	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.50	4.00	55.00	6.78	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.50	2.00	60.00	3.23	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.50	2.00	60.00	3.23	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.50	2.00	60.00	3.23	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.50	4.00	60.00	6.25	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.50	4.00	60.00	6.25	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	2.00	42.00	4.55	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	2.00	42.00	4.55	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	4.00	42.00	8.70	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	2.00	50.00	3.85	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	2.00	50.00	3.85	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	2.00	50.00	3.85	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	4.00	50.00	7.41	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	4.00	50.00	7.41	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	4.00	50.00	7.41	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	2.00	55.00	3.51	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	2.00	55.00	3.51	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	2.00	55.00	3.51	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	4.00	55.00	6.78	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	4.00	55.00	6.78	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	2.00	60.00	3.23	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	2.00	60.00	3.23	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	4.00	60.00	6.25	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	4.00	60.00	6.25	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	4.00	60.00	6.25	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.75	6.00	60.00	9.09	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	2.00	42.00	4.55	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	2.00	42.00	4.55	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	4.00	42.00	8.70	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	2.00	50.00	3.85	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	2.00	50.00	3.85	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	2.00	50.00	3.85	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	4.00	50.00	7.41	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	4.00	50.00	7.41	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	2.00	55.00	3.51	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	2.00	55.00	3.51	0	0	10	42	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Bolton	1999	1	0	Mexican-style	1	6.00	2.00	55.00	3.51	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	4.00	55.00	6.78	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	4.00	55.00	6.78	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	4.00	55.00	6.78	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	2.00	60.00	3.23	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	2.00	60.00	3.23	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	2.00	60.00	3.23	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	4.00	60.00	6.25	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	4.00	60.00	6.25	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.00	4.00	60.00	6.25	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	2.00	42.00	4.55	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	2.00	42.00	4.55	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	4.00	42.00	8.70	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	2.00	50.00	3.85	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	2.00	50.00	3.85	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	2.00	50.00	3.85	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	4.00	50.00	7.41	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	4.00	50.00	7.41	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	6.00	50.00	10.71	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	2.00	55.00	3.51	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	2.00	55.00	3.51	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	2.00	55.00	3.51	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	4.00	55.00	6.78	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	4.00	55.00	6.78	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	4.00	55.00	6.78	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	8.00	55.00	12.70	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	2.00	60.00	3.23	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	2.00	60.00	3.23	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	2.00	60.00	3.23	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	4.00	60.00	6.25	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	4.00	60.00	6.25	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	6.00	60.00	9.09	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	6.00	60.00	9.09	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	6.00	60.00	9.09	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	8.00	60.00	11.76	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	6.50	8.00	60.00	11.76	0	0	10	42	1
Bolton	1999	1	0	Mexican-style	1	5.00	2.00	42.00	4.55	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	2.00	42.00	4.55	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	2.00	42.00	4.55	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	4.00	42.00	8.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	4.00	42.00	8.70	0	0	10	42	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Bolton	1999	1	0	Mexican-style	1	5.00	4.00	42.00	8.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	2.00	50.00	3.85	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	2.00	50.00	3.85	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	2.00	50.00	3.85	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	4.00	50.00	7.41	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	4.00	50.00	7.41	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	4.00	50.00	7.41	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	2.00	55.00	3.51	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	2.00	55.00	3.51	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	2.00	55.00	3.51	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	4.00	55.00	6.78	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	4.00	55.00	6.78	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	4.00	55.00	6.78	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	2.00	60.00	3.23	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	2.00	60.00	3.23	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	2.00	60.00	3.23	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	4.00	60.00	6.25	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	4.00	60.00	6.25	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	4.00	60.00	6.25	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	6.00	60.00	9.09	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	6.00	60.00	9.09	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	6.00	60.00	9.09	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	8.00	60.00	11.76	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.00	8.00	60.00	11.76	0	0	10	42	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Bolton	1999	1	0	Mexican-style	1	5.00	8.00	60.00	11.76	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	2.00	42.00	4.55	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	2.00	42.00	4.55	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	2.00	42.00	4.55	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	4.00	42.00	8.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	4.00	42.00	8.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	2.00	50.00	3.85	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	2.00	50.00	3.85	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	4.00	50.00	7.41	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	4.00	50.00	7.41	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	4.00	50.00	7.41	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	2.00	55.00	3.51	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	2.00	55.00	3.51	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	4.00	55.00	6.78	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	4.00	55.00	6.78	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	4.00	55.00	6.78	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	2.00	60.00	3.23	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	2.00	60.00	3.23	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	2.00	60.00	3.23	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	4.00	60.00	6.25	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	4.00	60.00	6.25	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	4.00	60.00	6.25	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	6.00	60.00	9.09	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	6.00	60.00	9.09	0	0	10	42	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Bolton	1999	1	0	Mexican-style	1	5.25	6.00	60.00	9.09	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	8.00	60.00	11.76	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.25	8.00	60.00	11.76	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	2.00	42.00	4.55	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	2.00	42.00	4.55	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	4.00	42.00	8.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	4.00	42.00	8.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	2.00	50.00	3.85	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	2.00	50.00	3.85	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	4.00	50.00	7.41	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	4.00	50.00	7.41	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	2.00	55.00	3.51	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	2.00	55.00	3.51	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	4.00	55.00	6.78	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	4.00	55.00	6.78	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	4.00	60.00	6.25	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	6.00	60.00	9.09	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	6.00	60.00	9.09	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	6.00	60.00	9.09	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	8.00	60.00	11.76	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	8.00	60.00	11.76	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.50	8.00	60.00	11.76	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	2.00	42.00	4.55	0	0	10	42	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Bolton	1999	1	0	Mexican-style	1	5.75	4.00	42.00	8.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	4.00	42.00	8.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	4.00	55.00	6.78	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	2.00	60.00	3.23	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	6.00	60.00	9.09	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	6.00	60.00	9.09	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	8.00	60.00	11.76	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	8.00	60.00	11.76	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	5.75	8.00	60.00	11.76	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	2.00	42.00	4.55	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	4.00	42.00	8.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	4.00	42.00	8.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	4.00	50.00	7.41	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	8.00	50.00	13.79	0	0	10	42	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Bolton	1999	1	0	Mexican-style	1	6.00	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	6.00	60.00	9.09	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	6.00	60.00	9.09	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	6.00	60.00	9.09	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	8.00	60.00	11.76	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	8.00	60.00	11.76	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.00	8.00	60.00	11.76	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	2.00	42.00	4.55	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	4.00	42.00	8.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	4.00	42.00	8.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	6.00	42.00	12.50	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	8.00	42.00	16.00	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	4.00	50.00	7.41	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	6.00	50.00	10.71	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	8.00	50.00	13.79	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	6.00	55.00	9.84	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	8.00	55.00	12.70	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	4.00	60.00	6.25	0	0	10	42	0
Bolton	1999	1	0	Mexican-style	1	6.50	8.00	60.00	11.76	0	0	10	42	0
Buazzi	1991	0	0	Swiss	5	5.30	1.00	35.00	2.78	0	1	24	77	0
Buazzi	1991	0	0	Swiss	5	5.30	1.20	36.50	3.18	0	1	24	77	0
Buazzi	1991	0	0	Swiss	5	5.30	1.20	38.00	3.06	0	1	24	77	0
Buazzi	1991	0	0	Swiss	5	5.30	1.00	36.50	2.67	0	1	24	80	0
Buazzi	1991	0	0	Swiss	5	5.20	1.30	37.00	3.39	0	1	24	80	0

Author	Year	[1]Pre(0)/ Post(1)- Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Buazzi	1991	0	0	Swiss	5	5.30	1.40	35.80	3.76	0	1	24	80	0
Buazzi	1991	0	0	Swiss	5	5.30	1.40	37.00	3.65	0	1	24	66	0
Buazzi	1991	0	0	Swiss	5	5.20	1.00	39.00	2.50	0	1	24	66	0
Buazzi	1991	0	0	Swiss	5	5.40	1.00	39.00	2.50	0	1	24	66	0
Buyong	1998	0	0	Cheddar	5	5.20	1.30	32.00	3.90	0	1	8	184	0
Canillac and Mourney	2004	1	0	Cheese medium (semi-soft cheese, tryptone, NaCl, distilled water)	4	5.30	6.37	85.00	6.97	0	1	13	2.5	0
Cao-Hoang	2010	1	0	Mini Babybel	4	5.20	0.67	48.00	1.38	0	1	4	6	0
Carnio	2000	1	0	Model red-smear cheese (Weinkase type)	2	6.50	2.50	50.00	4.76	0	0	10	29	1
Carnio	2000	1	0	Model red-smear cheese (Weinkase type)	2	6.50	2.50	50.00	4.76	0	0	10	24	1
Cataldo	2006	1	0	Crescenza	1	5.60	4.50	57.50	7.26	0	0	4	14	1
Cataldo	2006	1	0	Crescenza	1	5.30	7.25	57.50	11.20	0	0	4	14	0
Cataldo	2006	1	0	Gorgonzola	4	6.40	6.00	42.00	12.50	0	1	4	14	0
Cataldo	2006	1	0	Gorgonzola	4	6.40	6.00	42.00	12.50	0	1	4	14	0
Cataldo	2006	1	0	Mozzarella	3	5.40	4.50	60.00	6.98	0	1	4	14	0
Cataldo	2006	1	0	Mozzarella	3	5.40	4.50	60.00	6.98	0	1	4	14	0
Cataldo	2006	1	0	Ricotta	2	6.35	1.00	80.00	1.23	0	0	4	14	0
Cataldo	2006	1	0	Ricotta	2	6.35	1.00	80.00	1.23	0	0	4	14	0
Cetinkaya	2004	0	1	Kashar	3	5.28	4.60	41.90	9.89	0	0	5	120	0
Cetinkaya	2004	0	1	Kashar	3	5.28	4.60	41.90	9.89	0	0	5	60	0
Cetinkaya	2004	0	1	Kashar	3	5.28	4.60	41.90	9.89	0	0	5	120	0
Cetinkaya	2004	0	1	Kashar	3	5.28	4.60	41.90	9.89	0	0	5	120	0
Cetinkaya	2006	0	0	Kashar	3	5.15	4.60	41.90	9.89	0	1	6	7	0
Cetinkaya	2006	0	0	Kashar	3	5.15	4.60	41.90	9.89	0	1	6	7	0
Cetinkaya	2006	0	0	Kashar	3	5.15	4.60	41.90	9.89	0	1	6	7	0
Cetinkaya	2006	0	0	Kashar	3	5.15	4.60	41.90	9.89	0	1	6	7	0
Chen and Hotchkiss	1993	0	0	Low-fat cottage cheese	2	5.14	0.50	80.00	0.62	0	0	4	63	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Chen and Hotchkiss	1993	0	0	Low-fat cottage cheese	2	5.14	0.50	80.00	0.62	0	0	7	14	1
Collins	2011	1	0	Cottage cheese	2	4.30	0.50	80.00	0.62	0	0	20	3	0
Collins	2011	1	0	Cottage cheese	2	4.30	0.50	80.00	0.62	0	0	20	3	0
Collins	2011	1	0	Cottage cheese	2	4.30	0.50	80.00	0.62	0	0	20	3	0
D'Amico	2008	1	0	Soft, surface-mold-ripened cheese made w/ GDL	1	4.80		65.70	3.90	0	1	4	70	1
D'Amico	2008	1	0	Soft, surface-mold-ripened cheese made w/ GDL	1	4.80		65.70	3.90	0	1	4	70	1
D'Amico	2008	1	0	Soft, surface-mold-ripened cheese made w/ GDL	1	4.80		69.65	3.10	0	1	4	70	1
D'Amico	2008	1	0	Soft, surface-mold-ripened cheese made w/ GDL	1	4.80		69.65	3.10	0	1	4	70	1
D'Amico	2008	1	0	Soft, surface-mold-ripened cheese made w/ GDL	1	4.80		66.15	3.53	0	1	4	70	1
D'Amico	2008	1	0	Soft, surface-mold-ripened cheese made w/ GDL	1	4.80		66.15	3.53	0	1	4	70	1
D'Amico	2008	1	1	Soft, surface-mold-ripened cheese made w/ GDL	1	4.80		64.81	3.34	0	1	4	70	1
D'Amico	2008	1	1	Soft, surface-mold-ripened cheese made w/ GDL	1	4.80		64.81	3.34	0	1	4	70	1

Author	Year	[1]Pre(0)/ Post(1)- Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
D'Amico	2008	1	1	Soft, surface-mold-ripened cheese made w/ GDL	1	4.80		67.13	3.30	0	1	4	70	1
D'Amico	2008	1	1	Soft, surface-mold-ripened cheese made w/ GDL	1	4.80		63.74	3.85	0	1	4	70	1
D'Amico	2008	1	1	Soft, surface-mold-ripened cheese made w/ GDL	1	4.80		63.74	3.85	0	1	4	70	1
D'Amico	2008	1	1	Soft, surface-mold-ripened cheese made w/ GDL	1	4.80		67.13	3.30	0	1	4	70	0
Davies	1997	1	1	Ricotta	2	5.80	0.75	75.00	0.99	0	0	7	63	1
Davies	1997	1	1	Ricotta	2	6.10	0.75	75.00	0.99	0	0	7	63	1
Dominguez	1987	0	0	Manchego-type semi-hard of sheep:goat:cow (15:35:50)	4	5.45		42.82	5.91	1	1	15	60	1
Dominguez	1987	0	0	Manchego-type semi-hard of sheep:goat:cow (15:35:50)	4	5.45		42.82	5.91	1	1	15	60	1
Dominguez	1987	0	0	Manchego-type semi-hard of sheep:goat:cow (15:35:50)	4	5.45		42.82	5.91	1	1	15	60	1
Dominguez	1987	0	0	Manchego-type semi-hard of sheep:goat:cow (15:35:50)	4	5.45		42.82	5.91	1	1	15	60	0
Durmaz	2009	0	1	Carra (raw milk + black cumin)	4	5.19	4.42	56.55	7.25	0	0	15	90	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
El-Ziney	1998	1	0	Cottage cheese	2	5.40	0.50	74.50	0.67	0	0	7	21	1
Ennahar	1994	1	1	Core of soft surface-ripened	3	5.10	2.00	50.00	3.85	0	0	4	32	0
Ennahar	1994	1	1	Rind of soft surface-ripened	3	6.20	2.00	50.00	3.85	0	0	4	32	1
Ennahar	1998	1	0	Muenster (smear-surface soft)	4	6.48	1.60	46.00	3.36	0	1	15	21	1
Eppert	1997	0	0	Model red-smear cheese (Weinkase type)	3	7.00	2.50	50.00	4.76	0	1	10	21	1
Erkmen	2000	0	0	Turkish white feta cheese w/ starter	3	4.77	1.50	60.00	2.44	0	1	4	90	0
Erkmen	2000	0	0	Turkish white feta cheese w/ starter	3	4.77	1.50	60.00	2.44	0	1	4	90	0
Erkmen	2000	0	0	Turkish white feta cheese w/ starter	3	4.77	1.50	60.00	2.44	0	1	4	90	0
Erkmen	2000	0	0	Turkish white feta cheese w/ starter	3	4.77	2.00	60.00	3.23	0	1	4	90	0
Erkmen	2000	0	0	Turkish white feta cheese w/ starter	3	4.77	2.00	60.00	3.23	0	1	4	90	0
Erkmen	2000	0	0	Turkish white feta cheese w/ starter	3	4.77	2.00	60.00	3.23	0	1	4	90	0
Erkmen	2000	0	0	Turkish white feta cheese w/o starter	3	5.87	1.50	60.00	2.44	0	0	4	90	0
Erkmen	2000	0	0	Turkish white feta cheese w/o starter	3	5.87	1.50	60.00	2.44	0	0	4	90	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Erkmen	2000	0	0	Turkish white feta cheese w/o starter	3	5.87	1.50	60.00	2.44	0	0	4	90	0
Erkmen	2000	0	0	Turkish white feta cheese w/o starter	3	5.87	2.00	60.00	3.23	0	0	4	90	0
Erkmen	2000	0	0	Turkish white feta cheese w/o starter	3	5.87	2.00	60.00	3.23	0	0	4	90	0
Erkmen	2000	0	0	Turkish white feta cheese w/o starter	3	5.87	2.00	60.00	3.23	0	0	4	90	0
Ferreira	1996	1	0	Cottage	2	4.65	1.00	80.00	1.23	0	0	20	7	0
Finazzi	2011	1	1	Water buffalo mozzarella	3	5.13	1.00	60.00	1.64	1	0	10	22	1
Finazzi	2011	1	1	Water buffalo mozzarella	3	4.63	1.00	60.00	1.64	1	0	10	9	1
Finazzi	2011	1	1	Water buffalo mozzarella	3	4.66	1.00	60.00	1.64	1	0	20	9	1
Finazzi	2011	1	1	Water buffalo mozzarella	3	4.79	1.00	60.00	1.64	1	0	5	22	0
Gahan	1996	1	0	Cottage cheese (commercial)	2	5.15	0.41	75.00	0.54	0	1	4	26	0
Gahan	1996	1	0	Cottage cheese (lab made)	2	4.71	0.41	75.00	0.54	0	1	4	15	0
Gahan	1996	1	0	Low-fat cheddar cheese	5	5.25	2.00	45.00	4.26	0	1	8	70	0
Gahan	1996	1	0	Mozzarella	3	5.60	0.63	50.00	1.24	0	0	4	28	0
Gahan	1996	1	0	Whole-fat cheddar cheese	5	5.16	1.70	38.00	4.47	0	1	8	70	0
Gamiero	2007	0	1	Ewe's milk cheese (Azeitao)	3	5.73	2.00	65.00	2.99	1	0	10	78	1
Gamiero	2007	0	1	Ewe's milk cheese (Azeitao)	3	5.73	2.00	65.00	2.99	1	0	10	78	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Gamiero	2007	0	1	Ewe's milk cheese (Azeitao)	3	5.73	2.00	65.00	2.99	1	0	10	78	1
Gamiero	2007	0	1	Ewe's milk cheese (Azeitao)	3	5.73	2.00	65.00	2.99	1	0	10	78	1
Gamiero	2007	0	1	Ewe's milk cheese (Azeitao)	3	5.73	2.00	65.00	2.99	1	0	12	78	1
Gamiero	2007	0	1	Ewe's milk cheese (Azeitao)	3	5.73	2.00	65.00	2.99	1	0	12	78	1
Gamiero	2007	0	1	Ewe's milk cheese (Azeitao)	3	5.73	2.00	65.00	2.99	1	0	12	78	0
Gamiero	2007	0	1	Ewe's milk cheese (Azeitao)	3	5.73	2.00	65.00	2.99	1	0	12	78	0
Gamiero	2007	0	1	Ewe's milk cheese (Azeitao)	3	5.73	2.00	65.00	2.99	1	0	12	78	0
Gay	2005	0	0	Camembert	3	6.00	1.60	56.00	2.78	0	1	4	40	1
Gay	2005	0	1	Camembert	3	5.75	1.60	56.00	2.78	0	1	4	40	1
Genigeorgis	1991	1	1	Blue cheese	4	5.10			6.10	0	1	4	36	0
Genigeorgis	1991	1	1	Blue cheese	4	5.10			6.10	0	1	8	18	0
Genigeorgis	1991	1	1	Blue cheese	4	5.10			6.10	0	1	30	18	0
Genigeorgis	1991	1	0	Brie (center)	3	6.00			2.50	0	1	4	6	1
Genigeorgis	1991	1	0	Brie (center)	3	7.40			3.58	0	1	4	19	1
Genigeorgis	1991	1	0	Brie (center)	3	6.00			2.50	0	1	8	4	1
Genigeorgis	1991	1	0	Brie (center)	3	7.40			3.58	0	1	8	9	1
Genigeorgis	1991	1	0	Brie (center)	3	6.00			2.50	0	1	30	2	1
Genigeorgis	1991	1	0	Brie (center)	3	7.40			3.58	0	1	30	1	1
Genigeorgis	1991	1	0	Brie (surface)	3	7.20			2.50	0	1	4	30	1
Genigeorgis	1991	1	0	Brie (surface)	3	7.70			3.58	0	1	4	14	1
Genigeorgis	1991	1	0	Brie (surface)	3	7.20			2.50	0	1	8	36	1
Genigeorgis	1991	1	0	Brie (surface)	3	7.70			3.58	0	1	8	9	1
Genigeorgis	1991	1	0	Brie (surface)	3	7.20			2.50	0	1	30	2	1
Genigeorgis	1991	1	0	Brie (surface)	3	7.70			3.58	0	1	30	3	1
Genigeorgis	1991	1	0	Camembert (center)	3	7.30			2.49	0	1	4	36	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Genigeorgis	1991	1	0	Camembert (center)	3	7.30			2.49	0	1	8	15	1
Genigeorgis	1991	1	0	Camembert (center)	3	7.30			2.49	0	1	30	2	1
Genigeorgis	1991	1	0	Camembert (surface)	3	7.30			2.49	0	1	4	36	1
Genigeorgis	1991	1	0	Camembert (surface)	3	7.30			2.49	0	1	8	22	1
Genigeorgis	1991	1	0	Camembert (surface)	3	7.30			2.49	0	1	30	2	1
Genigeorgis	1991	1	0	Cheddar (mild)	5	5.20			4.49	0	1	4	30	0
Genigeorgis	1991	1	0	Cheddar (mild)	5	5.20			4.49	0	1	8	30	0
Genigeorgis	1991	1	0	Cheddar (mild)	5	4.90			2.60	0	1	30	4	0
Genigeorgis	1991	1	0	Cheddar (mild)	5	5.20			4.49	0	1	30	7	0
Genigeorgis	1991	1	1	Cheddar (sharp)	5	5.20			4.77	0	1	4	36	0
Genigeorgis	1991	1	1	Cheddar (sharp)	5	5.60			5.40	0	1	8	4	0
Genigeorgis	1991	1	1	Cheddar (sharp)	5	5.20			4.77	0	1	8	36	0
Genigeorgis	1991	1	1	Cheddar (sharp)	5	5.60			5.40	0	1	30	4	0
Genigeorgis	1991	1	1	Cheddar (sharp)	5	5.20			4.77	0	1	30	3	0
Genigeorgis	1991	1	0	Colby	5	5.50			4.93	0	1	4	36	0
Genigeorgis	1991	1	0	Colby	5	5.50			4.93	0	1	8	36	0
Genigeorgis	1991	1	0	Colby	5	5.50			4.93	0	1	30	9	0
Genigeorgis	1991	1	0	Cotija	5	5.60			9.60	0	1	4	36	0
Genigeorgis	1991	1	0	Cotija	5	5.50			12.50	0	0	4	30	0
Genigeorgis	1991	1	0	Cotija	5	5.60			9.60	0	1	8	18	0
Genigeorgis	1991	1	0	Cotija	5	5.50			12.50	0	0	8	10	0
Genigeorgis	1991	1	0	Cotija	5	5.60			9.60	0	1	30	8	0
Genigeorgis	1991	1	0	Cotija	5	5.50			12.50	0	0	30	6	0
Genigeorgis	1991	1	0	Cottage cheese	2	4.90			1.00	0	1	4	24	1
Genigeorgis	1991	1	0	Cottage cheese	2	5.00			1.04	0	1	4	24	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Genigeorgis	1991	1	0	Cottage cheese	2	5.00			1.14	0	0	4	16	1
Genigeorgis	1991	1	0	Cottage cheese	2	5.10			1.13	0	0	4	36	1
Genigeorgis	1991	1	0	Cottage cheese	2	5.10			1.13	0	0	4	27	1
Genigeorgis	1991	1	0	Cottage cheese	2	4.90			1.00	0	1	8	18	1
Genigeorgis	1991	1	0	Cottage cheese	2	5.00			1.14	0	0	8	24	1
Genigeorgis	1991	1	0	Cottage cheese	2	5.10			1.13	0	0	8	8	1
Genigeorgis	1991	1	0	Cottage cheese	2	5.10			1.13	0	0	8	14	1
Genigeorgis	1991	1	0	Cottage cheese	2	5.10			1.13	0	0	30	4	1
Genigeorgis	1991	1	0	Cottage cheese	2	5.10			1.13	0	0	30	2	1
Genigeorgis	1991	1	0	Cottage cheese	2	5.00			1.04	0	1	8	36	0
Genigeorgis	1991	1	0	Cottage cheese	2	4.90			1.00	0	1	30	8	0
Genigeorgis	1991	1	0	Cottage cheese	2	5.00			1.04	0	1	30	8	0
Genigeorgis	1991	1	0	Cottage cheese	2	5.00			1.14	0	0	30	8	0
Genigeorgis	1991	1	0	Cottage cheese (with acetic acid)	2	5.00			1.16	0	0	4	8	0
Genigeorgis	1991	1	0	Cottage cheese (with acetic acid)	2	5.00			1.16	0	0	8	8	0
Genigeorgis	1991	1	0	Cottage cheese (with acetic acid)	2	5.00			1.16	0	0	30	8	0
Genigeorgis	1991	1	1	Cracker Barrel (cheddar)	5	5.20			5.00	0	1	4	24	0
Genigeorgis	1991	1	1	Cracker Barrel (cheddar)	5	5.20			5.00	0	1	8	30	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Genigeorgis	1991	1	1	Cracker Barrel (cheddar)	5	5.20			5.00	0	1	30	8	0
Genigeorgis	1991	1	0	Cream cheese	2	4.80			0.90	0	1	4	36	0
Genigeorgis	1991	1	0	Cream cheese	2	4.80			0.90	0	1	8	30	0
Genigeorgis	1991	1	0	Cream cheese	2	4.80			0.90	0	1	30	12	0
Genigeorgis	1991	1	0	Domestic Feta	3	4.30			7.50	0	1	4	8	0
Genigeorgis	1991	1	0	Domestic Feta	3	4.30			7.50	0	1	8	8	0
Genigeorgis	1991	1	0	Domestic Feta	3	4.30			2.20	0	1	8	8	0
Genigeorgis	1991	1	0	Domestic Feta	3	4.30			7.50	0	1	30	4	0
Genigeorgis	1991	1	0	Domestic Feta	3	4.30			2.20	0	1	30	4	0
Genigeorgis	1991	1	0	Domestic Kasseri	4	4.80			5.80	0	1	4	6	0
Genigeorgis	1991	1	0	Domestic Kasseri	4	4.80			5.80	0	1	8	8	0
Genigeorgis	1991	1	0	Domestic Kasseri	4	4.80			5.80	0	1	30	4	0
Genigeorgis	1991	1	0	Imported Feta	3	4.30			7.00	0	1	4	8	0
Genigeorgis	1991	1	0	Imported Feta	3	4.20			7.40	0	1	4	8	0
Genigeorgis	1991	1	0	Imported Feta	3	4.30			7.00	0	1	8	8	0
Genigeorgis	1991	1	0	Imported Feta	3	4.20			7.40	0	1	8	8	0
Genigeorgis	1991	1	0	Imported Feta	3	4.30			7.00	0	1	30	4	0
Genigeorgis	1991	1	0	Imported Feta	3	4.20			7.40	0	1	30	4	0
Genigeorgis	1991	1	0	Imported Kasseri	4	5.30			5.52	0	1	4	36	0
Genigeorgis	1991	1	0	Imported Kasseri	4	5.30			5.52	0	1	8	24	0
Genigeorgis	1991	1	0	Imported Kasseri	4	5.30			5.52	0	1	30	8	0
Genigeorgis	1991	1	0	Limburger	4	7.20			4.78	0	1	4	36	0
Genigeorgis	1991	1	0	Limburger	4	7.20			4.78	0	1	8	36	0
Genigeorgis	1991	1	0	Limburger	4	7.20			4.78	0	1	30	9	0
Genigeorgis	1991	1	1	Monterey Jack	4	5.00			1.28	0	1	4	30	0
Genigeorgis	1991	1	0	Monterey Jack	4	5.20			2.72	0	1	4	30	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Genigeorgis	1991	1	1	Monterey Jack	4	5.00			1.28	0	1	8	19	0
Genigeorgis	1991	1	0	Monterey Jack	4	5.20			2.72	0	1	8	30	0
Genigeorgis	1991	1	1	Monterey Jack	4	5.00			1.00	0	1	30	4	0
Genigeorgis	1991	1	1	Monterey Jack	4	5.00			1.28	0	1	30	13	0
Genigeorgis	1991	1	0	Monterey Jack	4	5.00			3.00	0	1	30	4	0
Genigeorgis	1991	1	0	Monterey Jack	4	5.20			2.72	0	1	30	13	0
Genigeorgis	1991	1	0	Muenster	4	5.50			3.80	0	1	4	36	0
Genigeorgis	1991	1	0	Muenster	4	5.50			3.80	0	1	8	36	0
Genigeorgis	1991	1	0	Muenster	4	5.50			3.80	0	1	30	9	0
Genigeorgis	1991	1	0	Provolone	4	5.60			4.62	0	1	4	36	0
Genigeorgis	1991	1	0	Provolone	4	5.60			4.62	0	1	8	36	0
Genigeorgis	1991	1	0	Provolone	4	5.60			4.62	0	1	30	9	0
Genigeorgis	1991	1	0	Queso Fresco	1	6.50			6.15	0	0	4	30	1
Genigeorgis	1991	1	0	Queso Fresco	1	6.50			6.15	0	0	8	6	1
Genigeorgis	1991	1	0	Queso Fresco	1	6.60			6.60	0	0	30	3	1
Genigeorgis	1991	1	0	Queso Fresco	1	6.60			4.50	0	0	30	6	1
Genigeorgis	1991	1	0	Queso Fresco	1	6.50			6.15	0	0	30	3	1
Genigeorgis	1991	1	0	Queso Fresco	1	6.60			6.60	0	0	4	30	0
Genigeorgis	1991	1	0	Queso Fresco	1	6.60			4.50	0	0	4	10	0
Genigeorgis	1991	1	0	Queso Fresco	1	6.60			6.60	0	0	8	14	0
Genigeorgis	1991	1	0	Queso Fresco	1	6.60			4.50	0	0	8	8	0
Genigeorgis	1991	1	0	Queso Panela	1	6.70			3.95	0	0	4	10	1
Genigeorgis	1991	1	0	Queso Panela	1	6.70			3.95	0	0	8	4	1
Genigeorgis	1991	1	0	Queso Panela	1	6.70			3.95	0	0	30	1	1
Genigeorgis	1991	1	0	Queso Panella	1	6.60			3.48	0	0	4	30	1
Genigeorgis	1991	1	0	Queso Panella	1	6.20			2.50	0	0	4	36	1
Genigeorgis	1991	1	0	Queso Panella	1	6.60			3.48	0	0	8	6	1
Genigeorgis	1991	1	0	Queso Panella	1	6.60			3.48	0	0	30	3	1
Genigeorgis	1991	1	0	Queso Panella	1	6.20			2.50	0	0	30	3	1
Genigeorgis	1991	1	0	Queso Panella	1	6.20			2.50	0	0	8	8	0
Genigeorgis	1991	1	0	Queso Ranchero	1	6.20			4.10	0	0	4	18	1
Genigeorgis	1991	1	0	Queso Ranchero	1	6.20			4.10	0	0	8	8	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Genigeorgis	1991	1	0	Queso Ranchero	1	6.20			4.10	0	0	30	1	1
Genigeorgis	1991	1	0	Ricotta (with K-sorbate and vinegar)	2	6.10			0.60	0	0	4	30	1
Genigeorgis	1991	1	0	Ricotta (with K-sorbate and vinegar)	2	6.10			0.60	0	0	8	8	1
Genigeorgis	1991	1	0	Ricotta (with K-sorbate and vinegar)	2	6.10			0.60	0	0	30	1	1
Genigeorgis	1991	1	0	Ricotta (with vinegar)	2	5.90			0.70	0	0	4	36	1
Genigeorgis	1991	1	0	Ricotta (with vinegar)	2	6.10			0.71	0	0	4	22	1
Genigeorgis	1991	1	0	Ricotta (with vinegar)	2	6.10			0.71	0	0	4	36	1
Genigeorgis	1991	1	0	Ricotta (with vinegar)	2	5.90			0.70	0	0	8	6	1
Genigeorgis	1991	1	0	Ricotta (with vinegar)	2	6.10			0.71	0	0	8	8	1
Genigeorgis	1991	1	0	Ricotta (with vinegar)	2	6.10			0.71	0	0	8	27	1
Genigeorgis	1991	1	0	Ricotta (with vinegar)	2	5.90			0.70	0	0	30	1	1
Genigeorgis	1991	1	0	Ricotta (with vinegar)	2	6.10			0.71	0	0	30	4	1
Genigeorgis	1991	1	0	Ricotta (with vinegar)	2	6.10			0.71	0	0	30	2	1
Genigeorgis	1991	1	0	String cheese	4	5.50			4.24	0	1	4	36	0
Genigeorgis	1991	1	0	String cheese	4	5.50			4.24	0	1	8	36	0
Genigeorgis	1991	1	0	String cheese	4	5.50			4.24	0	1	30	9	0
Genigeorgis	1991	1	1	Swiss	5	5.50			2.72	0	1	4	36	0
Genigeorgis	1991	1	1	Swiss	5	5.50			2.72	0	1	8	19	0
Genigeorgis	1991	1	1	Swiss	5	5.50			2.72	0	1	30	7	0
Genigeorgis	1991	1	0	Teleme	2	5.90			1.79	0	1	8	36	1
Genigeorgis	1991	1	0	Teleme	2	5.90			1.79	0	1	30	15	1
Genigeorgis	1991	1	0	Teleme	2	5.90			1.79	0	1	4	36	0
Genigeorgis	1991	1	1	Tilllamook (cheddar)	5	5.10			3.94	0	1	4	36	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Genigeorgis	1991	1	1	Tilllamook (cheddar)	5	5.10			3.94	0	1	8	36	0
Genigeorgis	1991	1	1	Tilllamook (cheddar)	5	5.20			5.60	0	1	30	8	0
Genigeorgis	1991	1	1	Tilllamook (cheddar)	5	5.10			3.94	0	1	30	3	0
Giannou	2009	1	0	Graviera w/ commercial starter	1	5.60	2.00	34.80	5.43	0	1	4	90	0
Giannou	2009	1	0	Graviera w/ commercial starter	1	5.60	2.00	34.80	5.43	0	1	4	90	0
Giannou	2009	1	0	Graviera w/ commercial starter	1	5.60	2.00	34.80	5.43	0	1	12	60	0
Giannou	2009	1	0	Graviera w/ commercial starter	1	5.60	2.00	34.80	5.43	0	1	12	60	0
Giannou	2009	1	0	Graviera w/ commercial starter	1	5.60	2.00	34.80	5.43	0	1	25	60	0
Giannou	2009	1	0	Graviera w/ commercial starter	1	5.60	2.00	34.80	5.43	0	1	25	60	0
Giannou	2009	1	0	Graviera w/ commercial starter + Enterococcus faecium Graviera isolate	1	5.60	2.00	34.80	5.43	0	1	4	90	0
Giannou	2009	1	0	Graviera w/ commercial starter + Enterococcus faecium Graviera isolate	1	5.60	2.00	34.80	5.43	0	1	4	90	0
Giannou	2009	1	0	Graviera w/ commercial starter +	1	5.60	2.00	34.80	5.43	0	1	12	60	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Giannou	2009	1	0	Enterococcus faecium Graviera isolate Graviera w/ commercial starter + Enterococcus faecium Graviera isolate	1	5.60	2.00	34.80	5.43	0	1	12	60	0
Giannou	2009	1	0	Graviera w/ commercial starter + Enterococcus faecium Graviera isolate	1	5.60	2.00	34.80	5.43	0	1	25	60	0
Giannou	2009	1	0	Graviera w/ commercial starter + Enterococcus faecium Graviera isolate	1	5.60	2.00	34.80	5.43	0	1	25	60	0
Glass, K.	1998	1	0	Process Cheese Slices	6	5.66	2.35	39.40	5.63	0	1	30	4	0
Glass, K.	1998	1	0	Process Cheese Slices	6	5.84	2.57	39.70	6.08	0	1	30	4	0
Glass, K.	1998	1	0	Process Cheese Slices	6	5.82	2.50	39.10	6.01	0	1	30	4	0
Glass, K.	1998	1	0	Process Cheese Slices	6	5.61	2.47	39.60	5.87	0	1	30	4	0
Glass, K.	1998	1	0	Process Cheese Slices	6	5.70	2.45	39.70	5.81	0	1	30	4	0
Glass, K.	1998	1	0	Process Cheese Slices	6	5.78	2.62	40.30	6.10	0	1	30	4	0
Goerges	2011	1	0	Tilsit (smeared)	4	7.00	3.30	38.50	7.89	0	1	13	28	1
Goerges	2011	1	0	Tilsit (smeared)	4	7.00	3.30	38.50	7.89	0	1	13	28	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Govaris	2002	0	0	Feta	3	4.60	2.20	53.40	3.96	0	1	4	28	0
Govaris	2002	0	0	Feta	3	4.60	2.27	52.90	4.11	0	1	4	28	0
Govaris	2002	0	0	Teleme	3	4.60	2.10	55.20	3.66	0	1	4	28	0
Govaris	2002	0	0	Teleme	3	4.60	2.15	54.50	3.80	0	1	4	28	0
Govaris	2011	1	0	Feta	3	4.55	2.14	53.40	3.85	0	1	4	30	0
Govaris	2011	1	0	Feta	3	4.55	2.14	53.40	3.85	0	1	4	32	0
Govaris	2011	1	0	Feta	3	4.55	2.14	53.40	3.85	0	1	4	32	0
Guenther	2011	0	0	Washed-rind cheese with red-smear surface (Limburger-type)	3	7.90		64.00	3.00	0	1	6	11	1
Guenther	2011	0	0	Washed-rind cheese with red-smear surface (Limburger-type)	3	7.90		64.00	3.00	0	1	6	11	1
Guenther	2011	0	0	Washed-rind cheese with red-smear surface (Limburger-type)	3	7.80		64.00	3.00	0	1	6	11	1
Guenther	2011	0	0	Washed-rind cheese with red-smear surface (Limburger-type)	3	7.80		64.00	3.00	0	1	6	11	1
Guenther	2011	0	0	Washed-rind cheese with red-smear surface (Limburger-type)	3	7.90		64.00	3.00	0	1	6	11	1
Guenther	2011	0	0	Washed-rind cheese with red-smear surface	3	7.50		64.00	3.00	0	1	6	11	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Guenther	2011	0	0	(Limburger-type) Washed-rind cheese with red-smear surface (Limburger-type)	3	7.60		64.00	3.00	0	1	6	11	1
Guenther	2011	0	0	White mold (Camembert-type)	3	7.00		64.00	3.00	0	1	6	10	1
Guenther	2011	0	0	White mold (Camembert-type)	3	7.70		64.00	3.00	0	1	6	10	1
Guenther	2011	0	0	White mold (Camembert-type)	3	7.70		64.00	3.00	0	1	6	10	1
Hicks	1991	1	0	Cottage cheese	2	5.06	1.00	80.00	1.23	0	1	4	14	0
Hicks	1991	1	0	Cottage cheese	2	4.69	1.00	80.00	1.23	0	1	4	14	0
Hicks	1991	1	0	Cottage cheese	2	4.75	1.00	80.00	1.23	0	1	4	14	0
Hicks	1991	1	0	Cottage cheese	2	5.06	1.00	80.00	1.23	0	1	8	14	0
Hicks	1991	1	0	Cottage cheese	2	4.69	1.00	80.00	1.23	0	1	8	14	0
Hicks	1991	1	0	Cottage cheese	2	4.75	1.00	80.00	1.23	0	1	8	14	0
Hicks	1991	1	0	Cottage cheese	2	5.06	1.00	80.00	1.23	0	1	12	14	0
Hicks	1991	1	0	Cottage cheese	2	4.69	1.00	80.00	1.23	0	1	12	14	0
Hicks	1991	1	0	Cottage cheese	2	4.75	1.00	80.00	1.23	0	1	12	14	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	5	30	0
Kagkli	2009	1	0	Katiki Domokou	2	4.50	1.00	75.00	1.32	0	0	5	30	0

Author	Year	[1]Pre(0)/ Post(1)- Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
				(Greek soft cream cheese)										
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	5	30	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	5	30	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	5	30	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	10	30	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	10	30	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	10	30	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	10	30	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	10	30	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	15	20	0
Kagkli	2009	1	0	Katiki Domokou	2	4.50	1.00	75.00	1.32	0	0	15	13	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
				(Greek soft cream cheese)										
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	15	15	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	15	15	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	15	15	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	20	10	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	20	9	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	20	10	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	20	9	0
Kagkli	2009	1	0	Katiki Domokou (Greek soft cream cheese)	2	4.50	1.00	75.00	1.32	0	0	20	10	0
Konteles	2009	0	0	Feta	3	4.58	2.83	56.39	4.78	0	1	4	30	0
Konteles	2009	0	0	Feta	3	4.58	2.83	56.39	4.78	0	1	4	30	0
Konteles	2009	0	0	Feta	3	4.58	2.83	56.39	4.78	0	1	4	30	0
Konteles	2009	0	0	Feta	3	4.58	2.83	56.39	4.78	0	1	4	30	0
Kovincic	1991	0	0	Trappist	4	5.42	1.14	43.90	2.53	0	1	10	90	1
Kovincic	1991	0	0	Trappist	4	5.02	0.71	42.87	1.63	0	1	10	90	1
Kovincic	1991	0	0	Trappist	4	5.00	1.10	51.14	2.11	0	1	10	90	1

Author	Year	[1]Pre(0)/ Post(1)- Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Kovincic	1991	0	0	Trappist	4	5.03	0.78	45.29	1.69	0	1	10	90	1
Kovincic	1991	0	0	Trappist	4	5.40	1.19	45.00	2.58	0	1	10	90	0
Laukova	2001	0	0	Saint-Paulin	4	5.42	1.50	48.00	3.03	0	1	14	56	0
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	21	1
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	28	1
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	28	1
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	21	1
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	28	0
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	28	0
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	21	0
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	28	0
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	28	0
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	21	0
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	28	0
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	28	0
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	28	0
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	21	0
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	28	0
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	21	0
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	28	0
Leuschner	2002	0	0	Soft cheese	1	4.50	1.00	75.00	1.32	0	1	4	28	0
Lin	2006	1	0	Queso Fresco (exudate)	1	6.00	3.00	48.00	5.88	0	0	4	84	1
Lin	2006	1	0	Queso Fresco (exudate)	1	6.00	3.00	48.00	5.88	0	0	4	84	1
Lin	2006	1	0	Queso Fresco (exudate)	1	6.00	3.00	48.00	5.88	0	0	12	21	1
Lin	2006	1	0	Queso Fresco (exudate)	1	6.00	3.00	48.00	5.88	0	0	12	21	1
Lin	2006	1	0	Queso Fresco (exudate)	1	6.00	3.00	48.00	5.88	0	0	21	10	1
Lin	2006	1	0	Queso Fresco (exudate)	1	6.00	3.00	48.00	5.88	0	0	21	10	0
Lin	2006	1	0	Queso Fresco (interior)	1	6.30	3.00	48.00	5.88	0	0	4	84	1
Lin	2006	1	0	Queso Fresco (interior)	1	6.30	3.00	48.00	5.88	0	0	4	84	1
Lin	2006	1	0	Queso Fresco (interior)	1	6.30	3.00	48.00	5.88	0	0	12	21	1
Lin	2006	1	0	Queso Fresco (interior)	1	6.30	3.00	48.00	5.88	0	0	12	21	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Lin	2006	1	0	Queso Fresco (interior)	1	6.30	3.00	48.00	5.88	0	0	21	10	1
Lin	2006	1	0	Queso Fresco (interior)	1	6.30	3.00	48.00	5.88	0	0	21	10	1
Lin	2006	1	0	Queso Fresco (surface)	1	6.30	3.00	48.00	5.88	0	0	4	84	1
Lin	2006	1	0	Queso Fresco (surface)	1	6.30	3.00	48.00	5.88	0	0	4	84	1
Lin	2006	1	0	Queso Fresco (surface)	1	6.30	3.00	48.00	5.88	0	0	12	21	1
Lin	2006	1	0	Queso Fresco (surface)	1	6.30	3.00	48.00	5.88	0	0	12	21	1
Lin	2006	1	0	Queso Fresco (surface)	1	6.30	3.00	48.00	5.88	0	0	21	10	1
Lin	2006	1	0	Queso Fresco (surface)	1	6.30	3.00	48.00	5.88	0	0	21	10	1
Linton	2008	0	1	Camembert from raw milk-high inoculum	3	5.61	2.71	54.35	4.75	0	1	13	14	1
Linton	2008	0	1	Camembert from raw milk-high inoculum + pressure trt	3	5.55	2.76	56.23	4.68	0	1	13	14	0
Linton	2008	0	1	Camembert from raw milk-low inoculum	3	5.61	2.71	54.35	4.75	0	1	13	14	1
Linton	2008	0	1	Camembert from raw milk-low inoculum + pressure trt	3	5.55	2.76	56.23	4.68	0	1	13	14	0
Liu	2006	0	0	Cottage cheese	2	4.90	4.50	80.00	5.33	0	1	4	2	0
Liu	2006	0	0	Cottage cheese	2	4.90	4.50	80.00	5.33	0	1	4	10	0
Liu	2006	0	0	Cottage cheese	2	4.90	4.50	80.00	5.33	0	1	4	15	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Liu	2006	0	0	Cottage cheese	2	4.90	4.50	80.00	5.33	0	1	4	15	0
Liu	2006	0	0	Cottage cheese	2	4.90	4.50	80.00	5.33	0	1	4	15	0
Liu	2006	0	0	Cottage cheese	2	4.90	4.50	80.00	5.33	0	1	4	15	0
Liu	2006	0	0	Cottage cheese	2	4.90	4.50	80.00	5.33	0	1	4	15	0
Liu	2006	0	0	Cottage cheese	2	4.90	4.50	80.00	5.33	0	1	4	15	0
Loessner	2003	0	0	Model red-smear cheese (Weinkase type)	1	7.00	2.50	50.00	4.76	0	1	10	36	1
Lopez-Pedemonte	2007	0	0	Model washed-curd cheese	4	5.00		45.00	1.50	0	1	8	30	0
Maisnier-Patin	1992	0	0	Camembert	3	6.80	1.50	50.40	2.89	0	1	11	42	1
Maisnier-Patin	1992	0	0	Camembert	3	6.80	1.50	50.40	2.89	0	1	11	42	1
Margolles	1997	0	0	Afeuga'l Pitu Cheese (acid coagulated Spanish cheese, soft) - pilot plant made	1	4.43	1.11	85.90	1.28	0	0	5	7	0
Margolles	1997	0	0	Afeuga'l Pitu Cheese (acid coagulated Spanish cheese, soft) - pilot plant made	1	4.09	1.11	68.97	1.58	0	0	5	7	0
Margolles	1997	0	0	Afeuga'l Pitu Cheese (acid coagulated Spanish cheese, soft) -	1	4.09	1.11	61.89	1.76	0	0	5	7	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Margolles	1997	0	0	pilot plant made Afeuga'l Pitu Cheese (acid coagulated Spanish cheese, soft) - Artisian	1	4.46	1.39	85.88	1.59	0	0	5	7	0
Margolles	1997	0	0	Afeuga'l Pitu Cheese (acid coagulated Spanish cheese, soft) - Artisian	1	4.03	1.39	67.37	2.02	0	0	5	7	0
Margolles	1997	0	0	Afeuga'l Pitu Cheese (acid coagulated Spanish cheese, soft) - Artisian	1	4.05	1.39	59.62	2.28	0	0	5	7	0
Martins	2010	0	0	Ricotta	2	5.47	0.50	53.00	0.93	0	0	4	28	1
McAuliffe	1999	0	0	Cottage cheese	2	5.20	1.00	80.00	1.23	0	1	4	7	0
McAuliffe	1999	0	0	Cottage cheese	2	5.20	1.00	80.00	1.23	0	1	18	7	0
McAuliffe	1999	0	0	Cottage cheese	2	5.20	1.00	80.00	1.23	0	1	30	7	0
Mehta	1993	0	0	Cheddar	5	5.12	1.95	40.76	4.57	0	1	7	140	0
Mehta	1993	0	0	Cheddar	5	5.16	2.30	42.40	5.15	0	1	7	140	0
Mehta	1993	0	0	Cheddar	5	5.22	1.98	35.99	5.21	0	1	7	140	0
Mehta	1993	0	0	Cheddar (reduced fat)	5	5.23	2.42	43.25	5.30	0	1	7	140	0
Mehta	1993	0	0	Cheddar (reduced fat)	5	5.21	2.29	43.41	5.01	0	1	7	140	0
Mehta	1993	0	0	Cheddar (reduced fat)	5	5.24	2.08	41.98	4.72	0	1	7	140	0
Mendoza-Yepes	1999	1	0	Queso Fresco w/ starter	1	6.50	3.00	48.00	5.88	0	1	3	22	0
Mendoza-Yepes	1999	1	0	Queso Fresco w/ starter	1	6.50	3.00	48.00	5.88	0	1	7	22	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Mendoza-Yepes	1999	1	0	Queso Fresco w/o starter	1	6.50	3.00	48.00	5.88	0	0	3	22	1
Mendoza-Yepes	1999	1	0	Queso Fresco w/o starter	1	6.50	3.00	48.00	5.88	0	0	7	22	1
Menon	2001	1	0	Sterilized commercial mozzarella	3	5.40	1.60	52.00	2.99	0	0	7	15	1
Menon	2001	1	0	Sterilized commercial mozzarella	3	5.40	1.60	52.00	2.99	0	0	7	15	1
Menon	2001	1	0	Sterilized commercial mozzarella	3	5.40	1.60	52.00	2.99	0	0	30	7	1
Menon	2001	1	0	Sterilized commercial mozzarella	3	5.40	1.60	52.00	2.99	0	0	30	7	1
Mills	2011	0	0	Gouda slurry	4	5.20	3.00	41.00	6.82	0	1	13	126	0
Mills	2011	0	0	Lab-scale rennet coagulated cheese	3	5.20	0.50	60.00	0.83	0	1	12	28	0
Mojgani	2010	1	1	Lab cheese w/ L. casei RN 78, w/o salt	4	5.94	0.00	40.00	0.00	0	1	4	90	0
Mojgani	2010	1	1	Lab cheese w/ L. casei RN 78, w/o salt	4	5.94	0.00	40.00	0.00	0	1	35	90	0
Mojgani	2010	1	1	Lab cheese w/ L. casei RN78, Lactocin RN78, w/o salt	4	5.91	0.00	40.00	0.00	0	1	4	90	0
Mojgani	2010	1	1	Lab cheese w/ L. casei RN78, Lactocin RN78, w/o salt	4	5.91	0.00	40.00	0.00	0	1	35	90	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Mojgani	2010	1	1	Lab cheese w/ Lactocin RN78, NaCl	4	5.57	3.00	40.00	6.98	0	1	4	90	0
Mojgani	2010	1	1	Lab cheese w/ Lactocin RN78, NaCl	4	5.57	3.00	40.00	6.98	0	1	35	90	0
Mojgani	2010	1	1	Lab cheese w/ Lactocin RN78, w/o salt	4	5.54	0.00	40.00	0.00	0	1	4	90	0
Mojgani	2010	1	1	Lab cheese w/ Lactocin RN78, w/o salt	4	5.54	0.00	40.00	0.00	0	1	35	90	0
Mojgani	2010	1	1	Lab cheese w/ salt	4	5.82	3.00	40.00	6.98	0	1	4	90	0
Mojgani	2010	1	1	Lab cheese w/ salt	4	5.82	3.00	40.00	6.98	0	1	35	90	0
Mojgani	2010	1	1	Lab cheese w/o salt	4	5.54	0.00	40.00	0.00	0	1	4	90	0
Mojgani	2010	1	1	Lab cheese w/o salt	4	5.54	0.00	40.00	0.00	0	1	35	90	0
Monnet	2010	1	0	Smear-ripened model cheese	1	6.23	0.00	80.00	0.00	0	1	12	21	1
Monnet	2010	1	0	Smear-ripened model cheese	1	6.05	0.00	80.00	0.00	0	1	12	21	1
Monnet	2010	1	0	Smear-ripened model cheese	1	5.33	0.00	80.00	0.00	0	1	12	21	1
Monnet	2010	1	0	Smear-ripened model cheese	1	6.20	0.00	80.00	0.00	0	1	12	21	1
Monnet	2010	1	0	Smear-ripened model cheese	1	5.93	0.00	80.00	0.00	0	1	12	21	1
Monnet	2010	1	0	Smear-ripened model cheese	1	5.76	0.00	80.00	0.00	0	1	12	21	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Monnet	2010	1	0	Smear-ripened model cheese	1	6.75	0.00	80.00	0.00	0	1	12	21	1
Monnet	2010	1	0	Smear-ripened model cheese	1	6.20	0.00	80.00	0.00	0	1	12	21	1
Monnet	2010	1	0	Smear-ripened model cheese	1	6.21	0.00	80.00	0.00	0	1	12	21	1
Monnet	2010	1	0	Smear-ripened model cheese	1	5.91	0.00	80.00	0.00	0	1	12	21	1
Monnet	2010	1	0	Smear-ripened model cheese	1	6.09	0.00	80.00	0.00	0	1	12	21	1
Monnet	2010	1	0	Smear-ripened model cheese	1	6.26	0.00	80.00	0.00	0	1	12	21	1
Monnet	2010	1	0	Smear-ripened model cheese	1	5.42	0.00	80.00	0.00	0	1	12	21	1
Monnet	2010	1	0	Smear-ripened model cheese	1	6.46	0.00	80.00	0.00	0	1	12	21	1
Morales	2006	1	0	Mahon	5	5.20	3.67	41.21	8.18	0	1	8	5	0
Morgan	2001	0	1	Soft lactic cheese made from raw goat milk (interior)	3	4.25		60.90	0.10	1	1	2	42	0
Morgan	2001	0	1	Soft lactic cheese made from raw goat milk (surface)	3	4.25		60.90	0.10	1	1	2	42	0
Morgan, S.M.	2001	1	0	Cottage cheese	2	5.20	1.00	80.00	1.23	0	1	30	5	0
Naldini	2009	0	0	Brazilian Minas Frescal cheese (direct acidification)	1	6.50	2.00	64.10	3.03	0	0	5	25	1
Naldini	2009	0	0	Brazilian Minas Frescal	1	6.39	2.00	64.10	3.03	0	0	10	25	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
				cheese (direct acidification)										
Naldini	2009	0	0	Brazilian Minas Frescal cheese (lactic culture method)	1	5.31	2.00	65.80	2.95	0	1	5	25	0
Naldini	2009	0	0	Brazilian Minas Frescal cheese (lactic culture method)	1	5.31	2.00	65.80	2.95	0	1	10	25	0
Nunez	1997	0	1	Manchego	5	6.78		42.82	5.91	0	1	12	60	1
Nunez	1997	0	1	Manchego	5	6.78		42.82	5.91	0	1	12	60	0
Nunez	1997	0	1	Manchego	5	6.78		42.82	5.91	0	1	12	60	0
Nunez	1997	0	1	Manchego	5	6.72		42.82	5.91	0	1	12	60	0
Nunez	1997	0	1	Manchego	5	6.72		42.82	5.91	0	1	12	60	0
Nunez	1997	0	1	Manchego	5	6.72		42.82	5.91	0	1	12	60	0
O'Sullivan	2005	1	0	Smear-ripened	3	5.92		50.00	4.78	0	1	16	15	1
Olarte	2002	0	0	Cameros (fresh goat cheese)	1	6.60	0.78	65.00	1.19	1	0	4	28	1
Ozturkoglu	2006	0	0	Turkish white cheese	3	4.92	5.96	60.23	9.00	0	1	4	45	0
Ozturkoglu	2006	0	0	Turkish white cheese	3	5.14	6.20	61.49	9.16	0	1	4	45	0
Panagou	2008	0	0	Katiki (Greek soft cheese)	1	4.40	2.00	75.00	2.60	0	0	5	40	0
Panagou	2008	0	0	Katiki (Greek soft cheese)	1	4.40	2.00	75.00	2.60	0	0	10	40	0
Panagou	2008	0	0	Katiki (Greek soft cheese)	1	4.40	2.00	75.00	2.60	0	0	15	40	0
Panagou	2008	0	0	Katiki (Greek soft cheese)	1	4.40	2.00	75.00	2.60	0	0	20	40	0
Papageorgiou	1989	0	0	Feta	3	4.60	2.21	54.08	3.93	0	1	4	90	0
Papageorgiou	1989	0	0	Feta	3	4.85	2.36	55.84	4.05	0	1	4	90	0
Papageorgiou	1989	0	0	Feta	3	4.60	2.50	54.82	4.36	0	1	4	90	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Papageorgiou	1989	0	0	Feta	3	4.65	2.36	53.92	4.19	0	1	4	90	0
Papageorgiou	1989	0	0	Feta	3	4.90	2.36	53.89	4.20	0	1	4	90	0
Papageorgiou	1989	0	0	Feta	3	4.80	2.21	55.67	3.82	0	1	4	90	0
Papageorgiou	1996	0	0	Anthotyros, fresh whey cheese	1	6.41	1.37	67.10	2.00	0	0	5	38	1
Papageorgiou	1996	0	0	Anthotyros, fresh whey cheese	1	6.41	1.37	67.80	1.98	0	0	5	38	1
Papageorgiou	1996	0	0	Anthotyros, fresh whey cheese	1	6.41	1.54	65.80	2.29	0	0	5	38	1
Papageorgiou	1996	0	0	Anthotyros, fresh whey cheese	1	6.41	1.37	67.10	2.00	0	0	12	16	1
Papageorgiou	1996	0	0	Anthotyros, fresh whey cheese	1	6.41	1.37	67.80	1.98	0	0	12	16	1
Papageorgiou	1996	0	0	Anthotyros, fresh whey cheese	1	6.41	1.54	65.80	2.29	0	0	12	16	1
Papageorgiou	1996	0	0	Anthotyros, fresh whey cheese	1	6.41	1.37	67.10	2.00	0	0	22	7	1
Papageorgiou	1996	0	0	Anthotyros, fresh whey cheese	1	6.41	1.37	67.80	1.98	0	0	22	7	1
Papageorgiou	1996	0	0	Anthotyros, fresh whey cheese	1	6.41	1.54	65.80	2.29	0	0	22	7	1
Papageorgiou	1996	0	0	Manouri, fresh whey cheese	1	6.30	2.23	50.60	4.22	0	0	5	38	1
Papageorgiou	1996	0	0	Manouri, fresh whey cheese	1	6.30	2.40	52.60	4.36	0	0	5	38	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Papageorgiou	1996	0	0	Manouri, fresh whey cheese	1	6.30	2.23	53.40	4.01	0	0	5	38	1
Papageorgiou	1996	0	0	Manouri, fresh whey cheese	1	6.30	2.23	50.60	4.22	0	0	12	16	1
Papageorgiou	1996	0	0	Manouri, fresh whey cheese	1	6.30	2.40	52.60	4.36	0	0	12	16	1
Papageorgiou	1996	0	0	Manouri, fresh whey cheese	1	6.30	2.23	53.40	4.01	0	0	12	16	1
Papageorgiou	1996	0	0	Manouri, fresh whey cheese	1	6.30	2.23	50.60	4.22	0	0	22	7	1
Papageorgiou	1996	0	0	Manouri, fresh whey cheese	1	6.30	2.40	52.60	4.36	0	0	22	7	1
Papageorgiou	1996	0	0	Manouri, fresh whey cheese	1	6.30	2.23	53.40	4.01	0	0	22	7	1
Papageorgiou	1996	0	0	Myzithra, fresh whey cheese	1	6.50	0.00	68.30	0.00	0	0	5	38	1
Papageorgiou	1996	0	0	Myzithra, fresh whey cheese	1	6.50	0.00	67.80	0.00	0	0	5	38	1
Papageorgiou	1996	0	0	Myzithra, fresh whey cheese	1	6.50	0.00	69.20	0.00	0	0	5	38	1
Papageorgiou	1996	0	0	Myzithra, fresh whey cheese	1	6.50	0.00	68.30	0.00	0	0	12	16	1
Papageorgiou	1996	0	0	Myzithra, fresh whey cheese	1	6.50	0.00	67.80	0.00	0	0	12	16	1
Papageorgiou	1996	0	0	Myzithra, fresh whey cheese	1	6.50	0.00	69.20	0.00	0	0	12	16	1

Author	Year	[1]Pre(0)/ Post(1)- Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Papageorgiou	1996	0	0	Myzithra, fresh whey cheese	1	6.50	0.00	68.30	0.00	0	0	22	7	1
Papageorgiou	1996	0	0	Myzithra, fresh whey cheese	1	6.50	0.00	67.80	0.00	0	0	22	7	1
Papageorgiou	1996	0	0	Myzithra, fresh whey cheese	1	6.50	0.00	69.20	0.00	0	0	22	7	1
Papageorgiou	1997	0	0	Feta	3	5.43	1.20	59.67	1.97	0	1	-38	225	0
Papageorgiou	1997	0	0	Feta	3	5.43	1.20	59.67	1.97	0	1	-38	225	0
Papageorgiou	1997	0	0	Feta	3	5.43	1.20	59.67	1.97	0	1	-38	225	0
Papageorgiou	1997	0	0	Feta	3	5.43	1.20	59.67	1.97	0	1	-38	225	0
Papageorgiou	1997	0	0	Feta	3	5.43	1.20	59.67	1.97	0	1	-18	225	0
Papageorgiou	1997	0	0	Feta	3	5.43	1.20	59.67	1.97	0	1	-18	225	0
Papageorgiou	1997	0	0	Feta	3	5.43	1.20	59.67	1.97	0	1	-18	225	0
Papageorgiou	1997	0	0	Feta	3	5.43	1.20	59.67	1.97	0	1	-18	225	0
Piccinin	1995	1	0	Cottage cheese (Canada)	2	4.88	1.00	80.00	1.23	0	1	5	24	0
Piccinin	1995	1	0	Cottage cheese (Canada)	2	4.90	1.00	80.00	1.23	0	1	5	24	0
Piccinin	1995	1	0	Cottage cheese (Canada)	2	5.00	1.00	80.00	1.23	0	1	5	24	0
Piccinin	1995	1	0	Cottage cheese (Canada)	2	4.85	1.00	80.00	1.23	0	1	5	24	0
Piccinin	1995	1	0	Cottage cheese (Canada)	2	4.83	1.00	80.00	1.23	0	1	5	24	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Piccinin	1995	1	0	Cottage cheese (US)	2	5.01	1.00	80.00	1.23	0	1	5	24	0
Piccinin	1995	1	0	Cottage cheese (US)	2	5.00	1.00	80.00	1.23	0	1	5	24	0
Piccinin	1995	1	0	Cottage cheese (US)	2	5.01	1.00	80.00	1.23	0	1	5	24	0
Piccinin	1995	1	0	Cottage cheese (US)	2	4.99	1.00	80.00	1.23	0	1	5	24	0
Piccinin	1995	1	0	Cottage cheese (US)	2	5.22	1.00	80.00	1.23	0	1	5	24	0
Ramsaran	1998	0	1	Camembert w/ nisin starter	3	4.90	2.00	50.00	3.85	0	1	2	64	1
Ramsaran	1998	0	1	Camembert w/ nisin starter	3	6.50	2.00	50.00	3.85	0	1	2	65	1
Ramsaran	1998	0	0	Camembert w/o nisin starter, pasteurized milk	3	4.90	2.00	50.00	3.85	0	1	2	64	1
Ramsaran	1998	0	0	Camembert w/o nisin starter, pasteurized milk	3	6.50	2.00	50.00	3.85	0	1	2	65	1
Ramsaran	1998	0	1	Camembert w/o nisin starter, raw milk	3	4.90	2.00	50.00	3.85	0	1	2	64	1
Ramsaran	1998	0	1	Camembert w/o nisin starter, raw milk	3	6.70	2.00	50.00	3.85	0	1	2	65	1
Ramsaran	1998	0	1	Feta w/ nisin starter	3	5.20	2.20	56.00	3.78	0	1	1	74	0
Ramsaran	1998	0	0	Feta w/o nisin starter, pasteurized milk	3	4.80	2.20	56.00	3.78	0	1	1	74	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Ramsaran	1998	0	0	Feta w/o nisin starter, pasteurized milk	3	6.50	2.20	56.00	3.78	0	1	1	75	1
Ramsaran	1998	0	1	Feta w/o nisin starter, raw milk	3	5.10	2.20	56.00	3.78	0	1	1	74	0
Retureau	2010	1	0	Saint-Nectaire	3	6.42	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.17	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.27	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.15	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.72	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.11	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.10	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.41	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.74	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.60	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.63	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.41	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.10	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.47	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.35	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.17	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.14	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.98	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.92	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.34	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.05	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.28	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.34	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.48	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.21	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.05	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.97	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.31	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.14	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.93	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.67	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.52	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.14	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.07	2.40	59.50	3.88	0	1	9	28	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Retureau	2010	1	0	Saint-Nectaire	3	6.42	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.45	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.67	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	6.35	2.40	59.50	3.88	0	1	9	28	1
Retureau	2010	1	0	Saint-Nectaire	3	7.29	2.40	59.50	3.88	0	1	9	28	1
Rodriguez	1998	0	1	Manchego	4	5.07		42.82	5.91	0	1	12	60	0
Rodriguez	1998	0	1	Manchego	4	5.11		42.82	5.91	0	1	12	60	0
Rodriguez	1998	0	1	Manchego	4	5.15		42.82	5.91	0	1	12	60	0
Rodriguez	1998	0	1	Manchego	4	6.03		42.82	5.91	0	1	12	60	0
Rodriguez	2001	0	1	Semi-hard cheese	4	4.99	1.40	54.00	2.53	0	1	12	60	0
Rodriguez	2001	0	1	Semi-hard cheese	4	4.88	1.40	54.00	2.53	0	1	12	60	0
Rodriguez	2005	0	1	Semi-hard cheese	4	5.04	1.40	54.00	2.53	0	1	12	30	0
Rodriguez	2005	0	1	Semi-hard cheese	4	4.91	1.40	54.00	2.53	0	1	12	30	0
Rogga	2005	1	0	Galotyri, traditional Greek soft acid-curd (commercial/artisan starter)	2	4.00	1.80	75.80	2.32	0	1	4	28	0
Rogga	2005	1	0	Galotyri, traditional Greek soft acid-curd (commercial/artisan starter)	2	4.00	1.80	75.80	2.32	0	1	4	28	0
Rogga	2005	1	0	Galotyri, traditional Greek soft acid-curd (commercial/artisan starter)	2	4.00	1.80	75.80	2.32	0	1	12	14	0
Rogga	2005	1	0	Galotyri, traditional Greek soft acid-curd (commercial/artisan starter)	2	4.00	1.80	75.80	2.32	0	1	12	14	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Rogga	2005	1	0	Galotyri, traditional Greek soft acid-curd (commercial/industrial starter)	2	3.80	1.80	76.90	2.29	0	1	4	28	0
Rogga	2005	1	0	Galotyri, traditional Greek soft acid-curd (commercial/industrial starter)	2	3.80	1.80	76.90	2.29	0	1	4	28	0
Rogga	2005	1	0	Galotyri, traditional Greek soft acid-curd (commercial/industrial starter)	2	3.80	1.80	76.90	2.29	0	1	12	14	0
Rogga	2005	1	0	Galotyri, traditional Greek soft acid-curd (commercial/industrial starter)	2	3.80	1.80	76.90	2.29	0	1	12	14	0
Rogga	2005	1	1	Galotyri, traditional Greek soft acid-curd (pilot/artisan starter)	2	4.36	3.10	77.50	3.85	0	1	4	28	0
Rogga	2005	1	1	Galotyri, traditional Greek soft acid-curd (pilot/artisan starter)	2	4.36	3.10	77.50	3.85	0	1	12	28	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Rogga	2005	1	1	Galotyri, traditional Greek soft acid-curd (pilot/industrial starter)	2	4.42	2.90	74.00	3.77	0	1	4	28	0
Rogga	2005	1	1	Galotyri, traditional Greek soft acid-curd (pilot/industrial starter)	2	4.42	2.90	74.00	3.77	0	1	12	28	0
Ryser	1987	0	0	Camembert	3	6.10	2.40	54.40	4.23	0	1	6	35	1
Ryser	1987	0	0	Camembert	3	6.10	2.40	54.40	4.23	0	1	6	35	1
Ryser	1987	0	0	Camembert	3	7.40	2.40	54.40	4.23	0	1	6	50	1
Ryser	1987	0	0	Camembert	3	7.40	2.40	54.40	4.23	0	1	6	50	1
Ryser	1987	0	0	Camembert	3	7.40	2.40	54.40	4.23	0	1	6	50	1
Ryser	1987	0	0	Camembert	3	7.40	2.40	54.40	4.23	0	1	6	50	1
Ryser	1987	0	0	Camembert	3	6.00	3.10	44.10	6.57	0	1	6	10	1
Ryser	1987	0	0	Camembert	3	6.00	2.30	55.30	3.99	0	1	6	10	1
Ryser	1987	0	0	Camembert	3	6.00	2.50	59.00	4.07	0	1	6	10	1
Ryser	1987	0	0	Camembert	3	6.00	3.70	56.60	6.14	0	1	6	10	1
Ryser	1987	0	0	Camembert	3	6.00	1.70	53.00	3.11	0	1	6	10	1
Ryser	1987	0	0	Camembert	3	6.00	2.10	60.00	3.38	0	1	6	10	1
Ryser	1987	0	0	Camembert	3	6.00	1.90	58.30	3.16	0	1	6	10	1
Ryser	1987	0	0	Camembert	3	6.00	2.20	52.60	4.01	0	1	6	10	1
Ryser	1987	0	0	Camembert	3	6.00	2.10	54.10	3.74	0	1	6	10	1
Ryser	1987	0	0	Camembert	3	6.00	2.30	53.10	4.15	0	1	6	10	1
Ryser	1987	0	0	Camembert	3	6.00	2.20	49.70	4.24	0	1	6	10	1
Ryser	1987	0	0	Camembert	3	6.00	2.20	57.00	3.72	0	1	6	10	1
Ryser	1987	0	0	Camembert	3	4.60	2.40	54.40	4.23	0	1	6	21	0
Ryser	1987	0	0	Camembert	3	4.60	2.40	54.40	4.23	0	1	6	21	0
Ryser	1987	0	0	Camembert	3	4.60	2.40	54.40	4.23	0	1	6	21	0
Ryser	1987	0	0	Camembert	3	4.60	2.40	54.40	4.23	0	1	6	21	0
Ryser	1987	0	0	Camembert	3	5.50	2.40	54.40	4.23	0	1	6	35	0
Ryser	1987	0	0	Camembert	3	5.50	2.40	54.40	4.23	0	1	6	35	0
Ryser	1987	0	0	Camembert	3	5.50	2.40	54.40	4.23	0	1	6	35	0
Ryser	1987	0	0	Camembert	3	5.50	2.40	54.40	4.23	0	1	6	35	0
Ryser	1987	0	0	Camembert	3	6.10	2.40	54.40	4.23	0	1	6	35	0
Ryser	1987	0	0	Camembert	3	6.10	2.40	54.40	4.23	0	1	6	35	0
Ryser	1987	0	0	Cheddar	5	5.08	1.40	37.00	3.65	0	1	6	70	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Ryser	1987	0	0	Cheddar	5	5.08	1.70	35.90	4.52	0	1	6	70	0
Ryser	1987	0	0	Cheddar	5	5.06	1.50	36.60	3.94	0	1	6	70	0
Ryser	1987	0	0	Cheddar	5	5.05	1.40	38.20	3.54	0	1	6	70	0
Ryser	1987	0	0	Cheddar	5	5.05	1.50	38.40	3.76	0	1	6	70	0
Ryser	1987	0	0	Cheddar	5	5.08	1.80	36.90	4.65	0	1	6	70	0
Ryser	1987	0	0	Cheddar	5	5.06	1.60	36.20	4.23	0	1	6	70	0
Ryser	1987	0	0	Cheddar	5	5.05	1.90	37.70	4.80	0	1	6	70	0
Ryser	1987	0	0	Cheddar	5	5.08	1.70	36.70	4.43	0	1	6	70	0
Ryser	1987	0	0	Cheddar	5	5.07	1.50	37.20	3.88	0	1	6	70	0
Ryser	1987	0	0	Cheddar	5	5.04	1.70	37.80	4.30	0	1	6	70	0
Ryser	1987	0	0	Cheddar	5	5.05	1.50	38.50	3.75	0	1	6	70	0
Ryser	1987	0	0	Cheddar	5	5.06	1.60	36.80	4.17	0	1	6	70	0
Ryser	1987	0	0	Cheddar	5	5.09	1.40	37.00	3.65	0	1	13	70	0
Ryser	1987	0	0	Cheddar	5	5.09	1.40	37.00	3.65	0	1	13	70	0
Ryser	1987	0	0	Cheddar	5	5.07	1.50	36.60	3.94	0	1	13	70	0
Ryser	1987	0	0	Cheddar	5	5.07	1.40	38.20	3.54	0	1	13	70	0
Ryser	1987	0	0	Cheddar	5	5.05	1.50	38.40	3.76	0	1	13	70	0
Ryser	1987	0	0	Cheddar	5	5.08	1.80	36.90	4.65	0	1	13	70	0
Ryser	1987	0	0	Cheddar	5	5.07	1.70	36.70	4.43	0	1	13	70	0
Ryser	1987	0	0	Cheddar	5	5.05	1.50	37.20	3.88	0	1	13	70	0
Ryser	1987	0	0	Cheddar	5	5.04	1.70	37.80	4.30	0	1	13	70	0
Ryser	1989	0	0	Brick	4	5.34	0.70	42.30	1.63	0	1	10	175	1
Ryser	1989	0	0	Brick	4	5.34	0.90	43.10	2.05	0	1	10	175	1
Ryser	1989	0	0	Brick	4	5.34	1.10	42.60	2.52	0	1	10	175	1
Ryser	1989	0	0	Brick	4	5.34	0.50	43.50	1.14	0	1	10	175	1
Ryser	1989	0	0	Brick	4	5.34	0.70	44.70	1.54	0	1	10	168	1
Ryser	1989	0	0	Brick	4	5.34	0.70	40.70	1.69	0	1	10	168	1
Ryser	1989	0	0	Brick	4	5.34	0.60	44.40	1.33	0	1	10	168	1
Ryser	1989	0	0	Brick	4	5.34	0.70	43.00	1.60	0	1	10	168	1
Samelis	2003	1	1	Anthotyros w/ 100 IU/g nisin starter to whey, traditional Greek whey cheese, direct acidification	1	7.08	0.50	73.90	0.67	0	0	4	45	1
Samelis	2003	1	1	Anthotyros w/ 100 IU/g nisin starter to whey,	1	7.14	0.50	74.50	0.67	0	0	4	45	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Samelis	2003	1	1	traditional Greek whey cheese, natural acidification Anthotyros w/ 500 IU/g nisin starter to cheese,	1	7.01	0.50	73.90	0.67	0	0	4	45	1
Samelis	2003	1	1	traditional Greek whey cheese, direct acidification Anthotyros w/ 500 IU/g nisin starter to cheese,	1	6.89	0.50	74.50	0.67	0	0	4	45	1
Samelis	2003	1	1	traditional Greek whey cheese, natural acidification Anthotyros w/ 500 IU/g nisin starter to whey,	1	7.11	0.50	73.90	0.67	0	0	4	45	1
Samelis	2003	1	1	traditional Greek whey cheese, direct acidification Anthotyros w/ 500 IU/g nisin starter to whey,	1	7.12	0.50	74.50	0.67	0	0	4	45	1
Samelis	2003	1	1	traditional Greek whey cheese, natural acidification Anthotyros w/o nisin	1	6.88	0.50	74.50	0.67	0	0	4	45	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
				starter, traditional Greek whey cheese Anthotyros w/o nisin										
Samelis	2003	1	1	starter, traditional Greek whey cheese	1	7.08	0.50	73.90	0.67	0	0	4	45	1
Samelis	2009	0	0	Graviera, traditional Greek hard cheese	5	6.60	1.00	40.50	2.41	0	1	4	60	0
Schaffer	1995	0	0	Blue cheese	4	5.85	4.40	42.60	9.36	0	1	4	70	0
Schaffer	1995	0	0	Blue cheese	4	5.85	4.60	43.40	9.58	0	1	4	70	0
Schaffer	1995	0	0	Blue cheese	4	5.85	4.20	45.80	8.40	0	1	4	70	0
Schaffer	1995	0	0	Blue cheese	4	5.85	5.10	42.10	10.81	0	1	4	70	0
Schaffer	1995	0	0	Blue cheese	4	5.85	5.50	40.80	11.88	0	1	4	70	0
Schaffer	1995	0	0	Blue cheese	4	5.85	5.10	40.30	11.23	0	1	4	70	0
Schaffer	1995	0	0	Cheddar	5	5.02	1.70	38.30	4.25	0	1	7	70	0
Schaffer	1995	0	0	Cheddar	5	5.20	1.70	37.60	4.33	0	1	7	70	0
Schaffer	1995	0	0	Cheddar	5	5.02	1.80	37.40	4.59	0	1	7	70	0
Schaffer	1995	0	0	Cheddar	5	5.16	1.70	38.30	4.25	0	1	7	70	0
Schaffer	1995	0	0	Cheddar	5	5.12	1.50	36.90	3.91	0	1	7	70	0
Schaffer	1995	0	0	Cheddar	5	5.22	1.50	38.30	3.77	0	1	7	70	0
Schvartzman	2010	0	0	Lab cheese w/o starter	1	5.60	0.00	75.00	0.00	0	0	21	0.66	1
Schvartzman	2010	0	0	Lab cheese w/o starter	1	5.60	3.00	75.00	3.85	0	0	21	0.66	1
Schvartzman	2010	0	0	Lab cheese w/o starter	1	5.90	0.00	75.00	0.00	0	0	21	0.66	1
Schvartzman	2010	0	0	Lab cheese w/o starter	1	5.90	3.00	75.00	3.85	0	0	21	0.66	1
Schvartzman	2010	0	0	Lab cheese w/o starter	1	6.10	0.00	75.00	0.00	0	0	21	0.66	1
Schvartzman	2010	0	0	Lab cheese w/o starter	1	6.10	3.00	75.00	3.85	0	0	21	0.66	1
Schvartzman	2010	0	0	Lab cheese w/o starter	1	6.50	0.00	75.00	0.00	0	0	21	0.66	1

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Schvartzman	2010	0	0	Lab cheese w/o starter	1	6.50	3.00	75.00	3.85	0	0	21	0.66	1
Schvartzman	2010	0	0	Lab cheese w/o starter	1	5.60	6.00	75.00	7.41	0	0	21	0.66	0
Schvartzman	2010	0	0	Lab cheese w/o starter	1	5.60	8.00	75.00	9.64	0	0	21	0.66	0
Schvartzman	2010	0	0	Lab cheese w/o starter	1	5.90	6.00	75.00	7.41	0	0	21	0.66	0
Schvartzman	2010	0	0	Lab cheese w/o starter	1	5.90	8.00	75.00	9.64	0	0	21	0.66	0
Schvartzman	2010	0	0	Lab cheese w/o starter	1	6.10	6.00	75.00	7.41	0	0	21	0.66	0
Schvartzman	2010	0	0	Lab cheese w/o starter	1	6.10	8.00	75.00	9.64	0	0	21	0.66	0
Schvartzman	2010	0	0	Lab cheese w/o starter	1	6.50	6.00	75.00	7.41	0	0	21	0.66	0
Schvartzman	2010	0	0	Lab cheese w/o starter	1	6.50	8.00	75.00	9.64	0	0	21	0.66	0
Schvartzman	2011	0	0	Lab cheese with bact, yeast smear (pasteurized milk) - rind	2	5.60	3.00	75.00	3.85	0	1	8	14	0
Schvartzman	2011	0	0	Lab cheese with bact, yeast smear (pasteurized milk) -core	2	5.20	3.00	75.00	3.85	0	1	8	14	0
Schvartzman	2011	0	0	Lab cheese with bact, yeast smear (raw milk) - core	2	5.30	3.00	75.00	3.85	0	1	8	14	0
Schvartzman	2011	0	0	Lab cheese with bact, yeast smear (raw milk) - rind	2	5.50	3.00	75.00	3.85	0	1	8	14	0
Shresta	2011	1	0	Cheddar	5	5.73	1.88	35.90	4.98	0	1	4	90	0
Shresta	2011	1	0	Cheddar	5	5.27	1.74	34.20	4.84	0	1	4	90	0
Shresta	2011	1	0	Cheddar	5	5.73	1.88	35.90	4.98	0	1	10	90	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Shresta	2011	1	0	Cheddar	5	5.27	1.74	34.20	4.84	0	1	10	90	0
Shresta	2011	1	0	Cheddar	5	5.73	1.88	35.90	4.98	0	1	21	30	0
Shresta	2011	1	0	Cheddar	5	5.27	1.74	34.20	4.84	0	1	21	30	0
Shresta	2011	1	0	Low-sodium cheddar	5	5.08	0.68	39.00	1.71	0	1	4	90	0
Shresta	2011	1	0	Low-sodium cheddar	5	5.45	0.70	39.30	1.75	0	1	4	90	0
Shresta	2011	1	0	Low-sodium cheddar	5	5.08	0.68	39.00	1.71	0	1	10	90	0
Shresta	2011	1	0	Low-sodium cheddar	5	5.45	0.70	39.30	1.75	0	1	10	90	0
Shresta	2011	1	0	Low-sodium cheddar	5	5.08	0.68	39.00	1.71	0	1	21	30	0
Shresta	2011	1	0	Low-sodium cheddar	5	5.45	0.70	39.30	1.75	0	1	21	30	0
Siafaras	2008	1	0	Kefalograviera (hard cheese)	5	6.10		26.50	6.00	1	1	9	90	0
Smith-Palmer	2001	1	0	Full-fat cream cheese	2	4.90	0.40	55.00	0.72	0	1	4	14	1
Smith-Palmer	2001	1	0	Low-fat cream cheese	2	5.10	0.40	70.00	0.57	0	1	4	14	1
Solano-Lopez	2000	0	0	Chihuahua	5	5.80	2.90	36.40	7.38	0	1	12	42	0
Solano-Lopez	2000	0	0	Manchego	4	5.40	2.90	42.10	6.44	0	1	12	5	0
Spanu	2011	1	0	Ricotta salata (paste)	3	6.09	0.85	50.18	1.67	0	0	4	180	0
Spanu	2011	1	0	Ricotta salata (rind)	3	6.09	0.85	50.18	1.67	0	0	4	180	1
Stecchini	1995	1	0	Mozzarella	3	5.40	1.60	52.00	2.99	0	1	5	21	1
Sulzer	1991	0	0	Camembert	3	6.20	2.50	52.00	4.59	0	1	6	48	1
Theodoridis	2006	0	1	Chevre Metsovo	4	5.18	3.01	35.51	7.81	1	1	4	60	0
Theodoridis	2006	0	0	Pichtogalo Chanion	3	4.23	1.25	64.45	1.90	1	1	4	60	0
Tsiotsias	2002	1	0	Anthotyros	1	6.40	0.60	65.00	0.91	0	0	4	42	1
Tsiotsias	2002	1	0	Anthotyros	1	6.40	0.60	65.00	0.91	0	0	10	42	1
Uhlich	2006	1	0	Queso Blanco slices	1	6.80	2.32	48.80	4.54	0	1	5	40	1

Author	Year	[1]Pre(0)/ Post(1)- Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Uhlich	2006	1	0	Queso Blanco slices	1	6.80	2.32	48.80	4.54	0	1	10	25	1
Uhlich	2006	1	0	Queso Blanco slices	1	6.80	2.32	48.80	4.54	0	1	15	12	1
Uhlich	2006	1	0	Queso Blanco slices	1	6.80	2.32	48.80	4.54	0	1	20	12	1
Uhlich	2006	1	0	Queso Blanco slices	1	6.80	2.32	48.80	4.54	0	1	25	6	1
Villani	1996	0	1	Mozzarella - water-buffalo	3	4.87	1.80	50.00	3.47	1	1	18	1	0
Villani	1996	0	1	Mozzarella - water-buffalo	3	4.87	1.80	50.00	3.47	1	1	18	1	0
Villani	1996	0	1	Mozzarella - water-buffalo	3	4.87	1.80	50.00	3.47	1	1	18	1	0
Villani	1996	0	1	Mozzarella - water-buffalo	3	4.87	1.80	50.00	3.47	1	1	18	1	0
Villani	1996	0	1	Mozzarella - water-buffalo	3	4.91	1.80	50.00	3.47	1	1	18	1	0
Villani	1996	0	1	Mozzarella - water-buffalo	3	4.91	1.80	50.00	3.47	1	1	18	1	0
Villani	1996	0	1	Mozzarella - water-buffalo	3	4.91	1.80	50.00	3.47	1	1	18	1	0
Villani	1996	0	1	Mozzarella - water-buffalo	3	4.91	1.80	50.00	3.47	1	1	18	1	0
Vytrasova	2010	1	0	Acid curd (Loose brand)	2	4.60	0.70	70.00	0.99	0	0	20	14	1
Vytrasova	2010	1	0	Acid curd (Loose brand)	2	4.60	0.70	70.00	0.99	0	1	5.5	14	0
Vytrasova	2010	1	0	Slovak-style string cheese	4	5.50		40.00	4.24	0	1	5.5	14	0
Vytrasova	2010	1	0	Slovak-style string cheese	4	5.50		40.00	4.24	0	1	20	14	0
Vytrasova	2010	1	0	Soft-ripened (Olomouc brand)	3	5.80	4.30	64.60	6.24	0	1	5.5	14	0
Vytrasova	2010	1	0	Soft-ripened (Olomouc brand)	3	5.80	4.30	64.60	6.24	0	1	20	14	0
Whitley	2000	1	0	Mold-ripened cheese	3	6.25	1.50	50.00	2.91	0	1	5.15	42	1
Yousef	1988	0	0	Colby	5	5.18	1.50	42.30	3.42	0	1	4	140	0

Author	Year	[1]Pre(0)/ Post(1)-Proc Contam	[2]Raw(1)/ Past(0) Milk	Cheese	[3]Cat	[4]pH	Salt (%)	[5]H_2O (%)	[6]WPS (%)	[7]Bovine(0) /Other(1)	[8]Starter(1) /None(0)	[9]Temp (°C)	Storage Time (days)	[10]G(1)/ NG(0)
Yousef	1988	0	0	Colby	5	5.00	1.40	42.60	3.18	0	1	4	140	0
Yousef	1988	0	0	Colby	5	5.18	1.60	38.60	3.98	0	1	4	140	0
Yousef	1988	0	0	Colby	5	5.13	1.40	38.30	3.53	0	1	4	140	0
Yousef	1988	0	0	Colby	5	5.19	1.40	39.70	3.41	0	1	4	140	0
Yousef	1988	0	0	Colby	5	5.05	1.40	38.20	3.54	0	1	4	140	0
Yousef	1990	0	0	Parmesan	5	5.05	1.50	30.10	4.75	0	1	12.8	60	0
Yousef	1990	0	0	Parmesan	5	5.05	1.60	31.40	4.85	0	1	12.8	60	0
Yousef	1990	0	0	Parmesan	5	5.10	1.70	31.20	5.17	0	1	12.8	60	0
Yousef	1990	0	0	Parmesan	5	5.05	1.60	30.70	4.95	0	1	12.8	60	0
Yousef	1990	0	0	Parmesan	5	5.00	1.80	30.20	5.63	0	1	12.8	60	0
Yousef	1990	0	0	Parmesan	5	5.10	1.80	31.00	5.49	0	1	12.8	60	0
Zottola	1994	0	0	Cold-pack cheese	6	5.10	1.00	60.00	1.64	0	1	4	56	0
Zottola	1994	0	0	Cold-pack cheese	6	5.10	0.50	44.00	1.12	0	1	4	56	0
Zottola	1994	0	0	Cold-pack cheese	6	5.10	1.00	60.00	1.64	0	1	23	56	0
Zottola	1994	0	0	Cold-pack cheese	6	5.10	0.50	44.00	1.12	0	1	23	56	0

Validation of *Listeria monocytogenes* Thermal Resistance Model in Whole Bovine Milk

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Summary

This report lays out a model of thermal death times (D-values) for *Listeria monocytogenes* in whole bovine milk between 55–71.7°C based on data from 18 published experimental studies and the researcher's own data. It first compares four methods of controlling for the varied experimental conditions in which the data were gathered before comparing three different model shapes. Its goal is to obtain a model of the $\log_{10}D$–temperature curve that maximizes predictive accuracy while minimizing the risk of under-prediction, particularly at lower temperatures (≤ 57.2°C). The major results are as follows:

Key Results

- Among the four modeling approaches considered, a mixed-effect model with a study-level random effect yielded the best fit for the data.
  - While the mixed-effect inverse-variance weighted model performed decently on the small sample-corrected Akaike information criterion ($AICc = 31.29$), it underperformed on the Bayesian information criterion ($BIC = 70.01$). The inverse-variance weighted model with no random effect performed poorly on both ($AICc = 62.48$, $BIC = 99.75$).
  - The mixed-effect model's AICc was the lowest among all candidate approaches ($AICc = 30.63$), closely followed by the ordinary least-squares (OLS) regression model ($AICc = 31.72$). The reverse was true of the models' BIC values (mixed-effect $BIC = 35.88$; OLS $BIC = 34.59$). AICc/BIC differences of this magnitude are inconclusive.
  - A likelihood-ratio test on the mixed-effect and OLS models yielded strong evidence that the mixed-effect model provides a better fit for the data ($p < 0.001$). This suggests that controlling for inter-study variability substantially improves model fit.
- The quadratic model performed best among candidate shapes in repeated 6-fold cross validation, yielding the smallest prediction errors and the lowest rate of under-prediction.
  - The quadratic model's mean squared prediction error (MSPE) was lowest among all candidate model shapes, both on the data used to fit the model (mean $MSPE = 0.0468$) and on novel data (mean $MSPE = 0.0530$).
  - The quadratic model's MSPE was found to be meaningfully lower than the piecewise model (mean difference =-0.0041; 95% CI: -0.0063, -0.0021), which was found to be the second-most-accurate model.
  - The difference between the quadratic model's in-sample and out-of-sample MSPE was not substantial (95% CI: -0.0418, 0.0207). Thus, there is not evidence that its predictive accuracy deteriorates when attempting to predict new $\log_{10}D$ values.
  - Likewise, its severity-weighted under-prediction ratio was lowest among all models both in-sample ($\kappa = 0.160$) and out-of-sample ($\kappa = 0.005$). This likewise does not decrease substantially on novel data (95% CI: -1.19, 0.199).
- The final mixed-effect quadratic model fits the data well (pseudo-$R^2 = 0.938$; $AICc = 20.62$) and under-predicts only 3 of 162 observations when using its 95% prediction interval, a rate of 1.9%. Two of these three points were found to be statistical outliers in preliminary analyses.

1 Introduction

Listeria monocytogenes is among the leading causes of death from foodborne illness in the United States. The relationship between D-value—the time required to achieve 1 log_{10} reduction in microorganisms present in a substance—and milk treatment temperature is central to controlling *L. monocytogenes* contamination in the production of milk and milk products. The goal of this report is to construct a model of the D-value–temperature relationship that most accurately predicts a range of plausible $\log_{10}$ D-values at cheesemaking thermitization temperatures (between 55–71.7°C) using data culled from experimental literature. In addition, it seeks a model that is conservative enough to ensure D-value underestimation is extremely rare. Section 2 details the data (2.1) before laying out the research goal and questions guiding my analyses (2.2), and the models and methods themselves (2.3). Section 3 gives the results of these analyses organized by research question: it first compares modeling approaches to find the method that best fits the data (3.1), and follows by comparing various model shapes to find the one that predicts $\log_{10}D$ most accurately (3.2). After presenting the final model (3.3), Section 4 concludes with a brief discussion of my analyses' strengths and limitations.

2 Materials & Methods

2.1 Data

From 18 experimental studies, the researcher compiled 162 published D-values for *L. monocytogenes* in whole bovine milk; D-values reported in other units (e.g., minutes) were converted to seconds and all were transformed into $\log_{10}D$ by the researcher. These data were all gathered from thermal inactivation trials conducted between 55–71.7°C, the range of thermitization temperatures for cheesemaking, under varied experimental conditions. Information about each observation's experimental conditions were provided, but were not used in modeling. (This decision is expanded upon in the Discussion section at the end of the report.) Also given were the citation for each study, its primary author, and its publication year. Of these 162 D-values, 96 came from from 10 studies where the values commonly of interest to meta-analysis—in particular, standard deviations or variances for each replicate set—were reported. As with D-values, some were given in minutes while others were in seconds. I will refer to observations with reported standard deviations as "complete" observations and those without such values "incomplete" observations. In addition, 8 D-values were obtained from experiments conducted by the researcher at temperatures between 57.2–65.6°C.

2.2 Research Questions

The overarching research goal was to find a model that most accurately predicts $\log_{10}$ D-value using only the temperature at which a batch of milk is treated while minimizing under-prediction, particularly at temperatures ≥ 57.2°C. For the purposes of my statistical analyses, this entailed tackling two sets of research questions:

1. Does measurement- or study-level variability affect the quality of model fit?
2. What is the relationship between temperature and $\log_{10}$ D-value?
   (a) What is the shape of the relationship? Is it linear or non-linear?
   (b) Is the relationship constant for all temperatures or does it vary, particularly at higher temperatures (e.g., $\geq 70°C$)?

2.3 Statistical Analyses

2.3.1 Data Preparation

For complete observations, standard deviations given in units other than seconds (e.g., minutes) were converted to seconds, and then all standard deviations were squared to obtain variances in units of seconds^2. For each study, reference author and year were combined to identify the data source of each set of observations—no author–year combinations are repeated across studies, so these labels uniquely refer to one study each. The data were then separated into data sets, a "full" data set with both complete and incomplete observations (162 observations), and a "complete-only" data set containing only complete observations (96 observations). The researcher's data (8 observations) were kept separate as it was only used for validation in my analyses.

Univariate outlier detection for numeric variables was carried out via visual inspection of boxplots. Multivariate outlier detection was conducted using Mahalanobis Distance as implemented in the `performance` `R` package with a threshold of 0.05 (that is, the 5% of observations that deviate most from others when all numeric variables are considered jointly) [1]. Six potential outliers were detected using this method, but none were removed. Three were unconcerning upon further inspection, while the others were sufficiently explained by the (experimentally-appropriate) conditions of their respective studies, were the mean of 3–4 replicates, and were included in prior meta analyses.

2.3.2 Statistical Models

Existing literature on meta-analytic modeling largely centers on two types of models: inverse-variance weighted models and mixed-effect models. These correspond to the two primary sources of confounding error when combining data gathered with varied methods across multiple studies: measurement- and study-level variability, respectively. To assess which modeling strategy was most appropriate, four models were fit using every combination of these two strategies, as shown in Table 1 below. Only linear models were fit for each strategy because the main metric used to compare model shapes (see next subsection) is a non-decreasing function of model complexity. In other words, the modeling approach that fits the data best with a linear model will almost surely be the same approach that fits best with a more complex model. Supplementary cross-validation was conducted to verify this theory and no notable exceptions were found; as such, these supplementary analyses will not be presented.

Table 1: Candidate modeling approaches.

		Variance Structure	
		Noise Only	Noise + Study Variability
Weighting Structure	Unweighted	*Ordinary Least Squares Model*	*Study Effect Model*
	Observation Weighted	*Inverse-Variance Weighted Model*	*Inverse-Variance Weighted Study Effect Model*

Ordinary least-squares (OLS) regression assumes a linear relationship between the effects of independent variables and the mean of the dependent variable, a normal distribution of noise, that the noise is uncorrelated between observations and with the values of the independent variables, and that the variability of this noise is constant across the range of dependent variable values being modeled. Inverse-variance weighted (IVW) least-squares makes the same assumptions with the exception of the latter two. In this model, observations influence the model's parameter estimates in inverse proportion to their measurement uncertainty (variance). This results in unequal variances that reflect each observation's measurement uncertainty, as well as model estimates that are influenced more by higher-precision observations (i.e., those from studies under more controlled conditions or with higher replicate counts) than lower-precision observations.

Finally, the study effect model shares the assumptions of OLS with the exception of uncorrelated errors. Instead, it is assumed that the errors of observations from different studies are uncorrelated, but that errors of observations within each study have some systematic correlation structure—for example, due to differences in agar used or the storage conditions of the strain—and that these errors have a normal distribution independent of the observation-level noise. It is assumed that this study-level noise has mean zero and that each study only changes the model's intercept. In other words, it models the effect of each study as a slight (random) shift in mean $\log_{10}D$ observed at all temperatures, and these shifts average out to zero when all studies are taken together.

To assess the shape of the $\log_{10}D$–temperature curve, three model shapes were fit: linear, piecewise, and quadratic. In the linear model, the change in $\log_{10}D$ when temperature changes by 1°C is constant for all temperatures. In the piecewise model, the effect of temperature is constant until a "break point" temperature, at which point

the line slope changes; thus changing temperature by 1°C has one constant effect on $\log_{10}D$ below the "break point" and a different (but still constant) effect afterwards. The location of this "break point" was determined by fitting linear models with break points at each 0.1°C increment between 55–57.2°C and 68–71.7°C—temperature regions at which potential non-linearities are suggested by visual inspection of the data—and picking the model that minimizes the residual sum of squared errors. In the quadratic model, the effect of temperature on $\log_{10}D$ changes as a function of temperature^2, meaning the slope of the regression line changes at a constant rate as temperature changes.

Assumption checks were carried out for all manually-fit models with no concerning deviations. Assumptions were not checked for each model in repeated 6-fold cross-validation (see next subsection), but the analyses done based on these models does not depend on the distribution of error terms and so is unaffected by assumption violations. In fact, the model comparison techniques I employed were chosen because none are particularly sensitive to violations of error structure assumptions, making them reliable even in the presence of minor model misspecification.

2.3.3 Statistical Analyses

Modeling Approach Comparisons

The goal of the first set of model comparisons was to identify the modeling approach most appropriate for the 'confounding' sources of variability in the data. The fit of the four candidate approaches outlined above was first assessed using the small sample-corrected Akaike information criterion (AICc) and the Bayesian information criterion (BIC). These are two similar but distinct metrics of fit and predictive accuracy that measure the information in the data that is 'lost' by a model; lower values imply better fit in the sense that the model preserves more information. Both AICc and BIC penalize overly-complex models in order to penalize models that preserve information by modeling too much of the data's 'noise'. In particular, AICc and BIC differ only in the 'penalty' applied to each added model coefficient—BIC penalizes model complexity more than AICc—due to their having different goals. Typically, AICc is thought to find the best predictive model while BIC aims at identifying the 'truest' model among a set of candidates [2]. These values can only be compared for models fit on the same data set, so every model was fit on the complete-only data set for these comparisons. Table 2 provides the common 'rules of thumb' used to evaluate the strength of evidence against higher AICc/BIC models.

Table 2: Information criteria 'rules of thumb' for the strength of evidence against the higher-AICc/BIC model.

AICc/BIC difference	Evidence against higher AICc/BIC
0 to 2	Inconclusive
2 to 6	Moderate
6 to 10	Strong
>10	Very Strong

The two metrics selected different models (that is, the candidate model with the smallest AICc did not also have the smallest BIC) due to their differing penalties for model complexity. Neither 'second-stage' candidate model involved inverse-variance weighting, so both were refit on the full data set to most accurately assess their relative fits. These two models—the OLS and study effect models—are "nested", meaning that the OLS model can be recovered from the study effect model by setting the study effect term to 0. For this reason, a likelihood-ratio test (LRT) was used to compare the models. Under the null hypothesis, the two models fit the data equally well, so the OLS model is preferable by parsimony. Under the alternative hypothesis, the study effect model fits the data significantly better than the OLS model, so the increase in complexity is justified. The LRT was chosen over the F test due to the latter's sensitivity to outliers, the existence of which was discussed above. Since the models differ by a (strictly non-negative) variance component, this LRT statistic does not have the typical χ^2_1 distribution; rather, it is distributed as a mixture of χ^2 distributions with 0 and 1 degrees of freedom [3]. The p-value for the LRT was calculated using this distribution.

Model Shape Comparisons

Once a modeling approach is obtained, the goal is to find the shape of model that predicts $\log_{10}D$ with the

smallest mean error. To assess the predictive accuracy of the three candidate model shapes, repeated stratified 6-fold cross-validation was performed using the full data set. This method was chosen because it allows for a realistic assessment of each model shape's performance on 'new' data by simulating the distribution of its predictive accuracy and conservatism. The fold value was chosen because it was an even divisor of the total number of observations and was the smallest common divisor greater than 5 (typically, the smallest number of folds used for cross-validation) that evenly divided the number of observations from multiple studies. Some studies had fewer than 6 observations; in this case, some observations were re-sampled multiple times in different folds. This results in certain studies' observations having slightly more influence on the cross-validation process. However, these observations span the range of temperatures and studies with multiple observations at a single temperature tend to have inter-observation variance below that of all observations at that temperature. Thus, these observations will not deteriorate the quality of the cross-validation overall and any effect they *do* have will be consistent across all models in a given fold.

The algorithm for this process was as follows:

1. Split data into 6 "folds" of (roughly) equal size under the constraint that observations from from every study be present in every fold.
2. Fit three study effect models (one of each candidate shape) using five of the six folds.
3. For each model, predict $\log_{10}D$ for:
    a. The five folds used to fit the model (in-sample predictions).
    b. The left-out fold and the researcher's 8 observations (out-of-sample predictions).
4. For each set of predictions, calculate:
    a. Accuracy: Mean squared prediction error (MSPE).
    b. Conservatism ratio (denoted κ): Absolute value of the mean ratio of underestimation to overestimation weighted by error 'severity' at a given temperature. The equation for this ratio and the severity values used in my analysis are given as an appendix.
5. Repeat steps 2–4 five more times, leaving out a different fold each time.
6. Repeat steps 1–5 a total of 1,000 times.

This resulted in 6000 MSPE and conservatism ratio values for in-sample and out-of-sample predictions for each model shape. Using these, I calculated the mean MSPE for each model shape separately for in-sample and out-of sample predictions, and obtained 95% confidence bounds as the 2.5% and 97.5% quantiles of the simulated values. I used the same methods to obtain the mean and 95% confidence bounds for the difference between in-sample and out-of-sample MSPE for each model shape. The latter was used to assess over-fitting; a prediction model's accuracy should not reduce significantly when faced with 'new' data. Likewise, I obtained the median conservatism for each model shape separately for in-sample and out-of sample predictions along with a 95% confidence interval, along with the median and 95% confidence bounds for the difference between in-sample and out-of-sample conservatism. Medians were chosen because the distribution of conservatism values was heavily skewed; medians are robust to skewness, while means are not. As with AICc and BIC, lower values of MSPE and conservatism are 'good' in the sense that they imply a model has smaller prediction errors (on average) and a lower rate of under-prediction.

2.3.4 Confidence & Prediction Intervals

The confidence and prediction intervals presented in this report are all 95% population-level intervals, which means that they were calculated without accounting for study effects or observation weighting. This is the interval most applicable to predicting new observations not found in the data set used to fit the models. In addition, asymptotic confidence and prediction intervals were employed for models with a study effect or observation weighting. These intervals use standard normal ($z_{\frac{\alpha}{2}}$) quantiles as opposed to the Student's T ($t_{\frac{\alpha}{2},\nu}$) quantiles (where ν is the degrees of freedom, typically calculated using Satterthwaite's approximation) and produce intervals equivalent to those obtained from the delta method as sample size grows to infinity [4].

This method was chosen for four reasons. First, all models' assumption checks found that the residuals were well-approximated by a normal distribution; in this case, the empirical distributions of confidence and prediction intervals converge to their asymptotic counterparts relatively quickly. Second, this method drastically reduces the complexity of obtaining distributional quantiles when calculating predictions or estimates at the cost of producing intervals that are, on average, optimistic (that is, too narrow) by a factor of 1.4–2.6%. Third, the intervals were not used as a criteria in any model comparisons, so the results were unaffected by this approximation. And fourth, the z quantile given in the interval formulas (found in the appendix) can easily be replaced by its t counterpart if this optimism is deemed unacceptable.

3 Results

This section first looks at the model fit criteria for each of the four modeling approaches outlined above before using the 'winning' model to compare the three candidate model shapes.

3.1 Modeling Method

Figure 1 shows the fitted regression lines produced by each of the four candidate modeling approaches with corresponding 95% prediction intervals. Visually, the differences appear marginal; the IVW model's line deviates most from the other three, especially at higher and lower temperatures. The tightness of the IVW–study effect combination model's prediction interval is due to the way its weighting structure interacts with its variance structure: once observations with large variances are down-weighted, the noise and study effect variance estimates have less "work" to do. This is not necessarily a good thing in the sense that observation-level weighting could alias inter-study variability, resulting in an anti-conservative (that is, overly optimistic) prediction interval. In other words, it risks removing so much noise that it also removes part of the signal, yielding an inaccurate picture of the model's precision.

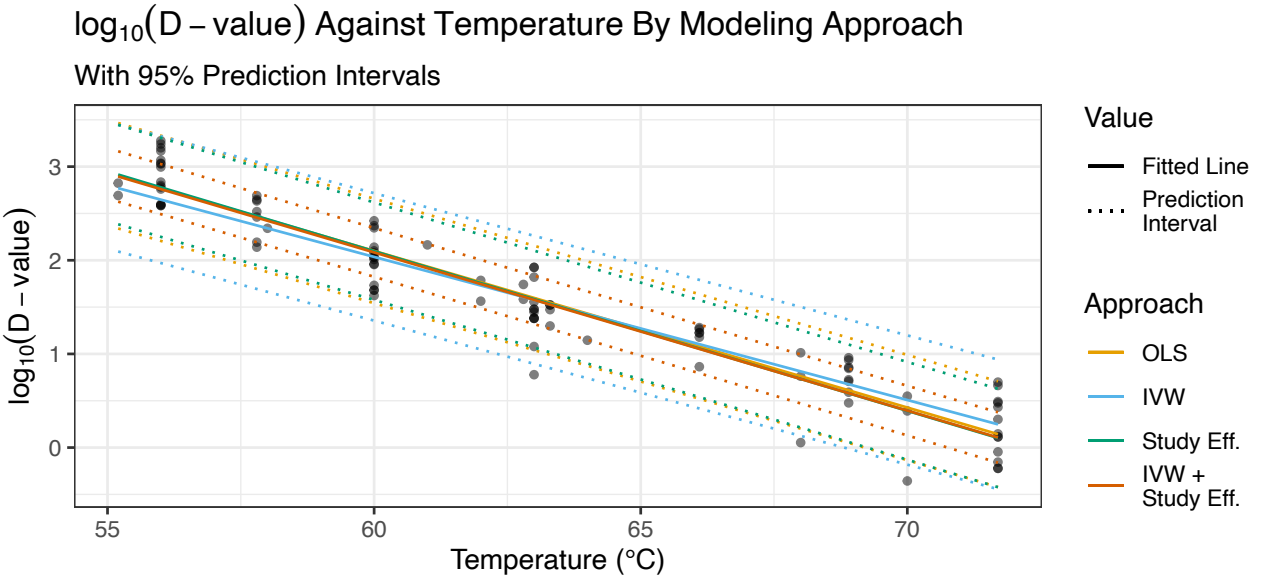

Figure 1: Candidate modeling approaches, all presented as linear models.

Table 3 gives the AICc and BIC for each candidate model. The Δ columns are the difference between each model's AICc/BIC and the minimum AICc/BIC among all candidate models. The study effect model yielded the smallest AICc, while the OLS model had the lowest BIC, though both differences are inconclusive. Though the IVW–study effect model's AICc fell between the AICc's of the OLS and study effect models, its BIC was substantially larger, suggesting that the added variance term was not 'pulling its weight' once variance was weighted out at the observation level. This is unsurprising, given that IVW and the study effect are different methods of accomplishing similar goals; the combination is, in this case, excessive. The subpar performance of the

IVW-only model, however, suggests that controlling for the observation-level variance alone is pound-for-pound less effective than either relying on the properties of OLS or modeling the variability at the study level.

Table 3: Information criteria values for candidate models on "complete" observations.

Model	AICc	Δ AICc	BIC	Δ BIC
OLS	**31.72**	**1.10**	**34.59**	**0.00**
Inv. Var.	62.48	31.85	99.75	65.16
Study Effect	**30.63**	**0.00**	**35.88**	**1.29**
Inv. Var. + Study Effect	31.29	0.66	70.01	35.42

The LRT statistic and p-value are given in Table 4, along with the AICc and BIC of the OLS and study effect models on the full data set. Note that AICc and BIC tend to increase as the number of observations used to fit a model increase, so the study effect model's relatively minor increases in these two metrics—while not directly meaningful—lends credence to its 'scalability'. The LRT test produced a p-value < 0.001, which suggests that there strong evidence of an improvement in model fit attributable to the additional study effect variance term. Thus, the study effect modeling approach will be used to assess the predictive accuracy of the three candidate model shapes.

Table 4: Comparison of OLS and study effect models using full data set.

Model	AICc	BIC	χ^2	p-value
OLS	44.50	53.62		
Study Effect	**32.93**	**45.02**	**13.68**	**<0.001**

3.2 Model Shape

Figure 2 shows the fitted regression lines produced by each of the three candidate model shapes with corresponding 95% prediction intervals. All models include a study effect variance term and were fit on the full data set. The quadratic model is slightly more conservative than the other shapes at low (≤ 57.5°C) and high (≥ 68°C) temperatures, while the reverse is true in the 62–64°C region.

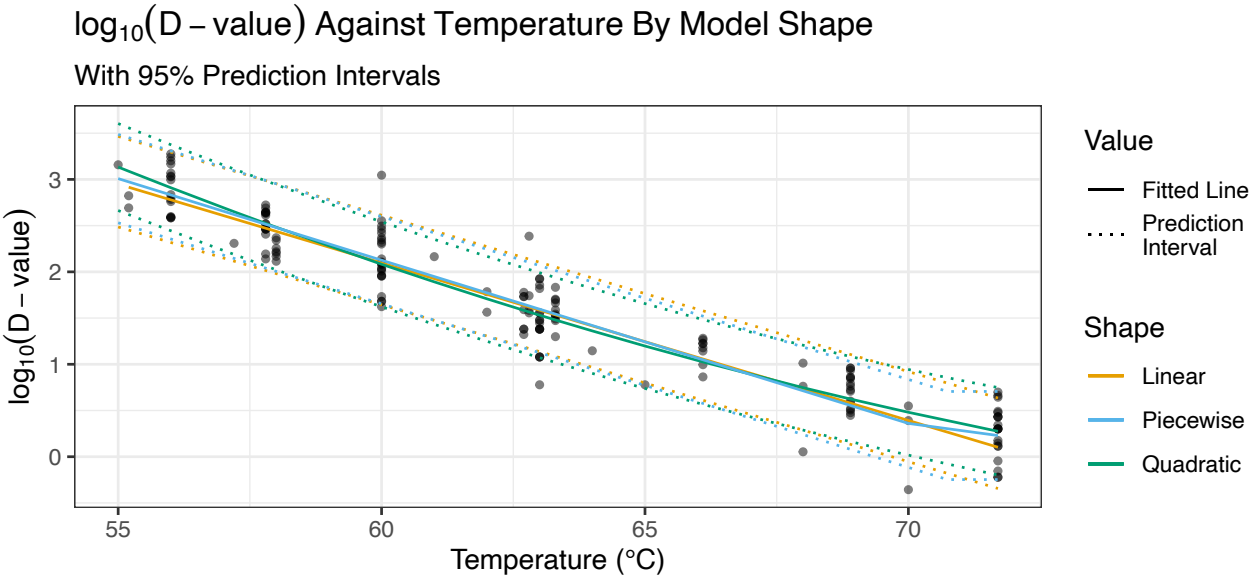

Figure 2: Fitted regression lines of candidate study effect model shapes with 95% prediction intervals.

The distribution of each shape's in-sample and out-of-sample predictive accuracy (as measured by mean squared prediction error) across the 1,000 replications of 6-fold cross-validation is shown in Figure 3. The inset text box gives the mean difference between in-sample and out-of-sample MSPE, denoted $\bar{\Delta}$, along with its 95% confidence interval. The quadratic model performed best both in-sample (mean $MSPE = 0.0468$) and out-of-sample (mean $MSPE = 0.053$). This is an unsurprising result; increasing model complexity will never increase in-sample MSPE and will only increase out-of-sample MSPE in cases of extreme over-fitting. Over-fit models will, however, experience a relatively substantial decline in out-of-sample MSPE *relative* to in-sample MSPE. The data suggest this was not the case for the quadratic model. Though it experienced a larger decline in MSPE than the other shapes ($\bar{\Delta}$ = -0.0061), the quadratic model's 95% confidence interval for mean MSPE difference (-0.0418, 0.0207) includes zero and thus does not suggest that this decline was significant enough to raise concerns about over-fitting. Further, the quadratic model's MSPE was significantly smaller than that of the piecewise model (mean difference=-0.0041, 95% CI: -0.0063—0.0021).

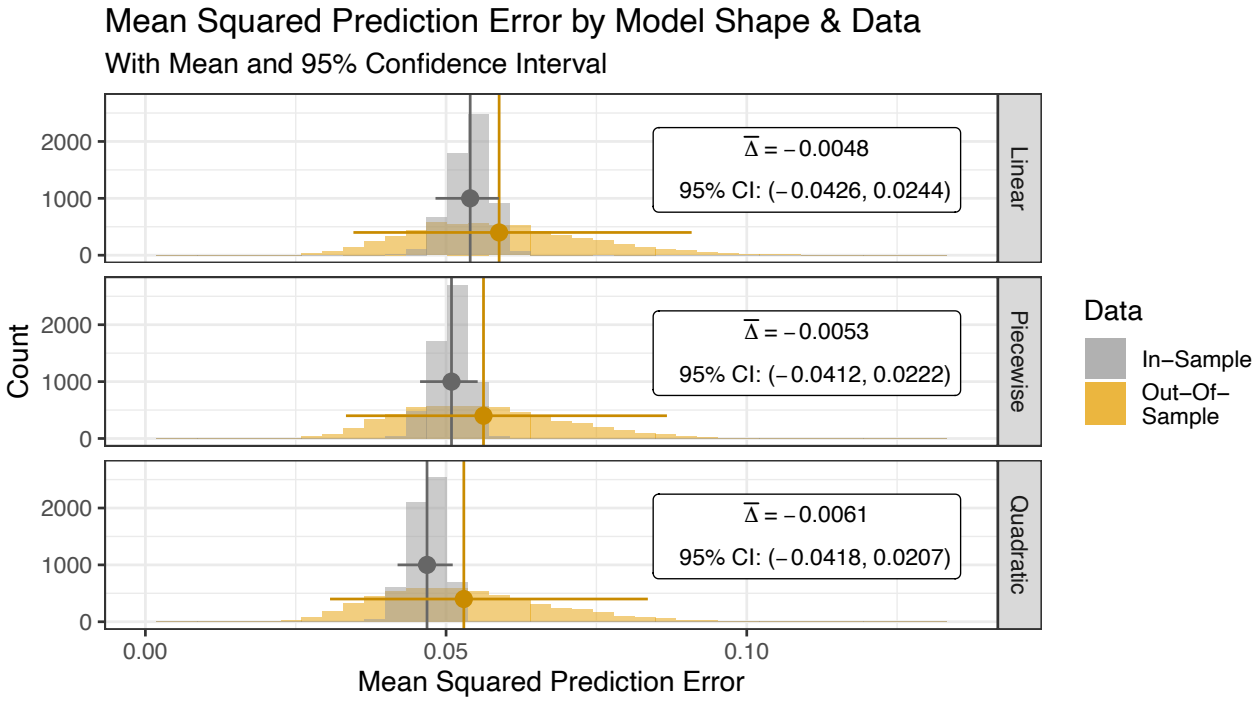

Figure 3: In-sample and out-of-sample mean squared prediction error for each candidate model shape with mean MSPE (vertical line) and 95% prediction intervals (horizontal line).

Figure 4 shows the distribution of in-sample and out-of-sample conservatism for each model shape. The median conservatism is denoted with a vertical bar and its 95% confidence interval is given as a horizontal bar. The inset text box displays the median difference between in-sample and out-of-sample conservatism, $\tilde{\Delta}$, with its 95% confidence interval. The heavy skewness of out-of-sample conservatism is worth noting and will be discussed more extensively in the next section. Still, the quadratic model produced both the least median under-prediction ratio both in-sample ($\kappa_{\text{in}} = 0.1597$) and out of sample ($\kappa_{\text{out}} = 0.0053$), as well as the smallest median difference in conservatism ($\tilde{\Delta} = 0.1518$). Thus, among the candidate model shapes, the quadratic model has both the smallest average prediction error and tends to be the most conservative in critical temperature regions.

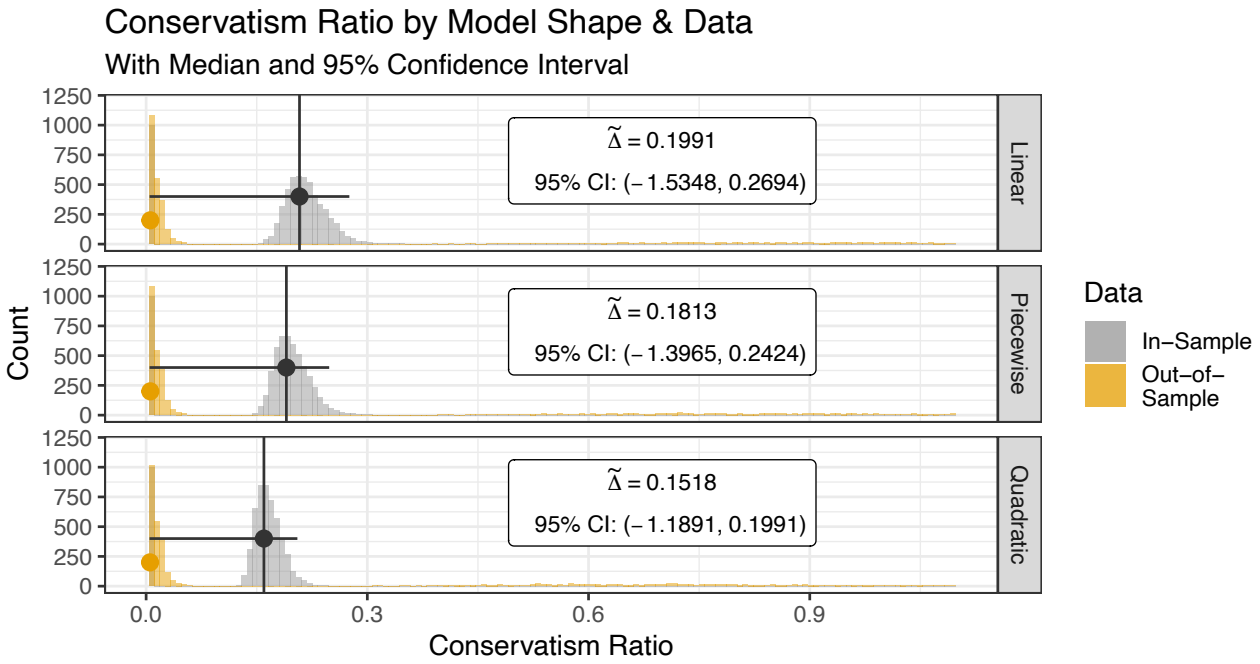

Figure 4: In-sample and out-of-sample conservatism ratio for each candidate model shape with median conservatism (vertical line) and 95% prediction intervals (horizontal line).

3.3 Final Model

The final model's fitted regression line, along with its 95% confidence and prediction intervals, is shown in Figure 5 below. It models $\log_{10} D$ as a quadratic function of temperature with a study-level random effect that accounts for the between-study variability in experimental conditions. Its AICc is 20.62, a substantial improvement over the linear model's AICc of 32.93. The inset text box provides the model equation and its (pseudo-)R^2, which measures the proportion of total variability—both between-study noise and random error—explained by the model. Equations for the confidence and predictions interval are provided as an appendix.

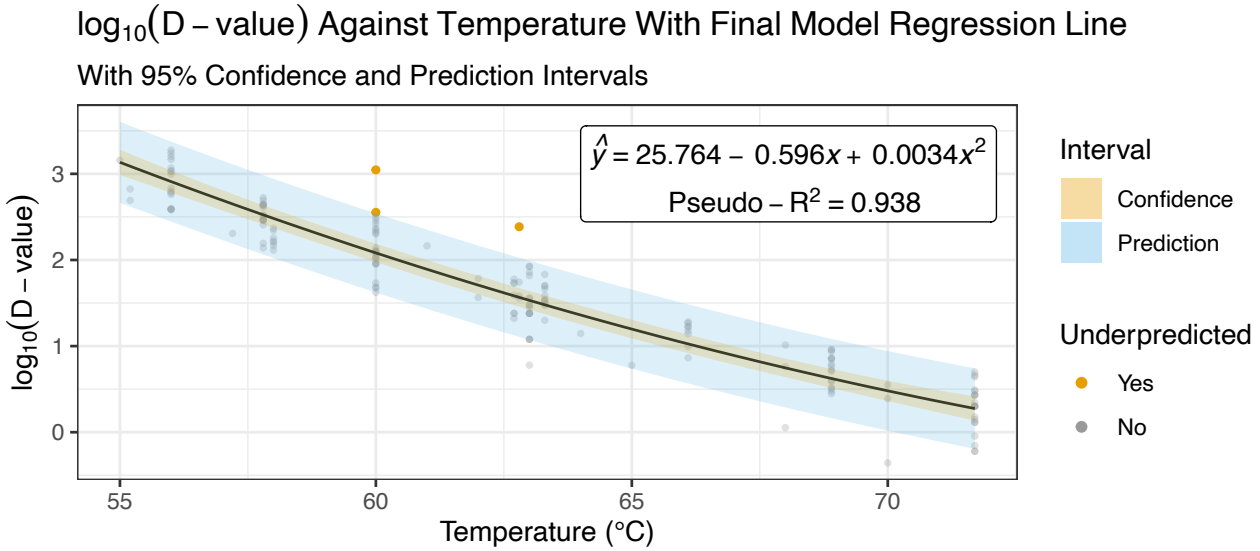

Figure 5: Final quadratic study effect model.

Using the upper bound of the prediction interval—which will be uniformly more conservative than the regression line itself—yields only three underpredicted observations, an underprediction rate of 1.9%. These observations are displayed in Table 5, along with the upper bound of the 95% prediction interval at that temperature and the magnitude of underprediction (denoted Δ). Note also that the first and last observations in the table below (141 and 153) were identified as outliers in preliminary analyses.

Table 5: Underpredicted values in the final model when using the upper bound of the 95% prediction interval.

Obs.	Study	Temp. (°C)	$\log_{10}D$	Upper Prediction Interval Bound	Δ
141	Knabel, 1990	62.8	2.39	2.03	-0.36
151	Fedio, 1989	60.0	2.55	2.54	-0.01
153	Fedio, 1989	60.0	3.05	2.54	-0.50

4 Discussion & Concluding Remarks

It is first worth laying out the implications of the study effect model 'winning out' over the other candidate approaches. This suggests the existence of some systematic variation in reported D-values that is not attributable to temperature or mere random noise. The obvious candidates for the source of this variation are the experimental conditions not controlled for by the model: the type of milk used, the storage and heating methods, and the culture media, for example. The study-level random effect compensates for the omission of these predictors to the extent that within-study experimental conditions are constant. In studies where reported D-values were obtained under only one set of experimental conditions (or a small number of highly similar conditions), the random effect term is a reasonable proxy for their effect on the D-value–temperature relationship; in studies that report D-values from a variety of experimental conditions, its compensatory ability is less clear. A model that explicitly controls for these conditions might yield a more accurate picture of the D-value–temperature relationship, but will likely succumb to over-fitting, reducing its predictive accuracy for D-values in novel conditions. In addition, it suggests that variability in reported D-values is not primarily attributable to variation in measurement accuracy for the meaned D-values. It is impossible to know whether this result would hold had more standard deviation data been reported in the literature.

Second, the quadratic shape of the final model deviates from similar meta-analytic models found in existing literature, which typically include only a linear temperature term. It is important to emphasize that quadratic models by nature perform worse than linear models when extrapolating, and thus the quadratic model here is only applicable for whole bovine milk thermitized between 55–71.7°C. To be sure, a linear fit provides a workable model for the D-value–temperature relationship and is likely more applicable outside of the temperature range modeled here. The goals guiding selection of the model presented here, however, differ from those of past work, and the linear model falls short of the predictive accuracy and conservatism offered by the quadratic model in this temperature range. The strong performance by the piecewise model is a somewhat surprising result given that it is no more complex than the linear model in terms of the number of parameters estimated. The viability of, for example, double-piecewise models (with two "break points") or piecewise–quadratic models is a topic for future research.

Third, I freely admit that the conservatism ratio, κ, employed to assess the model shapes' propensity to underpredict is imperfect. As presented here, the conservatism ratio was constructed to serve as a heuristic, not as a hard-and-fast criteria for model selection. Asymmetric loss functions tend to be model- and topic-specific; they are typically derived using a wealth of prior statistical and subject knowledge on the distribution of prediction errors and their 'costs' for a particular type of model applied to a particular field of research. This is doubly true for asymmetric loss functions that vary as a function of an independent variable. Deriving such a statistic was not possible both due to time constraints and my lack of familiarity with the relevant food microbiology literature. While the distribution of in-sample κ had some of the statistical properties desirable in a model selection statistic, the long-tailed distribution of out-of-sample κ suggests that the statistic I constructed is highly sensitive to sample size and the way data are randomly assigned to cross-validation folds, both of which are undesirable properties in a model selection statistic. These concerns did not influence my results, since the

model that performed best in terms of κ also performed best in terms of MSPE. However, it warrants against the application of κ generally.

Finally, it is worthwhile to address the tension between predictive accuracy and conservatism. Clearly, a model that produces a naive $\log_{10}D$ of, say, 4 regardless of temperature would be perfectly conservative, while a spline or quantile regression model that curves to accommodate even small variations in observed D-values at each temperature would yield predictions that are, on average very accurate. These goals lend themselves two fundamentally different modeling strategies, and 'splitting the difference' is far from trivial.

Still, the analyses I have presented provide good reason to believe that the final model 'splits the difference' best among the models considered. It generates predictions with high accuracy and rarely under-predicts when using its upper prediction interval bound, particularly outside of the 60–62.8°C region. The increase in model complexity brought about by the added quadratic temperature term was found to yield a non-trivial improvement in predictive accuracy relative to the linear models typical of similar meta-analyses and the piecewise model. Its accuracy does not deteriorate substantially when tasked with predicting new data, which suggests the model is not over-fit. These are desirable qualities in a prediction model.

5 References

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Appendix: Conservatism Ratio Function

The loss function ℓ used to calculate the conservatism ratio has the form

$$\ell(y_i, \hat{y}_i) = \begin{cases} \text{Temp.} \leq 57.2°\text{C} & \begin{cases} 3\text{c}^- \delta_i & \delta_i < 0 \\ \text{c}^+ \delta_i & \delta_i \geq 0 \end{cases} \\ 57.2°\text{C} < \text{Temp.} < 60°\text{C} & \begin{cases} \text{c}^- \delta_i & \delta_i < 0 \\ \text{c}^+ \delta_i & \delta_i \geq 0 \end{cases} \\ \text{Temp.} \geq 60°\text{C} & \begin{cases} \text{c}^- \delta_i & \frac{\delta_i}{y_i} < 0.1 \\ \frac{9}{10}\text{c}^- \delta_i & \frac{\delta_i}{y_i} \in [0.1, 0] \\ \text{c}^+ \delta_i & \delta_i > 0 \end{cases} \end{cases}$$

where $\delta_i = \hat{y}_i - y_i$, c^- is the 'severity' of underestimating (for my analyses, $\text{c}^- = 1.5$) and c^+ is the 'severity' of overestimating (for my analyses, $\text{c}^+ = 1$). And the conservatism ratio is

$$\kappa = \frac{1}{n} \times \left| \frac{\sum_{i=1}^{n} \ell(y_i, \hat{y}_i) \mathbb{1}_{\{\delta_i < 0\}}}{\sum_{i=1}^{n} \ell(y_i, \hat{y}_i) \mathbb{1}_{\{\delta_i \geq 0\}}} \right|$$

where n is the sample size and $\mathbb{1}$ is an indicator function that takes the value 1 if the condition in the $\{\}$ is true and is 0 otherwise.

Appendix: Confidence & Prediction Intervals

Let x_0 be the temperature (in °C) at which an estimated mean $\log_{10} D$ or a predicted $\log_{10} D$ is desired. Construct a row vector $\mathbf{x}_0 = (1,\ x_0,\ x_0^2)$. Denote the column vector of regression model coefficients by $\boldsymbol{\beta} = (\beta_0,\ \beta_1,\ \beta_2)^T$, its variance–covariance matrix by $\boldsymbol{V}$, and denote by $\hat{\sigma}_e^2$ the variance of the residuals (the values of these are given below). Finally, denote by $z_{\frac{\alpha}{2}}$ the $1 - \frac{\alpha}{2}$ (upper) quantile of the standard normal distribution. For a 95% interval, this quantile is 1.96.

Then the $100 \times \left(1 - \frac{\alpha}{2}\right)\%$ confidence interval for estimated mean $\log_{10} D$ at temperature x_0 is

$$\left[\mathbf{x}_0 \boldsymbol{\beta} \pm z_{\frac{\alpha}{2}} \sqrt{\mathbf{x}_0 \boldsymbol{V} \mathbf{x}_0^T} \right]$$

and the $100 \times \left(1 - \frac{\alpha}{2}\right)\%$ prediction interval for a new $\log_{10} D$ observation at temperature x_0 is

$$\left[\mathbf{x}_0 \boldsymbol{\beta} \pm z_{\frac{\alpha}{2}} \sqrt{\mathbf{x}_0 \boldsymbol{V} \mathbf{x}_0^T + \hat{\sigma}_e^2} \right]$$

If, instead, estimation/prediction are desired for multiple values at once, a matrix $\mathbf{X}_0$ should be constructed, with each row composed as in the vector $\mathbf{x}_0$. After obtaining the product $\mathbf{X}_0 \boldsymbol{V} \mathbf{X}_0^T$, the diagonal elements of the resulting matrix $diag(\mathbf{X}_0 \boldsymbol{V} \mathbf{X}_0^T)$ should be extracted and structured as a column vector so that they can be added to the column vector $\mathbf{x}_0 \boldsymbol{\beta}$.

The relevant values from the final model are

$$\boldsymbol{\beta} = \begin{bmatrix} 28.5176 \\ -0.595756 \\ 0.00335 \end{bmatrix} \qquad \boldsymbol{V} = \begin{bmatrix} 11.5408444 & -0.3643802 & 0.0028574 \\ -0.3643802 & 0.0115293 & -0.0000906 \\ 0.0028574 & -0.0000906 & 0.0000007 \end{bmatrix} \qquad \hat{\sigma}_e^2 = 0.05188$$