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Predictive model for growth of *Clostridium perfringens* at temperatures applicable to cooling of cooked meat†

V. K. Juneja[1]*, R. C. Whiting[1], H. M. Marks[2] and O. P. Snyder[3]

The objective of this study was to develop a model to predict the growth of Clostridium perfringens *from spores at temperatures applicable to the cooling of cooked meat products. The growth medium used was trypticase-peptone-glucose-yeast extract broth.* C. perfringens *population counts were determined at appropriate intervals by plating onto tryptose-sulfite-cycloserine agar.* C. perfringens *growth from spores was not observed at a temperature of 51°C for up to 3 weeks. It was found that, generally, for relative growth of more than* $\frac{1}{2}$ log_{10}, *the use of the logistic function provided a better prediction than the use of the Gompertz function. The two parameters: germination, outgrowth and lag (GOL) time, and exponential growth rate, EGR, were determined using the logistic function. The exponential growth rates and the reciprocal of the GOL times were fitted to the square root Ratkowsky model, using temperature as the independent variable. Applying multi-variate statistical procedures, confidence intervals were computed on the prediction of the amount of relative growth for a given temperature. Closed form equations are developed that allow for predicting growth for a general cooling scenario and the standard error of the prediction. These equations depend upon microbiological assumptions of the effect of history of the GOL times for gradual changes in temperature. For example, for cooling from 50°C to 10°C in 8 h, the equations predict a relative growth of 3·37 with an upper 97·5% confidence limit of 6·73.*

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[1]US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 E. Mermaid Lane, Wyndmoor, Pennsylvania 19038, USA
[2]US Department of Agriculture, Food Safety Inspection Service, 14th and Independence SW, Washington DC 20250, USA
[3]Hospitality Institute of Technology and Management, 670 Transfer road, Suite 21A, St. Paul, Minnesota 55114, USA

Introduction

Clostridium perfringens are widely distributed in a variety of foods, particularly meat and poultry. The organism has been implicated in numerous foodborne disease outbreaks and thus, continues to remain a major concern to the food industry world wide. (Anonymous 1996, Centers for Disease Control and Prevention 1985, Stringer et al. 1980, Todd et al. 1997). Illness results after the ingestion of a large number of viable vegetative cells which have grown in the implicated meat or poultry product and then survive stomach passage. The vegetative cells subsequently sporulate in the small intesine. The heat-labile enterotoxin, known as *C. perfringens* enterotoxin is

*Corresponding author. Fax: (215) 233-6406; E-mail: vjuneja@arserrc.gov.

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synthesized during sporulation and is released together with the mature spore during sporangial autolysis.

The abilities of *C. perfringens* to form heat-resistant spores and to grow at a very rapid rate at relatively high temperatures are the major contributing factors leading to food poisoning. The temperature range for growth of *C. perfringens*, 6–52·3°C, is well documented (Hall and Angelotti 1965, Johnson 1990, Shoemaker and Pierson 1976). A short generation time of 7·1 min in autoclaved ground beef means that after the spores have germinated, rapid cooling of foods is critical (Willardsen et al. 1979). The time/temperature guidelines for cooling cooked products recommends that the maximum internal temperature should not remain between 54·4 and 26·7°C for more than 1·5 h nor between 26·7 and 4·4°C for more than 5 h (USDA 1989). The US Food and Drug Administration (FDA) Division of Retail Food Protection recognized that inadequate cooling was a major food safety problem and established a recommendation that all food should be cooled from 60 to 21°C (140–70°F) in 2 h and from 21 to 5°C (70–41°F) in 4 h (FDA Code 1997). Utilizing *Clostridium perfringens* in cooked beef with cooling from 54·4°C to 7·2°C at rates varying from 6 to 18 h, Juneja et al. (1994) found that even a longer cooling period of up to 15 h prevented growth from a spore inoculum.

Blankenship et al. (1988) developed a model for the growth from spores of *C. perfringens* in cooked chilli during a 4 h and 6 h exponential cooling time for a temperature decline from 50°C to 25°C. In that previous study, the number of germinating spores were calculated every hour during the cooling period.

In the scientific literature, there is a lack of quantitative data on the growth of *C. perfringens* over the entire growth temperature range which foods must pass through during cooling after cooking. The heat-activated spores in such products are likely to germinate, outgrow and multiply if the rate and extent of cooling is insufficient. Inadequate cooling practices in retail food operations have been cited as a major cause of food poisoning with *C. perfringens* (Bryan 1978, Bean and Griffin 1990). Accordingly, the aim of the present work was to develop a model to predict the relative growth of *C. perfringens* from spores at temperatures relevant to the cooling of cooked products.

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) were used in this study. The origin and sources of the strains and spore production methods have been reported (Juneja et al. 1993). After the spore crop of each strain had been washed twice and resuspended in sterile distilled water, the stock spore suspensions were stored at 4°C. A spore cocktail containing all three strains of *C. perfringens* was prepared immediately prior to experimentation by mixing equal numbers of spores from each suspension. This spore mixture was heat-shocked for 20 min at 75°C prior to use.

Growth medium, inoculation and sampling

Trypticase-peptone-glucose-yeast extract (TPGY) containing (% w/v): 5% trypticase; 0·5% peptone; 2% yeast extract; 0·1% cysteine hydrochloride (Sigma Chemical Company, St. Louis, Missouri, USA); and 0.4% dextrose was used for determination of growth rates. Except as noted, ingredients were obtained from Difco (Detroit, Michigan, USA); trypticase was obtained from Baltimore Biological Laboratory (BBL, Cockeysville, Maryland, USA). The medium was dispensed in 50 ml portions into 250 ml trypsinizing flasks equipped with a rubber septum inserted in the side arm sampling port, and sterilized by autoclaving.

Dextrose and cysteine hydrochloride were dissolved in deionized water, filter sterilized (0·22-μm pore-size syringe filter, Nalge Company, Rochester, New York, USA) and added aseptically to the medium. Each flask received 0·5 ml of the heat-shocked spores to obtain an initial count of about 2–3 $\log_{10}$ spores ml^{-1} of the growth medium. The flasks were then

flushed with sterile N_2 for 10 min and sealed with a rubber stopper. All flasks were incubated on a rotary shaker (150 rpm) at 15–50°C (2°C increments). At intervals appropriate for each growth temperature, samples were withdrawn through the side arm septum with a syringe fitted with a hypodermic needle. Serial dilutions made in 0·1% peptone water (w/v) were surface-plated with a Spiral plater (Model D, Spiral Biotech, Bethesda, Maryland, USA) onto tryptose-sulfite-cycloserine agar without cycloserine, i.e., SFP agar and egg yolk enrichment. The lower limit of detection by this procedure is 21 cfu ml^{-1}. After overlaying with an additional 10 ml of SFP agar, the plates were allowed to solidify before placing into anaerobic jars. The total *C. perfringens* population was determined after 48 h of incubation at 37°C in a Gas Pak system (BBL).

Three replicate experiments were conducted at each temperature. For some sets of replicates there were problems with the incubator, samples were not measured for sufficient time to observe substantial growth, or there was an apparent sample contamination. In such cases, an additional three replicates were conducted and the results from the repeated experiments were used. Exceptions were made for 15°C and 28°C. For 15°C, only two replicates were conducted, and in one of them there was some growth. The results from this experiment were used for estimation purposes, as described below. For 28°C, two replicates were conducted for a second set of experiments, after an incubator problem was noted for one of the replicates of the first set of triplicate replicates. Thus, results from four replicates were used. In addition, four data values, whose measurements were made within 1·5 h from the start, were deleted because the measured values were an approximate 1 $\log_{10}$ or more above the minimum values obtained from the corresponding growth experiments of the questionable data values, and were clearly inconsistent with the measurements made before or after. One data value, at 42°C, measured at 7 h, was deleted because its measurement value was more than 1 $\log_{10}$ below the previously measured value and more than 4 $\log_{10}$ below the subsequent measured value.

Results

Fitting growth curves

The growth of an organism as a function of time, can be described by

$$L(t) = A + (P - A)f(t|M, B) \qquad (1)$$

where $L(t)$ is the common logarithm of $N(t)$, the number of organisms at time t, $f(t|M, B)$ is a non-decreasing function of time between 0 and 1, M and B are non-negative parameters that describe the slope and location of the curve along the t-axis and are functions of the relative growth rate and the GOL time, A is an asymptotic minimum value and P is an asymptotic maximum value and represents the maximum population density. For example functions that can be used for $f(t|M, B)$ are the Gompertz function: $g(t|M, B) = \exp(-\exp(-B(t-M))$, the logistic function: $h(t|M, B) = 1/(1 + \exp(-B(t - M)))$ or a generalization of the logistic which introduces a third parameter, v, such as the Richards extension, $r(t|M, B, v) = (1/(1 + v\exp(-B(t - M))^{1/v}$ (Zwietering et al. 1990). The Gompertz function has been shown to provide good predictions of growth in experimental situations for many organisms (Zwietering et al. 1990) and has been used for fitting growth for many organisms (Buchanan 1990). This function describes a slow initial growth, followed by a rapid increase in growth, and then asymptotically leveling off. The logistic function describes a more rapid initial growth than that of Gompertz. While these functions are continuous and have continuous derivatives, the parameters are difficult to interpret in biological terms. A simple function to interpret, which though is not biological plausible but still may provide sufficient predictions of growth, is the spine function, $s(t|M, B) = \min(1, \max(0, B(t - M)))$ (Buchanan et al. 1997).

In generating growth curves using data from controlled experiments, it is often assumed (Gibson et al. 1988, Buchanan 1990) that P is a constant quantity and $A = \log_{10}(N_0)$, where N_0 is the initial number of organisms. To estimate the parameters for equation 1, for a given temperature/replicate growth experiment, the

value of A was computed to be the average of the $\log_{10}$ transformed measured population densities that occurred at a time equal to or less than the time of the minimum measured population density. Data analysis on the maximum measured population densities obtained from the different experiments suggested that the maximum population density did not depend on temperature. Thus, P was considered a constant and was set equal to $10{\cdot}4\log_{10}$, which was the $\log_{10}$ of the maximum measured population density obtained from all the experiments. Estimates of B and M were derived using PC-SAS® system for windows, release 6·12, PROC NLIN procedure. For the spline function, $s(t|M,B)$, data for which $L(t)$ were greater than 7 were deleted.

The estimates parameters of the Richard and spline functions were highly variable among replicates experiments for a given temperature, or, for the Richard function, on occasion the estimation procedure did not converge to a solution. A reason for the instability is that the number of data values per experiment (≤ 10) was not sufficient to provide stable estimates of the three parameters. Thus, the Richards and spline functions were not used.

The use of either the Gompertz and logistic functions provides reasonably good predictions, so that the choice of which function to use should depend upon the behavior in regions for which it would be most important to have good predictions. Thus the data analysis concentrated on regions where there would be at least moderate growth rates or low *GOL* times. The growth curves generated in this research will be applied for predicting growth of *C. perfringens* during cooling of cooked ready-to-eat products. The concern is to limit the relative growth of *C. perfringens* to no more than certain amount, for example, 10-fold ($1\log_{10}$) (USDA 1996). Thus, for evaluating growth curves, particular attention is paid to the performance of the predictions of relative growth in the vicinity of $1\log_{10}$. Our data analysis did show that the use of the Gompertz function provided, on the average, better predictions of relative growth for the low temperatures ($\leq 22°C$) than use of the logistic function, but for temperatures above 22°C ($\leq 25°C$), on average, the logistic was better in the primary region of concern. For purposes of further analysis of the residuals, the data were divided into three regions defined by a measure of $\log_{10}$ relative growth, $Rg = L(t) - A$: 1) lag period, $Rg < 0{\cdot}5$; 2) early growth, $0{\cdot}5 \leq Rg < 2{\cdot}3$; and 3) later growth, $Rg \geq 2{\cdot}3$. The means, standard deviations of the residuals, and the correlation of the residuals with Rg in each of the three regions for the Gompertz and logistic functions identified above for temperatures $\geq 25°C$ are presented in Table 1. As can be seen, the use of the Gompertz function provides better predictions of the relative growth that the use of the logistic function in region 1, lag period, where the measured relative growth is small. However, for region 2, early growth, where the relative growth has begun to accelerate, the logistic function does better than the Gompertz. Both curves in this region underestimate growth, but the magnitude of the negative bias using the Gompertz function is approximately twice that when using the logistic function and the residuals (using the Gompertz function) are negatively correlated with Rg. Thus, for predicting relative growth as a function of time and temperature, the logistic function is used.

From estimates of M and B, estimates of *GOL* time and the exponential growth rate, *EGR*, were computed. The exponential growth rate, *EGR*, is defined to be the maximal relative

Table 1. Summary of residuals (observed $\log_{10}$(density) − predicted $\log_{10}$(density)) over temperatures $\geq 25°C$, by magnitude of $\log_{10}$ relative growth = ($\log_{10}$ (density) − A) where A is estimate of $\log_{10}$(density at time $t = 0$)

Log_{10} relative growth (Rg)	Residuals		
	Statistic	Logistic	Gompertz
$Rg < 0{\cdot}5$	Mean ($n = 60$)	−0·147	0·013
	s.d.	0·170	0·122
	Correlation[a]	0·444	0·767
$0{\cdot}5 \leq Rg < 2{\cdot}3$	Mean ($n = 54$)	0·091	0·192
	s.d.	0·287	0·365
	Correlation	0·057	−0·384
$2{\cdot}3 \leq Rg$	Mean ($n = 116$)	0·012	0·020
	s.d.	0·479	0·542
	Correlation	0·209	0·521

[a]Pearson correlation of residuals with Rg.

growth rate $d(L(t))/dt$, with units $\log_{10}$(cfu ml^{-1}) h^{-1} (Gibson et al. 1988, McKeekin et al. 1993). The *GOL* time is defined as the value of time at the point of intersection of the line containing the point $(M, L(M))$ with slope equal to the exponential growth rate and the horizontal line at L_0 (McKeekin et al. 1993). The growth characteristics, *EGR* and *GOL*, for the Gompertz function, $g(t)$, can be expressed as:

$$ERG = B(P - A)/e$$
$$GOL = M + (e \cdot g(0) - 1)/B \quad (2)$$

where $e = \exp(1)$, while those for the logistic function, $h(t)$, can be expressed as:

$$EGR = B(P - A)/4$$
$$GOL = M + (4h(0) - 2)/B \quad (3)$$

The estimated values of *GOL* and *EGR*, assuming $A = \log_{10}(N_0)$ (Gibson et al. 1988), derived from the experiments, using Gompertz and logistic growth curves, are presented in Table 2. The heading in Table 2, 'Approximate *GOL* time,' conveys that the calculations were made assuming $A = \log_{10}(N_0)$. This assumption leads

Table 2. Estimated *GOL* times (h) and exponential growth rate ($\log_{10}$(cfu ml^{-1})h^{-1}) of *Clostridium perfringens* in TPGY broth for Gompertz and logistic curves

Experiment	Temperature	Approximate[a] *GOL* time Gompertz	Approximate[a] *GOL* time logistic	Exponential growth rate Gompertz	Exponential growth rate logistic
1	15·0	−72·654	154·752	0·0033	0·0049
2	19·0	39·057	41·412	0·1383	0·1339
3	19·0	40·757	43·946	0·1226	0·1222
4	19·0	38·146	42·006	0·1245	0·1266
5	22·0	18·380	25·323	0·1582	0·1928
6	22·0	15·340	17·685	0·2042	0·2055
7	22·0	16·597	19·311	0·2015	0·2088
8	25·0	6·020	6·551	0·3141	0·3212
9	25·0	6·405	6·787	0·3222	0·3255
10	25·0	8·752	8·933	0·4357	0·4308
11	28·0	5·196	5·878	0·8441	0·9117
12	28·0	5·808	6·156	0·8573	0·8390
13	28·0	5·553	6·035	0·7616	0·7547
14	28·0	5·741	6·669	0·7942	0·9050
15	30·0	3·575	4·222	0·7833	0·8129
16	30·0	4·459	5·085	1·0368	1·1527
17	30·0	4·598	5·229	1·0635	1·2032
18	35·0	3·326	3·830	1·3014	1·4417
19	35·0	3·030	3·160	1·3555	1·3383
20	35·0	2·747	3·068	1·2642	1·3196
21	37·0	3·286	3·624	1·4145	1·4859
22	37·0	2·714	2·963	1·3512	1·3722
23	37·0	3·242	3·518	1·3889	1·4231
24	42·0	3·641	3·693	2·3024	2·2162
25	42·0	4·809	5·127	1·5778	1·6544
26	42·0	5·612	6·095	1·5720	1·7278
27	45·0	2·979	3·399	1·2136	1·3737
28	45·0	3·579	3·766	1·6499	1·7206
29	45·0	3·603	3·786	1·6838	1·7548
30	47·5	2·686	2·819	1·5155	1·5148
31	47·5	2·912	3·012	1·6915	1·6734
32	47·5	2·936	3·067	1·6314	1·6338
33	50·0	5·759	6·024	1·5356	1·6256
34	50·0	5·452	5·737	1·4677	1·5538
35	50·0	5·620	5·893	1·5313	1·6169

[a]Calculated assuming the initial number of organisms, at time = 0, is equal to minimum asymptote ($t \to -\infty$).

to a negative approximate estimate for the *GOL* time at 15°C using the Gompertz function. The exact calculation of the *GOL* time gives a positive answer and is close to the value obtained for the *GOL* time using the logistic function. As can be seen from Table 2, the estimated values of *GOL* time and *EGR* are similar for the logistic and Gompertz functions. However, for the Gompertz curve, when time is equal to the *GOL* time, the $\log_{10}$ transformation of the relative growth, $L(t) - A$, is equal to $(P - A)\exp(-e) \approx 0{\cdot}066\ (P - A)$, and for the logistic curve, it is equal to $(P - A)/(1 + \exp(2)) \approx 0{\cdot}119(P - A)$. Thus the estimate of the $\log_{10}$ transformation of the relative growth, $\log_{10}(N(t)/N_0)$, at lag time using a logistic curve is approximately twice that using Gompertz curve.

Modeling growth characteristics

The above equations apply for a constant temperature. However, for a cooling scenario, the temperature would be changing, so that, for predicting the amount of relative growth, $N(t)/N_0$, it is necessary to express the growth characteristics: *GOL* and *EGR*, as functions of temperature. For generic bacteria, it has been found by researchers (Ratkowsky et al. 1983) that the square root of the exponential growth rate, k, as the dependent variable, and the most general form of Ratkowsky model,

$$k^{1/2}(T) = a(T - T_{\min})\,(1 - \exp\,(b(T - T_{\max})))^{\alpha} \tag{4}$$

where a, b, $T_{\min}$ and $T_{\max}$, are unknown positive parameters, α is usually either 1 or $\frac{1}{2}$, provides a good statistical fit. The Ratkowsky equation describes a curve for which, starting from zero at temperature $T_{\min}$, there is a near linear increase of the dependent variable, k, with increasing temperature, until reaching a maximum value, followed then by a rapid decline to zero at temperature $T_{\max}$. The choice of α depends upon the curvature at the maximum level and the rapidity of the decline for high temperatures. If the maximum seems flat, then the exponent $\frac{1}{2}$ would be preferable. This appears to be the case for the data generated in this study. The root mean square error of the residuals from the regression when $\alpha = \frac{1}{2}$ was smaller than that for when $\alpha = 1$. Without many data points near the point of curvature, at temperatures less than and greater than the temperature for which the curve is at its maximum it is difficult to estimate well the curvature. The consequence, statistically, is that the estimate of α from a regression analysis is highly correlated with the estimates of the other parameters resulting in very unstable estimates. Thus α was not used as an unknown parameter and was set equal to $\frac{1}{2}$ for the regression analysis.

For modeling *GOL* and *EGR*, there are two equations, similar to the equations 4, with eight parameters. For temperature outside the interval $T_{\min}$ to $T_{\max}$ the *GOL* time and the exponential growth rates are not defined. Assuming different $T_{\min}$ and $T_{\max}$ values for *GOL* and *EGR* and using the same estimation procedures as described below, the estimate of $T_{\min}$ $(EGR) = 10{\cdot}75°\,\mathrm{C} > T_{\min}(GOL) = 8{\cdot}26°\mathrm{C}$, while both $T_{\max}(GOL)$ and $T_{\max}(EGR)$ were approximately equal to 51°C. The two estimates, $T_{\min}$ (GOL) and $T_{\min}(EGR)$, are not statistically significantly different. For the final model, it will be assumed that $T_{\min}(GOL) = T_{\min}(EGR)$ and $T_{\max}(GOL) = T_{\max}(EGR)$. This assumption decreases the number of parameters from eight to six and thus increases the stability of the estimates and the degrees of freedom associated with the estimates. These assumptions are also considered reasonable because the cells are assumed to begin in stationary phase and thus it is necessary for them to go through the germination, outgrowth and lag phase before exponential growth could begin. By definition once they leave the lag phase, they are in the exponential phase of growth. In addition, as mentioned above, the maximum population density, MPD is considered constant over the range of temperatures for which growth was observed, and was set equal to the maximum measured value. Thus, for modeling the growth characteristics, there are two equations with 6 unknown parameters, a_1, b_1, a_g, b_g, $T_{\min}$ and $T_{\max}$.

$$\begin{aligned} 1/GOL^{1/2} &= a_1(T - T_{\min}) \\ &\quad \times (1 - \exp(b_1(T - T_{\max})))^{1/2} \\ EGR^{1/2} &= a_g(T - T_{\min}) \\ &\quad \times (1 - \exp(b_g(T - T_{\max})))^{1/2} \end{aligned} \tag{5}$$

For each temperature, the means of the estimated *GOL* times and exponential growth rates were calculated (Table 3). The regression analyses were performed on the means which could reasonably be assumed to be independent and closer to having a normal distribution than the individual replicate measurements. Using the mean values rather than the individual replicate values helps simplify calculations of standard errors and confidence intervals.

At the temperature 51°C, no growth of *C. perfringens* was observed for over 3 weeks, or approximately 504 h. Thus, *GOL* times and exponential growth rates could not be estimated. As a result, it is necessary to impute values for these observations. First, relative growth at time equal to the *GOL* time is greater than zero, thus, the *GOL* time would be greater than 504 h. For estimating the parameters of the regression equations, the *GOL* time was assumed to be 5 weeks, or 840 h at temperature 51°C. In addition, since no growth was observed at $t = 504$ h, then, from equation 1, $L(504) = (P - A)/(1 + \exp(-B(504 - M))) \approx 0$. For determining an imputed value for the exponential growth rate, *EGR*, it was assumed, arbitrarily, that there was a relatively small relative growth, equal to 20%. Thus, it is assumed that $L(504) = \log_{10}(1{\cdot}2) = 0{\cdot}079$. The value of $P - A = (P - \log_{10}(N_0))$ in our application, is approximately 8, so that $B(504 - M) = -\ln(8/0{\cdot}079 - 1) = -4{\cdot}6055$. Using equation 3 for the *GOL* time gives a second equation in B and M. Solving yields $B = 0{\cdot}00775$, and thus, $EGR = B(P - A)/4 = 0{\cdot}0155 \log_{10}(\text{cfu ml}^{-1})\text{h}^{-1}$. The inclusion of the observation of no growth at 51°C 'forces' the estimate of T_{max} to be close to 51°C. However, the estimate of T_{max} itself, given the observation of no growth at 51°C is included, is not affected much by small changes, say 50%, of the imputed value for this observation from the one selected. Thus, because this observation has relatively more influence than other data points on the estimates of the parameters, particularly that of T_{max}, and, for a lesser degree, the value assigned is imputed, the weight for this observation in the regression was set equal to 0·5.

Estimates of the six parameters were made, simultaneously, using the seemingly unrelated regression (SUR) procedure of the PROC MODEL routine on PC-SAS®, release 6·12. This estimation procedure minimizes a function of the squares of the residuals weighted by the inverse of the variance–covariance matrix of the residuals. The advantage of estimating the six parameters directly from the two equations using the SAS® PROC MODEL routine is that this procedure takes into consideration the correlations that exist between the parameter estimates and computes directly their variance–covariance matrix. However the determination of degrees of freedom to assign the estimates of the parameters and linear combinations of them is not easy. It suffices to say that direct approximations of the degrees of freedom would depend upon the magnitudes of the weights used in the linear combinations and the underlying correlations between variables. To avoid such complications, the SAS® program assigns degrees of freedom for equations using a simple formula, specifically, in our case, $df = N - p_1 - p_2/2$, where p_1 is the number of parameters that are not in common to both equations, p_2 are the number of parameters that are in common and N is the number of observations. For us, $N = 13$, $p_1 = 2$ and $p_2 = 2$. Thus 10 degrees of freedom are associated with the estimate of the standard error of the residuals for each equation. Based on another formula that SAS® uses, the t-distribution that is used to compute confidence intervals for the both types of parameters has 10 degrees of freedom. Finally, for computing the confidence intervals of predictions, SAS® uses an average based on the degrees of freedom associated with the parameters in the equation. Since all the parameters for out equations have 10 degrees of freedom, thus all estimates would have 10 degrees of freedom.

The mean observed and predicted *GOL* times and exponential growth rates together with standard errors of the predictions derived from the regression and the correlations between them are presented in Table 4. The covariance matrix was computed by approximating the *GOL* time and the exponential growth rates as functions of the parameters using the linear (first partial derivatives) terms of a Taylor series (Rao 1973). Confidence intervals are approximated by using the t-distribution with

Table 3. Mean of estimated *GOL* times (h) and exponential growth rate, *EGR*, ($\log_{10}$(cfu ml^{-1})h^{-1}) of *Clostridium perfringens* in TPGY broth, and corresponding predictions from regressions

Temperature (°C)	Estimate of mean *GOL*	Predicted *GOL*	Standard error of *GOL* prediction	Estimate of mean *EGR*	Predicted *EGR*	Standard error of *EGR* prediction	Correlation of *EGR* and *GOL*
15·0	154·75	100·89	69·23	0·00	0·05	0·03	−0·98
19·0	42·45	30·47	9·11	0·13	0·15	0·05	−0·87
22·0	20·77	17·05	3·38	0·20	0·27	0·05	−0·70
25·0	7·42	10·90	1·69	0·36	0·42	0·06	−0·49
28·0	6·18	7·59	1·04	0·85	0·61	0·07	−0·34
30·0	4·85	6·17	0·82	1·06	0·75	0·08	−0·31
35·0	3·35	4·06	0·49	1·37	1·17	0·12	−0·38
37·0	3·37	3·56	0·40	1·43	1·37	0·15	−0·41
42·0	4·97	2·87	0·28	1·87	1·88	0·19	−0·42
45·0	3·65	2·89	0·32	1·62	2·12	0·19	−0·34
47·5	2·97	3·51	0·53	1·61	2·04	0·23	−0·33
50·0	5·88	8·53	1·84	1·60	1·05	0·23	−0·34
51·0	840·00[a]	376·40	877·00	0·02[a]	0·03	0·06	−0·99

[a] Imputed values.

Table 4. Estimates, standard errors and 95% confidence intervals of parameters used for estimating growth characteristics

Parameter[a]	Estimate	Standard error	Lower limit[b]	Upper limit[b]
a_1	0·020	0·003	0·014	0·027
b_1	0·190	0·076	0·022	0·359
a_g	0·044	0·005	0·032	0·055
b_g	0·419	0·159	0·065	0·773
T_{min}(°C)[c]	10·126	2·223	6·209	16·514
T_{max}(°C)	51·020	0·047	50·915	51·125

[a] $1/GOL^{1/2} = a_1(t - T_{min})(1 - \exp(b_1(t - T_{max}))^{1/2}, ERG^{1/2} = a_g(t - T_{min})(1 - \exp(b_g(t - T_{max}))^{1/2}$ where *EGR* is exponential growth rate.
[b] Confidence limits computed using 97·5th percentile of t − distribution with 10 degrees of freedom.
[c] Based on estimate of natural log transformation, to assure that lower limit exceeds 0°C.

10 degrees of freedom. Thus, 95% confidence intervals are obtained by adding and subtracting 2·228 times the standard error (the 97·5th percentile of the t-distribution with 10 degrees of freedom is 2·228). From Figs 1 and 2, it can be seen that some of the experimentally determined values are outside of the computed 95% confidence interval. While the figures appear to show a 'reasonable' agreement between the predicted and observed transformed values, there are large standard errors of prediction of *GOL* time at temperatures near T_{min} and T_{max}. To help assure estimates of relative growth that would provide an adequate margin of public

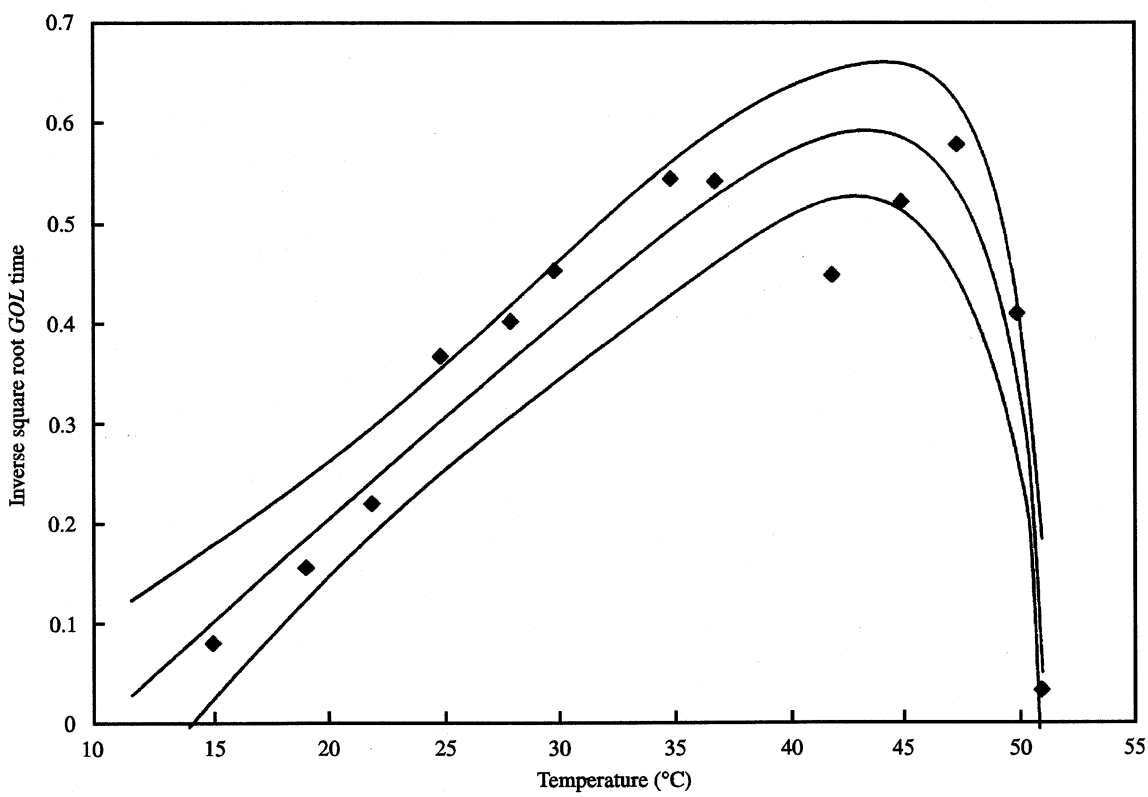

Figure 1. The square root of the reciprocal of the *GOL* time (h) of *C. perfringens* (◆) from this study, predictions and 95% confidence limits (——) vs. temperature (°C).

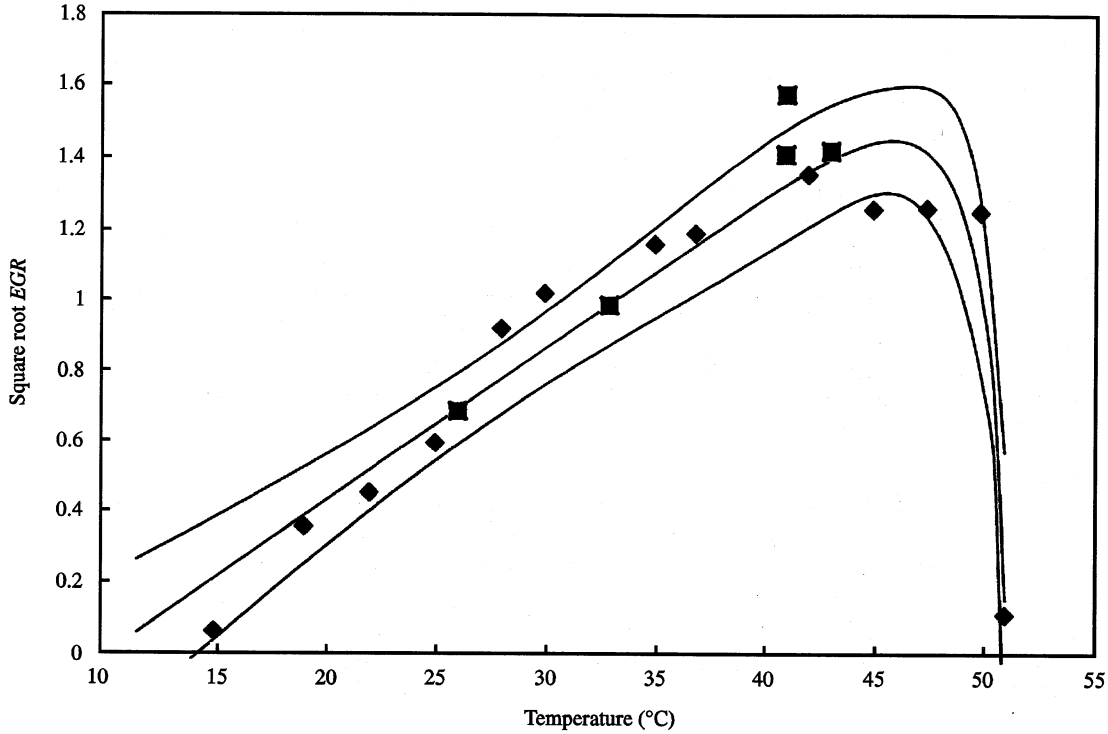

Figure 2. The square root of the exponential growth rate, *EGR*, ($\log_{10}$(cfu ml^{-1})h^{-1}) of *C. perfringens* (◆) from this study, reported values from other studies (■), predictions and 95% confidence limits (——) vs. temperature (°C).

safety, upper confidence limits of growth should be used.

The estimate of T_{min} was 10·1°C, with a 95% confidence interval of 6·2°C to 16·5°C. The relatively low standard error associated with the parameter estimate T_{max} compared to that of T_{min} is because of the significant decline in growth at 51°C compared to the growth at temperatures only slightly lower than 51°C. The model, in this respect, is consistent with other studies which have observed growth at low temperatures (Hall and Angelotti 1965, Shoemaker and Pierson 1976). The exponential growth rates obtained in this study in broth were, in general, consistent with those reported in the literature for meat, as can be seen in Fig. 2. Willardsen et al. (1978) reported that *C. perfringens* strain NCTC 8238 exhibited exponential growth rates of 1·03 and 2·54 $\log_{10}$(cfu ml^{-1})h^{-1} at 33 and 41°C, respectively, in autoclaved ground beef stored aerobically. In the same study, a composite of various strains of *C. perfringens* vegetative cells in autoclaved ground beef exhibited exponential growth rates of 0·46, 0·93 and 2·03$\log_{10}$(cfu ml^{-1})h^{-1} at 26, 33, and 41°C, respectively. From the model (Eqn 5) given in this paper the predicted exponential growth rates and 95% confidence intervals of them, in parentheses, are: for 26°C, 0·48 (0·35, 0·63); for 33°C, 0·99 (0·77, 1·24); and for 41°C, 1·78 (1·39, 2·22). In another study, an estimated exponential growth rate of a *C. perfringens* eight-strain composite in autoclaved ground beef under air at 41°C was 2·05 $\log_{10}$ (cfu ml^{-1})h^{-1} (Willardsen et al. 1979). Labbe and Huang (1995) reported an exponential growth rate of 2·06 $\log_{10}$ (cfu ml^{-1}) h^{-1} in fluid thioglycollate medium plus beef (Difco) at 43°C, which is close to the prediction obtained from Eqn 5: 1·98 with 95% confidence interval of (1·57, 2·43).

Discussion

The model developed in the previous section can be used to predict growth or relative growth for specified temperatures throughout

the entire cooling temperature range of the cooked foods. For the experiments performed for this study all environmental conditions were constant, except for temperature. The pH of the broth was 6·8. It is worth emphasizing that the equations for *C. perfringens* presented in this report, are appropriate for foods of neutral pH, high water activity and absence of other microbial inhibitors.

To apply the equations, it is necessary to specify the initial level or density of organisms, since, from Eqn 1, the prediction of the amount of relative growth depends upon the number or density of organisms, N_0, before growth commences. For example, if $\log_{10}(N_0) = 2$ then the coefficient, $P - A$, in Eqn 1 would be estimated to be 8·4, while if $\log_{10}(N_0) = 4$, then it would be estimated to be 6·4. Thus, when increasing $\log_{10}(N_0)$ by 2, the estimate of the $\log_{10}$ of the relative growth would decrease by approximately 25%, or by close to one generation.

Fig. 3 represents an example prediction curve, together with a 97·5% upper confidence limit, of the $\log_{10}$ transformation of relative growth, $L(t|T)$, at a temperature, T, of 37°C (98·6°F), assuming an initial density of 10^4 spores (so that $P - A = 6{\cdot}4$). The confidence limit was computed by approximating the standard errors using linear terms of the Taylor series and the covariance matrix of the estimates of the growth parameters, and assuming 10 degrees of freedom. To prevent lower confidence limits being less than 0, confidence limits are computed for the natural logarithm of the $L(t|T)$. For 37°C, at a prediction of 1 $\log_{10}$ relative growth a 97·5% upper confidence limit of prediction is approximately 1·73 $\log_{10}$ relative growth. It is predicted that the amount of time for a 1 $\log_{10}$ relative growth would be 3·93 h. A 97·5% lower limit for the time to obtain a predicted 1 $\log_{10}$ relative growth is approximately 3·21 h.

For temperatures below T_{min} or above T_{max} there is predicted to be no growth, and the *GOL* time and the exponential growth rate are not defined. However, because of the

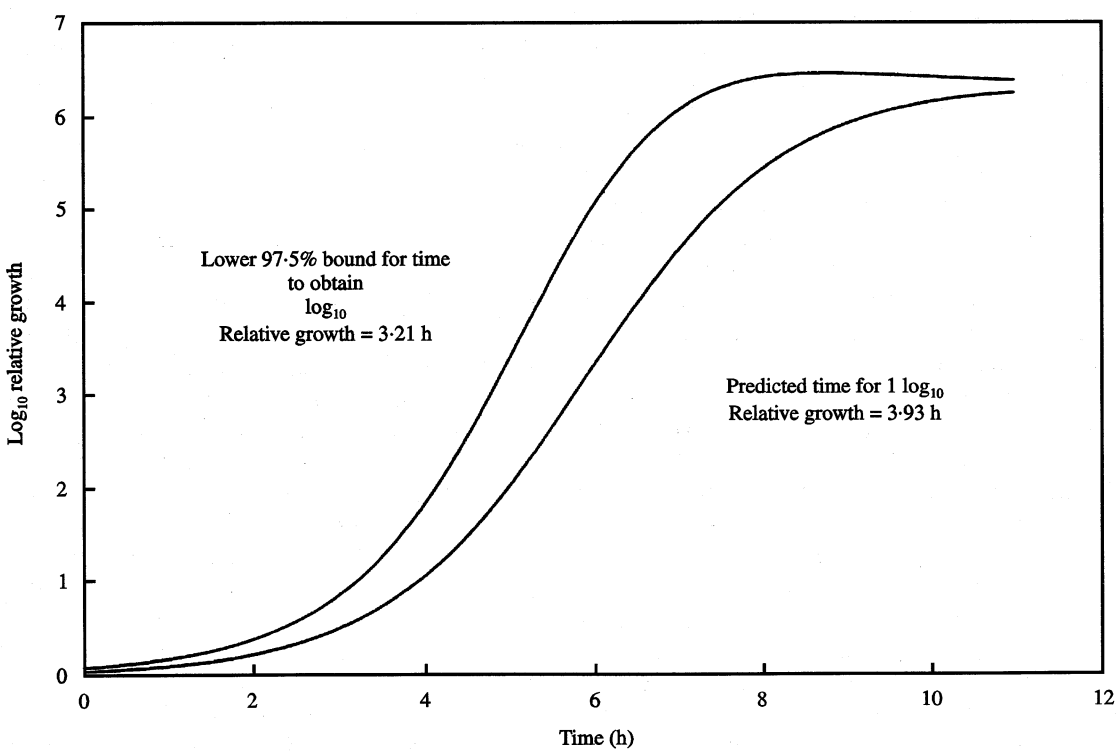

Figure 3. Plot of predicted relative growth with initial density = 10^4 cells, and upper 97·5% confidence limit. Temperature = 37°C (98·6°F); *GOL* time-3·56 h; exponential growth rate = 1·37 $\log_{10}$(cfu ml^{-1})h^{-1}.

uncertainty of the estimates of T_{min} or T_{max}, there is the possibility that there would be growth at temperatures outside the interval (T_{min}, T_{max}). The variance approximation technique using the Taylor series for temperatures outside the interval (T_{min}, T_{max}) cannot be applied. To estimate an upper confidence limit for the predictions of growth for temperatures outside the interval (T_{min}, T_{max}) it is necessary, therefore, to use a more direct and complicated calculation. A brief description is given in an appendix.

For general cooling scenarios, the temperature is changing constantly. Discontinuous cooling followed by a rise in temperature (often due to equipment malfunction or electrical outage) and subsequent continuation of cooling may occur in the food industry and retail food service establishments. The regulatory agencies and the personnel involved in the food preparation need to determine if the product remains safe after such cooling deviations. Thus an expression for determining growth for cooling scenarios is needed. In addition, for the purpose of calculating directly an estimate of the standard error of the predictions, a closed form expression of the predicted relative growth as a function of the parameters estimated from the Ratkowsky equations is needed.

In the usual scenario, temperatures of the warmest section of the product, where organisms might reside, would be monitored. Between times for which temperatures are known, assuming that the ambient air temperature remains nearly the same, it is assumed that temperatures change exponentially (Juneja et al. 1994). Thus, it is assumed that the temperature, T, changes with time by some known continuous function of time, $q(t)$. However, because Eqns 1 and 3 apply for constant temperature one cannot just apply the calculus for calculating relative growth without some assumptions or actual knowledge regarding the impact of prior environmental history on the growth parameters. It is generally assumed that the exponential growth rate is not dependent upon previous environments, while the *GOL* time does depend upon previous environments (Baranyi and Roberts 1994). As the temperature, T, changes, the growth curve, $\varepsilon(T)$, changes. Thus, for example, suppose the initial temperature was T_0, and then, after a time, t_0, the temperature of the product changes suddenly to T_1. From the above assumption, the exponential growth rate can be assumed to be equal to the one associated with curve $\varepsilon(T_1)$, however, the *GOL* time would not be equal to that of curve $\varepsilon(T_1)$. In order to maintain continuity in the estimate of the relative growth moving from one growth curve, $\varepsilon(T)_0$, to the other curve, $\varepsilon(T_1)$, the change on $\varepsilon(T_1)$ could be added to the value of the relative growth at t_0, before the temperature change. The problem is where (for what value of time) do we begin to measure the change on $\varepsilon(T_1)$? Because the *GOL* time is expressed as an additive constant to time, it is proposed that the beginning value for measuring change on $\varepsilon(T_1)$ is just a translation of time proportional to the distance from a specified 'pivot' point, $z(T_1)$, of the curve, which is a function of the temperature, such as *GOL* or *M*. Thus, the beginning point for measuring change on $\varepsilon(T_1)$, t_1^*, is at a distance from $z(T_1)$, which is proportional to the distance that t_0 is from $z(T_0)$. Thus $t_1^* - z(T_1) = (1 - p_0)z(T_1)$ where $p_0 = t_0/z(T_0)$. Continuing, suppose at time t_1 the temperature changes to T_2 and that $p_1 = (t_1 - t_1^*)/z(T_1)$. The starting time, t_2^*, on the growth curve $\varepsilon(T_2)$ is defined by $t_2^* - z(T_2) = (1 - p_0 - p_1)z(T_2)$. Fig. 4 depicts three hypothetical growth curves and shows the 'starting points' on the second and third curves after changes in temperature at time t_0 and t_1. For the ith curve, the starting point, t_i^*, is given by $t_i^* - z(T_i) = (1 - \Sigma p_j)z(T_i)$, where the subscript j is from 0 to $i - 1$.

More generally, assume that $L(t|T)$ can be written as a function $H(k \cdot (t - z(T), \eta)$ where k, z and η are functions of temperature. Let τ be the time such that $q(\tau) = T$. Define $f(\tau)$ to be a function defining the translation from the pivot point $z(q(\tau))$ along the time axis reflecting the accumulated ratios of length of times to pivot points 'spent' on previous growth curves up to time τ, that is, $f(\tau) = z(q(\tau))(1 - \int_0^\tau z^{-1}(q(s))\mathrm{d}s$. Note, this function will be negative once the time exceeds 100% of the *GOL* times. Using the standard calculus (Toralballa 1967) it can be shown that the expected

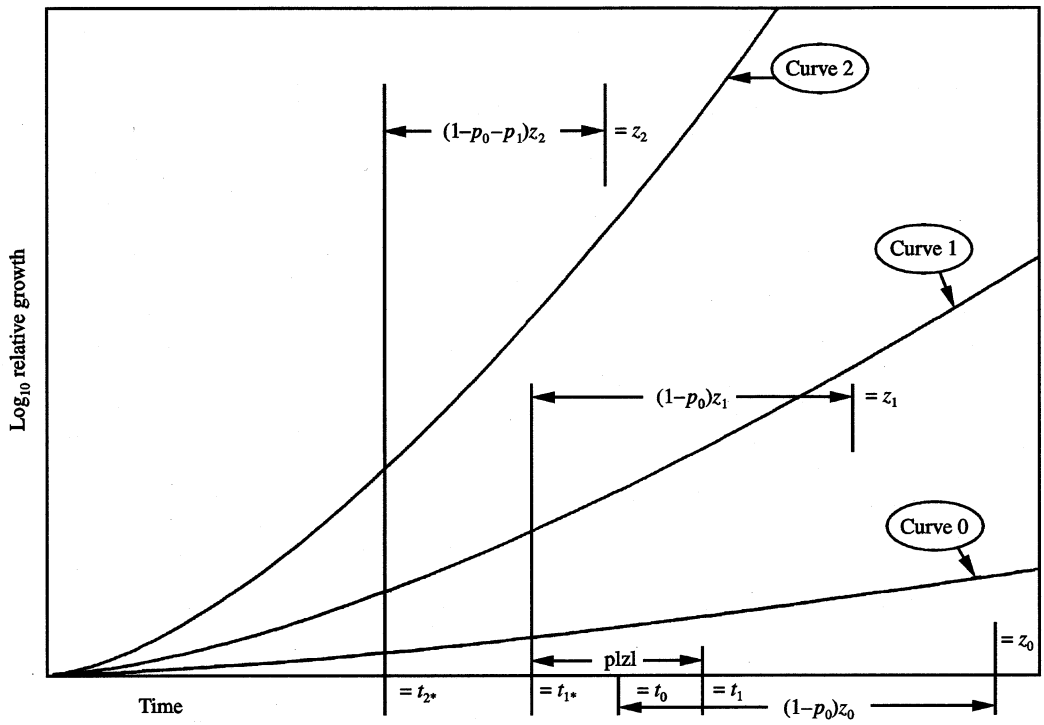

Figure 4. Hypothetical relative growth curves depicting determination of starting times, t_i^*, on curves for measuring relative growth when there are changes in temperature. z = 'pivot' points from which distances on the time axis are measured.

value of $\log_{10}(N(t)/N_0) = E(L(t))$, can be expressed as:

$$E(L(t)) = \int_0^t H'(k(g(\tau))) \cdot f(z(\tau), \eta(g(\tau)))\, d\tau \qquad (6)$$

where H' is the derivative of H with respect to time evaluated at time zero and holding temperature constant. The variance of $E(L(t))$, reflecting the uncertainty of the estimated parameters derived from the Ratkowsky equations, can now be determined by computing partial derivatives of the expression in Eqn 6 with respect to the parameters that are estimated in Eqn 5 and using the Taylor series linear approximation, as described above. While the calculations look formidable, they can be performed with relative ease on computer programs that have mathematical language and calculation capability.

Two suggested pivot points are: M, the time for which the rate of relative growth is equal to the maximal rate of relative growth, *EGR*, or the *GOL* time. Our calculations (performed on Mathcad®, version 7) indicate the choice of a pivot point may not be too critical as would be expected since the slope of the growth curve for times greater than the *GOL* time is close to the *EGR*. For sudden changes in temperature, it is possible that the *GOL* time for the new temperature changes by more than a proportional amount of time spent at the *GOL* time for the old temperature (Zwietering et al. 1994). However, for continuous or slow temperature change, it would be expected that the *GOL* times changes proportionally, as described above by the function f. Experiments are being planned to validate the translation function f, or to determine a more appropriate function. For our calculations, Eqn 6 is used with the function, f, defined above, that uses the *GOL* time as the pivot point. In order to reflect the uncertainty of the estimates, a 97·5% upper confidence limit is calculated by adding to the predicted value 2·228 times the

estimated standard error of prediction obtained from the Taylor series approximation. For a hypothetical cooling of product from 50°C to 10°C in 8 h, the predicted relative growth is 3·37 and the 97·5% upper limit is 6·73.

In summary, this paper presents Ratkowsky type equations (models) for predicting the effect of temperature on *GOL* and *EGR* of *C. perfringens* during cooling of certain cooked meat products. From these equations and assumptions, a closed form expression for the expected relative growth that would occur with changing temperatures during cooling of meat products was developed. The use of this closed form equation enables direct calculation of the predicted relative growth and approximate confidence limits of these predictions. Research is being planned to validate assumptions and equations presented in this paper.

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Appendix A

Calculation of confidence interval of estimated relative growth at temperatures outside interval $(T_{\min}, T_{\max})$.

For a given temperature T, compute $L(t|T, v)$ for every vector, v, in the six-dimensional space ρ_6 that corresponds to the six parameters given in Eqn 3. If $T < v(T_{\min})$ or $T > v(T_{\max})$, where $v(T_{\min})$ or $v(T_{\max})$ represent the component of the vector, v, along the $(T_{\min})$ axis or $(T_{\max})$ axis, respectively, then $L(t|T, v)$ is defined to be equal to 0. To each point, v, a probability measure, $\rho\,(v)$, is assigned. The probability measure is based on the density of the multivariate T^2-distribution, where $T^2 = (v - v_0)'S^{-1}(v - v_0)$ (Anderson 1958), with mean, v_0, equal to the values of the parameters defined in Table 3 and covariance matrix, S, equal to the covariance matrix of the estimates of the growth parameters, assumed to have 10 degrees of freedom. Accordingly, the value $T^2/10[(5/6)]$ is distributed as a central F-distribution with six and five degrees of freedom. Thus, for each vector v, there is curve, $L(t|T, v)$, which depends upon the specified temperature, T, and a probability measure, $\rho(v)$. For given time, t, the elements of the probability distribution: $(L(t|T, v), \rho(v))$ can be used to compute upper confidence limits.