J. Dairy Sci. 104:12332–12341
https://doi.org/10.3168/jds.2021-20350

Determination and validation of D-values for *Listeria monocytogenes* and Shiga toxin–producing *Escherichia coli* in cheese milk

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ABSTRACT

Certain cheeses can be legally produced in the United States using raw milk, but they must be aged for at least 60 d to reduce pathogen risks. However, some varieties, even when aged for 60 d, have been shown to support growth of *Listeria monocytogenes* or survival of Shiga toxin–producing *Escherichia coli* (STEC). Thermization, as a subpasteurization heat treatment, has been proposed as a control to reduce the risk of pathogens in raw cheese milk while retaining some quality attributes in the cheese. However, the temperature and time combinations needed to enhance safety have not been well characterized. The objective of this research was to determine and validate decimal reduction values (D-values) for *L. monocytogenes* and STEC at thermization temperatures 65.6, 62.8, and 60.0°C; a D-value at 57.2°C was also determined for *L. monocytogenes* only. Nonhomogenized, pasteurized whole-milk samples (1 mL) were inoculated with 8-log cfu/mL *L. monocytogenes* or STEC (5- or 7-strain mixtures, respectively), vacuum-sealed in moisture-impermeable pouches, and heated via water bath submersion. Duplicate samples were removed at appropriate intervals and immediately cooled in an ice bath. Surviving bacteria 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 of 17.1 and 7.2, 33.8 and 16.9, and 146.6 and 60.0 s were found for *L. monocytogenes* and STEC, respectively, and a $D_{57.2°C}$-value of 909.1 s was determined for *L. monocytogenes*. Triplicate validation trials were conducted for each test temperature using 100 mL of milk inoculated with 3 to 4 log cfu/mL of each pathogen cocktail, A 3-log reduction of each pathogen was achieved faster in larger volumes than what was predicted by D-values (D-values were fail-safe). Data were additionally compared with published results from 21 scientific studies investigating *L. monocytogenes* and STEC in whole milk heated to thermization temperatures (55.0–71.7°C). These data can be used to give producers of artisanal raw-milk cheese flexibility in designing thermal processes to reduce *L. monocytogenes* and STEC populations to levels that are not infectious to consumers.

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

INTRODUCTION

Artisanal cheese sales in the United States 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 (The Specialty Food Association, 2016). Among >900 US artisan, farmstead, and specialty cheese producers surveyed in 2018, 50% were using raw and 17% were using thermized cheese milk, representing substantial increases from 2016 survey numbers (32% and 6%, respectively; ACS, 2016, 2018). Although only an estimated 1.6% of the US population consumes unpasteurized-milk cheeses, consumption of unpasteurized dairy products poses significantly higher risks for foodborne illness and hospitalization compared with pasteurized products (Costard et al., 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 natural cheeses made from raw milk (Fernandes, 2009; Panthi et al., 2017; Donnelly, 2018). *Listeria monocytogenes* and STEC have been classified as especially high-risk pathogens in raw-milk cheeses due to the severity of their associated illnesses (Condron et al., 2009; Donnelly, 2018), their ability to grow (Ryser and Marth, 1987; Sanaa et al., 2004) or survive (Reitsma and Henning, 1996; Schlesser et al., 2006; D'Amico et al., 2010) in certain raw-milk cheeses even when initially present at low levels in the cheese

Received February 22, 2021.
Accepted August 18, 2021.
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milk, and their resistance to starter cultures and native microbiota found in raw-milk cheese compared with other vegetative pathogens (Pereira et al., 2009; Montel et al., 2014).

Current US regulations (Code of Federal Regulations Title 21 Part 133) allow for 47 cheeses varieties, including surface ripened cheeses [21 Code of Federal Regulations 133.182(a)], to be legally manufactured using unpasteurized milk provided that they are aged a minimum of 60 d at ≥2°C (Donnelly, 2018). High moisture, high pH cheeses, including those made with pasteurized milk if recontaminated, are at particular risk of supporting growth of *L. monocytogenes* and causing illness (Ryser and Marth, 1987; Jackson et al., 2018). However, soft (high moisture) ripened cheeses carry an estimated 50- to 160-fold greater risk for listeriosis per serving when made with unpasteurized versus pasteurized milk (Jackson et al., 2018) and disproportionately account for a high number of listeriosis outbreaks implicating dairy foods in the United States (Langer et al., 2012; CDC, 2017). Shiga toxin–producing *Escherichia coli* accounted for 11% of US unpasteurized cheese outbreaks from 1998 to 2011 (Gould et al., 2014), and for 6 of 8 unpasteurized-milk cheese outbreaks from 1983 to 2018 in the United Kingdom (Donnelly, 2018). Additionally, STEC are especially hazardous due to their low infectious dose (<100 cfu; Farrokh et al., 2013; Donnelly, 2018) and ability to survive long periods in aged cheeses (Reitsma and Henning, 1996; Ramsaran et al., 1998; Maher et al., 2001; D'Amico et al., 2010).

Except for cheeses involving extensive acidification or curd-cooking in manufacture, 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 et al., 2009; Donnelly, 2018). Milk thermization has been proposed as a strategy to make cheeses made from unpasteurized milk safer while still meeting labeling requirements for raw-milk cheese designation in the United States (Johnson et al., 1990). Following 3 North American STEC outbreaks associated with raw-milk Gouda and Gouda-like cheeses aged ≥60 d, Canadian regulators recommended thermization of milk before the production of Gouda or Gouda-like cheeses to increase their microbial safety (Boyd et al., 2021).

Several working definitions of thermization exist, but no established definition in US regulations has been published, and the ability of thermization to reduce pathogens such as *L. monocytogenes* and STEC in milk have not been thoroughly described. Thermization is usually understood to result in a general microbial reduction of 3 to 4 log (CAC, 2004) and a positive phosphatase test for the treated cheese milk (Eugster and Jakob, 2019). A joint US Food and Drug Administration and 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 for soft-ripened cheeses approximately 7.2- to 10-fold compared with cheeses made from made from non-thermized raw milk (US FDA/Health Canada, 2015). Although work that specifically describes the thermal inactivation of the 7 key STEC serogroups (O26, O45, O103, O111, O121, O145, and O157) in whole milk has not been published, STEC are less heat resistant than *L. monocytogenes*; therefore, thermization treatment should reduce STEC at equal or higher rates as *L. monocytogenes* (Fernandes, 2009; Sörqvist, 2003; van Asselt and Zwietering, 2006; King et al., 2014).

The objective of the current study was to close knowledge gaps by determining decimal reduction values (D-values) for *L. monocytogenes* and STEC (multistrain mixtures) in whole milk to ultimately establish process lethality guidelines for whole-milk cheese thermization. Using the D-values determined in this study, predicted times to a 3-log reduction in *L. monocytogenes* and STEC levels at different temperatures in whole milk were subsequently validated using methodology simulating vat heat treatment. Results obtained in this study were compared with values published in the scientific literature. These data can be used to develop temperature-time tables for thermization that can be used by cheesemakers to manage risks associated with cheeses made from unpasteurized milk.

MATERIALS AND METHODS

Literature Analysis

Thermal lethality data from 18 studies investigating *L. monocytogenes* and 3 studies investigating STEC in whole-milk samples heated to thermization temperatures (55–71.7°C) were compiled. The temperature range for thermization was based on published definitions of thermization specific to cheesemaking (Lindsay et al., 2021). Data sets included bovine whole-milk samples only with any level of homogenization, pasteurization, sterilization, and heat treatment by any methodology; many publications used single strains rather than mixtures of strains including multiple serotypes. A total of 162 and 25 D-values for *L. monocytogenes* (Supplemental Table A1) and STEC (Supplemental Table A2; Appendix A, https://minds.wisconsin.edu/handle/1793/82300), respectively, were included. Log D-values versus heat treatment temperature were graphed, with individual thermal destruction curves constructed for *L. monocytogenes* (Figure 1) and STEC (Figure 2).

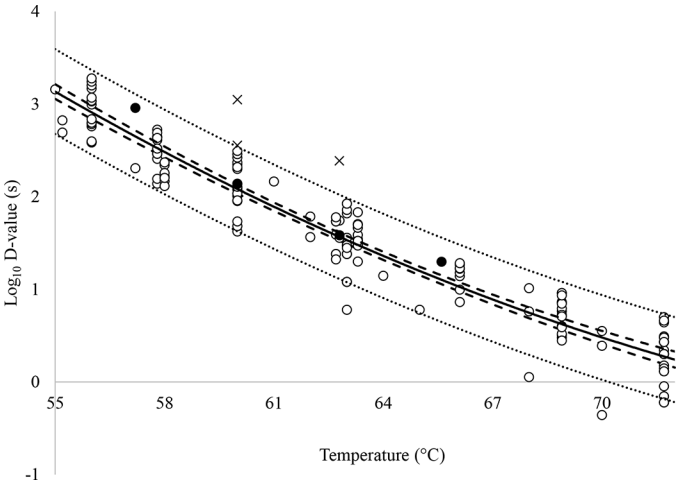

Figure 1. Literature (○) and study (●) log D-values for *Listeria monocytogenes* in inoculated whole milks versus test temperature. Literature values were compiled for whole bovine milks heated to thermization temperatures (55–71.7°C) and based on data given in Supplemental Table A1 (Appendix A, https://minds.wisconsin.edu/handle/1793/82300). Fitted thermal destruction curve with the equation log D-value = $0.00335095078067352x^2 - 0.595756448366535x + 25.763869392577$ (—; $R^2 = 0.94$; x represents temperature in °C) shown with 95% confidence (- -) and prediction (⋯) intervals. Literature D-values underpredicted by thermal destruction curve (×) are additionally displayed.

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 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 (https://www.r-project.org/) with a threshold of 0.05. To assess the predictive accuracy of the 3 candidate model shapes, repeated study-stratified 6-fold cross validation was performed on the full data set. D-values from the current study (8 observations, 2 for each test temperature) were used to validate models in each fold of each repetition.

Inoculum Preparation

Five *L. monocytogenes* and 7 STEC strains (Table 1) were grown individually in 10 mL of trypticase soy broth (BBL BD) at 37°C for 18 to 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 to 24 h. Cells were harvested via scraping plate surfaces with a sterile inoculating loop and by suspending the lawns from each individual strain in 4.5 mL of 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 the multistrain mixture of *L. monocytogenes* or STEC. Strain purities were verified by streaking on modified Oxford agar (**MOX**; *Listeria* selective agar base, Difco, BD) or sorbitol MacConkey agar (BBL BD) for *L. monocytogenes* and STEC, respectively, as well as on TSA, whereas cocktail populations were enumerated by spread plating on MOX and sorbitol MacConkey agar for *L. monocytogenes* and STEC, respectively. Time 0 (pre-cook) samples were assayed to identify rate of recovery from the inoculated product. The log reduction was calculated using the average populations of *L. monocytogenes* or STEC recovered from triplicate uncooked, inoculated samples.

Sample Inoculation

Fifty milliliters of nonhomogenized, pasteurized whole milk (SuperNatural Organic Whole Milk, Kalona Organics) was hand-shaken to distribute the cream layer and pipetted into a sterile 50-mL centrifuge tube (Falcon, BD Biosciences) before inoculation at a 1.0%

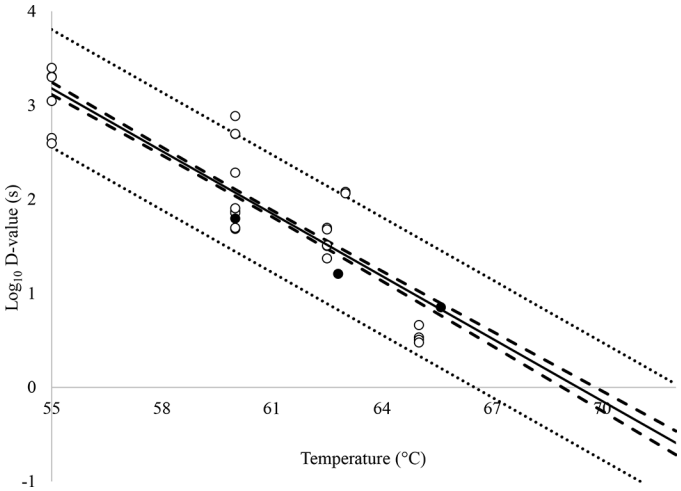

Figure 2. Literature (○) and study (●) log D-values for Shiga toxin–producing *Escherichia coli* in inoculated whole bovine milk samples. Literature values were compiled for whole bovine milks heated to thermization temperatures (55–71.7°C) and based on data given in Supplemental Table A2 (Appendix A, https://minds.wisconsin.edu/handle/1793/82300). Fitted thermal destruction curve with the equation log D-value = $-0.2224x + 15.3830$ (—; $R^2 = 0.79$; x represents temperature in °C) shown with 95% confidence (- -) and prediction (⋯) intervals.

Table 1. *Listeria monocytogenes* and Shiga toxin–producing *Escherichia coli* (STEC) strain cocktails used in D-value determination and validation studies

Organism	Strain	Serotype	Source
L. monocytogenes	108	1/2b	Hard salami isolate
L. monocytogenes	301	1/2a	Cheddar cheese isolate
L. monocytogenes	310	4b	Goat's milk cheese isolate associated with illness
L. monocytogenes	R2–500	4b	Soft Hispanic-style cheese isolate
L. monocytogenes	R2–501	4b	Clinical isolate associated with soft Hispanic-style cheese
STEC	00–3142	O111:H8	Clinical isolate
STEC	01–3002	O103:H2	Clinical isolate
STEC	01–3434	O121:H9	Clinical isolate
STEC	01–3510	O45:H2	Clinical isolate
STEC	99–3311	O145:NM	Clinical isolate
STEC	H30	O26:H11	Clinical isolate
STEC	ATCC 43895	O157:H7	Clinical isolate

(vol/vol) level with *L. monocytogenes* or STEC strain cocktail to yield approximately 8 log cfu/mL milk. After vortexing, 1-mL aliquots of inoculated milk were pipetted into moisture- and gas-impermeable pouches [3-mil high barrier ethylene-vinyl alcohol (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; Win-Pak] and vacuum-packaged (Multivac AGW). Pouches were flattened to a uniform thickness before heating.

Heating of Pouched Samples

Sample pouches were attached to a fabricated sampling rack to provide even distribution of samples bags within a water bath and to allow for simultaneous and efficient immersion. The sampling rack was submerged in a circulating water bath (Magniwhirl Constant Temp Bath, Blue M Electric Company) heated to target temperatures (65.6, 62.8, and 60°C; in addition, experiments with *L. monocytogenes* were conducted at 57.2°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) 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) 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 s. Time 0 samples were not removed from the water bath until samples reached the specified target temperature. At each sampling point, inoculated samples (triplicate for *L. monocytogene*s, duplicate for STEC) were removed and immediately submerged in an ice-water bath for a minimum of 2 min to reach ≤4°C. Chilled sample pouches were removed from the ice-water bath, dried, and sanitized (outside of the bag) with 70% ethanol before opening. We added 9.0 mL of BPW to each pouch (1:10 dilution) before stomaching (Neutec Masticator, Neutec Group Inc.) samples for 30 s. Trials for each temperature-pathogen combination were conducted in duplicate.

Heating of Flasked Validation Samples

D-values determined from heating milk in vacuum-sealed bags in a water bath were subsequently validated by heating of milk in sterilized flasks. We pipetted 100-mL samples of nonhomogenized, pasteurized whole milk (Kalona brand) into sterilized 250-mL Büchner flasks containing a stir bar. Milk was prewarmed in a water bath to target temperature with constant stirring before inoculation by individually immersing flasks in a stainless-steel pan water bath over a magnetic hotplate stirrer (Corning, model PC-620D), with water reaching >2 inches (5.08 cm) above the milk surface. Once the target temperature of the milk was stabilized, milk was inoculated with a target of 3 to 4 log cfu/mL *L. monocytogenes* or STEC (1% vol/vol; 5–6 log cfu/mL cell suspension serially diluted with BPW), 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 removed from the flask by pipetting into a 15-mL centrifuge tube (Falcon, BD Biosciences) fully submerged in an ice-water bath. Immediately following sampling, each sample was shaken vigorously in the ice-water bath for

10 s to cool the sample to ≤4°C. Chilled milk sample tubes were removed from the ice-water bath, dried, and sanitized with 70% ethanol before opening. Samples were diluted and plated as described below. Independent trials for each temperature-pathogen combination were conducted in triplicate.

Enumeration and Data Analysis

Samples were serially diluted with BPW and surface plated on MOX and SMAC agars overlaid with TSA for *L. monocytogenes* and STEC, respectively, to aid recovery of heat-injured cells (Kang and Fung, 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 including at least 5 sampling points for each pathogen-temperature-trial were fitted using 8 relevant models (e.g., linear regression, Weibull, biphasic) in the GInaFiT add-on for Excel (Geeraerd et al., 2005; Microsoft Excel 2016). Final D-values were calculated from linear regression of log reductions of each trial, as linear regression returned the highest R^2 values among models fitted as well as the most conservative D-values. 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 to 65.6°C to compare relative heat resistance between the 2 pathogens. The z-values were determined by graphing log D-value versus temperature for each pathogen (Figure 3 and 4 for *L. monocytogenes* and STEC, respectively), with z-values equaling the absolute inverse of the slope of each linear regression line.

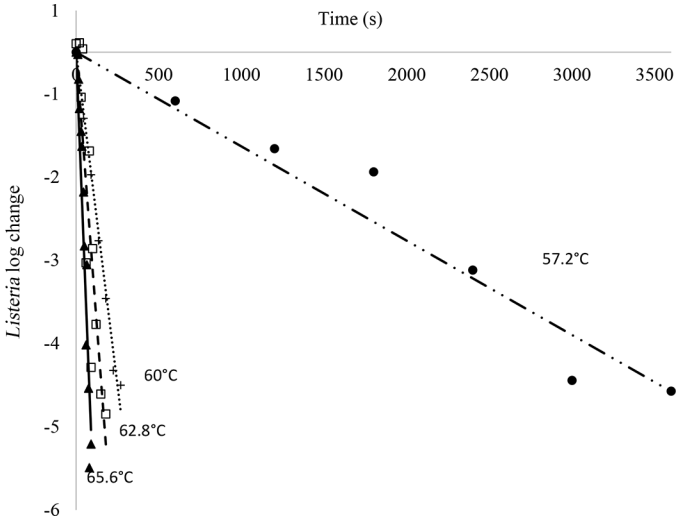

Figure 3. Change log populations of *Listeria monocytogenes* versus time in nonhomogenized, whole bovine milk heated at 57.2, 60.0, 62.8, and 65.6°C. Pooled results from duplicate trials are shown. Linear regression for survival curves were used for D-value calculation.

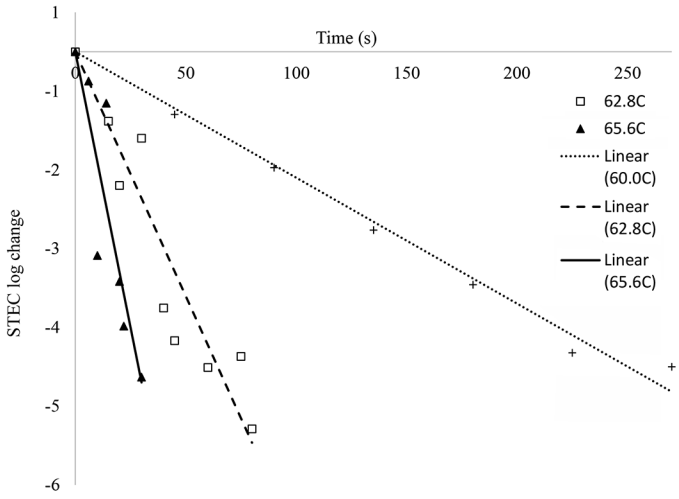

Figure 4. Change log populations for Shiga toxin–producing *Escherichia coli* (STEC) versus time in nonhomogenized, whole bovine milk heated at 60.0, 62.8, and 65.6°C. Pooled results from duplicate trials are shown. Linear regression for survival curves were used for D-value calculation.

RESULTS

This study investigated thermization (i.e., subpasteurization) treatments necessary for the reduction of *L. monocytogenes* and STEC in nonhomogenized whole bovine milk to levels where they would not be infectious to consumers if applied to raw-milk cheesemaking. Following determination of D- and z-values for each pathogen, D-values were validated in a larger volume (100 mL vs. 1 mL in the D-value work) of inoculated milk with lower inoculum (3–4 log cfu/mL) to confirm the time to 3-log kill in vat pasteurization being equal to or shorter than predicted using the D-values. Additionally, compiled published lethality data on *L. monocytogenes* in whole-milk samples heated to thermization temperatures were modeled, finding nonlinearity of fit over thermization temperatures of 55 to 71.7°C (Figure 1), although a linear response was observed for temperatures of 60.0 to 65.6°C for both pathogens (Figures 1 and 2).

Table 2. D- and z-values for *Listeria monocytogenes* and Shiga toxin–producing *Escherichia coli* (STEC) in nonhomogenized, whole bovine milk (experimental values) and from models derived from combined literature values

		D-value (s) for Temperature				
Pathogen	Value source	57.2°C	60.0°C	62.8°C	65.6°C	z-value
L. monocytogenes	Experimental values[1]	909.1	137.0	38.2	19.8	6.7°C[2]
	Literature values[3]	447.1	120.8	36.8	12.7	NC[4]
STEC	Experimental values[5]	Not tested	62.5	16.1	7.15	6.0°C
	Literature values[6]	458.9	109.4	26.1	6.2	4.5°C

[1]R^2 of *L. monocytogenes* inactivation regression models 0.98, 0.87, 0.92, 0.96 at 57.2, 60.0, 62.8, 65.6°C, respectively.
[2]Due to nonlinearity of regression for D-values below 60°C, z-value calculated using temperatures from 60.0 to 65.6°C only.
[3]R^2 of *L. monocytogenes* literature model = 0.94.
[4]NC = unable to calculate z-value from nonlinear model.
[5]R^2 of STEC inactivation regression models 0.99, 0.97, 0.92 at 60.0, 62.8, 65.6°C, respectively.
[6]R^2 of STEC literature model = 0.79.

Modeling of Published D-values

Thermal lethality data from 18 studies investigating *L. monocytogenes* and 3 studies investigating STEC in whole-milk samples heated to thermization temperatures (55–71.7°C) were used for modeling. Experimental conditions including growth of test bacterial strains, inoculation procedures, details on milk samples used, and methodology for heating and enumeration are given in Supplemental Tables A1 and A2 (Appendix A, https://minds.wisconsin.edu/handle/1793/82300) for literature data sets for *L. monocytogenes* and STEC, respectively. Among the modeling approaches considered for fitting published *L. monocytogenes* 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 underprediction (to ensure model was fail-safe). Quadratic fitting of the data set 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 the linear model (20.62 vs. 32.93), an indication of better model fit. The final model was found to underpredict 3 of 162 observations (Figure 1, R^2 = 0.94), a rate of 1.9%, though 2 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 et al. (1990), and Fedio and Jackson (1989), respectively. Elevated *L. monocytogenes* inoculum growth temperatures of 43°C and 48°C were used by the 2 authors, which has been shown to alter lipid and protein biosynthesis, membrane composition, and subsequently increase *L. monocytogenes* thermal resistance in liquid dairy products (Pagán et al., 1998; Doyle et al., 2001). Due to the nonlinearity of the model, a z-value cannot be calculated using standard methods. D-values were calculated from the model as 447.1, 120.8, 36.8, and 12.7 s at 57.2, 60.0, 62.8 and 65.6°C, respectively.

In contrast to the wealth of prior data for *L. monocytogenes*, relatively few published studies report thermal lethality of STEC in milk, particularly non-O157 strains. As a result, the predictive model fitted for STEC inactivation was less robust; the linear model had an R^2 of 0.79 (Figure 2 and Table 2); D-values were calculated as 458.9, 109.4, 26.1, and 6.2 s at 57.2, 60.0, 62.8 and 65.6°C, respectively.

Determination and Validation of D- and Z-values in Cheese Milk

Survival curves for *L. monocytogenes* and STEC in nonhomogenized whole milk revealed a linear decrease in populations at each test temperature across trials (Figures 3 and 4). As expected, D-values for STEC were lower than those for *L. monocytogenes* at each test temperature (Tables 2 and 3). Due to the nonlinearity of thermal inactivation for temperatures less than 60°C for *L. monocytogenes* found in the model for previously published data, z-values were calculated with values from 60 to 65.6°C; the z-values in the whole-milk matrix were 6.7 and 6.0°C for *L. monocytogenes* and STEC, respectively.

In the validation experiments, *L. monocytogenes* populations decreased from ~3.5-log cfu/mL to undetectable levels (<0.48 log cfu/mL) in 15 to 26 s, 60 to 90 s, 180 to 210 s, and 1,200 to 1,800 s at test temperatures of 65.6, 62.8, 60.0, and 57.2°C, respectively, whereas >3.5-log reduction of STEC to undetectable levels were observed in 5 to 10 s, 12 to 25 s, 94 s, and

Table 3. Time (in min:s) and temperature combinations for 3-log reduction of *Listeria monocytogenes* and Shiga toxin–producing *Escherichia coli* (STEC) in nonhomogenized, whole milk based on determined D-values in thin layer thermal death time experiments (TDT; duplicate trials) and time to >3 log kill based on validation in flasks [triplicate trials; time to undetectable levels < 0.48-log cfu/mL, from starting average 4.12 ± 0.29 (SD) log cfu/mL of STEC or 3.57 ± 0.55 log cfu/mL *L. monocytogenes*; rounded up to nearest 5-s interval]

	Time (min:s) to 3-log decrease			
	L. monocytogenes		STEC	
Temperature (°C)	Predicted time based on TDT experiments	Time based on flask validation	Predicted time TDT experiments	Time based on flask validation
57.2	45:30	30:00	Not tested	3:10
60.0	6:54	3:00	3:06	1:35
62.8	1:54	1:30	0:48	0:25
65.6	1:00	0:30	0:24	0:10

189 s at the same test temperatures (Table 3). The observed lethality in the validation experiments was faster than that predicted based on the D-value experimental data in the small scale (thin layer) thermal death time experiments. This finding suggested that the D-values determined in the current study are fail-safe when heating larger volumes of nonhomogenized whole milk, as would occur during typical batch thermization treatments. However, to ensure sufficient lethality, particularly when using HTST equipment, establishing a thermal process based on the more conservative D- and z-values (Table 2; Figures 3 and 4) will deliver a greater margin of safety compared with using the values from the validation study alone (Table 3).

DISCUSSION

This study determined D- and z-values for multiserotype mixtures of *L. monocytogenes* and STEC in whole milk and developed models for thermal inactivation for both pathogens from values derived from the literature at temperatures relevant to thermization of milk for cheesemaking. As expected, the new experimental data from this study revealed that *L. monocytogenes* requires longer time for thermal inactivation at any given temperature than does STEC. Although subpasteurization heating of milk is not as reliable as pasteurization to ensure the safety of cheeses, it can reduce the risk of pathogens in cheese made with unpasteurized milk, especially when used in conjunction with other preventive controls, including supply control (using milk with low microbial loads), good manufacturing practices and sanitation, employee hygiene, and consideration of the effect of cheese composition (moisture, water activity, pH) on microbial survival or growth during aging.

Published thermal inactivation data in microbiological media and dairy products suggest that temperatures in the lower range recommended for cheese milk thermization (e.g., 55–57°C) are lethal to STEC but are either sublethal to *L. monocytogenes* or require long periods of heating for significant lethality (Arocha et al., 1992; Ercolini et al., 2005; Skandamis et al., 2009; Peng et al., 2012; Pöntinen et al., 2017). Nevertheless, 8 of 40 published definitions for milk thermization specific to cheese milk included temperatures ≤57°C, suggesting that many currently-used thermization practices would result in little, if any, *L. monocytogenes* reduction, depending on the treatment time (Lindsay et al., 2021). Additionally, the time required to reduce *L. monocytogenes* by 3-log may be longer than practical at these lower treatment temperatures, as thermization times as long as 30 min are required temperatures 55 to 68°C (Lindsay et al., 2021). In agreement with these conclusions, regulators have suggested milk thermization at temperatures ≤58°C are inadequate or less effective than higher thermization temperatures (Condron et al., 2009). Additionally, mild thermization treatments resulting in 3 log reductions of either organism in milk may be insufficient to ensure safety of the subsequent cheese; greater lethality standards may be needed in the case of mild temperature abuse, especially given the ability of both *L. monocytogenes* and STEC to grow at low temperatures in permissive food matrices (King et al., 2014).

The curvature of the compiled *L. monocytogenes* log D-value versus temperature plots in milk samples heated to temperatures 52 to 75°C was also observed by Mackey and Bratchell (1989). The authors attributed the 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 or 7.4°C, respectively (Mackey and Bratchell, 1989). Conversely, van Lieverloo et al. (2011) 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 (laboratory-scale pasteurizer with flow) and worst-case (large sample volume heated in

water bath) methodologies. Regardless of the cause for the nonlinear results for our data and for published D-values at temperatures less than 57°C, the inconsistent lethality of *L. monocytogenes* also suggests that lower thermization temperatures should be used with caution and may require additional validation testing.

Although researchers have reported generic *E. coli* thermal inactivation models based on compiled D-values in microbiological media and foodstuffs that included milk and dairy data sets (Sörqvist, 2003; van Asselt and Zwietering, 2006), no other published research to date has specifically compiled STEC log D-values in milk samples. The thermal inactivation data available in the published literature for STEC in cheese milk was insufficient to develop a robust model to predict D- and z-values that could be used for thermization. The model developed from the literature data suggested that STEC is more thermal resistant than *L. monocytogenes* at lower temperatures, which is counter to findings in laboratory media and foods (International Commission on Microbiological Specifications for Foods, 1996). Therefore, the experimental data developed in this study is more representative of expected D and z-values for STEC and *L. monocytogenes* in bovine cheese milk.

Because calculated z-values are dependent on the temperature range included in their determination (van Doornmalen and Kopinga, 2009), z-values should be interpreted with caution and used solely as a reference (Peng et al., 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 et al. (2013). Z-values for our experimental data were 6.7 and 6.0°C for *L. monocytogenes* and STEC, which similar to ranges in other published studies. *Listeria monocytogenes* z-values of 5.7 to 7.0°C were reported in predictive models constructed from data in microbiological medium and a variety of foodstuffs including dairy (Mackey and Bratchell, 1989; Sörqvist, 2003; van Asselt and Zwietering, 2006; van Lieverloo et al., 2011, 2013). Though no predictive models specifically for STEC have been published to date, generic *E. coli* z-values of 6.0 and 10.6°C were suggested by Sörqvist (2003) and van Asselt and Zwietering (2006), respectively, from their meta-analyses in liquid foods and media.

Although D- and z-values for *L. monocytogenes* and STEC vary depending on the matrix, strains used, and methodology, the values identified in our current study (Table 2) can be used as guidelines to develop thermal processing parameters (temperature-hold time) to inactivate pathogens in cheese milk. Supplemental files (Appendix B, https://minds.wisconsin.edu/handle/1793/82300) associated with this manuscript provide examples of temperature-time combinations to reduce pathogen levels in thermized milk, which should be used in combination with appropriate preventive controls and aging requirements. Use of full pasteurization (e.g., 71.1°C, 15 s) is intended to reliably eliminate pathogenic vegetative bacteria in cheese milk, but achieving a minimum 3-log reduction will reduce the risk of illness associated with cheeses made with unpasteurized milk (US FDA/Health Canada, 2015).

CONCLUSIONS

As the popularity of artisanal raw-milk cheeses continues to grow in the United States, control measures to enhance their safety must be scientifically evaluated and optimized. Though hygienic controls can help to reduce microbial populations in raw cheese milk, heat treatment is the most important process used for eliminating vegetative bacterial pathogens from the finished product. Thermization permits some beneficial quality attributes of raw cheese milk to be retained while improving safety. This study identified thermization conditions necessary for 3-log reductions of *L. monocytogenes* and STEC in whole milk. These conditions will provide cheesemakers with flexibility in designing 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 cheese 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.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of University of Wisconsin-Madison Food Research Institute staff including Tanner Bilstad, Max Golden, Quinn Huibregtse, Yinuo Jin, Ellie Leafgren, and Brandon Wanless for media preparation, and Kristin Schill, Wendy Bedale, and Jie Yin Lim for manuscript editing. We appreciate the helpful discussions with Chad Galer and Tim Stubbs, National Dairy Council (Rosemont, IL), and Bob Wills, Clock Shadow Creamery (Milwaukee, WI). This research was funded by the National Dairy Council, the Robert H. and Carol L. Deibel Distinguished Graduate Fellowship in Food Safety Research (University of Wisconsin–Madison), and by unrestricted gifts from the industry to the Food Research Institute, University of Wisconsin–Madison. The authors have not stated any conflicts of interest.

REFERENCES

American Cheese Society (ACS). 2018. 2018 State of the U.S. Artisan/Specialty Cheese Industry: Food Safety Report. Accessed Sep. 11, 2021. https://www.cheesesociety.org/wp-content/uploads/2019/01/ACS-2018-Food-Safety-Report_TOC_ExecSummary_Intro.pdf.

American Cheese Society. 2016. 2016 State of the U.S. Artisan/Specialty Cheese Industry: Report of Key Findings. Accessed Sep. 11, 2021. https://1sskha48hhbhl0oc61bhsagh-wpengine.netdna-ssl.com/wp-content/uploads/2017/02/2016-State-of-the-US-Artisan-and-Specialty-Cheese-Industry-Summary-Report.pdf.

Arocha, M. M., M. McVey, S. D. Loder, J. H. Rupnow, and L. Bullerman. 1992. Behavior of hemorrhagic E*scherichia coli* O157:H7 during the manufacture of cottage cheese. J. Food Prot. 55:379–381. https://doi.org/10.4315/0362-028X-55.5.379.

Boyd, E., A. Trmcic, M. Taylor, S. Shyng, P. Hasselback, S. Man, C. Tchao, J. Stone, L. Janz, L. Hoang, and E. Galanis. 2021. *Escherichia coli* O121 outbreak associated with raw milk Gouda-like cheese in British Columbia, Canada, 2018. Can. Commun. Dis. Rep. 47:11–16. https://doi.org/10.14745/ccdr.v47i01a03.

CAC (Codex Alimentarius Commission). 2004. Code of Hygienic Practice for Milk and Milk Products. Codex Alimentarius Commission. Accessed Jan. 24, 2021. http://www.fao.org/fileadmin/user_upload/livestockgov/documents/CXP_057e.pdf.

CDC (Centers for Disease Control and Prevention). 2017. Foodborne illness source attribution estimates for 2013 for *Salmonella, Escherichia coli* O157, *Listeria monocytogenes*, and *Campylobacter* using multi-year outbreak surveillance data. Accessed Jan. 24, 2021. https://www.cdc.gov/foodsafety/pdfs/IFSAC-2013FoodborneillnessSourceEstimates-508.pdf.

Condron, R., P. Desmarchelier, R. Dyson, D. Eddy, L. Hammond, M. Kirk, R. MacBean, J O'Regan, S. Rice, J. Robertson, and N. Willman. 2009. Microbiological risk assessment of raw milk cheese. Food Standards Australia New Zealand 1–264. Accessed Sep 11, 2021. https://www.foodstandards.gov.au/code/proposals/documents/P1007%20PPPS%20for%20raw%20milk%201AR%20SD3%20Cheese%20Risk%20Assessment.pdf.

Costard, S., L. Espejo, H. Groenendaal, and F. J. Zagmutt. 2017. Outbreak-related disease burden associated with consumption of unpasteurized cow's milk and cheese, United States, 2009–2014. Emerg. Infect. Dis. 23:957–964. https://doi.org/10.3201/eid2306.151603.

D'Amico, D. J., M. J. Druart, and C. W. Donnelly. 2010. Behavior of *Escherichia coli* O157:H7 during the manufacture and aging of Gouda and stirred-curd Cheddar cheeses manufactured from raw milk. J. Food Prot. 73:2217–2224. https://doi.org/10.4315/0362-028X-73.12.2217.

Donnelly, C. 2018. Review of controls for pathogen risks in Scottish artisan cheeses made from unpasteurized milk. Food Standards Scotland 1–146. Accessed Sep. 11, 2021. https://www.foodstandards.gov.scot/downloads/FSS_2017_015_-_Control_of_pathogens_in_unpasteurised_milk_cheese_-_Final_report_v_4.7_-_20th_November_2018_.pdf.

Doyle, M. E., A. S. Mazzotta, T. Wang, D. W. Wiseman, and V. N. Scott. 2001. Heat resistance of *Listeria monocytogenes*. J. Food Prot. 64:410–429. https://doi.org/10.4315/0362-028X-64.3.410.

Ercolini, D., V. Fusco, G. Blaiotta, F. Sarghini, and S. Coppola. 2005. Response of *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella* Typhimurium, and *Staphylococcus aureus* to the thermal stress occurring in model manufactures of Grana Padano cheese. J. Dairy Sci. 88:3818–3825. https://doi.org/10.3168/jds.S0022-0302(05)73067-8.

Eugster, E., and E. Jakob. 2019. Pre-treatments of milk and their effect on the food safety of cheese. Milchwissenschaft 72:45–52.

Farrokh, C., K. Jordan, F. Auvray, K. Glass, H. Oppegaard, S. Raynaud, D. Thevenot, R. Condron, K. De Reu, A. Govaris, K. Heggum, M. Heyndrickx, J. Hummerjohann, D. Lindsay, S. Miszczycha, S. Moussiegt, K. Verstraete, and O. Cerf. 2013. Review of Shiga-toxin-producing *Escherichia coli* (STEC) and their significance in dairy production. Int. J. Food Microbiol. 162:190–212. https://doi.org/10.1016/j.ijfoodmicro.2012.08.008.

Fedio, W. M., and H. Jackson. 1989. Effect of tempering on the heat resistance of *Listeria monocytogenes*. Lett. Appl. Microbiol. 9:157–160. https://doi.org/10.1111/j.1472-765X.1989.tb00313.x.

Fernandes, R. 2009. Liquid milk products; Cheese. Pages 1–20 and 61–76 in Microbiology Handbook Dairy Products. Leatherhead Publishing and Royal Society of Chemistry.

Geeraerd, A. H., V. P. Valdramidis, and J. F. Van Impe. 2005. GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. Int. J. Food Microbiol. 102:95–105. https://doi.org/10.1016/j.ijfoodmicro.2004.11.038.

Gould, L. H., E. Mungai, and C. B. Behravesh. 2014. Outbreaks attributed to cheese: differences between outbreaks caused by unpasteurized and pasteurized dairy products, United States, 1998–2011. Foodborne Pathog. Dis. 11:545–551. https://doi.org/10.1089/fpd.2013.1650.

International Commission on Microbiological Specifications for Foods. 1996. Characteristics of microbial pathogens. Pages 129, 145–164 in Microorganisms in Foods 5: Characteristics of Microbial Pathogens. Blackie Academic & Professional.

Jackson, K. A., L. H. Gould, J. C. Hunter, Z. Kucerova, and B. Jackson. 2018. Listeriosis outbreaks associated with soft cheeses, United States, 1998–2014. Emerg. Infect. Dis. 24:1116–1118. https://doi.org/10.3201/eid2406.171051.

Johnson, E. A., J. H. Nelson, and M. Johnson. 1990. Microbiological safety of cheese made from heat-treated milk, part III. Technology, discussion, recommendations, bibliography. J. Food Prot. 53:610–623. https://doi.org/10.4315/0362-028X-53.7.610.

Kang, D. H., and D. Y. C. Fung. 2000. Application of thin agar layer method for recovery of injured *Salmonella* Typhimurium. Int. J. Food Microbiol. 54:127–132. https://doi.org/10.1016/S0168-1605(99)00174-9.

King, N., R. Lake, P. Cressey, and A. Hudson. 2014. Risk profile: Shiga toxin-producing *Escherichia coli* in raw milk. MPI Technical Paper No: 2014/14. Accessed Jan. 30, 2021. https://www.mpi.govt.nz/dmsdocument/1121/direct.

Knabel, S. J., H. W. Walker, P. A. Hartman, and A. F. Mendonca. 1990. Effects of growth temperature and strictly anaerobic recovery on the survival of *Listeria monocytogenes* during pasteurization. Appl. Environ. Microbiol. 56:370–376. https://doi.org/10.1128/aem.56.2.370-376.1990.

Langer, A. J., T. Ayers, J. Grass, M. Lynch, F. J. Angulo, and B. F. Mahon. 2012. Nonpasteurized dairy products, disease outbreaks, and state laws – United States, 1993 – 2006. Emerg. Infect. Dis. 18:385–391. https://doi.org/10.3201/eid1803.111370.

Lindsay, D., R. Robertson, R. Fraser, S. Engstrom, and K. Jordan. 2021. Heat induced inactivation of microorganisms in milk and dairy products. Int. Dairy J. 121:105096. https://doi.org/10.1016/j.idairyj.2021.105096.

Mackey, B. M., and N. Bratchell. 1989. The heat resistance of *Listeria monocytogenes*. Lett. Appl. Microbiol. 9:89–94. https://doi.org/10.1111/j.1472-765X.1989.tb00298.x.

Maher, M. M., K. N. Jordan, M. E. Upton, and A. Coffey. 2001. Growth and survival of *E. coli* O157:H7 during the manufacture and ripening of a smear-ripened cheese produced from raw milk. J. Appl. Microbiol. 90:201–207. https://doi.org/10.1046/j.1365-2672.2001.01232.x.

Montel, M. C., S. Buchin, A. Mallet, C. Delbes-Paus, D. A. Vuitton, N. Desmasures, and F. Berthier. 2014. Traditional cheeses: Rich and diverse microbiota with associated benefits. Int. J. Food Microbiol. 177:136–154. https://doi.org/10.1016/j.ijfoodmicro.2014.02.019.

Pagán, R., P. Manas, I. Alvarez, and F. J. Sala. 1998. Heat resistance in different heating media of *Listeria monocytogenes* ATCC 15313 grown at different temperatures. J. Food Saf. 18:205–219. https://doi.org/10.1111/j.1745-4565.1998.tb00215.x.

Panthi, R. R., K. N. Jordan, A. L. Kelly, and J. J. Sheehan. 2017. Selection and treatment of milk for cheesemaking. Pages 23–50 in Cheese: Chemistry, physics, and microbiology (Vol 1), 4th ed. P. L.

H. McSweeney, P. F. Fox, P. Cotter, and D. Everett, ed. Elsevier Academic Press.

Peng, S., J. Hummerjohann, R. Stephan, and P. Hammer. 2013. Heat resistance of *Escherichia coli* strains in raw milk at different subpasteurization conditions. J. Dairy Sci. 96:3543–3546. https://doi.org/10.3168/jds.2012-6174.

Peng, S., R. Stephan, J. Hummerjohann, J. Blanco, and C. Zweifel. 2012. *In vitro* characterization of Shiga toxin-producing and generic *Escherichia coli* in respect of cheese-production relevant stresses. J. Food Saf. Food Qual. 63:136–141. https://doi.org/10.2376/0003-925X-63-136.

Pereira, C. I., J. A. Graca, N. S. Ogando, A. M. P. Gomes, and F. X. Malcata. 2009. Bacterial dynamics in model cheese systems, aiming at safety and quality of Portuguese-style traditional ewe's cheeses. J. Food Prot. 72:2243–2251. https://doi.org/10.4315/0362-028X-72.11.2243.

Pöntinen, A., M. Aalto-Araneda, M. Lindström, and H. Korkeala. 2017. Heat resistance mediated by pLM58 plasmid-borne ClpL in *Listeria monocytogenes.* MSphere 2. https://doi.org/10.1128/mSphere.00364-17.

Ramsaran, H., J. Chen, B. Brunke, A. Hill, and M. W. Griffiths. 1998. Survival of bioluminescent *Listeria monocytogenes* and *Escherichia coli* O157:H7 in soft cheeses. J. Dairy Sci. 81:1810–1817. https://doi.org/10.3168/jds.S0022-0302(98)75750-9.

Reitsma, C. J., and D. R. Henning. 1996. Survival of enterohemorrhagic *Escherichia coli* O157:H7 during the manufacture and curing of cheddar cheese. J. Food Prot. 59:460–464. https://doi.org/10.4315/0362-028X-59.5.460.

Ryser, E. T., and E. H. Marth. 1987. Fate of *Listeria monocytogenes* during the manufacture and ripening of Camembert cheese. J. Food Prot. 50:372–378. https://doi.org/10.4315/0362-028X-50.5.372.

Sanaa, M., L. Coroller, and O. Cerf. 2004. Risk assessment of listeriosis linked to the consumption of two soft cheeses made from raw milk: Camembert of Normandy and Brie of Meaux. Risk Anal. 24:389–399. https://doi.org/10.1111/j.0272-4332.2004.00440.x.

Schlesser, J. E., R. Gerdes, S. Ravishankar, K. Madsen, J. Mowbray, and A. Y. L. Teo. 2006. Survival of a five-strain cocktail of *Escherichia coli* O157:H7 during the 60-day aging period of Cheddar cheese made from unpasteurized milk. J. Food Prot. 69:990–998. https://doi.org/10.4315/0362-028X-69.5.990.

Skandamis, P. N., J. D. Stopforth, Y. Yoon, P. A. Kendall, and J. N. Sofos. 2009. Heat and acid tolerance responses of *Listeria monocytogenes* as affected by sequential exposure to hurdles during growth. J. Food Prot. 72:1412–1418. https://doi.org/10.4315/0362-028X-72.7.1412.

Sörqvist, S. 2003. Heat resistance in liquids of *Enterococcus* spp., *Listeria* spp., *Escherichia coli, Yersinia enterocolitica, Salmonella* spp. and *Campylobacter* spp. Acta Vet. Scand. 44:1–19. https://doi.org/10.1186/1751-0147-44-1.

The Specialty Food Association. 2016. The state of the specialty food industry 2016–Summary report. Accessed Jan. 24, 2021. https://www.specialtyfood.com/news/article/state-specialty-food-industry-2016/.

US FDA (Food and Drug Administration)/Health Canada. 2015. Joint FDA/Health Canada quantitative assessment of the risk of listeriosis from soft-ripened cheese consumption in the United States and Canada: report. Accessed Jan. 24, 2021. https://www.fda.gov/media/90493/download.

Usajewicz, I., and B. Nalepa. 2006. Survival of *Escherichia coli* O157:H7 in milk exposed to high temperatures and high pressure. Food Technol. Biotechnol. 44:33–39.

van Asselt, E. D., and M. H. Zwietering. 2006. A systematic approach to determine global thermal inactivation parameters for various food pathogens. Int. J. Food Microbiol. 107:73–82. https://doi.org/10.1016/j.ijfoodmicro.2005.08.014.

van Doornmalen, J. P., and K. Kopinga. 2009. Temperature dependence of F-, D- and z-values used in steam sterilization processes. J. Appl. Microbiol. 107:1054–1060. https://doi.org/10.1111/j.1365-2672.2009.04290.x.

van Lieverloo, J. H. M., M. de Roode, M. B. Fox, M. H. Zwietering, and M. H. J. Wells-Bennik. 2011. Multivariate regression model of thermal inactivation of *Listeria monocytogenes* in liquid food products Pages 202–205 in Predictive Modelling of Food Quality and Safety - Conference Proceedings. E. Cummins, J. M. Frías and V.P. Valdramidis, eds. UCD, DIT, Teagasc.

van Lieverloo, J. H. M., M. de Roode, M. B. Fox, M. H. Zwietering, and M. H. J. Wells-Bennik. 2013. Multiple regression model for thermal inactivation of *Listeria monocytogenes* in liquid food products. Food Control 29:394–400. https://doi.org/10.1016/j.foodcont.2012.05.078.

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