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Predictive Model for Growth of *Clostridium perfringens* during Cooling of Cooked Beef Supplemented with NaCl, Sodium Nitrite and Sodium Pyrophosphate

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Abstract

This paper presents a model for predicting relative growth of *Clostridium perfringens* in ground beef products at different percentages of salt (0 to 3%), sodium pyrophosphate (0 to 0.3%), and nitrite (0 and 200 ppm). The results of the experiments indicates that salt was the primary variable affecting the amount of growth, and that growth in general, was significantly affected by the presence of nitrite. The inclusion of SPP did not significantly improve the model's fit with observed results. The primary growth model, derived from growth experiments at constants temperatures, is based on a common form of Baranyi's growth curves and the secondary model is based on cardinal temperatures, relating maximum specific growth rates as a function of temperature. When product is heated and then cooled, the model predicts an initial decline in the number of cells. The model's predictions compared well with results obtained from dynamic temperatures experiments, for salt concentrations up to 1.5% and nitrite, to 200 ppm, when samples were initially heated and then cooled. The model can be used by processors to evaluate the risk of *C. perfringens* spore germination and outgrowth during cooling (stabilization) deviations or in custom cooling schedules in case the processors cannot follow the USDA FSIS Compliance Guidelines (Appendix A) for Cooling of Heat-Treated Meat and Poultry Products (Stabilization).

Keywords: Cardinal temperature model; Specific growth rates; Spore germination; Spore outgrowth; *C. perfringens*; Cooling models

Introduction

Clostridium perfringens is one of the most commonly reported bacterial agents of foodborne illness in the United States. The United States Centers for Disease Control and Prevention (CDC) estimated that *C. perfringens* causes almost 1 million cases of domestically acquired (USA) foodborne illness annually [1]. The spores of *C. perfringens* are widely distributed in soil, water, air and food and often contaminate raw meat and poultry during slaughter operations. The USDA-FSIS [2] nationwide microbiological baseline data indicate *C. perfringens* prevalences of 10.4, 8.4 and 2.6% in market hogs, cows and bulls, and steers and heifers, respectively. The report does not distinguish, however, between the vegetative cells and the spores, an important consideration for processors who use meat from these species for preparation of processed meat products. Subsequent reports by Kalinowski et al. [3] and Taormina et al. [4] reported lower prevalence (1 and 1.62%, respectively) of *C. perfringens* spores in raw meat blends sampled in meat processing establishments in the U.S. Direct comparison of these measurements is not readily possible because of the different methods of sampling and analytical methods used for the different studies. These estimates in any case show the ubiquitous occurrence of *C. perfringens* that in turn present a hazard that producers should address in their food processing.

The present USDA-FSIS performance standards for lethality prescribe a minimum 6.5-$\log_{10}$ reduction for *Salmonella* spp. in certain meat products containing beef and a minimum 7.0-$\log_{10}$ reduction for *Salmonella* spp for poultry products [5]. Juneja et al. [6] reported D-values at 58°C of 1.15-1.60 min for 10 strains of vegetative cells of *C. perfringens* in a model beef gravy system. These D-values are less than the $D_{58°C}$ values for *Salmonella* spp. (5.4 min in roast beef; 4). Thus, time and temperatures (thermal processes) designed to achieve a 6.5-7.0D reduction in *Salmonella* spp. should also be adequate to destroy vegetative cells of *C. perfringens* in so far as levels of *C. perfringens* generally are not larger than 7log. However, spores of this organism, if present, can survive the traditional heat processing schedules employed by the meat industry. The heat-activated surviving spores become vegetative and thus can germinate, outgrow and multiply during subsequent chilling operations, especially when the cooling is not sufficiently rapid. When food containing large number of vegetative cells is ingested, some cells may survive the acidic stomach environment and sporulate in the intestine, releasing an enterotoxin that is responsible for typical symptoms of food poisoning, i.e., diarrhea and abdominal pain.

The USDA-FSIS compliance guidelines [5-7] for cooling of thermally processed meat and poultry products state the products should be chilled following the prescribed chilling rates, or that customized cooling (stabilization) procedures be validated to control the relative growth of *C. perfringens*. The guidelines state that if cooling of uncured products from 54.4 to 26.7°C occurs within 1.5 h and cooling from 26.7-4.4°C occurs within 5 h [7], the product should not

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have unsafe levels of *C. perfringens*. However, if meat processors are unable to meet the guidance time-temperature cooling schedule, they must be able to document that the alternative or customized cooling schedule used will result in germination and outgrowth of <1.0log$_{10}$ CFU/g for *C. perfringens* and no growth of *C. botulinum* in the finished product. Part of the documentation includes the use of predictive microbiology models to predict the relative growth of *C. perfringens* from surviving heat activated spores, through lag, exponential and stationary phases of growth, at temperatures normally associated with the cooling schedules that have been developed.

Growth kinetics of organisms within food matrices are affected by the addition of chemical additives. Incorporation of salt (NaCl) into meat formulations was shown to inhibit *C. perfringens* spore germination and outgrowth. Zaika [8] reported that increasing salt content of cooked ground beef from 0 to 3% resulted in complete inhibition of *C. perfringens* spore germination and outgrowth even during abusive cooling (exponential) from 54.4°C to ≤8.5°C within 21 h. Phosphate salts are traditionally used in processed meats to enhance the water holding capacity of meat and thus, improve the texture of the meat products. Singh et al. [9] reported that incorporation of sodium acid pyrophosphate (0.3%) into ham resulted in significant inhibition of *C. perfringens* spore germination and outgrowth compared to the control (no phosphate) product. However, Singh et al. [9] reported that the inhibitory effect of the phosphate was minimized when acid and alkaline phosphate (sodium pyrophosphate, tetrabasic) are blended and used in preparation of the ham. The inhibition of sodium acid pyrophosphate can be attributed to the reduction in pH of the meat product when used at normal concentrations (up to 0.6%). Meat processors commonly use blends of pyro- and poly-phosphates (alkaline) to improve the water holding capacity of the meat products through increase of the meat pH beyond the isoelectric point of myofibrillar proteins of the meat. Sodium nitrite is added to processed meat products primarily to fix cured color, through formation of stable nitrosomyoglobin. In addition, research has shown that nitrites have significant anti-botulinal activity and have traditionally been used in the manufacture of canned meat products [10]. The mode of action of nitrite against spore forming bacteria is through inhibition of spore outgrowth subsequent to germination.

In a study by Juneja et al. [11], the growth medium used to determine growth was trypticase-peptone-glucose-yeast extract broth. Later on, predictive models pertaining to the behavior of surviving *C. perfringens* spores during cooling of cooked cured and uncured beef, chicken, and pork were developed [12-23]. To include other environmental factors, Le Marc et al. [24] developed a dynamic model for the effects of temperature, pH and NaCl concentration on the growth of *C. perfringens*. However, there is need for a dynamic model regarding the growth from spores during cooling of cooked beef supplemented with various intrinsic parameters. Accordingly, the aim of the work reported here was to assess the effects and interactions of salt (NaCl), sodium nitrite and sodium pyrophosphate (SPP) on *C. perfringens* growth during cooling that takes place after thermal treatment of ready-to-eat beef products. In addition, the aim was to develop a dynamic model that processors could use to help determine the safety of beef products that have been cooled after thermal treatment.

Materials and Methods

Test compounds, organisms and spore production

Sodium chloride (NaCl), nitrite, and sodium pyrophosphate (SPP) were obtained from Sigma-Aldrich (St. Louis, MO). *Clostridium perfringens* strains NCTC 8238, NCTC 8239 and ATCC 10288 were obtained from the culture collection maintained at the Eastern Regional Research Center, Wyndmoor, PA. Active cultures of each strain were produced in 10 ml of freshly prepared fluid thioglycollate medium in screw-cap tubes. Duncan and Strong sporulation medium was used for spore production as previously described [25]. Spore crop of each strain was heat-shocked at 75°C for 20 min and the total population was determined by spiral-plating (Autoplate 4000 Spiral Plater, Gaithersburg, MD) appropriate dilutions (in 0.1% peptone water), in duplicate, on tryptose-sulfite-cycloserine (TSC) agar (Difco) without added cycloserine as described previously [26]. Each spore crop was washed twice and then, the spore suspensions in sterile distilled water were stored at 4°C. A mixture of equal number of all three strains of *C. perfringens* spores was prepared immediately prior to use. This spore strains composite was heat-shocked prior to conducting experiments.

Preparation and inoculation of meat, sampling times and bacterial enumeration

Ground beef (93% lean) was obtained from a local grocery store and stored under refrigeration for a maximum of two-three days prior to use. The meat was separated into batches for different treatments and mixed thoroughly using a Kitchen Aid mixer (Kitchen Aid Co., St. Joseph, MI) with the additives to be tested. Each batch received various concentrations of sodium chloride (NaCl; 0 to 3%, wt/wt), sodium pyrophosphate (SPP; 0 to 0.3%, wt/wt), and/or sodium nitrite (0 or 200 ppm, wt/wt). Thereafter, the meat with additives was stored at -5°C until used (approx. 40 d). A day prior to the experiment, the meat were thawed overnight at ~4°C in a refrigerator. Duplicate 5 g-samples were aseptically weighed into low-oxygen transmission Whirl Pak bags (18-oz/532-ml capacity; 4.5" W × 9.0" L; 11 cm × 23 cm; barrier film 0.125 cc oxygen transmission per 100-in. square in 24 h [Part no. B01300; Nasco, Modesto, CA]) and aseptically inoculated with 0.1 ml of the heat-shocked three strain spore cocktail of *C. perfringens* to attain a final spore populations of ca. 3.0log$_{10}$ CFU/g. The bags were first massaged manually and then, blended with a stomacher (Interscience Mini Mix, St. Nom, France) for 2 min to ensure even distribution of the spores in the meat samples. Negative controls included bags containing meat samples inoculated with 0.1 ml of 0.1% (w/v) peptone water with no bacterial spores. After meat in the bags was flattened by pressing against a flat surface, the bags were evacuated to a negative pressure of 1000 millibars and heat-sealed using a Multivac gas-packaging machine (Model A300/16, Multivac Inc., Kansas City, MO). Both uninoculated raw meat and 5 g heat-shocked samples were used to confirm the absence of naturally occurring *C. perfringens*.

For assessing growth of spore inoculum at constant temperatures (isothermal experiments), all samples were incubated in a constant temperature water bath maintained at 15, 20, 25, 30, 34, 37, 40, 43, 46, 49, and 51°C. Two independent trials/replications were carried out at each temperature. Two bags for each replicate were then removed at predetermined time intervals. The total number of sampling times was about 8-10 at each temperature and the sampling frequency was based on growth temperature. The samples were analyzed for *C. perfringens* count enumeration by spiral-plating on tryptose-sulfite-cycloserine (TSC) agar (Difco) as described above, and the plates were incubated for 48 h at 37°C in a Bactron anaerobic chamber (Bactron IV, Sheldon Laboratories, Cornelius, OR). *C. perfringens* colonies were enumerated and the counts were recorded as CFU/gram of meat. For each experiment, at a given time, two plating counts were recorded and the data was used for estimates of the growth kinetics. In total there were 4,244 plate counts.

To validate the model, 16 dynamic temperature experiments, at levels of salt of 0% and 1.5%; nitrite, 0 ppm and 200 pm; and SPP of 0% and 0.3%, where specific cooling rates were varied (temperatures were programmed to decrease exponentially with time), where, for each over time, plate counts were measured, as described above. Model predictions were compared with the obtained results.

Statistical methods

Growth model from isothermal experiments: Let N(t) be the expected level of cells (cfu/ml) for a sample for a given temperature, T, and time, t. For each growth experiment, the primary growth model used was:

$$\ln\left(N(t)\right) = f\left(t|\theta\right) + \varepsilon$$
$$N(t) = \lambda(t)/r \quad (1)$$

x(t) ~ Poisson (λ(t))

where f(t|θ) is the primary growth model described below, θ are unknown fixed parameters whose values are estimated from the data, x(t) was the observed sample count at time t (the sum of the two plate counts for the bag), r is a factor that transforms plate counts to levels (cfu/ml) and depends on the dilution and the fractional portions of the plate that were counted in order to get countable numbers of colony forming units for the sample, λ(t) is the expected number of cfu for sampled portions at time t, and ε is an error term representing the between-sample error, assumed to be normally distributed with zero mean and standard deviation σ. The function f(t|θ) that we used is based on a model developed by Baranyi and Roberts [27].

$$f\left(t|\theta\right) = y(0) + \mu A(t) - \ln\left(1 + \frac{e^{\mu A(t)} - 1}{e^{m - y(0)}}\right) \quad (2)$$

where

$$A(t) = t + \mu^{-1}\ln\left(\frac{e^{-ut} + q_0}{1 + q_0}\right) \quad (3)$$

and y(0) is the value of f(t|) at t=0, representing the natural log of the initial level, μ=μ(T) is the maximum specific growth rate at temperature T, and q_0=q_0(T) is a constant that serves as an initial value in a set of differential equations that express the growth dynamically. The two parameters μ and q_0 are related to the lag phase duration, Lag, for the population of cells as follows [28]:

$$Lag = \mu^{-1}\ln\left(1 + 1/q_0\right) \quad (4)$$

Thus, the product of μ and Lag is a function of q_0 alone. It is this product, or more precisely the natural logarithm of this product, ζ, that was used in the subsequent analysis to determine a model for μ.

The maximum specific growth rate, μ(T), as a function of temperature, have been modeled [29]:

$$\mu(T)^{1/2} = a\left(T - T_{min}\right)\left[1 - \exp\left(b\left(T - T_{max}\right)\right)\right]^{1/2} \quad (5)$$

or (Rosso et al., 1995):

$$\mu(T) = \frac{\mu_0\left(T - T_{min}\right)^2\left(T - T_{max}\right)}{\left(T_0 - T_{min}\right)\left[\left(T_0 - T_{min}\right)\left(T - T_0\right) - \left(T_0 - T_{max}\right)\left(T_0 + T_{min} - 2T\right)\right]} \quad (6)$$

where *a*, *b*, T_{min}, and T_{max} for eq. (5) or μ_0, T_0, T_{min}, and T_{max} for eq. (6) are constants, for given matrix conditions, whose values are estimated from the isothermal experiments. The temperature parameters, T_x, x=0, min, max, referred to as the cardinal temperatures (thus the model referred to as the cardinal temperature model), are the temperatures for which, respectively, the maximum specific growth rate is largest, at any temperature below there is no growth, and for which any temperature above there is no growth. Thus, for temperatures less than T_{min} or greater than T_{max}, μ(T) was set equal to zero. The two sets of curves (eqs. (5) and (6)) are for the most part indistinguishable. In this paper we use eq. (6) to describe μ(T), and account for the effects of salt, SPP and nitrite by assuming the cardinal temperatures and μ_0 are simple functions of these independent variables. Some opinions consider the parameters T_{min} and T_{max} to be universal constants, independent of matrix effects. We do not believe that this constraint is necessary for purposes of modeling growth; rather, allowing them to be functions of matrix effects might provide better fits to the data and thus provide overall predictions that are more accurate. In describing the effects of the independent variables of salt, SPP and nitrite levels on the parameters of μ and q_0 various simple data analyses were performed to determine which of the variables might be important for predicting growth. The variables considered as dependent variables were φ=ln(μ), ψ=$\mu^{1/2}$, and the ζ=ln(μLag). In terms of ζ, q_0=[exp(e^{ζ}) − 1]$^{-1}$. From eqs. (2) and (3), f(t), for a given t, is an increasing function of q_0, and thus since q_0 is a decreasing function of ζ, f(t) is a decreasing function of ζ. The value of q_0 is sensitive to values of ζ; even what might appear to be small changes in the value of ζ (within a standard deviation unit), can have a large impact on q_0.

We also examined the secondary model presented in Le Marc et al. [24] based on eq. (5), but with multiplicative terms accounting for the effect of salt. This model involves first calculating water activity (aw) as a function of salt (S): aw=1-S(5.2471+1.2066S)/10^3 [18], and then multiplying eq. (5) by[(aw-aw_{min})(2-aw-aw_{min})]$^{1/2}$, where aw_{min} is a parameter with value estimated through statistical analysis. The parameter aw_{min} represents the water activity value for which at values less, there would be no growth.

Estimates of parameter values were obtained by maximum likelihood estimation (MLE), assuming ψ and ζ are distributed as a bivariate normal distribution. Univariate analyses for ψ and μ were also performed and, while nearly the same estimates for ψ were obtained, the standard errors of them were smaller within the bivariate model.

From the model for the specific growth rate, μ, estimates of growth for different scenarios were made, assuming temperature declines with specified specific cooling rates. The value of q_0 being conceptualized as a characteristic of the cells before growth commences and can depend upon the surrounding environment, was set at a value given in Juneja et al. [30], q_0=0.005628.

Validation: Because samples are initially heated, we explored the possibility that there would be an initial decline in the number of cells. For modeling inactivation, we assumed the Weibull inactivation model. The model predictions were based the Baranyi and Roberts [28] differential equations with an adjustment for inactivation,

$$\frac{dy(t)}{dt} = \mu(t|)\left(\frac{1}{1 + e^{-Q(t)}}\right)(1 - \exp(y(t) - m)) - b\theta t^{b-1}$$
$$\frac{dQ(t)}{dt} = \mu(t|)) \quad (7)$$

where y(t) is the natural logarithm of the level at time t, μ is the maximum specific growth rate, Q(t) is a function of factors that control the growth [27], m is the natural logarithm of the maximum population level, and b and θ are positive valued parameters describing the inactivation. Boundary conditions are at time=0, a known number of cells in the population and the value of q_0=exp(Q_0), given above. The

parameters μ, θ and b are possibly functions of temperature and the other food chemistry parameters. For calculating predicted levels using eq. (7), we used the trapezoidal rule with equal subintervals of 0.0005 h.

Analyses were performed using SAS® version, 9.2 and 9.3, in particular PROC NLMIXED. Tests for comparative goodness-of-fit for different models were based on the various information criteria measures (e.g., Akaike Information Criteria, AIC) and the deviance (=-2log likelihood) that are standard in the SAS® output, and the likelihood ratio test when models were nested. Graphs were constructed using S-Plus®, version 8.

Results and Discussion

Primary model

C. perfringens spore germination and outgrowth was not observed at temperatures ≥51°C, whereas significant growth was observed at 15°C for all isothermal experiments except for those that had a salt level of 3%, SPP level of 0, and nitrite level of 200 ppm. For all isothermal growth curves using the model given in eqs. (1)-(3), convergence, with computable variance error matrices, were obtained when parameters were estimated to be non-zero (two experiments had estimated lag time=0). Data from the two experiments (one at 30°C and the other at 46°C) with no measurable lag times were deleted from the analyses.

Secondary model

For modeling the maximum specific growth rates, a preliminary simple analysis was conducted to fit ordinary least squares (OLS) cubic equations to $\psi=\mu^{1/2}$ by each level of a variable to evaluate the relationships. Figures 1-3 provide plots of experiment-specific estimates of ψ and fitted OLS cubic curves for each level of each specified variable. These curves are displayed only to help the reader see trends in the data. Figure 1 shows distinct, nearly non-overlapping, curves that are in a decreasing relationship with salt levels where the lowest curves (with the lowest specific growth rates) are associated with the largest salt values. For SPP (Figure 2), this relationship was not observed. For nitrite there are two, nearly non-overlapping, distinct curves, where the lower curve is for nitrite=200 ppm (Figure 3), but their separation is not as discernible as the one observed for sodium

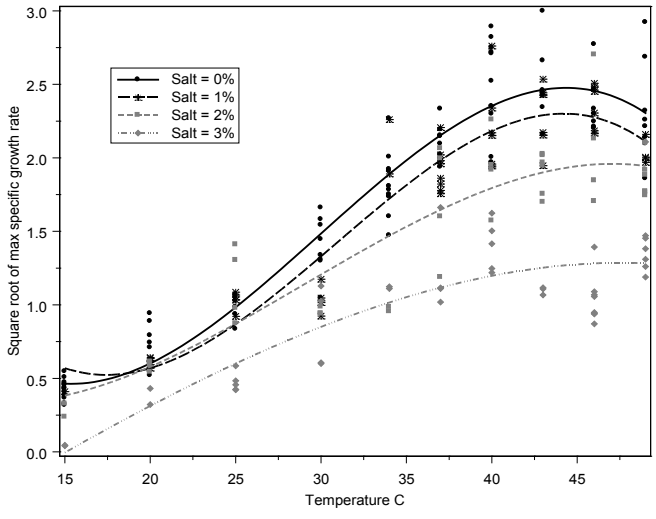

Figure 1: ψ - the square root of the experiment specific growth rate, versus temp. °C, by salt concentration.

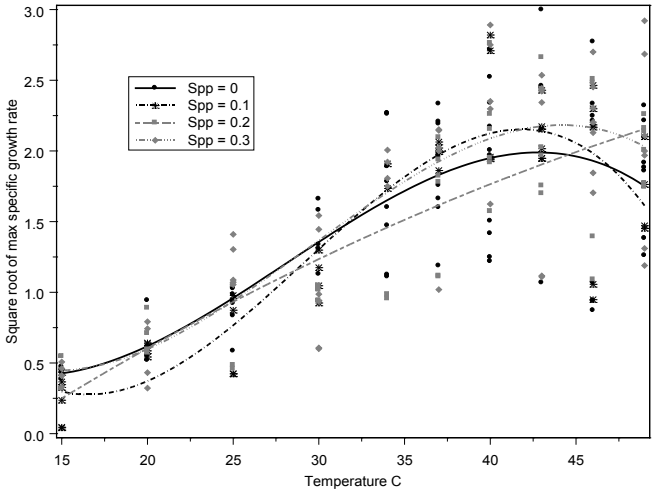

Figure 2: ψ - the square root of the experiment specific growth rate, versus temp. °C, by SPP concentration.

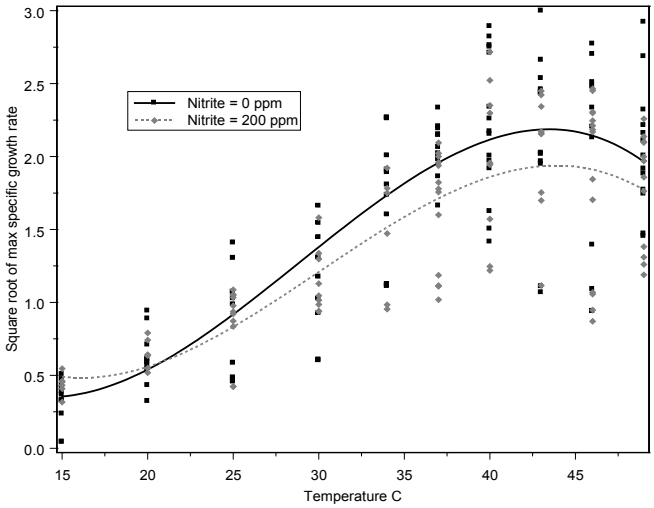

Figure 3: ψ - the square root of the experiment specific growth rate, versus temp. °C, by nitrite concentration.

chloride. The implication is that higher levels of sodium nitrite would reduce the maximum specific growth rates, only a limited amount.

Regarding ζ, for temperature, sodium chloride, and SPP, taken individually, there were not significant linear relationships with ζ. Figure 4 presents the plot of the averages of the values of ζ for each design combination, versus temperature, where the data points are distinguished by levels of sodium nitrite (0 ppm and 200 ppm). The horizontal lines are at the means for the two nitrite levels. Analysis of variance with the design-specific average values of ζ as the dependent variable indicated a statistical significant nitrite effect (p-value=0.007, after discarding two values that were less than -1).

Another simple analysis was conducted to determine the impact of one variable in a 'direct' fashion. The Spearman correlations were computed between the levels of a variable and estimates of ζ and φ (averaged over the two replicate experiments within a design point), and the signs of these correlations were used to test for trends: the numbers of positive and negative Spearman correlations were computed and

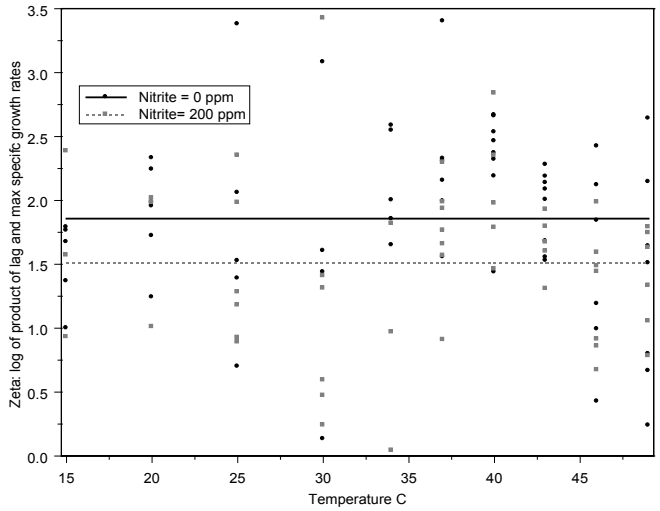

Figure 4: ζ - the natural log of the product of μ and Lag, a function of the physiological constant, q_0, versus temperature °C, by nitrite.

were tested against the null hypothesis of a binomial distribution with probability parameter equal to ½. Table 1 presents the results of the analysis. Of particular note is the comparison for nitrite levels with ζ: 14 of the 23 comparisons had negative correlation possibly suggesting larger values of ζ for nitrite=0 ppm compared to those for 200 ppm, everything else being equal, though the relationship is not statistically significant.

An initial model was developed based the observations made above. Subsequent to the development of the model, improvements to the model were attempted by adding or subtracting terms and using the likelihood ratio test as a goodness-of-fit criterion.

The first issue is the function of μ to be used as the dependent variable of regressions. It is natural to consider, φ=ln(μ) as the dependent variable because this often homogenizes the variance over the range of experiments (homoscedastic). The problem though is at 15°C, where some of the values of μ are close to zero, and thus the values of φ become negative with large absolute value and thus highly influential on the model. It is for this reason that $\psi=\mu^{1/2}$ was selected to be the dependent variable. The error structure thus was assumed to depend on the temperature or expected value of ψ. While many functions could be used, the function we used was,

$$\sigma_\psi = f + ge^{-\exp(\rho m)} \quad (8)$$

where m is the predicted expected value of μ given the values of the independent variables, and f, g and ρ are constants estimated (3 parameters). This was found to provide a satisfactory fit (as shown below).

Experiments with the same values for the independent variables (same design point) were run in duplicate (two replicates). The estimated values of ψ and ζ for the duplicate experiments were averaged, (denoted as ψ_a and ζ_a, respectively) and used as the dependent variable. The function used for μ is given in eq. (6). Thus it is necessary to specify the parameters: T_{min}, μ_0, T_{max}, and T_o as functions of the independent variables. There was no growth of *C. perfringens* for salt=3% and nitrite=200 ppm at 15°C whereas for levels less than 3% salt there was growth whether or not the nitrite level was at 200 ppm; thus, it is reasonable for initial analyses to assume T_{min} is function of salt level alone; nitrite was not included as a variable for T_{min}. From Figure 1, it appears that the relationship of salt and T_{min} might not be linear, so a non-linear increasing relationship is assumed (3 parameters), of the form:

$$T_{min} = h_1 + be^{cSalt} \quad (9)$$

where h_1, b and c are constants to be estimated. To be sure that b and c are positive, the actual parameters used in the model was h_2=ln(b) and h_3=ln(c). Eq. (9) ensures that T_{min} is an increasing function of salt. Also, from Figures 1 and 3, it seems reasonable to initially assume that μ_0 is a quadratic function of salt and with an effect for nitrite (4 parameters):

$$\mu_0 = h_4 + h_5 s + h_6 s^2 + h_7 Nit \quad (10)$$

where s and Nit are the (standardized) salt and nitrite levels, respectively, and $\{h_j, j=4,\ldots,7\}$ are parameters, with unknown values. The standardized value for salt is obtained by subtracting 1.5 from the salt value, and the standardized value of nitrite is obtained by subtracting 100 from the nitrite value and dividing the difference by 100. From Figure 1, it appears that T_0 increases with salt levels, thus T_0 was assumed to be a linear function of salt (2 parameters):

$$T_0 = h_8 + h_9 s \quad (11)$$

where h_8 and h_9 are constants, and s is the standardized salt value. The value of T_{max} is more difficult to ascertain from our experiments other than it is most likely near 51°C, and was assumed constant for all experimental conditions (=h_{10}). Thus, for this basic model for ψ, there are 10 fixed parameter values, $\{h_j, j=1, \ldots,10\}$, plus 3 parameters associated with the variance structure, giving a total of 13 parameters. For ζ, three parameters are used: two for describing the expected value of ζ: a constant plus an effect for nitrite, and one for the residual standard deviation, σ_ζ. One more parameter, η, is needed to describe the correlation between ψ and ζ. Thus, the initial model has 17 parameters, 10 fixed ones that define the specific - growth model.

For the above basic model, convergence was obtained. Assuming σ constant increased the deviance measure by 33.4, which, by the likelihood ratio test with 2 degrees of freedom, is statistically significant (p-value=5.6×10^{-8}). Figure 5 presents a Q-Q plot of the standardized residuals for $\psi = (\psi_a - \hat{\psi})/\hat{\sigma}_\psi$ where $\hat{x}$ is the estimate of x (where x is a parameter). Tests for non-normality were marginally significant with the Shapiro-Wilk test p-value equal to 0.014, and the Kolmogorov-Smirnov p-value equal to 0.069. Re-running the model without the observation associated with the point in the upper left of Figure 5 decreases the deviance measure by 16.4, which, using the Bonferroni approximation, has p-value of 0.0057. The Shapiro-Wilk test for non-normality of the standardized residuals for the rerun model (deleting the outlier data point) for ψ has p-value equal to 0.56, thus normality of these residuals can be accepted. For the standardized residuals for ζ, the Shapiro-Wilk test for non-normality has p-value equal to 0.25, while the other tests' p-values range from 0.15 to 0.17. Thus, with the one

	Variable		
Dependent variable	Sodium nitrite (N=23)	Sodium chloride(N=39)	SPP (N=39)
φ	(-)3.47	(-)0.024	(+)52.2
ζ	(-)40.5	(+)52.2	(+)100[a]

[a]19 correlations were negative and 20 were positive.

Table 1: Two-sided p-values (%) for sign test of Spearman correlations of dependent variable and specified independent variable, holding the others constant. The direction of the correlation[a] is given in parentheses: (-) means that most of the correlations were negative implying a decreasing relationship of dependent variable.

outlier data point eliminated, the underlying assumption of a bivariate normal distribution for ψ and ζ with eq. (7) describing σ_ψ provides a good fit. Figure 6 provides plots of the predicted standard deviation, σ_ψ, as a function of the predicted values of ψ (eq. (8)), together with the standard deviations of the residuals within intervals of length 0.25 of the predicted values of ψ. The plot shows a good fit. The correlation, η, of the residual errors associated with ψ_a and ζ_a was estimated to be 0.8477 with standard error of 0.0278. Figure 7 provides plots of the residuals versus the predicted values of ψ for the model given by eqs. (6)-(11). The plot does not indicate any obvious outlier data points.

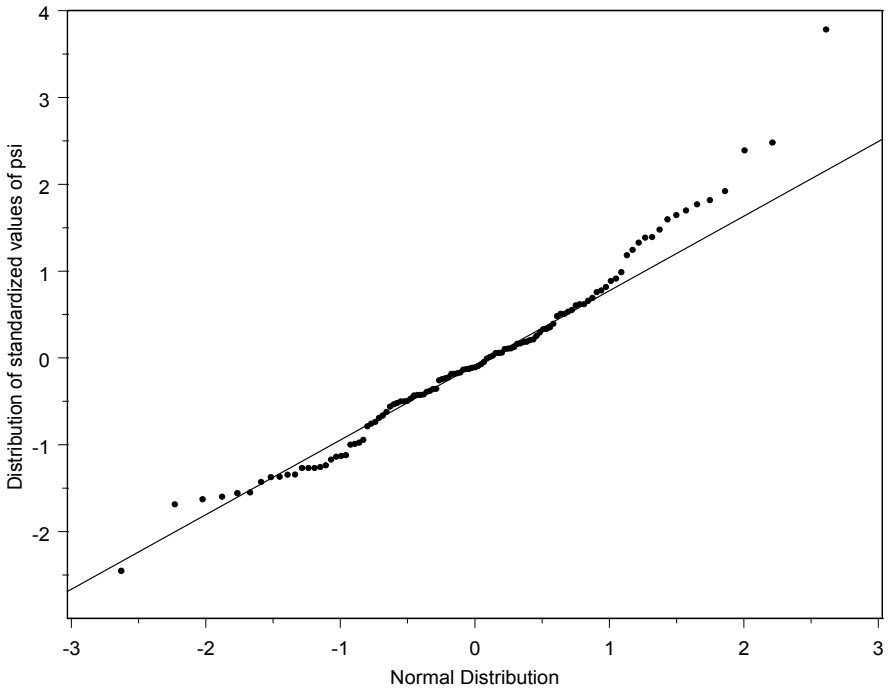

Figure 5: Q-Q plot for standardized residual for secondary model of ψ.

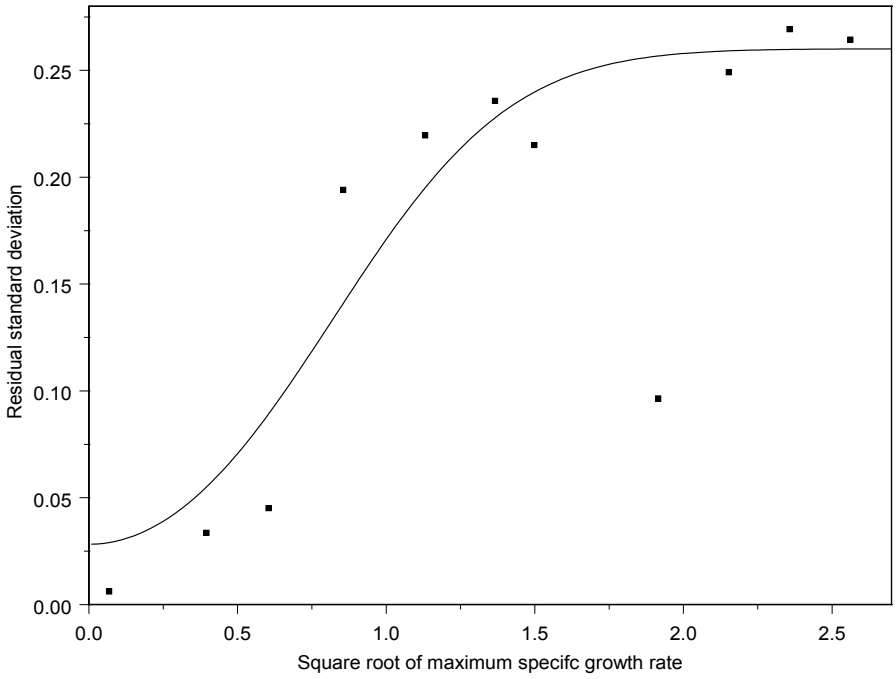

Figure 6: Predicted residual standard deviation, σ_ψ, as a function of the predicted values of ψ (eq. (8)), together with the standard deviations of the residuals within intervals of length 0.25 of the predicted values of ψ.

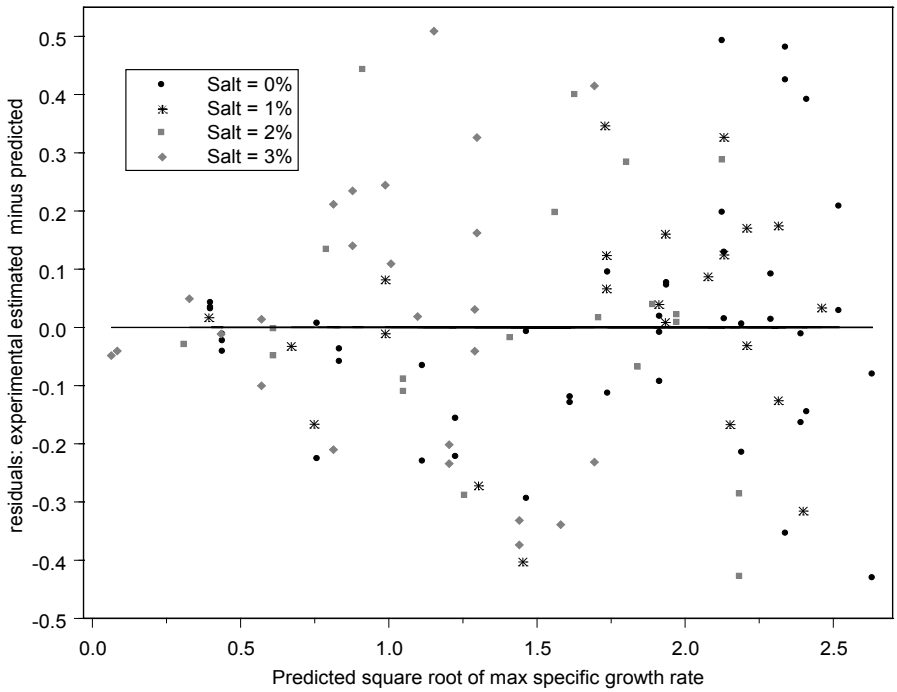

Figure 7: Plots of the residuals of ψ versus the predicted values of ψ for the model given by eqs. (9)-(11).

The quadratic coefficient, γ, in eq. (10) for μ_0, was estimated to be -0.302 with standard error of 0.0733 and was statistically significant with p-value about 2×10^{-4} by the likelihood ratio test; the fitted quadratic function over the range of salt from 0 to 3% was decreasing as we assume it should be. Adding nitrite interactions to the linear and quadratic terms in eq. (10) for μ_0 in the basic model was not significant with p-value=0.38 for the linear interaction term alone, and 0.63 for both (with 2 degrees of freedoms). Adding nitrite effects for T_0 in the basic model did not provide significant improvement, with p-value=0.26. Assuming T_{min} (eq. 9) constant in the basic model increased the deviance measure by 20.3, which has associated p-value of about 4×10^{-5} with 2 degrees of freedom.

A model based on that given in Le Marc [24] was also fitted by multiplying eq. (5) by $(aw\text{-}aw_{min})(2\text{-}aw\text{-}aw_{min})$, as explained in the *Statistical Methods* section. This model includes the same assumptions used in the model developed above based on eq. (6) for the minimum and maximum growth temperature, T_{min} and T_{max}, ζ, and the error structure. For eq. (5), describing the maximum specific growth, there are 4 parameters besides T_{min} and T_{max}: two associated with the parameter *a* in eq. (5) to account for the two levels of nitrite (0 and 200 ppm), *b*, and aw_{min}. For the model developed above based on eq. (6), there are 6 parameters (eqs. (9) and (10)), besides T_{min} and T_{max}, describing the maximum specific growth. Thus, the model based on eq. (5) has two fewer parameters than the model developed above, based on eq. (6). The AIC measure of goodness of fit for the model based on eq. (5) is greater than that for the model based on eq. (6) by about 29 implying a substantially poorer fit to the data obtained by the Le Marc model based on eq. (5), compared to that obtained by the model based on eq. (6). An example of the impact of this poorer fit for the Le Marc model based on eq. (5) is the derived estimate of 54.7°C, with a standard error of 1.84°C, for the maximum temperature, T_{max}, for which growth occurs. This estimated maximum growth temperature is much greater than that expected based on previous research (REF); this together with the large standard error, indicates that Le-Marc model applied to these data is providing the possibility of maximum growth temperature exceeding 58°C. This compares to the obtained estimate of T_{max}, from the model described in the previous paragraphs based on eq. (6), of 50.5°C with standard error of 0.84°C, thereby providing a range of estimated maximum growth temperatures aligned with previous research results (REF). Because of the substantially poorer fit obtained by the Le Marc model based on eq. (5), we rejected this model and selected the model based on eq. (6).

Table 2 provides the estimates of the values of the 10 fixed parameters, $\{h_j, j=1,\ldots,10\}$, defined from eqs. (9)-(11), used to describe the maximum specific growth rates, μ. Also included are the standard errors of each estimated parameter value and the correlation error matrix.

Regarding the relationship of ζ to the nitrite level, the model based estimated mean values were 1.46 and 1.83, respectively for nitrite levels of 0 and 200 pm, with a residual standard deviation σ_ζ=0.642. The average of the two estimates is about 1.65. The corresponding values of q_0 for the average is 0.00556 (with standard error of 0.00177), which is close to the value of q_0 derived for beef matrices for dynamic temperature change starting at 54°C of 0.005628 [30]. Because the different means of values of ζ for nitrite at 0 ppm and 200 ppm are not that largely different compared to the estimated residual standard deviation, σ_ζ (=0.643), and the trends here and from Table 1 are in opposite directions where the latter was not statistically significant, our model does not account for a possible nitrite effect on ζ for estimating relative growth. For applying the model derived in this paper, we assume q_0 constant, equal to 0.005628 - the value derived from dynamic experiments (REF), rather than the value derived from these experiments because the latter value of q_0 was estimated from data that reflects the dynamic environment for which this model is to be used. In summary, this model for predicting the log of the relative growth has 11 fixed parameters: the 10 given by eqs. (8)-(10) (first row

of Table 2 (Estimates)) and a value of q_0=0.005628, the physiological constant (eq. (1)).

Figure 8 depicts predicted growth curves for values of salt=0, 1, 2, and 3% and for nitrite=0 ppm and 200 ppm, assuming exponential cooling, where temperature as a function of time is determined from:

$$T(t) = T_a + (T_i - T_a)e^{-kt} \quad (12)$$

where T_a is the ambient temperature, T_i is the initial temperature and k is the exponential cooling rate. In the example used to generate the predicted growth curves of Figure 8, T_a=3°C, T_i=54.4°C, and k=0.3808. For this temperature curve, the temperature declines from 54.4°C to 27°C in 2 hours, and to 4°C in another 8.35 h.

Comparison of predictions of growth during dynamic temperature change

A commonly used model to evaluate the safety of processed meat products involved in cooling process (stabilization) deviations is the one from USDA-ARS-Pathogen Modeling Program (PMP; cooked uncured ground beef and beef broth models). We compare predictions of relative growth obtained from our model and from the PMP for four temperature profiles, given in Figure 9. The PMP does not have a model for salt levels greater than 0%; for the cured product, nitrite levels were 120 ppm rather than 200 ppm used in this study.

The USDA-Food Safety and Inspection Service (FSIS) compliance guidelines for cooling of cooked, ready-to-eat (RTE) meat and poultry products [7] recommend cooling from 54.4°C to 26.7°C within 1.5 h and subsequently, from 26.7°C to 4.4°C within 5 h or, alternatively, from 48.9°C to 12.8°C within 6 h when cooling has started within 90 min after the end of the cooking cycle. Considering these Compliance Guidelines, the four profiles for which we make relative growth predictions can be considered significant deviations from the guidelines. USDA-FSIS recommends using predictive models to evaluate the safety of the products involved in such process deviations under certain circumstances.

Parameter	h1	h2	h3	h4	h5	h6	h7	h8	h9	h10
Estimate	9.44117	-5.45785	0.81901	4.98941	-1.35775	-0.30155	-0.60403	47.88823	0.95446	50.54300
Std. err	0.27584	4.52272	0.65804	0.20238	0.10400	0.07336	0.09729	0.34864	0.31920	0.82008
h1	1.00000	-0.52524	0.51424	-0.27416	0.24655	0.10004	0.05188	-0.05913	0.45068	0.49370
h2	-0.52524	1.00000	-0.99955	0.18963	-0.10538	-0.34760	0.05108	-0.24612	-0.32257	-0.09051
h3	0.51424	-0.99955	1.00000	-0.18965	0.10412	0.35366	-0.05682	0.24333	0.31402	0.08507
h4	-0.27416	0.18963	-0.18965	1.00000	-0.27754	-0.56329	-0.17363	0.29207	-0.07675	-0.43655
h5	0.24655	-0.10538	0.10412	-0.27754	1.00000	-0.21570	-0.19301	0.36695	0.75586	0.65819
h6	0.10004	-0.34760	0.35366	-0.56329	-0.21570	1.00000	-0.07780	-0.05382	-0.10899	-0.12956
h7	0.05188	0.05108	-0.05682	-0.17363	-0.19301	-0.07780	1.00000	-0.28646	-0.17654	0.04962
h8	-0.05913	-0.24612	0.24333	0.29207	0.36695	-0.05382	-0.28646	1.00000	0.56581	0.05973
h9	0.45068	-0.32257	0.31402	-0.07675	0.75586	-0.10899	-0.17654	0.56581	1.00000	0.59087
h10	0.49370	-0.09051	0.08507	-0.43655	0.65819	-0.12956	0.04962	0.05973	0.59087	1.00000

Table 2: Estimated parameter values for estimating maximum specific growth rates (eqs. (9)-(11)), together with standard errors and error correlation matrix.

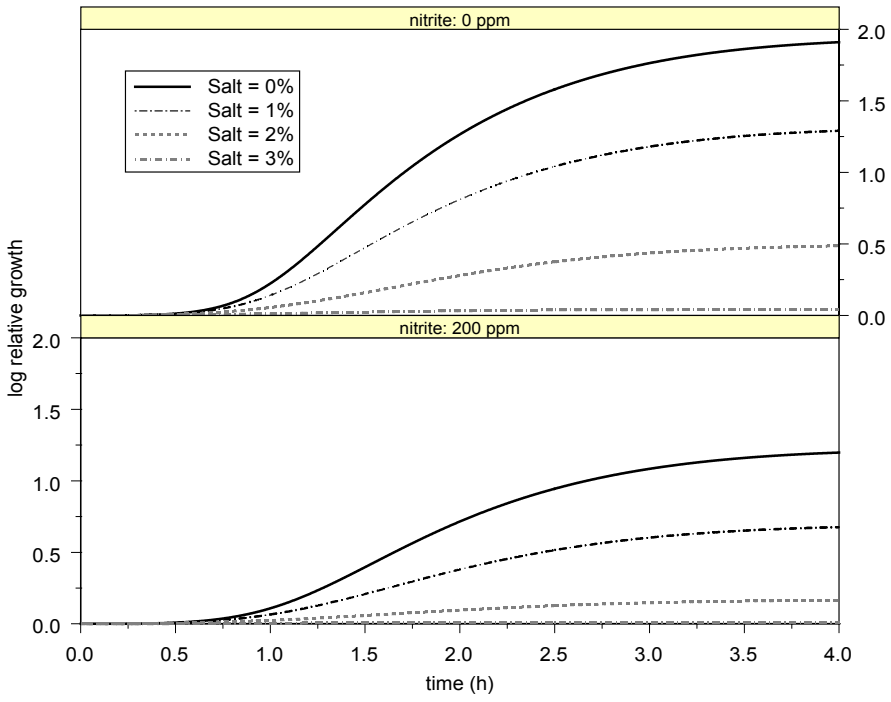

Figure 8: Predicted growth curves (log10 relative growth versus time (h)) using model of this paper (eq. (8)-(10)) for various salt and nitrite combinations, with exponential cooling, such that the temperature declines from 54.4°C to 27°C in 2 hours, and to 4°C in another 8.35 h.

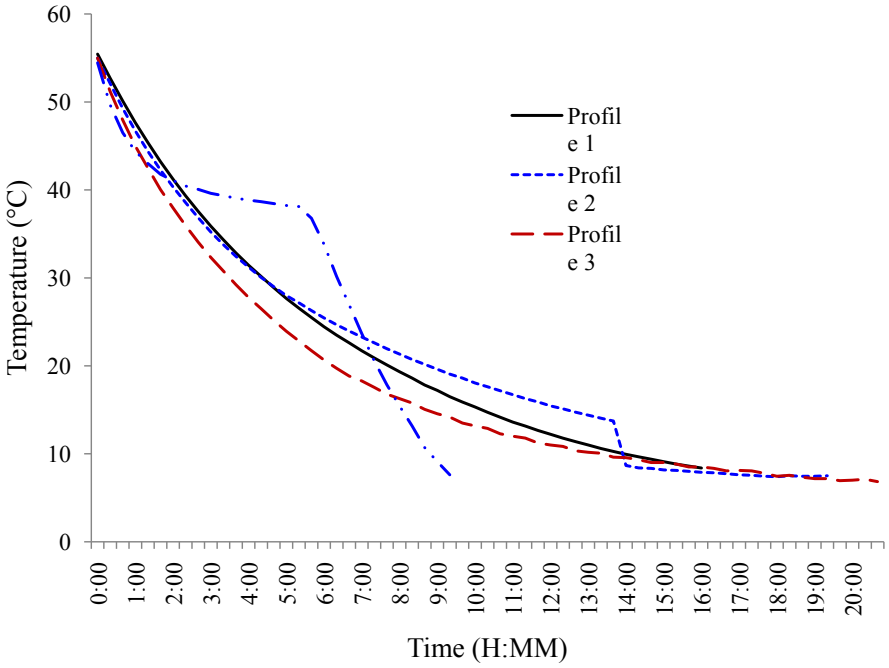

Figure 9: Temperature profiles used to make comparisons of predicted growth curves derived from the model of this paper and the PMP.

Temperature profile	log increase	Our model (h)	PMP[a] (h)	PMP[b] (h)
1	1	2.14	1.75	4.00
1	2	2.66	2.21	16.00
2	1	2.04	1.68	4.00
2	2	2.46	2.17	8.00
3	1	1.94	1.62	5.00
3	2	2.40	2.17	>20.00
4	1	1.77	1.49	3.50
4	2	2.16	1.94	4.00

PMP[a]: Ground Beef Model; PMP[b]: Beef Gravy Model.

Table 3: Predicted times to 1- and 2-log growth of *C. perfringens* in beef products as predicted by our model, PMP-ground beef model and PMP-beef gravy model, following four assumed process deviations.

Table 3 shows the predicted times for *C. perfringens* spore germination and outgrowth for 1- and 2-log growth for the PMP ground beef, PMP beef gravy and our present model (assuming zero salt and zero nitrite, with initial level of 1log). This model's predicted times for 1- and 2-log growth of *C. perfringens* from spore population were greater than those obtained from the PMP model from ground beef (for all the temperature profiles) but by only about 15 to 30 minutes, depending on the profile. The PMP beef broth model predicted times that were much greater than the times predicted by our present model and the PMP ground beef model, at minimum almost 2 hours longer. Figure 10, as an example, shows the growth curves predicted from the model of this paper and the PMP ground beef model for uncured product (salt=0% and nitrite=0 ppm) for the first temperature profile, assuming salt=0% and nitrite=0 ppm (the other profiles are similar and are not shown). Comparing the predicted log growth for 3 hours for the 4 temperature profiles, the predictions for this model are: 2.99, 3.15, 3.08, and 4.06log, respectively. From the PMP ground beef model, the corresponding predictions are 3.48, 3.45, 3.19, and 4.36log, respectively. The differences of predicted times and growths are not large, where the predictions from the model in this paper suggest slightly less growth for a given time.

Considering comparisons, however, when nitrites are not zero, the differences are not small, and in the opposite direction from that seen for the uncured product. For example, comparing the predicted log growth for 3 hours, assuming 200 ppm for the model of this paper, for the 4 temperature profiles, the predictions are: 2.13, 2.26, 2.20, and 2.97log, respectively. Assuming salt=0% and nitrite=120 ppm, from the PMP cured beef model, the corresponding predictions are: 0.41, 0.45, 0.43, and 0.74log, respectively. The model of this paper thus does not predict nitrite to be as effective in inhibiting growth, as does the PMP model. In this sense, the model of this paper provides more conservative (fail-safe) estimates of growth than those that would be obtained using the PMP in that if the former were used in designing processes or for determining the safety of product in light of a cooling deviation, the estimates of growth would be larger, and thus decisions based on the former model would better ensure safe levels of *C. perfringens* in cooled ready-to-eat beef products.

Validation results

For the 16 first measurements of level (at 2 hours) after the initial measurements, 10 were less than the measured initial levels, with an average reduction (on the log scale) of about 0.17log. For the salt

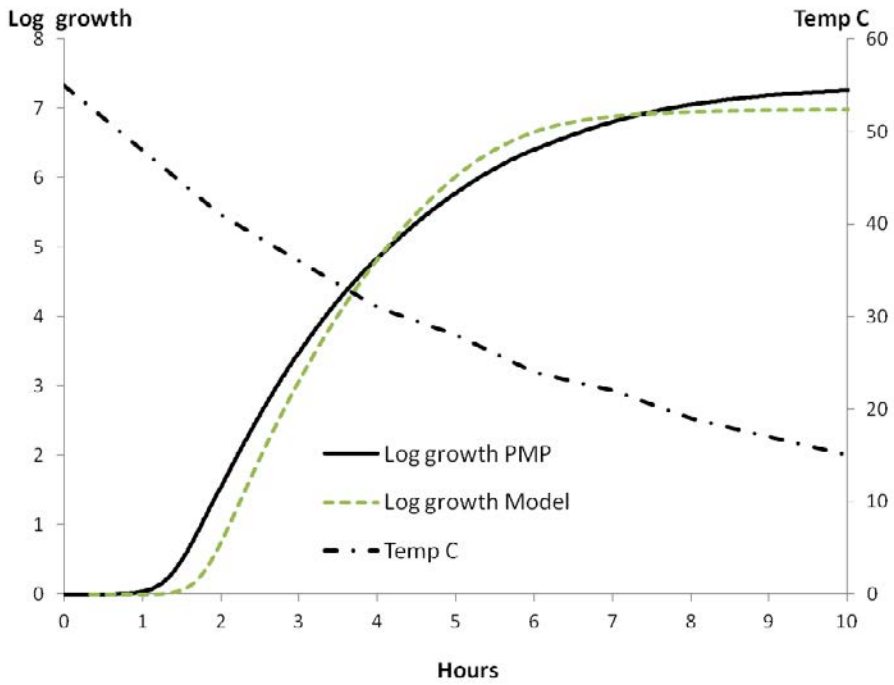

Figure 10: Growth curves for uncured product (salt=0% and nitrite=0 ppm) for the first temperature profile, assuming salt=0% and nitrite=0 ppm. For model in paper (dashed light line) and PMP ground beef (solid dark line).

Variable	α_1	α_2	$\ln(\alpha_3)$	α_4
Estimate	-1.0716	1.3812	-2.7652	-0.3058
Standard	0.9364	0.1981	0.6262	0.04737
α_1	1	-0.5238	0.4061	-0.2027
α_2	-0.5238	1	0.5283	-0.143
$\ln(\alpha_3)$	0.4061	0.5283	1	-0.1489
α_4	-0.2027	-0.143	-0.1489	1

Table 4: Estimates of parameter values for inactivation model, eq. (13). The first two rows are the point estimates and estimated standard errors. The last four rows are the error correlation matrix.

level of 1.5%, 8 of the 12 measurements were negative, while for the 4 measurements with salt level equal to 0%, 2 of the four were negative. The percentages of negative results (observed reduction) increased with increasing cooling rates. These results are consistent with the premise of initial die-off of cells, that rate of which is a function of the salt level and environmental temperature. The model for b and θ (eq. (7)) selected was:

$$\log it(b) = \alpha_1$$
$$\ln(\theta) = \alpha_2 + \alpha_3(T - 54.4) + \alpha_4 S \quad (13)$$

where T is the temperature (°C), S is the salt level, and α_j, j=1,…, 4 are parameters whose values are estimated using maximum likelihood estimation. Temperatures were controlled to decrease in time according to eq. (12) with ambient temperature (T_a) equal to 0°C and k=0.169, 0.202, 0.253, or 0.337. We assume that the inactivation effects would dissipate over time. To reflect this, we assume a convex shaped Weibull inactivation model given in eq. (7), with b<1. Because of our assumed restriction 0<b<1, we modeled the logit of b to help avoid possible boundary problems in estimation. In addition, for the same reason, we modeled the natural log of θ and the natural log of α_3.

The deviance measure for the model given in eq. (13) was 66.8. This compares to the deviance measure of 258 for the model that does not assume inactivation of cells; the difference of 191.7, with 4 degrees of freedom is highly significant. Without a term accounting for Salt (assuming α_4=0), the deviance was 96.8, thus including salt provides a statistically significant improvement of fit. When separately adding a linear effect for SPP and nitrite for the ln(θ), the improvements of goodness of fit were not statistically significant (p-value=0.46, and p-value=0.15, respectively). When adding both variables, the p-value for the improvement of fit was 0.21, not statistically significant. The p-value for adding a linear effect for the salt for estimating logit(b) was 0.83. Therefore, the model given in eq. (7) was accepted.

Table 4 provides the estimates of the parameters and their asymptotic standard errors and error correlation matrix. The estimate of the Weibull shape parameter, b=0.25510 and α_3=0.06296. Figure 11 provides a plot of the residual versus time, and Figure 12 provides the observed and the predicted curves of the logarithm of the levels divided by the levels at time=0 versus time. The data point associated with the large residual of nearly 1.25 was not included in the analysis; including it in the analysis increased model deviance to 76.9 from 66.8, with one degree of freedom, which has p-value of 0.0014. There are 80 data points, so that accounting for multiple comparisons this rejected data point is an outlier at less than a p-value of 0.115.

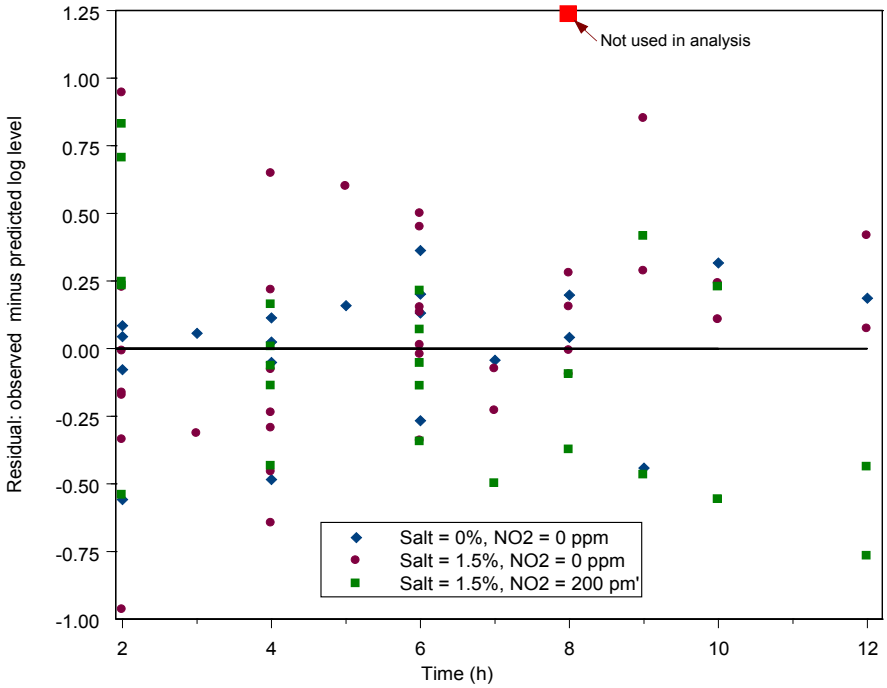

Figure 11: Residuals (observed minus predicted log levels) versus time (h) for dynamic temperature model (eq. (12)).

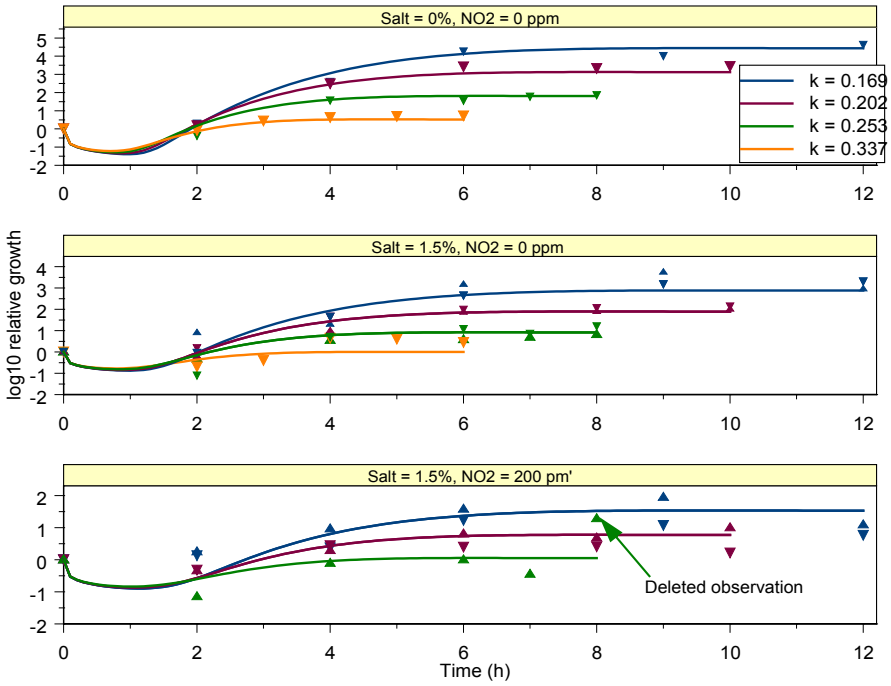

Figure 12: Observed and predicted curves of log of ratio of level to initial level (t=0) versus time (h) for different dynamic growth experiments. Downward triangles represent data for which SPP was 0%, upward triangles represent data for which SPP=0.3%.

Conclusion

This paper presents a model for estimates of growth of *C. perfringens* during cooling of cooked ground beef. The model is based on a common form of the Baranyi and Roberts [27] growth model for describing primary growth for given environmental conditions, the cardinal temperature model for describing the maximum specific growth for different environmental conditions, and the Weibull inactivation model accounting for possible die-off before growth takes place. We have not seen this last feature in models depicting the growth of *C. perfringens* during cooling of ready-to eat product but including

it enables the model to make accurate predictions of growth during the cooling scenarios examined in this paper. Our results show that the addition of salt has an inhibiting effect on the growth of *C. perfringens*, though not as strong as implied by Zaika [8] who reported that increasing salt content of cooked ground beef from 0 to 3% resulted in complete inhibition of growth. The results though did not show, consistently, an inhibiting effect of sodium pyrophosphate (SPP) on the specific growth rates, at least in comparison to that of salt and, to a lesser extent, sodium nitrite. Without the inactivation component, the model in this paper provided estimates of growth that are conservative (fail-safe) compared to those obtained from the pathogen modeling program of USDA-ARS. The model can be used by the processors to evaluate the risk of *C. perfringens* spore germination and outgrowth during thermal process deviations during cooling (stabilization) or in custom cooling schedules in case the processors cannot follow the USDA FSIS Compliance Guidelines for Cooling of Heat-Treated Meat and Poultry Products (Stabilization). The model though strictly applies to the food matrix that was studied; processors should validate this model for their specific cooked/heat-treated, not shelf stable meat and/or poultry products.

The results of this paper suggest further research is needed regarding the expected behavior of vegetative *C. perfringens* cells once thermally treated product begins to cool. After spores become vegetative, they are still in an environment subjected to heat that could result in, and our model predicts, cell inactivation. This cell die-off could be significant in predicting levels of *C. perfringens* in product and gauging the amount of growth that took place particularly if the product's temperature remains above the maximum temperature for *C. perfringens* growth for a long period. Without accounting for this, models could over predict levels in product. For risk assessments, this could lead to inaccurate predictions of risk due to *C. perfringens* in ready-to eat product.

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