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**Title:** Predictive Model for Growth of *Clostridium Perfringens* During Cooling of Cooked Cured Chicken

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**Citation:** Food Microbiology (2002) 19: 313-327

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**Number:** 7177

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# Predictive model for growth of *Clostridium perfringens* during cooling of cooked cured chicken<sup>†</sup>

Vijay K. Juneja<sup>1,\*</sup> and Harry M. Marks<sup>2</sup>

*Estimates of the growth kinetics of Clostridium perfringens from spores at temperatures applicable to the cooling of cooked cured chicken products are presented. A model for predicting relative growth of C. perfringens from spores during cooling of cured chicken is derived using a nonlinear mixed effects analysis of the data. This statistical procedure has not been used in the predictive microbiology literature that has been written for microbiologists. However, recently software systems have been including this statistical procedure. The primary growth curves, based on the stages of cell development, identify two parameters: (1) germination, outgrowth, and lag (GOL) time, or lag phase time; and (2) exponential growth rate, egr. The mixed effects model does not consider GOL and egr as constants, but as random variables that would, in all likelihood, differ for different cooling events with the same temperature. As such, it is estimated that the egr, for a given temperature, has a CV of approximately 19%. The model obtained by the mixed effects model is compared to the one obtained by the more traditional two-stage approach. The estimated parameters from the derived models are virtually the same. The model predicts, for example, a geometric mean relative growth of about 9.4 with an upper 95% confidence limit of 21.3 when cooling the product from 51°C to 12°C in 8 h, assuming log linear decline in temperature with time. C. perfringens growth from spores was not observed at a temperature of 12°C for up to 3 weeks.*

## Introduction

The genus *Clostridium perfringens* is one of the most common bacterial agents in foodborne-disease outbreaks throughout the world (Stringer et al. 1980). The Center for Disease Control (CDC) estimates that the organism has been implicated in an estimated yearly average (from 1982 to 1992) of about 1/4 million cases

of foodborne illnesses with an average of 41 hospitalizations and seven deaths per year in the United States (Mead et al. 1999). The pathogen ranks third behind *Salmonella* and *Campylobacter* in terms of total numbers of foodborne illness cases. In 1994, the total cost of illness due to *C. perfringens* was estimated at \$123 million in the US (Anonymous 1995).

*Clostridium perfringens* are widely distributed in a variety of foods, especially meat and poultry, although other foods are occasionally implicated (Duncan 1970, Genigeorgis 1975). The vehicle of transmission is usually a cooked food in which heat-resistant spores of this pathogen will survive cooking temperatures. The heat-activated surviving spores germinate,

Received:  
24 September 2001

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outgrow, and multiply during post-cook handling, primarily under conditions conducive to germination such as when the cooling of cooked foods is not rapid or when the products are not properly refrigerated or are temperature abused. Improper cooling (40-9%) of food products have been cited as the most common cause of *C. perfringens* outbreaks (Angulo et al. 1998). The foods involved in these outbreaks are generally thought to contain large number of viable vegetative cells, some of which survive passage through the stomach and eventually sporulate in the intestine, where the enterotoxin responsible for the characteristic food-poisoning symptoms (diarrhea and abdominal cramps) is synthesized and released during sporulation (Duncan and Strong 1969, Duncan et al. 1972).

Sufficient evidence exists to document that *C. perfringens* can grow in media supplemented with various levels of curing salts. While growth was not inhibited by 4% w/v NaCl, some strains do not grow in 5-6% NaCl and most strains failed to grow in 7-8% NaCl (Roberts and Derrick 1978). Gough and Alford (1965) reported that *C. perfringens* growth was not inhibited at 8000 ppm of sodium nitrite but was inhibited when the concentration was increased to 12000 ppm. It is worth mentioning that the inhibitory effect of sodium nitrite is enhanced when it is heated (Davidson and Juneja 1990). Gibson and Roberts (1986) reported that the inhibitory concentrations of sodium nitrite can be lowered if combined with other curing salts. In their study, *C. perfringens* growth at 20°C is inhibited by 200 µg of nitrite ml<sup>-1</sup> and 3% salt or 50 µg of nitrite ml<sup>-1</sup> and 4% salt at pH 6.2 in a laboratory medium. In another study, the levels of sodium nitrite necessary to inhibit the strains tested dropped from 300 to 25 ppm when the concentration of NaCl was increased from 3 to 6% (Roberts and Derrick 1978).

Mathematical models to predict the relative growth of *C. perfringens* from spores, through lag, exponential and stationary phases of growth, at temperatures normally associated with the cooling schedules have been developed (Juneja et al. 1999). The growth medium used to measure growth in the study by Juneja et al. (1999) was trypticase-peptone-glucose-

yeast extract broth. Recently, a predictive model pertaining to the behavior of surviving *C. perfringens* spores during cooling of cooked beef supplemented with preservatives was developed (Juneja et al. 2001). However, a model regarding the growth from spores during cooling of cooked chicken supplemented with preservatives has not been published in the scientific literature. Accordingly, in the work reported here, the objective was to develop a model that can be used to help determine the safety of cured chicken products or those supplemented with low levels of preservatives, which have been cooled and subjected to temperature abuse conditions.

Because of the potential food safety hazard associated with cooling cooked foods, discussed above, the United States Department of Agriculture (USDA) requires that cooked meat products be cooled according to specific guidelines in order to control risks from *C. perfringens* and *Clostridium botulinum*. Performance standards for the cooling of meat products, which were published in the Federal Register 66(39) on February 27, 2001, specify that there should not be more than log<sub>10</sub> 1 multiplication of *C. perfringens* within the product during cooling (USDA 2001). Thus, the primary application of the model developed in this paper would be to predict small to moderate amounts of relative growth of *C. perfringens* from spores during cooling of cooked cured chicken products. There exists evidence that, for certain situations, lag times can be a function of cell densities, through intercellular communication enhanced by substances produced by the cells (Kaprelyants and Kell 1996). However, for large densities (>10 cells g<sup>-1</sup>) there appears to be nearly a constant cell lag time (Kaprelyants and Kell 1996). In the application of models for predicting relative growth of *C. perfringens* cell populations with respect to the aforementioned USDA requirement, the initial densities are assumed to be at least in the range of 10 to 10<sup>4</sup> cells g<sup>-1</sup>, corresponding to the approximate range of initial levels used in this study. Consequently, the model used in this paper assume that relative growth is not dependent on the initial levels, and is based on one that has been developed recently (Baranyi 1998) for predicting small relative growth of a

population of cells initially in lag phase. The predictions of these models apply only to a population of cells initially in lag and remain in the exponential phase of growth.

A common approach for determining a model for predicting growth, referred to here as the standard approach (van Gwern and Zwietering 1998), is to fit, for each experimental condition,  $E$ , a growth curve (called the primary model),  $\omega(t|\theta)$ , as a function of time,  $t$ , characterized by a set of estimated parameters,  $\theta$ . Then, the estimates of these parameters are considered to be functions of a set of environmental variables,  $\theta(E)$ , reflecting some conditions of the environment of growth, in this paper, temperature,  $T$ . A secondary model of  $\theta(T)$  is derived through regression analysis so that, for an arbitrary temperature, estimates of the parameters that characterize the growth curve can be made and in turn an estimate of the amount of growth can be made for that temperature. For models that we have seen presented in the predictive microbiology literature, it has been assumed that the parameters that characterize the growth curves, for given environmental conditions, are constant. However, it is possible to visualize them as random variables with expected values and standard deviations that are functions of the environmental conditions. The variance of a predicted value for a given cooling event would be the sum of the variance of the expected value,  $\text{var}(E(\omega(t|\theta)))$ , an additional variance,  $E(\text{var}(\omega(t|\theta)))$ , that is due to the variation of the growth characteristics,  $\theta$ . Using the standard approach, data analysis including analyses of variances would be needed to determine or estimate the values of these standard deviations. An alternative approach is to treat the set of environmental variables as independent variables in a single system of equations with the observed or measured levels as the dependent variable (Membré et al. 1996). However, to account for the standard deviations of the parameter values and for the correlations that exist among the values of the dependent variable, a nonlinear mixed-effects regression is needed (Lindstrom and Bates 1990). Estimating the parameters using this alternative approach involves complex calculations, which we imagine is the reason why this alternative approach has not

been used (to our knowledge) in the predictive microbiology literature for microbiologists. However, software systems S-plus<sup>®</sup> and, more recently, SAS<sup>®</sup> in release 8.0 have procedures that permit estimates of parameters for such models. The output of these regression procedures include estimates of the parameters, with confidence intervals, variances that are associated with design or other environmental factors as well as the covariance matrix of all the estimates. Thus, use of a reliable statistical package offers a simple approach for estimating parameters of complex models, deriving confidence intervals of parameters and predictions of growth. In this paper, a comparison of both approaches is made.

## Materials and Methods

### *Test organisms and spore production*

Three strains of *C. perfringens*, NCTC 8238 (Hobbs serotype 2), NCTC 8239 (Hobbs serotype 3), and NCTC 10240 (Hobbs serotype 13), obtained from our culture collection, were used in this study. The spores were produced in a modified formulation of Duncan and Strong sporulation medium as previously described (Juneja et al. 1993). After the spore crop of each strain had been washed twice and re-suspended in sterile distilled water, the spore suspensions were stored at 4°C. Spores of individual strains at equal numbers were then pooled to prepare a cocktail. This composite of spore strains was not heat-shocked prior to use.

### *Growth medium/products*

Ground chicken was obtained from Hatfield Quality Meats, Inc. (Hatfield, Pennsylvania, USA). The proximate analysis of meats performed by the supplier indicated that the chicken contained 12% fat, 71% moisture, 2% ash and 15% protein. Brine (3.5%) was thoroughly mixed in the chicken before the meat was placed into stomacher 400 polyethylene bags (100 g bag<sup>-1</sup>) and vacuum sealed. Thereafter, five of these bags were vacuum sealed in barrier pouches (Bell Fibre products Corp.,

Columbus, Georgia, USA), frozen at  $-40^{\circ}\text{C}$  and irradiated (42 kGy) to eliminate indigenous microflora. Random samples were tested to verify elimination of microflora by diluting in 0.1% (wt/vol) peptone water (PW), spiral plating (Spiral Biotech, Bethesda, Maryland, USA; Model D) on Tryptic soy agar (TSA; Difco) and then incubating, both aerobically and anaerobically, at  $37^{\circ}\text{C}$  for 48 h.

### Sample preparation, and inoculation

Sodium nitrite (120 ppm) and the cocktail of three strains of *C. perfringens* were added (1 ml) to 100 g of the thawed irradiated beef products. Thereafter, the inoculated meat was blended with a Seward laboratory Stomacher 400 for 5 min to ensure even distribution of sodium nitrite and the organisms in the meat sample. Duplicate 5 g ground meat samples were then weighed aseptically into  $30 \times 19$  cm sterile filtered Stomacher bags (Spiral Biotech, Bethesda, Maryland, USA). Negative controls included bags containing meat samples inoculated with 0.1 ml of 0.1% (w/v) peptone water with no bacterial spores. Thereafter, the bags were compressed into a thin layer (approximately 0.5–1 mm thick) by pressing against a flat surface, excluding most of the air, and then heat-sealed under vacuum (negative pressure of 1000 millibars). One bag, randomly selected, was opened and heat shocked at  $75^{\circ}\text{C}$  for 20 min. The samples were serially diluted in 0.1% peptone water (wt/vol), surface plated with a Spiral plater (Model D, Spiral Biotech, Bethesda, Maryland, USA) on Shahidi-Ferguson perfringens agar as described earlier (Juneja et al. 1996). The lower limit of detection by this procedure is  $21 \text{ cfu ml}^{-1}$ . The total *C. perfringens* population was determined after 48 h of anaerobic incubation. This was recorded as the initial inoculated numbers of bacterial spores before cooking.

### Incubation temperatures, sampling times and bacterial enumeration

Simulating the conditions that occur in the retail food industry and institutional food service settings, the vacuum-packaged bags containing the meat samples were immersed

in a programmable water bath (Techne, ESRB, Cambridge, UK) at  $10^{\circ}\text{C}$ . The temperature of the water-bath was programmed to increase in a linear fashion to achieve  $60^{\circ}\text{C}$  in a period of 1 h. This process simulated the cooking of rare roast beef and heat-shocked the spores. Samples after cooking were enumerated and the data were recorded as spore numbers after cooking. Thereafter, all samples were incubated in constant temperature water bath stabilized at  $10.0^{\circ}\text{C}$ ,  $12.0^{\circ}\text{C}$ ,  $15.6^{\circ}\text{C}$ ,  $19.0^{\circ}\text{C}$ ,  $21.1^{\circ}\text{C}$ ,  $23.9^{\circ}\text{C}$ ,  $26.7^{\circ}\text{C}$ ,  $29.4^{\circ}\text{C}$ ,  $32.2^{\circ}\text{C}$ ,  $35.0^{\circ}\text{C}$ ,  $37.8^{\circ}\text{C}$ ,  $40.6^{\circ}\text{C}$ ,  $43.3^{\circ}\text{C}$ ,  $46.1^{\circ}\text{C}$ , or  $48.9^{\circ}\text{C}$ . At frequent intervals, appropriate for each growth temperature, samples were taken out for *C. perfringens* count as described above. Two independent experiments were done at each temperature. For each replicate experiment, an average  $\text{cfu g}^{-1}$  of two platings of each sampling point were used to determine estimates of the growth kinetics.

## Statistical Methodology

### Primary relative growth model

The procedures used for determining predictive models for relative growth during the lag and exponential phases of growth have been presented in an earlier paper (Juneja et al. 2001). A description of the model is given here. Baranyi (1998) developed a model by keeping track of the status of original cells, O, and "new" cells, D, where a D cell is either a result of completing an acclimation or lag phase of an O cell and is 'ready' to divide, or is an offspring cell from a D cell. Define  $\lambda(t)$  to be the infinitesimal cell 'death' rate of the O cell, and  $\mu(t)$  to be the infinitesimal cell 'birth' rate of a D cell. Let  $m_A(t)$  represent the number of cells at time  $t$ . The following set of differential equation

$$\begin{aligned} \dot{m}_O(t) &= -\lambda(t)m_O(t), \\ \dot{m}_D(t) &= \lambda(t)m_O(t) + \mu(t)m_D(t) \end{aligned} \quad \text{Eqn (1)}$$

with boundary conditions,  $m_O(0) = N_0$  and  $m_D(0) = 0$ , are derived reflecting the assumptions that the population of O cells follows a first-order kinetic decay and the D cells increase due to two sources: (1) the death of O cells, and (2) the birth of D cells. Equation (1)

corresponds to equations used by Baranyi (1998) with the exception here that the transition rates,  $\lambda(t)$  and  $\mu(t)$ , are time dependent. Defining  $v(t) = \int_0^t \mu(\tau) d\tau$  and  $\gamma(t) = \int_0^t \lambda(\tau) d\tau$ , the solution to Eqn (1) is

$$m_o(t) = N_0 e^{-\gamma(t)},$$

$$m_D(t) = N_0 e^{-\nu(t)} \int_0^t \lambda(\tau) e^{-(\gamma(\tau)+\nu(\tau))} d\tau. \quad \text{Eqn (2)}$$

The relative growth,  $r(t) = (m_o(t) + m_D(t)) / N_0$ , therefore, can be expressed as

$$r(t) = e^{-\gamma(t)} + e^{\nu(t)} \int_0^t \lambda(\tau) e^{-(\gamma(\tau)+\nu(\tau))} d\tau. \quad \text{Eqn (3)}$$

When it is assumed that the transition rates are constant:  $\mu(t) = \mu$ , and  $\lambda(t) = \lambda$ , for a given growth curve, it is derived that

$$r(t) = \frac{\mu e^{-\lambda t} + \lambda e^{\mu t}}{\lambda + \mu}, \quad \text{Eqn (4)}$$

which is the same equation derived by Baranyi (1998) (Eqn (23)). There are three parameters:  $N_0$ ,  $\lambda$ , and  $\mu$ . A mathematical definition of lag time for a nonlinear microbiological growth curve ( $\log_{10}(\text{cfu unit}^{-1})$  vs time) has been defined as the intersection of the horizontal line,  $y = \log_{10}(N_0)$ , and the tangent line of the curve with slope equal to the maximum derivative (McMeekin et al. 1993, Baranyi 1998). Applying this definition to Eqn (4), the mathematical lag time is defined as the intersection of the horizontal line,  $y = \log_{10}(N_0)$ , and the asymptotic line of the curve as  $t$  approaches infinity. The mathematical lag time, lagTime, is thus

$$\text{lagTime} = \frac{\ln(1 + \mu/\lambda)}{\mu} \quad \text{Eqn (5)}$$

corresponding to Eqn (28) of Baranyi (1998).

A complete growth curve includes cells that are in stationary phase, depicted when the rate of growth decreases and approaches zero. Generally, it is not too hard to select observations that represent cells that are in lag or exponential phase by looking at the linear portion of the growth curve, and excluding observed high levels that are not included in this portion;

most reasonable selections would provide estimates of the set of parameters  $\mu$  and  $\lambda$  that would be close to each other, relative to the differences that arise between independent experiments and between measured and predicted values from secondary models. To help us in the selection, estimates of a complete growth curve were made using a model developed by Baranyi et al. (1993), which can be described by the following differential equation:

$$\dot{x}(t) = \alpha(t)\eta x(t)(1 - x(t)/M), \quad \text{Eqn (6)}$$

where  $x(t)$  is the number of cells at time  $t$ ,  $\eta$  is a constant representing the maximum exponential growth rate,  $\alpha(t)$  is a factor which represents a lag phase,  $0 < \alpha(t) < 1$ , and  $M$  is the assumed maximum level at the stationary phase. The solution to Eqn (6) is of the form  $f(A(t))$  where  $A(t)$  is the anti-derivative of  $\alpha(t)$ , that is,  $dA(t)/dt = \alpha(t)$  and  $f$  satisfies the differential equation of Eqn (6) without  $\alpha(t)$  (or assuming  $\alpha(t) \equiv 1$  for all  $t$ ), so that  $f$  is the logistic function. Baranyi et al. (1993) identified a family of functions  $\{\alpha_n(t)\}$ , motivated by Michaelis-Menten kinetics; here, we select a simple function when  $n = 1$ ,  $A(t) = t - \ln(1 + t/\kappa)$ , where  $\kappa$  is a constant. Thus Eqn (6) is a function of four parameters:  $x(0)$ , the initial level at time = 0;  $\eta$ ;  $\kappa$ ; and  $M$ .

### Secondary models

The above equations apply for a constant temperature,  $T$ . To derive equations that apply for arbitrary temperatures, the values for the derived parameters are considered to be functions of temperature, and statistical regression procedures are used to derive secondary models that express the parameter values as a function of temperature (van Gerwen and Zwietering 1998). Following customary procedures, the actual secondary model is performed with the exponential growth rate expressed in the common log (base 10) scale,  $\text{egr} = \mu/\ln(10)$ . For generalizing egr, the Ratkowsky function (McMeekin et al. 1993) of the form

$$\text{egr}^{1/2}(T) = a(T - T_{\min})[1 - e^{b(T - T_{\max})}]^{1/2} \quad \text{Eqn (7)}$$

is used, where  $a$ ,  $b$ ,  $T_{\min}$ , and  $T_{\max}$  are the parameter values determined from a regression

analysis. For  $T > T_{\min}$ , or  $T < T_{\max}$ , egr is defined to be zero.

For generalizing lag times, the ratios of the lag times to the generation times for D cells (the time needed for doubling the population =  $\ln(2)/\mu$ ) have been considered (Ross 1999). From Eqn (5) this ratio, for a given temperature, is

$$\text{Rat} = \frac{\ln(1 + \mu/\lambda)}{\ln(2)}. \quad \text{Eqn (8)}$$

The time an O cell takes to become acclimated and transform to a D cell depends upon many factors related to the environment and history of the cells (Baranyi and Roberts 1994). However, an extensive data analysis of this ratio (Ross 1999) found that it is nearly constant, for practical purposes, over a range of temperatures. Assuming that the expected value of this ratio is constant, not dependent on temperature, Eqns (4), (7) and (8) can be used to define a model for predicting the relative growth for a given temperature. To complete the identification of the model, random error terms, or factors, need to be defined. For a given experiment, it is assumed that  $\mu$  and  $\lambda$  are constants. However, between experiments, these parameters may not be identical, but differ by small, random, amounts. Thus, as discussed in the Introduction, it is assumed that the parameters identified in Eqns (7) and (8) are not constants but random variables with given expected values and variances. The within-experiment variance of an estimated parameter is calculated from the regression of Eqn (4), by using the linear terms of the Taylor series expansion of Eqn (7) or (8), and the variance matrix of the estimated parameters from the regression (Rao 1973). The between-experiment variance for the standard approach is estimated from an analysis of the variance.

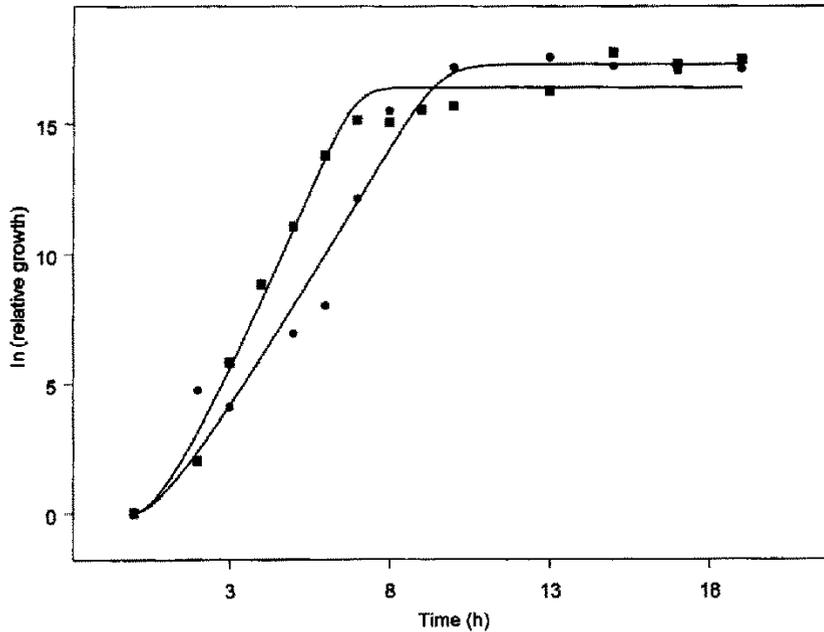
For the alternative approach, the parameters are estimated directly from the mixed effects model. It is assumed that, for a given estimate at time,  $t$ , of the cfu  $\text{ml}^{-1}$ ,  $n(t)$ ,  $\log_{10}(n(t))$  is measured with error,  $\varepsilon_0$ , that has expected value equal to zero and standard deviation equal to  $\sigma$ . For Eqn (7), it is assumed that  $\ln(\text{egr})$  has between experimental error  $\varepsilon_1$ , with expected value equal to zero, and standard deviation equal

to  $\sigma_1$ , and similarly for Eqn (8), it is assumed that  $\log_{10}(\text{Rat})$  has between experimental error  $\varepsilon_2$ , with expected value equal to zero, and standard deviation equal to  $\sigma_2$ . In addition, it is assumed that  $\varepsilon_1$  and  $\varepsilon_2$  have covariance,  $\sigma_{12}$ . In the Results section, it is shown that the expected value of  $\log_{10}(\text{Rat})$  can be assumed not to be dependent upon temperature, so that the system of equations

$$\begin{aligned} \log_{10}(n(t)) &= n_0 + \log_{10}\left(\frac{\mu e^{-\lambda t} + \lambda e^{\mu t}}{\lambda + \mu}\right) + \varepsilon_0, \\ \ln(u(T)) &= \ln(\ln(10)) + 2 \ln(a) + 2 \ln(T - T_{\min}) \\ &\quad + \ln\left[1 - e^{b(T - T_{\max})}\right] + \varepsilon_1, \\ \log_{10}\left[\frac{\ln(1 + \mu/\lambda)}{\ln(2)}\right] &= k + \varepsilon_2, \\ E(\varepsilon_j) &= 0, \quad \text{var}(\varepsilon_j) = \sigma_j^2, \\ j = 0, 1, 2, \text{cov}(\varepsilon_1, \varepsilon_2) &= \sigma_{12} \end{aligned} \quad \text{Eqn (9)}$$

defines the model. The above system identifies the primary parameters:  $a$ ,  $b$ ,  $T_{\min}$ ,  $T_{\max}$ ,  $k$ ,  $\sigma_j$ ,  $j = 0, 1, 2$ , and  $\sigma_{12}$ , that describe the model; the parameters,  $n_0$ , are nuisance parameters. All these parameters are estimated using the nonlinear regression, with mixed effects procedure (PROC NLMIXED) of SAS<sup>®</sup> for windows, release 8.00. The procedure of estimation is based on a maximum likelihood estimation (MLE) procedure of the marginal distribution of the dependent variable (Lindstrom and Bates 1990, Wolfinger, SAS<sup>®</sup>). Consequently, variance estimates would be slightly negatively biased (in a similar fashion that a MLE of a population variance is equal to the sample variance divided by the number of samples,  $s^2/n$ , whereas the unbiased estimate is  $s^2/(n-1)$ ).

Statistical analyses were performed using SAS<sup>®</sup> for windows, release 8.00; figures were created using Microsoft<sup>®</sup> Excel 97 SR-21 and S-Plus<sup>®</sup> 2000 release 3, and some calculations were performed using Mathcad<sup>®</sup> 7 Professional. Nonlinear regressions, other than the mixed effect model, were performed on SAS<sup>®</sup> using the procedure PROC MODEL; when parameters from more than one equation were being estimated, the seemingly unrelated regression (SUR) option was used, otherwise the ordinary least-squares option was selected. The SUR option accounts for the correlations



**Figure 1.** Plots of observed common logarithmic levels of *C. perfringens* vs time (h) and estimated complete growth curves, for temperature = 40.6°C.

that exist among the dependent variables that are used in defining the secondary model equations.

## Results

### Primary model

For the temperatures 10°C, 12°C, 21.1°C and 23.9°C very little growth was observed; for the latter two temperatures, it appears that the measurement times selected were not sufficient to observe growth. Growth curves for these three temperatures were not estimated. At 19°C, growth was observed but there was no discernable lag period and only one observed data point between the lag and stationary phases. Thus, for 19°C, growth curves were not estimated. At 29.4°C, also a linear portion of the curves could not be clearly identified, thus growth curves for these data were also not estimated. Growth curves were estimated for each replicate at the nine remaining temperatures (for a total of 20 curves: two replicates for all temperatures except for 35°C and 37.8°C where

there were three replicates). The function given in Eqn (4) applies for cells in the lag and exponential phases of growth. Thus, data observations which appeared to be representing cells in stationary phase were deleted. In most of the curves, these data were not clearly identifiable as on the linear portion of the growth curve. An exception seemed to be for the observed growth curves at 40.6°C. Figure 1 represents the observed and fitted log (relative) growth curves for 40.6°C using Eqn (6). The linear portion, beyond the lag phase, of the growth curve for replicate 1 (observed data marked by squares) appears to include the data up to and including time = 7 h for the second replicate, the linear portion appears to include observed data up to and including time = 10 h. Including these points, the estimates of  $\text{egr}$  from Eqn 4 are 1.14 and 0.77  $\log_{10}/\text{h}$  for the two replicates. Excluding the data at time = 7 h for the first replicate and time = 10 h for the second replicate, the estimates of  $\text{egr}$  are  $\log_{10}$  1.25 and 0.77  $\text{h}^{-1}$ . For the second replicate, deleting the point at time = 2 h (studentized residual = 2.36 when using Eqn (6)), the estimate of  $\text{egr}$ , including the data for time = 10 h, is 0.90 and, excluding this data point is 0.98. The differences

between the egr values with and without the data at time = 7 and 10 h are relatively small, at least, compared to the larger difference seen when comparing the estimates for the second replicate with and without the data at time = 2 h. In the estimates given below, the data at time = 7 h for the first replicate and time = 10 h for the second replicate are included.

Table 1 presents the estimated exponential growth rate, egr, and lagTime using Eqns (4) and (5), the generation time, and common logarithm of the ratio of lagTime to the generation time,  $\log_{10}(\text{Rat})$ , and its standard error.  $R^2$ 's were generally greater than approximately 0.95, with a few exceptions that were caused by unusual patterns of results. Of interest are the results for the second replicate at temperature 40.6°C. Using all the data, the standard error of the  $\log_{10}(\text{Rat})$  is 1.51, but when the data point at time = 2 h is deleted the standard error is reduced to 0.35. Consequently, in subsequent analyses, the results used are those obtained when deleting data at time = 2 h. Figure 2 depicts

the measured levels ( $\log_{10}(\text{cfu g}^{-1})$ ) and fitted curves using Eqn (4) for each temperature studied from 15.6 to 48.9°C, except for those listed above. The assumed stationary phase points excluded from the analysis can be identified from the graphs of Fig. 2 as the ones that do not have a predicted curve passing by them.

### Secondary models

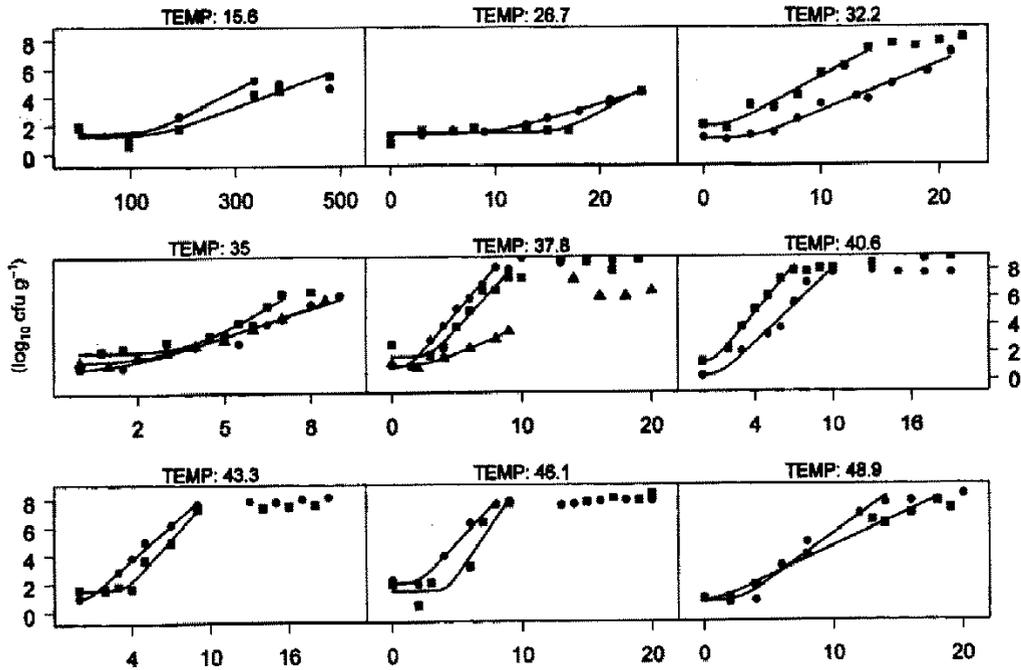
Figure 3 is a scatterplot of the square root of the exponential growth rate, egr, vs temperature, together with the fitted Ratkowsky function (Eqn (7)). For the highest temperature studied (48.9°C), there was substantial growth, so that this high temperature does not provide an approximation to the  $T_{\text{max}}$  parameter of Eqn (8). From a study of the relative growth of *C. perfringens* in broth (Juneja et al. 1999), an estimate of  $T_{\text{max}}$  was 51°C. For determining the parameter values of the Ratkowsky curve, thus, it was assumed that  $T_{\text{max}} = 51^\circ\text{C}$ . The other estimated parameter values (with standard errors)

**Table 1.** Summary statistics of fitted curves, egr, the exponential growth rate, lag time, generation time (h) and the  $\log_{10}$  of the ratio of lag time to the generation time, and asymptotic standard error of  $\log_{10}$  of the ratio

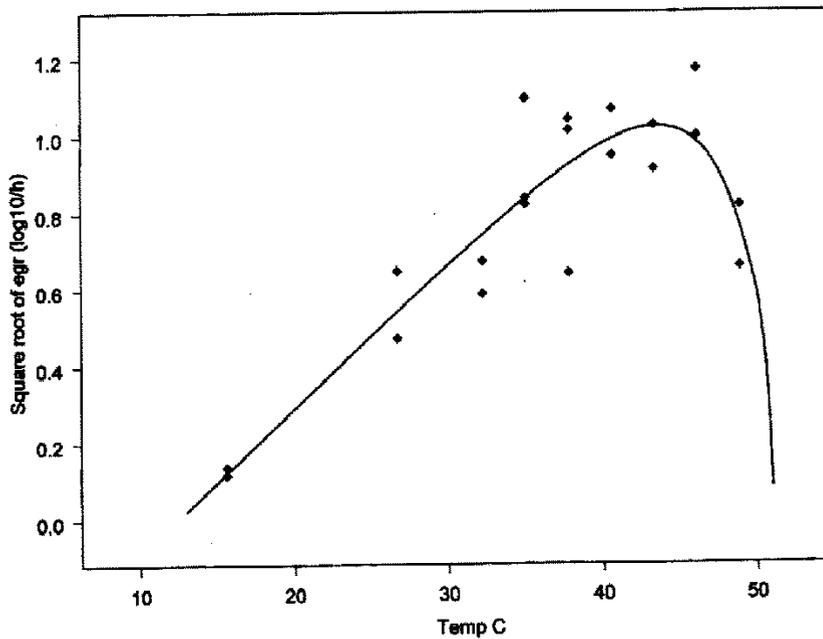
Temp. (°C)	egr ( $\log_{10} \text{ h}^{-1}$ )	lag time (h)	Generation	$\log_{10}(\text{lag gen}^{-1})$	std error $\log_{10}$
15.6	0.01	149.9	22.22	0.83	0.33
15.6	0.02	139.9	15.83	0.95	0.35
26.7	0.42	16.67	0.72	1.36	0.15
26.7	0.22	10.54	1.36	0.89	0.05
32.2	0.45	2.25	0.67	0.53	0.32
32.2	0.34	4.40	0.88	0.70	0.14
35.0	1.20	3.79	0.25	1.18	0.17
35.0	0.67	1.59	0.45	0.55	0.30
35.0	0.70	2.46	0.43	0.76	0.10
37.8	1.00	2.12	0.30	0.85	0.17
37.8	1.08	1.28	0.28	0.66	0.11
37.8	0.41	3.02	0.74	0.61	0.20
40.6	1.14	0.88	0.27	0.52	0.23
40.6	0.77	0.36	0.39	-0.03	1.51
40.6 <sup>a</sup>	0.90	1.36	0.34	0.61	0.35
43.3	1.05	3.57	0.29	1.09	0.10
43.3	0.83	0.70	0.36	0.29	0.31
46.1	1.37	4.21	0.22	1.28	0.28
46.1	0.99	2.09	0.30	0.84	0.21
48.9	0.43	1.16	0.70	0.22	0.44
48.9	0.66	2.78	0.45	0.79	0.24

<sup>a</sup>Second replicate at 40.6°C excluding observation at time = 2 h. These results are used in subsequent analysis.

Predictive model for growth of *Clostridium perfringens*



**Figure 2.** Plots of observed common logarithmic levels of *C. perfringens* vs time (h) and estimated growth curves, up to and including exponential phase, for studied temperatures.



**Figure 3.** Plot of square root of the exponential growth rate ( $\log_{10} \text{h}^{-1}$ ), egr, vs temperature, together with fitted Ratkowsky curve:  $\text{egr}^{1/2} = a(T - T_{\min})[1 - \exp(b(T - T_{\max}))]^{1/2}$ .

**Table 2.** Estimates of parameters for growth model<sup>a</sup> using primary and secondary models (the standard approach, using seemingly unrelated regression for the secondary model) and nonlinear mixed effects of MLE procedure where the natural log of the observed relative growth is the dependent variable

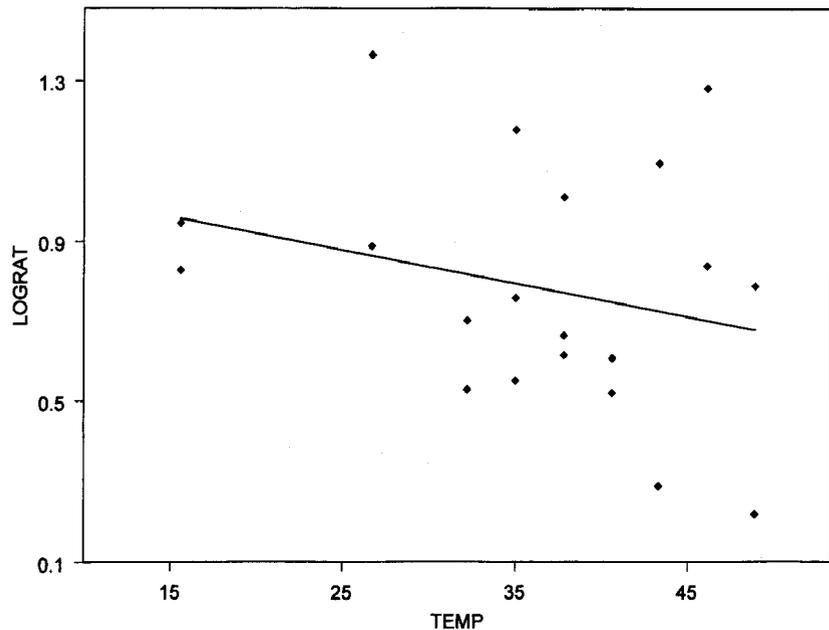
Parameter	Standard approach	Nonlinear mixed effect
<i>a</i>	0.0366(0.0023)	0.0358(0.0019)
<i>b</i>	0.217(0.056)	0.201(0.041)
<i>T</i> <sub>min</sub>	12.8(0.398)	12.3(0.354)
log <sub>10</sub> (Rat)	0.783(0.068)	0.760(0.062)
σ <sup>2</sup> <sub>blnegr</sub>	0.0367(0.0156) <sup>b</sup>	0.0348(0.0154)
σ <sup>2</sup> <sub>blog(Rat)</sub>	0.0313(0.0102) <sup>c</sup>	0.0266(0.0160)
σ <sup>2</sup> <sub>res</sub>	0.175 <sup>d</sup>	0.208(0.0270)

<sup>a</sup>Parameter values are defined in Eqn (9). The parameters, *a*, *b*, and *T*<sub>min</sub> are those used in a Ratkowsky equation for the exponential growth rate:  $egr = a(T - T_{min})[1 - \exp(b(T - 51^\circ C))]^{0.5}$ . The parameter Rat is the ratio of the lag time to the generation time. The parameters: σ<sup>2</sup><sub>blnegr</sub>, σ<sup>2</sup><sub>blog(Rat)</sub> refer to between experiment variances for the ln(egr) and log<sub>10</sub>(Rat), and σ<sup>2</sup><sub>res</sub> refers to the variance of the residuals.

<sup>b</sup>Standard error based on chi-square approximation with 11 degrees of freedom.

<sup>c</sup>Standard error based on chi-square approximation with 19 degrees of freedom.

<sup>d</sup>Pooled from individual regressions (115 degrees of freedom).



**Figure 4.** Plot of common log of ratio of lag time to generation time and linear regression line for temperatures.

are given in Table 2. The mean square error (MSE) of the residuals is 0.1037.

Figure 4 is a scatterplot of the estimated values of log<sub>10</sub>(Rat) vs temperatures together with the linear regression line, the slope of which is not significant (*P*-value=0.26).

An analysis of variance of the log ratio values did not indicate a statistical significant temperature effect (*P*-value = 0.48). The distribution of the log ratio values (assuming no temperature effect) is approximately normal (*P*-values for various tests for normality

>0.15), with mean,  $m$ , of 0.783 (corresponding geometric mean of about 6.07) and standard deviation,  $s$ , of 0.305. Thus, the standard error of the mean,  $m$ , is 0.0682. The lower 90% confidence limit of the geometric mean is 4.9, and the upper 90% confidence limit is 7.5.

The above analysis determines the expected values of the parameters defined by Eqns (7) and (8). To completely characterize the model, it is necessary to determine if these parameters can be considered constants for any cooling event or experiment with the same conditions, or rather as random variables with expected values given by the Eqns (7) and (8) and non-zero standard deviations and correlations. To do this, analyses of variances are performed. For  $\ln(\text{egr})$ , the between-experiment variance,  $\sigma_{\text{blnegr}}^2$ , calculated by subtracting the within-experiment variance from the within-temperature mean square error is estimated to be 0.037, which, based on 11 degrees of freedom, is significantly greater than zero, at the 0.15 significance level. An estimate of the coefficient of variation, CV, of  $\text{egr}$  can be approximated as 100% times  $\sigma_{\text{blnegr}}$ , which is equal to about 19%. For  $\log_{10}(\text{Rat})$  ignoring temperature and replication factors, an estimate of the between experiment variance,  $\sigma_{\text{blog(Rat)}}^2$ , of approximately 0.031 is derived. This implies that the CV of  $\text{Rat}$  is about twice that of  $\text{egr}$ .

As a comparison, a nonlinear mixed effect model is used to estimate the parameters identified in Eqn (9). For all experiments, the dependent variable was the log of the relative growth, obtained by subtracting the observed value at time = 0 from the other observed values. The parameter  $n_0$  was thus considered to be a constant, and would contribute to the residual error of the model. For the full model defined in Eqn (9), the covariance term,  $\sigma_{12}$ , was not significantly different from zero, and thus in the final model was assumed to be zero. The estimated parameters and standard errors are given in Table 2. These estimates are very close to the ones derived by the standard approach. It should be pointed out that the correlations of the estimates of the variances:  $\sigma_{\text{blnegr}}^2$ ,  $\sigma_{\text{blog(Rat)}}^2$ , with the estimates of the other parameters are small in absolute value.

## Discussion

The equations developed above apply for arbitrary, but fixed temperatures. As discussed in Juneja et al. (2001), applying equations developed for fixed temperatures when temperatures are changing could be problematic. Research (Zwietering et al. 1994) shows that the lag time can increase over that expected at a given temperature, after adjusting for the time spent in lag at a prior temperature. This is interpreted to mean that  $\lambda(t)$ , the infinitesimal cell 'death' rate of original O cells and becoming D cells, can decrease when the cells experience a temperature change. That is, the lag phase times would not decrease proportionally over time as the temperature changes. For purposes of investigating the effect of temperature changes on the growth curve, a simple assumption that  $\lambda(t)$  is proportional to the exponential cooling rate,  $k(t)$  could be considered (Juneja et al. 2001). Thus, a possible model could assume that, for a given exponential cooling rate,  $k$ , the infinitesimal death rate of original cells is given

$$\lambda_k(t) = \lambda(t)\rho_k, \quad \text{Eqn (10)}$$

where  $\rho_k$  is a decreasing function of  $k$ , such that  $\rho_0 = 1$  and  $\rho_\infty = \omega > 0$ . Growth curves could be determined for different constant exponential rates of temperature change to explore the relationship of  $\rho_k$  as a function of  $k$  and to evaluate if the above model can provide approximate estimates of relative growth for constant exponential rates of temperature change. Research to do this is being planned.

Equation (2) is used, assuming that  $\rho_k = 1$ , for calculating the relative growth for a hypothetical cooling of product from 51°C to 12°C in  $t_x$  hours, where temperature,  $T$ , at time  $t$ , is given by

$$g(t) = (T_1 - T_a)e^{-\psi t} + T_a. \quad \text{Eqn(11)}$$

$T_1$  is the initial temperature of the product,  $T_a$  is the ambient air temperature (here assumed equal to 0°C), and  $\psi$  is the exponential cooling rate. As discussed in the Introduction section, the variance of the predicted  $\log_{10}$  relative growth is expressed as a sum of the variance of the expected value plus the expected

value of the variance due to the variation of the defined parameters. The former variance is calculated by expressing the  $\log_{10}$  relative growth as a function of the parameters:  $a$ ,  $b$ ,  $T_{\min}$ , and  $k$  of Eqn (9), and using the linear terms of a Taylor series expansion with respect to these parameters and their covariance matrix. The square root of this variance is the standard error of the prediction. The variance due to the variation of the defined parameters is calculated by expressing the  $\log_{10}$  relative growth as a function of  $\mu$  and  $k$ , and linearizing using the Taylor series. The square root of the sum of the two variances is the standard deviation of the prediction, and can be used to create probability intervals for predicted amounts of growth for a given cooling event.

A word needs to be said about calculating the standard deviation, as described in the previous paragraph, from Eqn (2). Since  $k$  is assumed to be constant, it is a straightforward matter to compute the partial derivative with respect to  $k$  and use it to approximate the variance. However, the exponential growth rates,  $\mu$ , are not constant as the temperature changes, and thus, in actuality, the partial derivative that is desired is with respect to the function  $\mu(t)$ . However, it is assumed the variance of the error associated with  $\ln(\mu)$  is the same for all temperatures, so that for a given cooling event, or cooling scenario, it is reasonable to assume that  $\mu(t) = \eta\mu_0(t)$ , where  $\eta$  is a random variable, such that  $E(\ln(\eta)) = 0$  and  $\text{var}(\ln(\eta)) = \sigma^2_{\ln\eta}$  and  $E(\ln(\mu(t))) = \ln(\mu_0(t))$  for all  $t$ . Thus, for calculating the variance, Eqn (2) can be expressed as a function of  $k$  and  $\eta$ , and the partial derivatives used to approximate the variance are with respect to the random variables  $k$  and  $\eta$ , where it is assumed that

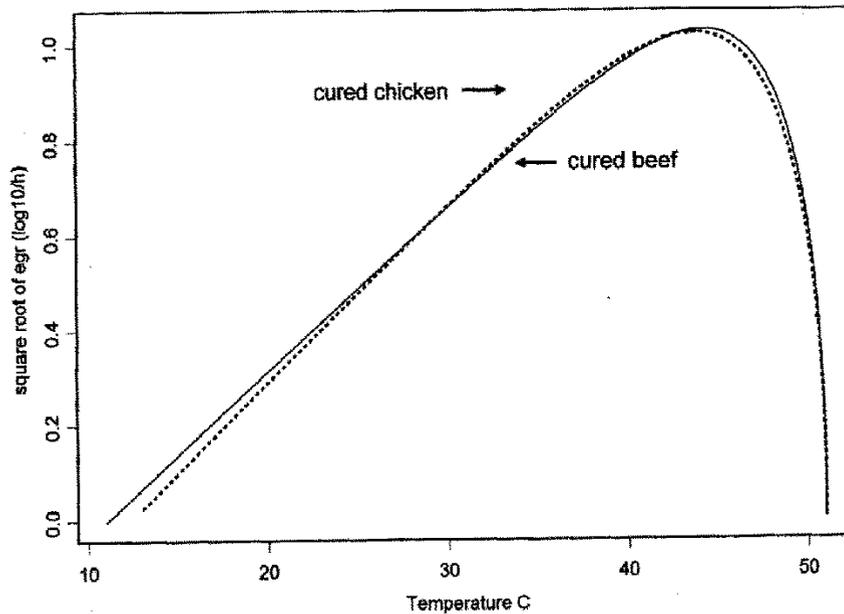
the variance of  $k$  is  $\sigma^2_{\ln(k)}$  and that of  $\ln(\eta)$  is  $\sigma^2_{\ln\eta}$ , and their correlation is zero.

Table 3 provides the predicted common logarithm of the expected relative growth from Eqn (2), for  $t_x = 6, 8$  and  $10$  h, the standard error, and the standard deviations for the two approaches of estimating the parameters. As can be seen from this table, the predictions and their measures of variability are similar for the two approaches of estimation. The standard errors are sufficiently small to enable construction of confidence intervals for the expected log of the relative growth, or the geometric mean of relative growth, for a given cooling scenario. Thus, for example, at 8 h, using the standard approach, the geometric mean relative growth is estimated to be 9.4, with 90% confidence interval, based on 17 degrees of freedom, of (1.33, 21.3). On the other hand, the standard deviations are comparable to the predicted geometric mean values, suggesting that the possible range of the amount of growth is large for a particular cooling event. For example, if it is assumed that the distribution is lognormal, then, for the 8 h cooling, a 90% probability interval for the relative growth is (0.32, 276). For 6 h, a 90% probability interval is (0.29, 24). For the 8 h cooling event, 66% of the variance is due to the variation in  $k$ , for 6 h, the percentage is 77%, and for 10 h, it is 56%. Since the lag phase duration would be more influential to the predicted amount of growth for small times, it is expected that the percentage would be a decreasing function of time.

A similar study as this one was conducted for cured beef (Juneja et al. 2001). Figure 5 presents the fitted curves from Eqn (7) of the square root of the egr vs temperature for the two studies. Both curves reach maximum levels

**Table 3.** Predicted common logarithm of expected relative growth of *C. perfringens* in cured chicken when cooled, log linearly, from 51°C to 12°C in 6, 8 and 10 h, the standard error of these predictions, and the standard deviation of predictions taking into account the between experiment variability of the growth characteristics

Hours	Standard approach			Mixed effects model		
	Predicted	s.e.	s.d.	Predicted	s.e.	s.d.
6	0.424	0.142	0.550	0.398	0.137	0.473
8	0.973	0.205	0.844	0.899	0.202	0.739
10	1.63	0.234	1.00	1.51	0.232	0.891



**Figure 5.** Plot of Ratkowsky curves derived for cured beef (Juneja et al. 2002) and cured chicken from this study.

of about 1 at near or slightly above 40°C; the minimum temperatures for both curves are about 11–12°C. For practical purposes, the two curves are nearly identical. However, the estimated geometric means of the ratios of the lag times to the generation times (Eqn (8)) are different; where, in the study on cured beef, the geometric mean was estimated to be 8.1, compared to the 6.1 value estimated in the cured chicken study. Using the data from the two studies, the statistical significance of the difference between these two estimates has a two-sided *P*-value of 0.16, suggesting that the estimated difference might not reflect a true difference between the lag phase times for the two products.

## Conclusion

The present study has assessed the growth of *C. perfringens* from spores inocula, in chicken supplemented with curing salts, when the chicken was cooked slowly to 60°C and then cooled rapidly to a specified temperature. Under these conditions, the growth of *C. perfringens* from spores in a period of 21 days

at 10°C and 12°C was not observed. These observations are in agreement with previous studies: Geopfert and Kim (1975) reported that *C. perfringens* growth does not begin in foods stored at 15°C or below, even after extended storage. However, this may not hold true when hot foods are cooled and the rate of cooling is not sufficiently fast. Solberg and Elkind (1970) reported that *C. perfringens* vegetative cells increased by 3 log cycles in 3 days at 15°C and in 5 days at 12°C but growth was restricted at 10 and 5°C. It is possible that growth kinetics at other temperatures would also be affected by the rate of cooling.

The assumptions that the infinitesimal cell death rates of O cells over time are constant, and that the cells that leave the GOL phase have the 'same' growth characteristics as new offspring cells may not be innocuous. For the latter concern, it might be that the developmental events leading to a cell leaving the GOL phase and the events needed for cell division are operating closer in parallel rather than in series or sequentially, as was assumed for the model used in this paper. The consequence, of course,

is that the microbiological interpretation of the model's parameters is questioned, even though the model provides an adequate fit of observed data. The types of experiments needed to test these types of hypotheses would involve many observations near the estimated times where the cells would be leaving GOL phase, and perhaps microscopic examination of the cells.

This paper presents a model for predicting small-to-moderate relative growth of *C. perfringens* during cooling of certain cooked cured chicken products, which is assumed not to be dependent upon the initial levels of the population when between  $10$  and  $10^4$  cfu  $g^{-1}$ . Rather than assuming that the parameters that describe growth kinetics are constant for a given temperature, it is assumed that they are random variables with expected values and standard deviations for a given cooling event. Estimates of the parameters were made by two procedures: the standard two-stage approach that is commonly used in predictive microbiology, and a nonlinear mixed effects procedure, based on a system of equations with the observed plate counts as the dependent variable. The standard procedure involved using SUR regression procedures and analyses of variances. The derived estimates of the two procedures were very close. The similarity would favor using the nonlinear mixed effects procedures because of its simplicity when used in software systems such as S-Plus® and SAS®. However, the standard approach allows the analyst to examine the data more closely, getting a better 'feel' for the data. We recommend that nonlinear mixed effects analyses be used because these account for the correlations that exist in microbiological data of the type in this study, but that such analyses be accompanied by a less formal data analyses that are used in the two-stage approach to help formulate the model.

Finally, the predictions are sensitive to an assumed value for the ratio of the lag to generation times; the between-experimental variability associated with this statistic is relatively large compared to that of the exponential growth rate. More research is needed to obtain better understanding of this ratio.

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