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A mathematical model of inactivation kinetics for a four-strain composite of *Salmonella* Enteritidis and Oranienburg in commercial liquid egg yolk☆

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ABSTRACT

The goal of this study was to develop a general model of inactivation of salmonellae in commercial liquid egg yolk for temperatures ranging from 58 °C to 66 °C by studying the inactivation kinetics of *Salmonella* in liquid egg yolk. Heat-resistant salmonellae (three serovars of Enteritidis [two of phage type 8 and one PT 13] and one Oranienburg) were grown to stationary phase in Tryptic Soy Broth and concentrated 10-fold by centrifugation. Each inoculum was added to liquid egg yolk and mixed thoroughly, resulting in a final population of ca. 7 log CFU/ml egg yolk. Inoculated yolk was injected into sterile glass capillary tubes, flame-sealed and heated in a water bath at 58, 60, 62, 64, and 66 °C. Capillary tubes were ethanol sanitized, rinsed, and contents were extracted. Yolk was diluted, surface plated onto Tryptic Soy Agar + 0.1% sodium pyruvate and 50 μg/ml nalidixic acid and incubated at 37 °C for 24 h before colonies were enumerated. Decimal reduction values were calculated from survivor curves with a minimum inactivation of 6 log CFU/ml at each temperature. Survival curves (except for 66 °C) featured initial lag periods before first order linear inactivation. Estimated asymptotic *D*-values were 1.83 min at 58 °C, 0.69 min at 60 °C, 0.26 min at 62 °C, 0.096 min at 64 °C and 0.036 min at 66 °C. The estimate of the asymptotic *z*-value was ca. 4.7 °C with standard error of 0.07 °C. A linear relationship between the $\log_{10}$ of the lag times and temperature was observed. A general kinetic model of inactivation was developed. The results of the study provide information that can be used by processors to aid in producing safe pasteurized egg yolk products and for satisfying pasteurization performance standards and developing industry guidance.

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1. Introduction

Current liquid egg pasteurization regulatory requirements are based on command and control prescribed time/temperature combinations for specified egg products. The USDA, Food Safety and Inspection Service (FSIS) suggested, within a recently completed risk assessment, that current pasteurization standards might not be completely effective in eliminating *Salmonella* from all liquid egg products and estimated that 5500 cases of salmonellosis/year occur from liquid egg (Coleman et al., 2005; Latimer et al., 2008). Although there have been no foodborne outbreaks associated with liquid egg in the U.S. since 1969, there have been 2 outbreaks in the U.K. from liquid egg in the past two years (UK FSA, 2007a,b) and 2 outbreaks in Japan between 1998 and 2003 (Hara-Kudo and Takatori, 2009).

United States liquid egg pasteurization requirements are found in the Code of Federal Regulations, Title 9, Ch. III, Sec. 590.570 (USDA, 1971) and require egg yolk to be held at 60 °C (140 F) for 6.2 min or 61.1 °C (142 F) for 3.5 min. These standards were based on data for the inactivation of salmonellae in liquid egg products acquired prior to 1970 and are currently being reevaluated in light of recent risk assessments (Latimer et al., 2008). In addition, changes in industrial practices such as in-line egg processing, which affect the pH during pasteurization, and variation in current egg product formulations not represented in the current regulation imply a need to study the inactivation of salmonellae in liquid egg products.

There have been few studies of *Salmonella* inactivation in liquid egg yolk products. Earlier studies (e.g., Osborne et al., 1954; Licciardello et al., 1965; Garibaldi et al., 1969) that report *D*-values

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in egg yolk imply linear, first order kinetics; however, a more recent study indicated nonlinear survival curves (Michalski et al., 1999). Some of these were characterized by tailing, while others were characterized by "shoulders" indicating "lag" times before first order kinetics commenced. Thus, the use of *D*-values by themselves might result in under- or over-estimating the actual lethality obtained. Some recent inactivation models (Peleg, 2000; Aragao et al., 2007) do not admit to asymptotic first order kinetics, making the notion of a *D*-value inoperable. Consequently, it is sometimes difficult for processors to use these models for predicting because of the complicated nature of the mathematical form of the equations used in the models.

Presently, to our knowledge, there are no published models that can be used for predicting log reductions for *Salmonella* levels in egg yolk. However, such models are needed since new technologies are being applied to liquid egg products indicating a greater need for flexibility with regard to the possible ranges of temperatures that can be used for pasteurization. In particular, temperatures as high as 66 °C are being used in industrial applications. Thus, the goal of this study was to develop a general model for estimating $\log_{10}$ reductions of salmonellae for temperatures between 58 and 66 °C. This was accomplished by studying the inactivation kinetics of a four-strain composite of thermally resistant *Salmonella* inoculated into commercially processed liquid egg yolk. Specifically included in the objectives of this study was to determine the general shape of thermal inactivation survival curves of *Salmonella* in liquid egg yolk and, at the same time, to develop a simple model that can be readily used by processors for reliably predicting log reductions of *Salmonella*.

2. Materials and methods

2.1. Selection of strains

Thirty strains of *Salmonella enterica*. subsp. *enterica* were screened for thermal resistance in liquid egg prior to experimentation. Resistance was determined by estimating asymptotic *D*-values at 58 °C in liquid whole egg. Four thermally resistant strains of *Salmonella* were then chosen for this study (Table 1). These isolates were selected for spontaneous mutants resistant to 50 ppm nalidixic acid and used to create a four-strain composite inoculum. While Mañas et al. (2003) reported that pasteurization of liquid whole egg at 60, 64 and 70 °C for 3.5, 2.5, and 1.5 min, respectively, only inactivated *S.* Senftenberg strain 775W by <1, <2, and <4 log CFU/ml, for our purposes, the use of *S.* Senftenberg 775W in egg pasteurization studies is not warranted: *Salmonella* Senftenberg 775W is an unusually heat-resistant strain of *Salmonella*, which has never been reported as a contaminant in eggs. This was also the conclusion reached by Osborne et al. (1954) in their discussion of which strains were to be included in liquid egg pasteurization challenge studies. The inclusion of *S.* Oranienburg in our studies is appropriate based on its known resistance to thermal inactivation, its inclusion in historical studies, and the possibility of the presence of this serovar in liquid egg today or in the future. The use of a four-strain cocktail is in keeping with recommendations made by The National Advisory Committee on the Microbiological Criteria for Foods (2010) for bacterial inactivation challenge studies.

2.2. Preparation of strains

Each *Salmonella* strain was grown separately in 10 ml of Tryptic Soy Broth (TSB, EMD, Merck KGaA, Darmstadt, Germany) with 50 ppm nalidixic acid at 42 °C for 24 h. A 10 μl loop transfer was performed and the strains were grown at 42 °C for another 24 h to achieve ca. 9 log CFU/ml. Each strain was serially diluted and plated in duplicate on Tryptic Soy Agar (Difco Laboratories, Detroit, MI) with 0.1% sodium pyruvate and 50 ppm nalidixic acid (TSAPN) to confirm populations. Strains were centrifuged at $2000 \times g$ and 20 °C for 10 min, supernatant fluid decanted, and the pellet was resuspended in 0.5 ml of 0.1% bacteriological peptone water by vortexing for 1 min to effect a 10-fold concentration of cells.

2.3. Preparation of commercial liquid egg yolk

Commercially processed liquid egg yolk was obtained from Michael Foods (Gaylord, MN) and 38 ml quantities were stored at −80 °C in individual sterile 50 ml conical tubes. Liquid egg yolk was thawed at 4 °C for 24 h prior to use. The pH of liquid egg yolk was measured with a single junction, gelled Ag/AgCl, flat surface electrode (Accumet, Fisher Scientific, Pittsburgh, PA) connected to a bench top pH meter (Denver Instrument model UB-5, Denver, CO). Background contamination of the product was determined via duplicate plating of 0.25 ml of uninoculated liquid egg yolk on TSAPN Suspensions (0.5 ml) of each strain of *Salmonella* were added to 38 ml of liquid egg yolk (21 °C) in a sterile beaker and hand mixed for 2 min with a sterile stainless steel spatula. Inoculated egg yolk (ca. 200 μl) was injected into 250 μl capillary tubes (Drummond Scientific, Co., Broomall, PA) using a sterile 6 ml Monoject Luer Lock Syringe (Tyco/Healthcare Kendall, Mansfield, MA) with a 16-gauge, 12.7 cm (5 in) injection needle (Air-Tite Products Co., Inc, Virginia Beach, VA) and flame-sealed with a Bunson burner.

2.4. Physical quality determinations

Liquid egg yolk samples were tested for physical quality attributes and proximate composition. Yolk total solids were determined in triplicate on four separate subsets of the liquid egg yolk

Table 1
Salmonella enterica subsp. *enterica* strains used in this study.

Strain	Designation	Origin	Background	Reference
Enteritidis, Phage type (Pt) 13	NVSL-96-15835	Jayne Stratton University of Nebraska Dept. of Food Science-Technology, Lincoln, NE. Originally from the USDA Veterinary Service Laboratories, Ames, IA.	Poultry isolate, Included in the 2002 International Egg Pasteurization Manual studies	Froning et al. (2002)
Enteritidis, Pt 8	C398	Keith Lampel, FDA, Washington D.C. Originally from Charles Benson, University of Pennsylvania, *Salmonella* Reference Center, New Bolton Center.	Chicken ovary, 39-Mda plasmid	Shah et al. (1991)
Enteritidis, Pt 8	C405	Keith Lampel, FDA, Washington D.C. Originally from Charles Benson, University of Pennsylvania, *Salmonella* Reference Center, New Bolton Center.	Egg yolk, New England salmonellosis outbreak	Shah et al. (1991)
Oranienburg	DD2229	Eastern Regional Research Center, FSIT Research Unit Stock Collection, Wyndmoor, PA. Originally from DuPont *Salmonella* library, Wilmington, DE.	Ribosomal spacer and heteroduplex polymorphism (RS/HP) type 2229	Jensen and Hubner (1996)

utilized for this study according to the methods described by Jones (2007). Percent fat of the sample was measured with a Soxtec HT-12 fat extractor (Foss North America, Inc., Eden Prairie, MN) utilizing a procedure recommended by the instrument manufacturer (Foss Tecator Application Sub Note 3165, Rev 3.0). Quadruplicate determinations of approximately 1 g of sample with 4 g of sand as a dispersing agent were made from each of the four subsets of liquid egg yolk ($n = 16$). The four subsets of liquid egg yolk were further tested in triplicate for the determination of apparent viscosity ($n = 12$). A RheoStress 600 rheometer (Thermo Material Characterization, Newington, NH) equipped with a 35 mm cone and plate (C35 2 degree Ti, Thermo Material Characterization) set at 0.105 mm gap and 23 °C was used. A controlled rate linear ramp from 4.00 1/s to 800 1/s over 45 s protocol was run with apparent viscosity determined at 300, 400 and 500 1/s. The program was calibrated with S200 standard (Cannon Instrument Co., State College, PA). All samples were sealed with food grade mineral oil to prevent sample drying during testing.

2.5. Thermal inactivation

Capillary tubes were inserted in holes of aluminum tape covering a test tube rack and immersed in a circulating water bath (Thermo Fisher Scientific NESLAB RTE17 Digital Plus, Newington, NH) at 58 °C for up to 23 min, 60 °C for up to 9 min, 62 °C for up to 140 s, 64 °C for up to 60 s, and 66 °C for up to 18 s. Digisense Thermocouple Flexible High Temperature Wire Probe Type K (Oakton Instruments, Vernon Hills, IL) connected to a Fluke 54 II Dual Input Thermometer (Everett, WA) was placed in the water bath as well as affixed inside a capillary tube containing uninoculated egg yolk, which monitored the come-up time and treatment temperature. At the end of each heating period, capillary tubes were immediately immersed in an ice water bath for 10 s.

2.6. Surface sanitization and plating

Capillary tubes were surface sanitized by immersing into 70% ethanol followed by two sterile deionized water rinses. The ends of the capillary tube were removed using ethanol-flamed wire cutters and the contents were expelled in a sterile sampling cup using a sterile 3 mm pipe cleaner. Serial dilutions were made and plated in duplicate on TSAPN using a Don Whitley Wasp II® Spiral Plater (Microbiology International, Frederick, MD) and incubated at 37 °C for 24 h. Undiluted egg samples (50 μl/plate) were also hand plated with sterile cell spreaders on TSAPN. Serially diluted sample plates were counted using a Synbiosis åCOLyte Supercount automated colony counting system (Microbiology International, Frederick, MD) and colony counts were recorded. Plates from undiluted samples were counted by hand. Presumptive positive *Salmonella* colonies were randomly confirmed by streaking on Xylose Lysine Tergitol agar (XLT4) and by serological agglutination (Difco™ Salmonella O Antiserum Poly A-I & Vi Becton, Dickinson & Company, Sparks, Maryland).

2.7. Study design

Data used in this study are from 19 separate survival curves; each one was generated from an experiment that consisted of a series of samples taken from time = 0, right before pasteurization, and at selected times during pasteurization. The numbers of sampling times for each experiment ranged from 8 to 10, with one exception, where there were 6 times. Some of these measurements for the larger times were non-detects. These inactivation curves were designed so as to yield final measurable population reductions of at least 6 $\log_{10}$ CFU/ml *Salmonella*. Duplicate plates for each sampling time were analyzed. The experiments were conducted on different days that are designated as "blocks." For temperatures 58–64 °C, there were 5 blocks, delineated as follows: block 1 (58, 60, 64 °C); block 2 (58, 60, 62, 64 °C); block 3 (62, 64 °C); block 4 (58, 60, 62, 64 °C); block 5 (64 °C). Experiments conducted at 66 °C were conducted over 4 different days, where the first three were conducted on separate days, and the last two conducted on the same day. In summary, the study consisted of 19 experiments within 9 bocks, with a total of 340 plate measurements. Initial levels were, on average, about 7.6 $\log_{10}$, the lowest reportable level (plate count = 1 CFU) was 1.3 $\log_{10}$, and almost all experiments concluded with samples that were non-detect. Thus, the observed range of $\log_{10}$ reductions was for the most part greater than 6 $\log_{10}$.

2.8. Statistical analysis and mathematical modeling

Two plate counts were made for each sample. The distribution of cells (colonies) in the sample before plating would be expected to be uniformly distributed, since the samples are mixed during preparation. A uniform distribution permits an assumption for the distribution of counts to be Poisson, which is used for the model. Thus, for two reasons the uniformity of the distribution of *Salmonella* cells within samples tested using duplicate plate counts: viz., (1) for quality assurance, to help assure that the results were correctly recorded or otherwise not in error; and (2) to verify that the distribution of counts is Poisson. Likelihood ratio tests were computed for the samples assuming the null hypothesis of Poisson distributions. That is, for each sample, the likelihood ratio statistic, 2χ, assuming that x and y are the two plate counts, where $x > 0$ and χ is given by

$$\chi = \begin{cases} x\ln(x) + y\ln(y) - (x+y)\ln((x+y)/2) & \text{if } \min(x,y) > 0 \\ x\ln(2) & \text{otherwise} \end{cases} \quad (1)$$

was computed. It is assumed that 2χ is distributed as a chi-square with 1 degree of freedom. The significances of this statistic (p-values), over all the samples based on the chi-square distribution with 1 degree of freedom, would be distributed uniformly if the null hypothesis that the underlying Poisson distributions for the two plates were the same is true. By examining the distribution of these p-values, an assessment of whether the null hypotheses can be accepted was made.

Various inactivation models were examined. Plots of the survival curves were initially examined graphically to determine features with respect to "shoulders" and "tailing." Next, various primary inactivation curves, $f_j(t|T)$, where the index j indicates the functional form of the curve, as a function of time, t, for a given temperature, T, were selected with features congruent to those of the observed survival curves. These curves were fitted using mixed effects, nonlinear regression, and the best fitting curve was selected. When the nonlinear model, based on a function $f(t|T)$ has the feature that, asymptotically as time get large, $f(t|T)$ approaches a straight line that is not parallel to the time-axis, then the negative of the inverse of the slope of the asymptotic line is referred to as the asymptotic D-value. (Asymptotic) thermal death curves can be plotted using the $\log_{10}$ of the asymptotic D-values versus temperature, and if the relationship is linear, the negative of the inverse of the slope of this line is referred to as the asymptotic z-value. Secondary models were developed by initially examining graphs of the estimated parameter values of the selected primary survival curve versus temperature, and choosing appropriate functions that captured the features of the observed relationships. From these results, a general model of predicting $\log_{10}$ reductions for any given temperature within the range of 58–66 °C, together with standard errors of the predictions, was developed using multivariate, mixed

effects, models. Estimated parameter values for models were obtained using PROC NLMIXED or PROC MIXED, as appropriate, of SAS® (version 9.1). For determining the structure (variances and fixed parameters) of the model, likelihood ratio tests were used. Graphs were created in S-PLUS® (version 8.0).

3. Results

3.1. Come-up and cool down times, and physical attributes

Come-up times for liquid egg yolk in capillary tubes at 58, 60, 62, 64, and 66 °C ranged from 5 to 8 s. The cool down times for all temperatures were 3 s. The mean pH of liquid egg yolk samples was 6.34. Other physical properties analyses determined proximate compositions of 43.73 ± 0.04% total solids and 27.58 ± 0.98% fat, apparent viscosities were determined to be 3.05 ± 0.11 at 100 1/s shear rate, 1.70 ± 0.05 at 200 1/s shear rate, 1.24 ± 0.05 at 300 1/s shear rate, 0.99 ± 0.02 at 400 1/s shear rate and 0.84 ± 0.02 at 500 1/s shear rate.

3.2. Control samples and salmonellae confirmation

Uninoculated egg yolk showed no bacterial contamination on TSAPN after 24 h of incubation at 37 °C. Inoculated liquid egg yolk contained a mean of 7.1 log CFU/ml of *Salmonella* before heat treatment. Serological agglutination and colonies streaked on XLT4 agar were confirmed to be *Salmonella*.

3.3. Sample inoculum uniformity and distribution

Fig. 1 provides a plot of the difference in sample-specific plate counts versus the mean of the plate counts, on a $\log_{10}$ scale for both *x*- and *y*-axes, where for a negative difference, minus the $\log_{10}$ of the absolute value of the difference was plotted. The upper straight lines forming a sideways "V" represent the maximum possible difference for a given average value. The two lines between the V represent demarcation lines for significance at the 0.05 level (two-sided) based on the statistic given in Eq. (1). Results between these lines and the lines of the V indicate statistically significant differences, contrary to the assumption of uniformity (of cells' distribution within the sample). The Kolmogorov–Smirnov test statistic was 0.075, with a *p*-value of 0.33, indicating that the uniform distribution can be accepted. Accordingly, the assumption of Poisson distribution of plate counts within samples is accepted and used for modeling.

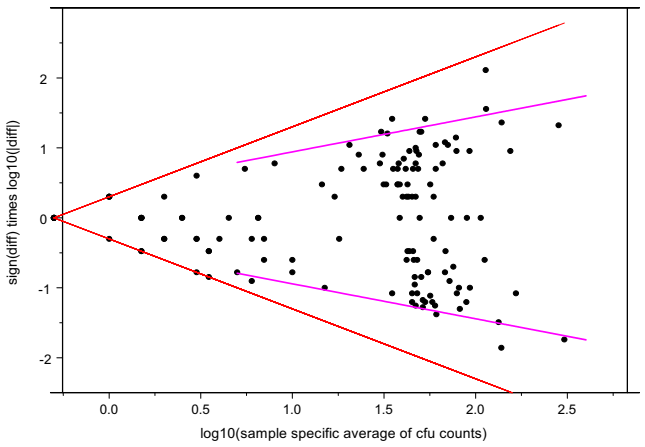

Fig. 1. Plot of sample-specific differences of two plate counts versus average ($\log_{10}$ scale). If a difference is negative, then the negative of the $\log_{10}$ of the absolute value of the difference is plotted. Bottom and top lines, forming a horizontal "V", are the maximum difference for a given average. Lines in the middle demark significance at <0.05.

3.4. Primary model of survival curves for thermal treatment

An examination of the observed survival curves (Fig. 2) indicates the existence of shoulders, or lag times, for all temperatures, except for 66 °C, prior to logarithmic (first order, kinetic) inactivation. Survival curves at 66 °C appeared to be log-linear in shape (Fig. 2). One data point at 66 °C time = 0 was deleted as an outlier, where it is clear that the point did not lie near the projection of the linear regression line derived from the other points. No other survival curve displayed a substantial degree of convexity as this survival curve would if the data point at time equal to 0 were included and thus it was assumed that the measurement for this point was in error and thus was deleted.

Various functions for fitting survival curves were tested. The first function was based on the Weibull function (Aragao et al., 2007), and the survival curve function is:

$$S(t) = n_0 - at^b \tag{2}$$

where $S(t)$ is the logarithm base 10 of the expected number of cells at time t, $N(t)$, and a, b, and n_0 are parameters whose values are estimated from the data. The value of b determines the general shape of the survival curve: convex ($b < 1$), log-linear ($b = 1$), and concave ($b > 1$). Only when $b = 1$ does a *D*-value exist because only in that case is the derivative of $S(t)$ with respect to t equal a constant; in the other cases, the derivative does not approach a non-zero constant as t gets large, thus asymptotically, *D*-values do not exist. A second function considered was:

$$\ln(N(t)) = n_0 - ct + \ln\left(1 + c/w\left(e^{wt} - 1\right)\right) \tag{3}$$

where c and w are positive constants (Juneja et al., 2001). This function is derived as a solution to differential equations that reflect a two-step inactivation process, where, for the cells in the population, the first stage reflects a lag period before entering a second stage of exponential (first order kinetic inactivation). The derivative of this function as t approaches infinity approaches the value of c, and at $t = 0$ the derivative is 0. As w approaches zero, the argument of the ln( ) function approaches $1 + ct$. A modification of this equation leads to a more flexible equation to estimate:

$$S(t) = n_0 - ct + \log10(1 + bt) \tag{4}$$

where $c > 0$ and $b \geq 0$ are parameters. The derivative of this function is $-c + \log_{10}(e)b/(1 + bt)$, thus as t gets large the survival curve approaches a straight line with slope = c. The derivative of this function at $t = 0$ is $-c + \log_{10}(e)b$. If it is assumed that the survival curve is non-increasing, then $c \geq b\log_{10}(e)$. Finally, a spline equation:

$$S(t) = n_0 - c\max(0, t - t_0) \tag{5}$$

was considered, where c and t_0 are parameters ($c > 0$ and $t_0 \geq 0$). For this model, for times less than t_0 there is predicted no decline of levels, and for times greater than t_0 the predicted decline is log-linear, with *D*-value equal to $1/c$. The last three equations all are characterized by a shoulder (for Eq. (4), the levels can be modeled to increase for $t > 0$ before declining) and asymptotic *D*-values, equal to $d = 1/c$.

For each experiment, parameter values were estimated using a Poisson, mixed effect, nonlinear regression, where it was assumed that the sample at a specific time was a random factor with standard deviation equal to σ. The specific model assumed that the

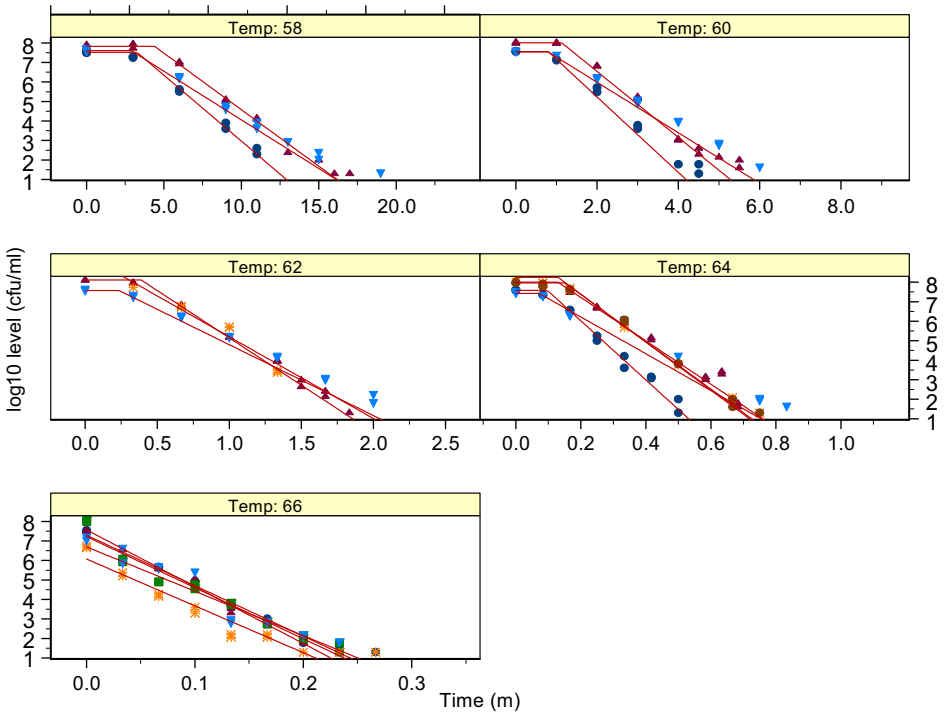

Fig. 2. Plot of fitted primary survival curve (spline equation, Eq. (4)) for inactivation of a four-strain composite of *Salmonella* Enteritidis and Oranienburg in commercial liquid egg yolk. (Replicates are represented by variously colored symbols.)

measurement, $y(t)$, at a specified time was made on a plate where the dilution, represented as 10^{-r}, and the fractional volume of the sample, f, for which counts were made were known. Hence, the estimate of the level (CFU/ml), $x(t)$, is computed as $10^{r}y(t)/f$ and the model is:

$$\begin{aligned} \log_{10}(x(t)) &= S(t) + \varepsilon_t \\ \lambda(t) &= x(t)/(f10^{r}) \\ y(t) &\sim \text{Poisson}(\lambda(t)) \end{aligned} \tag{6}$$

where ε_t is an error associated with the sample at t, assumed to be normally distributed with mean of 0 and standard deviation σ.

Table 2 provides the log of the likelihood ratio statistics given in the SAS® program output for the various models. Eq. (4) was fit with the elimination of the boundary condition, $c \geq b \log_{10}(e)$, since this restriction lead to estimates of either $b = 0$ or $d = 0$. The best fitting model was obtained for Eq. (4), the spline function, as evidenced by the lower average of the log-likelihood ratio statistics (Table 2). The 'reason' the spline function provided a better fit was that many measured levels for the first non-zero time were nearly equal to the measured levels at time = 0; that is, the first derivative at $t = 0$ would be estimated to be close to 0. Apparently, the spline equation was able to capture the imagined shape of the observed

Table 2
Likelihood ratio statistics using Eqs. (2)–(5) for estimating survival curves, and differences between them.

Temp (°C)	LLR Weibull Eq. (2)	LLR 2-stage Eq. (3)	LLR Eq. (4)	LLR Spline Eq. (5)	Difference Weibull (Eq. (2)) minus spline (Eq. (5))	Difference 2-stage (Eq. (3) minus spline (Eq. (5))	Difference Eq. (4) minus spline (Eq. (5))
58	59.1	68.5	49.8	57.6	1.4	10.8	−7.8
58	116.1	119.1	111.5	107.9	8.2	11.2	3.6
58	83.9	85.0	79.4	77.2	6.7	7.9	2.3
60	75.4	76.8	67.9	71.3	4.2	5.5	−3.4
60	131.0	130.9	126.6	125.9	5.1	5.0	0.7
60	100.8	99.8	93.1	84.2	16.6	15.6	8.9
62	119.2	125.8	113.1	113.4	5.8	12.4	−0.2
62	68.7			77.2	−8.6	–	–
62	108.6	107.3	98.9	94.3	14.3	13.0	4.6
64	87.2	89.1	79.9	83.3	3.9	5.8	−3.4
64	148.2	151.1	148.7	148.4	−0.2	2.7	0.2
64	96.1	101.3	96.7	92.8	3.3	8.5	3.9
64	119.9	120.5	120.3	121.3	−1.4	−0.8	−1.0
64	91.2	99.5	93.9	87.9	3.3	11.6	6.0
66	118.9	–	–	119.6	−0.7	–	–
66	129.7	–	–	130.2	−0.6	–	–
66	111.1	–	–	111.3	−0.1	–	–
66	85.8	–	–	95.8	−10.0	–	–
66	255.8	–	–	255.9	−0.1	–	–
					51.3	109.3	14.6

results better than the other equations. The Weibull on average had lower likelihood ratio test values than those for Eq. (3). The square root of the mean of squares of the estimated standard deviations, σ, using the spline function (Eq. (5)) over the experiments was 0.225 $\log_{10}$; for the Weibull function (Eq. (2)), the corresponding value was 0.226 $\log_{10}$, and for Eq. (2), 0.228.

A graphical comparison of the goodness-of-fits of the models using the Weibull function (Eq. (2)) and the spline function (Eq. (5)) is presented in Fig. 3. This figure presents plots of residuals that are equal to the differences of using the estimated $\log_{10}$ levels as the "observed" values and the $\log_{10}$ of the estimated values of $x(t)$ in Eq. (6) as the predicted values versus "standardized" times, which are the ratios of the times divided by the asymptotic D-value that was estimated by the spline model (Eq. (5)). In addition, on the graph are smoothed lines for each models' residuals produced by the S-Plus® graphics procedure, based on a weighted kernel estimate with Parzen weights and bandwidth 1. The smoothed line for the model using the spline function is, on average, closer to the x-axis than the smoothed line for the model using the Weibull function thus indicating a better fit to the data for the former.

The one "seemingly" outlier value (upper right corner of Fig. 3) occurs because the actual plate count was 1 CFU and the model predicts that a plate count of 1 or more has a probability of occurring equal to about 2%. Thus, in actuality, this point should not, we believe, be considered an outlier data point. The residuals in the right side of the graph represent data points for which the plate counts were low, but do not include plate counts that were zero. It is for this reason that the residuals depicted on the right side of the graphs tend to be greater than zero. For all 65 zero plate counts, the medians of the probabilities of obtaining a zero were about 96%, with the lowest near 1% for both models. Thus, no data were deleted as outliers, and for practical purpose the two models provide similar distributions of residuals.

Thus, based on the above data analysis cornering the comparison of the likelihood ratio statistics and the examination of residuals, the spline function can be accepted for modeling purposes. Accepting Eq. (5) implies that asymptotic D-values can be assumed to exist. The estimated asymptotic D-values for Eqs. (4) and (5) were similar. Fig. 3 provides plots of the observed and fitted curves using the spline function (Eq. (5)). In (Table 3) are the estimated lag times and asymptotic of D-values for each experiment.

Table 3
Estimated lag times (min) and asymptotic D-values for each experiment using Spline function (Eq. (5)).

Experiment	Temp (°C)	Day	Lag time Eq. (4) (min)	Log_{10} lag time	Asym. D-value spline Eq. (4) (min)	Log_{10} D-value
1	58	1	3.26	0.51	1.529	0.18
2	58	2	4.38	0.64	1.714	0.23
3	58	4	2.86	0.46	2.163	0.34
4	60	1	0.80	−0.10	0.572	−0.24
5	60	2	1.13	0.05	0.637	−0.20
6	60	4	0.80	−0.10	0.877	−0.06
7	62	2	0.39	−0.41	0.220	−0.66
8	62	3	–	–	0.238	−0.62
9	62	4	0.24	−0.63	0.316	−0.50
10	64	1	0.10	−1.01	0.071	−1.15
11	64	2	0.13	−0.89	0.097	−1.01
12	64	3	0.13	−0.89	0.085	−1.07
13	64	4	0.07	−1.14	0.121	−0.92
14	64	5	0.15	−0.82	0.086	−1.06
15	66	6	0.00	–	0.039	−1.41
16	66	7	0.00	–	0.034	−1.46
17	66	8	0.00	–	0.044	−1.36
18	66	9	0.00	–	0.042	−1.38
19	66	9	0.00	–	0.038	−1.42

3.5. Secondary model

Using asymptotic D-values estimated from Eq. (5), the asymptotic thermal death curve (TDC) – plot of the $\log_{10}$ asymptotic D-values versus temperature (°C) – (Fig. 4) appears linear. Also the $\log_{10}$ of the lag time, t_0, versus temperature appears to be linear (Fig. 5). Thus, the number of parameters for prediction is 4. The model for predicting log reductions (or lethalities) for a given time and temperature, or to determine the time needed to achieve a given lethality for a given temperature, involves estimating seemingly unrelated regression equations for the $\log_{10}$ of the D-value and $\log_{10}$ of the lag time. The statistical issue involves determining the error structure of the residuals. There are two nested levels: the within block level, and the between-block level. At each level there are three possible non-zero parameters: the two variances and the correlation of the variance matrix; thus in total there are 6 parameters associated with the residual errors.

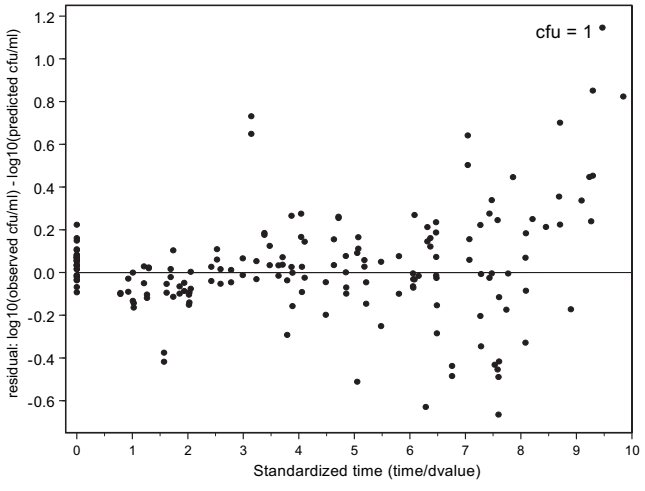

Fig. 3. Plot of residuals ($\log_{10}$ observed minus $\log_{10}$ predicted level) from models using spline and Weibull survival functions, versus standardized time (computed as the ratio of the actual time divided by asymptotic D-value using spline model (Eq. (5))).

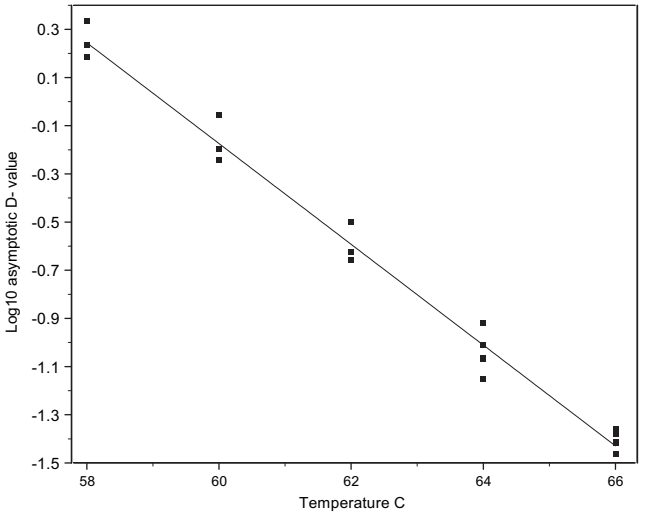

Fig. 4. Plot of $\log_{10}$ asymptotic D-values (min) versus temperature (°C) with linear ordinary least square regression line.

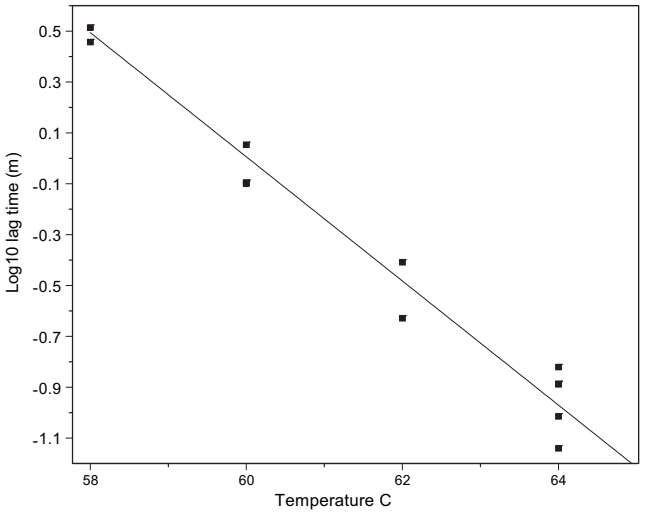

Fig. 5. Plot of $\log_{10}$ lag time (min) versus temperature (°C) with linear ordinary least square regression line.

There is an issue of whether or not the two correlations can be assumed to be zero. The number of experiments and replicates are too small to ascertain with high confidence whether such assumptions can be made. *A priori* we would expect negative correlations if measurement error were the dominate source contributing to variability of results for ostensibly the same conditions. Errors leading to an overestimate of lag times would tend to steeper estimated slopes and thus lead to underestimates of the asymptotic *D*-values. Estimates of the correlations derived from PROC MIXED of SAS®, using maximum likelihood estimation (MLE) for multivariate mixed effect models indicated no significant non-zero correlation, with estimated correlations of −0.48 (p-value = 0.31) for the between-block level, and −0.19 (p-value = 0.58) for the within block level. However we are not able to provide a reason why an assumption of a zero correlation would be sensible and there is no justifiable reason for us to assume that they are zero.

The actual model thus is:

$$\text{Log}_{10}(D) = \alpha + \beta(T-61) + \varepsilon_{b,D} + \varepsilon_{w,D}$$

$$\text{Log}_{10}(\text{lagTime}) = \alpha + \nu + (\beta+\varphi)(T-61) + \varepsilon_{b,L} + \varepsilon_{w,L} \qquad (7)$$

where T is temperature (°C), α, β, ν, and φ are parameter whose values are estimated from the data; $\varepsilon_{b,x}$ for $x = D$ and L, represent between-day error terms, assumed correlated, with standard deviations, $\sigma_{b,x}$ for $x = D$, L; and similarly $\varepsilon_{w,x}$ is defined for the within-day error terms. The likelihood ratio test indicated a significant between-day error (p-value <0.001) based on MLE estimators. Thus the model has 10 parameters, 4 that are fixed that are used for predicting the relationship for lethalities and times for fixed temperature. Table 4 provides the estimates of the fixed parameters of the model for predicting the relationship between times and lethalities for fixed temperatures, using REML. The estimates of the parameter values, α, β, ν and φ are given with their standard errors, as well as the correlations among them, as a matrix. The estimated asymptotic z-value is $-\beta^{-1}$ which is equal to 4.694 °C, with standard error estimated as 0.0696, using the value of $z^{-2}\sigma_\beta$ as the estimate, where σ_β is the standard error of the estimate of β (based on the linear terms of the Taylor series approximation for z in terms of β). Table 5 provides a comparison of the predicted $\log_{10}$ of the asymptotic *D*-values from the model and the average of the observed values, as well as the predicted asymptotic *D*-values and standard errors of the predictions.

Table 4
Estimated parameter values for the model using the spline function (Eqs. (5) and (7)) and associated error matrix. The matrix underneath double line is the error correlation matrix.

Variable	α	β	ν	φ
Estimate	−0.3766	−0.2131	0.1591	−0.04231
Standard error	0.02365	0.003162	0.06396	0.007113
α	1.0000	−0.3537	−0.6560	0.2122
β	−0.3537	1.0000	0.2165	−0.5971
ν	−0.6560	0.2165	1.0000	−0.2044
φ	0.2122	−0.5971	−0.2044	1.0000

Table 5
Average of observed $\log_{10}$ asymptotic *D*-values at each temperature and corresponding predicted values derived from model. Also given are predicted asymptotic *D*-values, obtained from 10^x where x is the estimated value, and standard errors of predictions.

Temp (°C)	Mean $\log_{10}$ *D*-values	Geometric mean observed *D*-values	Model predicted $\log_{10}$ *D*-value	Model predicted *D*-value	Std. error predicted *D*-value
58	0.25	1.78	0.26	1.83	0.058
60	−0.17	0.68	−0.16	0.69	0.031
61.1	–	–	−0.40	0.40	0.023
62	−0.59	0.25	−0.59	0.26	0.017
64	−1.04	0.09	−1.02	0.10	0.010
66	−1.41	0.04	−1.44	0.04	0.007

4. Discussion

The relationship between the time and lethalities for a given temperature is given as follows: if Leth(t) represents the obtained lethality at time t minutes, where lethality is defined as the $\log_{10}$ reduction that would be obtained if the initial level of cells was of sufficient magnitude, then

$$\text{Leth}(t) = \left(t - t_{\text{lag}}(T)\right) \Big/ D(T) \qquad (8)$$

where $D(T) = 10^{\alpha+\beta(T-61)}$ and $t_{\text{lag}}(T) = 10^{\alpha+\nu+(\beta+\varphi)(T-61)}$. This can be rewritten as:

$$\text{Leth}(t) = t10^{-\alpha-\beta(T-61)} + 10^{\nu+\varphi(T-61)} \qquad (9)$$

When predicting lethality, as a function of t, Eq. (8) provides a simple equation for estimating the standard error of the prediction, using linear terms of the Taylor series expansion.

Current USDA guidelines (9 CFR 590.570) mandate that liquid egg yolk must be pasteurized at 60 °C for 6.2 min or 61.1 °C for 3.5 min. The predicted lethalities for these two time/temperature

Table 6
Predicted times (min) to achieve given lethality at fixed temperatures (F), together with standard error of predictions.

Lethality ($\log_{10}$ CFU/ml)		Temperature °C (F)		
		60 (140)	61.1 (142)	62.2 (144)
6.0	Time (min)	5.21	2.97	1.70
	Std Error	0.23	0.12	0.07
6.3	Time (min)	5.41	3.09	1.77
	Std Error	0.24	0.13	0.07
6.7	Time (min)	5.69	3.25	1.86
	Std Error	0.25	0.14	0.08
7.0	Time (min)	5.89	3.37	1.93
	Std Error	0.26	0.14	0.08
7.5	Time (min)	6.24	3.57	2.05
	Std Error	0.28	0.15	0.09

Table 7
Estimated asymptotic *D*- and *z*-values *Salmonella* in liquid egg yolk in this study compared with *D*- and *z*-values reported in previous studies.[g]

Salmonella serotype	*D*-value (min) at temperature (°C)					*z*-Value (°C)
	55	58.8	60	61.1	62.2	
Enteritidis + Oranienburg (this study)[h]	NA	1.24	0.69	0.40	0.23	4.69
Typhimurium + Enteritidis			0.28[a]	0.16[a]	0.087[a]	4.33
Typhimurium		0.70[b]	0.40[b]			4.40
Derby, Newport, and Typhimurium			0.2–0.5[c]			
Typhimurium			0.67[d]	0.20[d]	0.14[d]	3.24
Senftenberg, not 775W			0.73[d]	0.28[d]	0.21[d]	4.07
Enteritidis			0.55–0.75[d]	0.27–0.35[d]	0.21–0.30[d]	4.60–6.60
Typhimurium	8.0[e]		0.80[e]			4.60
Meleagridis			0.92[f]			
Enteritidis	21.0[e]		1.10[e]			
Six unspecified strains isolated from dried egg yolk			0.8–1.2[f]			
Senftenberg 775W	42.0[e]		11.8[e]			

[a] Schuman and Sheldon (1997).
[b] Garibaldi et al. (1969).
[c] Licciardello et al. (1965).
[d] Palumbo et al. (1995).
[e] Humphrey et al. (1990).
[f] Osborne et al. (1954).
[g] Table modified from Doyle and Mazzotta (2000).
[h] *D*-values for our study are asymptotic *D*-values accounting for lag times.

combinations are somewhat close to each other, with estimates of 7.32 $\log_{10}$ (standard error = 0.372) for $T = 61.1$ oC and $t = 3.5$ m, and 7.45 $\log_{10}$ (standard error = 0.402) for $T = 60$ oC and $t = 6.2$ m. The degrees of freedom that are assigned to this estimate is not all together clear: for the 19 experiments for which there were *D*-values estimated, there were 2 fixed parameters plus random effects associated with the nested error structure. SAS® assigns 16 degrees of freedom to the estimates. However, the lag time would have less degrees of freedom because at 66 oC actual estimates were not used. We can think of the actual degrees of freedom falling somewhere between 10 and 16. Using this range of degrees of freedom, a 99% lower bound would be obtained by multiplying the standard error by 2.58–2.76, reflecting the range of degrees of freedom. In this paper, thus, we shall use a factor of 2.8, reflecting the fewest degrees of freedom. Thus a lower 99% confidence bound of the obtained lethality for 3.5 min at 61.1 oC is 7.32−2.8(0.372) = 6.28 $\log_{10}$; for 6.2 min at 60 °C, the lower bound is 6.32 $\log_{10}$.

Producers might want to design processes to ensure a minimal lethality. The results given in (Table 6) show the estimated needed times at fixed temperatures to obtained specified lethalities, together with standard errors derived using the linear terms of the Taylor series approximation. The amount of time necessary to reduce *Salmonella* by up to 7 log CFU/ml at 61.1 °C is estimated to be 3.37 m, with a standard error of 0.14. Multiplying the standard error by 2.8 provides a 99% upper bound estimate of the needed time of 3.77 m, rounding to 3.8 m.

The results of our study are somewhat at variance with inactivation kinetics quoted elsewhere in the published literature. *D*- and *z*-values of *Salmonella* pasteurized in liquid egg yolk reported in the literature are listed in Table 7 and compared to the asymptotic ones estimated in this study. In all but one case, the asymptotic *z*-value that was estimated in our study is larger than *z*-values reported in other studies. Similarly, most of the *D*-values reported in Table 7 for the other studies are less than the asymptotic *D*-values that were estimated in this study. The exceptions are for *D*- and *z*-values reported for *S.* Enteritidis, where the asymptotic *D*-values estimated in this study are in the range of *D*-values reported for one study (Palumbo et al., 1995) and less in the other study (Humphrey et al., 1990).

A simple way of comparing our results to those reported in other papers is to examine those obtained at one temperature. The most common temperature studied has been 60 °C (Table 7). Garibaldi et al. (1969) reported *D*-values of *Salmonella* in egg yolk to be 0.4 min at 60 °C (140 F) using *S.* Typhimurium TM-1. Licciardello et al. (1965) and Osborne et al. (1954) reported $D_{60°C}$ values for *Salmonella* in egg yolk that ranged from 0.2 to 0.5 min, and from 0.8 to 1.2 min, respectively. The results reported by Licciardello et al. (1965) are lower than the estimate of 0.69 min derived in our present study, given in Table 5, while the results reported by Osborne et al. (1954) are greater. Schuman and Sheldon (1997) reported *D*-values for egg yolk that ranged from 0.087 min at 62.2 °C and 0.28 at 60 °C, which are much lower than the asymptotic *D*-values of 0.26 and 0.69 min at 62 and 60 °C, respectively, given in Table 5. These differences could be explained by differences of strain-specific inactivation kinetics.

5. Conclusions

This paper presents a model of inactivation of *Salmonella* in commercial liquid egg yolk product for temperatures between 58 and 66 °C. Thermal inactivation experiments were conducted using a four-strain cocktail consisting of *Salmonella* Enteritidis and Oranienburg. A summary of our findings are: (1) Survival curves for temperatures less than 66 °C displayed shoulder effects, with distinct lag times before onset of first order kinetic inactivation; (2) estimated asymptotic *D*-values (in minutes) were: 1.83 (58 °C), 0.69 (60 °C), 0.40 (61.1 °C) 0.26 (62 °C), which are comparable to other reported values in the literature for *S.* Enteritidis and generally larger than those for other serotypes reported in the literature; (3) the estimated asymptotic *z*-value for *Salmonella* heated in liquid egg yolk between 58 and 66 °C was ca. 4.7 °C, which is generally larger than reported values in the literature; and (4) current USDA pasteurization regulations for liquid egg yolk (60 °C for 3.5 min, or 61.1 °C for 6.2 min) provide greater than a 6.3 log reduction for the *Salmonella* servovars used in this study. Our model is based on nonlinear inactivation that includes parameters describing the "lag time" and asymptotic *D*-value, and is applicable to as high as 66 °C. This model can help processors design their pasteurization systems to ensure production of safe liquid egg yolk product.

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