Predictive Models for Growth of Foodborne Pathogenic Spore-Formers at Temperatures Applicable to Cooling of Cooked Meat

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Abstract

Inadequate cooling of foods in retail food operations is a major safety problem. Accordingly, the objectives of these studies were to determine safe cooling rate for cooked beef and develop models to predict the germination, outgrowth and lag (GOL), and exponential growth rates (EGR) of *Clostridium perfringens* and *Clostridium botulinum* from spores. *C. perfringens* and *C. botulinum* growth from spores was not observed at a temperature of < 15 _C or > 51_ C and < 12 _C or > 48_C, respectively, for up to three weeks. First, we demonstrated the effectiveness and validity of "square- root model" under non-isothermal conditions. Next, we developed two models, one each for *C. perfringens* and proteolytic *C. botulinum,* to predict their growth from spores at temperatures applicable to the cooling of cooked meat. It was found that for *C. perfringens*, the use of the logistic function provided a better prediction of relative growth than the use of the Gompertz function. For *C. botulinum,* growth curves were determined by fitting Gompertz functions to the data. From the parameters of the Gompertz or logistic function the growth characteristics, GOL times and EGR, were calculated. These growth characteristics were subsequently described by Ratkowsky functions using temperature as the independent variable. By applying multivariate statistical procedures, the standard errors and confidence intervals were computed on the predictions of relative growth for a given temperature. Closed form equations were developed that allow prediction of growth for a general cooling scenario. The predictive models should aid in evaluating the safety of cooked product after cooling and thus, with the disposition of products subject to cooling deviations.

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Introduction

Clostridium perfringens and *C. botulinum* are anaerobic, gram-positive, spore-forming, rod-shaped, bacteria. *C. perfringens* continues to remain a major cause of foodborne illness which results after the ingestion of a large number of viable vegetative cells which have grown in the implicated meat or poultry product. *C. botulinum* is the most hazardous spore-forming foodborne pathogen because it produces deadly neurotoxic protein, known as botulinum neurotoxin, which causes a neuroparalytic disease known as botulism.

Improper storage and/or inadequate cooling practices in retail food operations have been cited as cause of food poisoning for 97% of *C. perfringens* and 34% of *C. botulinum* outbreaks (Bean and Griffin 1990). Such outbreaks clearly stress the importance of cooling foods quickly after cooking. The time/temperature compliance guidelines for cooling cooked meat and poultry products recommends that the maximum internal temperature should not remain between 130F (54.4C) and 80F (26.7C) for more than 1.5 h nor between 80F (26.7C) and 40F (4.4C) for more than 5 h (USDA 1999). The U. S. 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 21C (140 to 70F) in 2 h and from 21 to 5C (70 to 41F) in 4 h (FDA Code 1999).

The aim of the present work was to acquire quantitative data on the growth from spores of *C. perfringens* and *C. botulinum* over the entire growth temperature range which foods must pass through during cooling after cooking and developed models to predict the relative growth of the pathogens from spores at temperatures relevant to the cooling of cooked products.

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Materials and Methods

Test Organisms and Spore Production

Three strains of *Clostridium perfringens*, NCTC 8238 (Hobbs serotype 2), NCTC 8239 (Hobbs serotype 3), and NCTC 10240 (Hobbs serotype 13) and two strains each of Proteolytic *C. botulinum* type A and B strains were used in this study. *Clostridium perfringens* spore were produced in Duncan and Strong sporulation medium as described previously (Juneja et al. 1993). Proteolytic *C. botulinum* type A and B spores were prepared by anaerobically growing each strain in BAM broth (Huhtanen 1975) at 35°C for 3 weeks. Each spore preparation was stored at 4°C in sterile distilled water. Spore population was enumerated by spiral plating (Spiral Biotech, Bethesda, MD; Model D) appropriate dilutions (in 0.1% peptone water), in duplicate, on tryptose-sulfite-cycloserine agar without cycloserine, i.e., SFP agar for *C. perfringens* or Reinforced clostridial medium (RCM) for *C. botulinum* followed by incubation of plates anaerobically for 48 h at 35°C. A spore cocktail was prepared immediately prior to experimentation by mixing equal numbers of spores of *C. perfringens* (heat-shocked, 20 min/ 75°C) or *C. botulinum* (heat-shocked, 10min/ 80C) from each suspension.

Sample Preparation and Inoculation Cooking and Cooling Procedures

Duplicate 3 g ground beef samples were aseptically weighed into 15 × 22.9 cm sterile whirl-pak sampling bags (Model B736, NASCO Modesto, CA) and inoculated with heat-shocked *C. perfringens* or *C. botulinum* spore cocktail so that the final concentration of spores was approximately 1.5 $\log_{10}$ cfu/g. Thereafter, the bags were manually mixed to ensure even distribution of the organisms in the meat sample. The bags were evacuated to a negative pressure of 1,000 millibars and heat sealed using a Multivac Model A300/16 packaging machine (Germany). Racks holding the ground beef samples were fully submerged in 4.4°C water in a water circulating bath (Exacal, Model Ex-251HT, NESLAB Instruments, Inc., Newington, NH). To initiate cooking, the bath temperature was raised in a linear fashion to 60°C within 1 h. The cooling study was performed through the temperature range of 54.4°C to 7.2°C by adding ice to the stirred water bath at varying rates in order to simulate the desired cooling rate (Table 1).

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, Saint Louis, MO); and 0.4% dextrose was used for determination of growth rates of *C. perfringens*. 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. For *C. botulinum*, Reinforced clostridial medium (RCM; Difco) was used for determination of growth rates. The medium (50 ml) was dispensed in tubes and sterilized by autoclaving. The medium was supplemented with oxyrase (0.1 ml/5 ml broth medium) and then incubated at 35°C for 30 min prior to the experiments. Each flask/tube received 0.5 ml of the heat-shocked spores to obtain an initial count of about 3-4 $\log_{10}$ spores/ml of the growth medium. The flasks were then flushed with sterile N_2 for 10 min and sealed with a rubber stopper. All samples were incubated at temperatures ranging from 10-50°C (2°C increments). Samples were withdrawn periodically, serial dilutions made in 0.1% peptone water (wt/vol) (supplemented with oxyrase, 0.1 ml/ 5 ml, for *C. botulinum*), and then surface plated with a Spiral plater (Model D, Spiral Biotech, Bethesda, MD) onto appropriate media mentioned above. The total *C. perfringens*/*C. botulinum* population was determined after 48 h of incubation at 37°C in a Bactron anaerobic chamber (Sheldon Manufact. Inc., Cornelius, OR).

Results and Discussion

When hot cooked food is allowed to cool, the temperature must pass through a range that is favorable for pathogenic spore germination and multiplication of the vegetative cells. Safe and hazardous cooling times and temperatures for cooked ground beef containing *C. perfringens* spores are shown in Table 1. When *C. perfringens* spores in cooked ground beef were cooled from54.4 to 7.2°C in 12 h, and 15 h, no out-

TABLE 1. Safe and Hazardous Cooling Times and Temperatures for Cooked Ground Beef Containing C. perfringens Spores

Elapsed	Cooling time-temperature	
Time (h)	54.4 to 7.2°C in 15 h[a]	54.4 to 7.2°C in 18 h[b]
1	46.4	47.6
2	39.6	41.7
3	33.9	36.6
4	29.1	32.2
5	25.0	28.3
6	21.6	25.0
7	18.7	22.1
8	16.2	19.6
9	14.2	17.4
10	12.5	15.6
11	11.1	13.9
12	9.8	12.5
13	8.8	11.3
14	7.9	10.2
15	7.2	9.3
16	—	8.5
17	—	7.8
18	—	7.2

[a] Safe cooling time
[b] Hazardous cooling time

growth of the spores was observed. However, *C. perfringens* spores germinated and multiplied when a 18 h cooling time was followed. Rapid growth occurred between 22.1°C (7 h) and 15.6°C (10 h); the generation time was 25.5 min (Juneja et al. 1994).

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 the Ratkowsky model,

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

where a, b, T_{min}, and T_{max}, are unknown positive parameters, is usually either 1 or ½, provides a good statistical fit. we demonstrated the effectiveness and validity of this "square-root model" in predicting spore outgrowth during cooling(Figure 1). In a similar study, when ground beef samples inoculated with spores of *C. botulinum* were cooled from 54.4 to 7.2°C using cooling times varying from 6 to 21 h, spores germinated and grew, and the population densities increased by 1 log unit in 21 h (Juneja et al.1997).

Fitting Growth Curves

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

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

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)), or the logistic function: h(t|M, B) = 1/(1+exp(–B(t – M))).

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). For *C. perfringens*, 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 (≤ 22°C) than use of the logistic function, but for temperatures above 22°C (≥25°C), on the average, the logistic was better in the primary region of concern. Thus, for predicting relative growth as a function of time and temperature, the logistic function is used. For *C. botulinum,* the averages of the residuals (observed minus predicted relative growth) were: –0.065 using the logistic function and 0.025 using the Gompertz function. Thus, the Gompertz function is used for modeling the relative growth.

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 growth rate, d(L(t))/dt, with units $\log_{10}$(cfu/ml)/h (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:

$$\begin{aligned} egr &= B(P - A)/e \\ GOL &= M + (e \cdot g(0) - 1)/B \end{aligned} \quad (3)$$

TABLE 2. Estimated GOL Times (h) and Exponential Growth Rate ($\log_{10}$(CFU/ml)/h) of *Clostridium perfringens* in TPGY Broth for Gompertz and Logistic Curves; and *Clostridium botulinum* in RCM broth for Gompertz Curves

Temperature	Approximate[a] GOL time Gompertz	Approximate[a] GOL time logistic	Exponential growth rate Gompertz	Exponential growth rate logistic
		Clostridium perfringens		
15.0	-72.654	154.752	0.0033	0.0049
19.0	39.057	41.412	0.1383	0.1339
22.0	18.380	25.323	0.1582	0.1928
47.5	2.686	2.819	1.5155	1.5148
50.0	5.759	6.024	1.5356	1.6256
		Clostridium botulinum		
12	66.597	—	0.0610	—
15	34.652	—	0.0681	—
40	6.306	—	1.2112	—
46	2.943	—	0.4723	—
48	2.425	—	0.5840	—

TABLE 3. 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
		Clostridium botulinum		
a_1	0.015	0.002	0.011	0.019
b_1[c]	0.361	0.231	0.088	1.478
a_g	0.033	0.005	0.022	0.044
b_g[c]	0.167	0.096	0.047	0.592
T_{min}[c]	6.299	1.301	3.997	9.926
T_{max}	50.012	0.009	49.993	50.031

[a] $1/GOL^{1/2} = a_l(t\text{-}T_{min})(1\text{-}exp(b_l(t\text{-}T_{max}))^{½}$, where GOL is the germination, outgrowth and lag time (h), $EGR^{1/2} = a_g(t\text{-}T_{min})(1\text{-}exp(b_g(t\text{-}T_{max}))^{½}$, where EGR is exponential growth rate(log_{10}(cfu/ml)/h) .
[b] Confidence limits computed with 11 degrees of freedom.
[c] Based on estimate of natural log transformation, to assure positive confidence limits.

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

$$1/GOL^{1/2} = a_l(T - T_{min})(exp(b_l(T - T_{max}))^{1/2}$$
$$EGR^{1/2} = a_g(T - T_{min})(exp(b_g(T - T_{max}))^{1/2} \quad (4)$$

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. As can be seen from Table 2, the estimated values of GOL time and EGR are similar for the logistic and Gompertz functions for *C. perfringens*.

TABLE 4. Mean of Estimated GOL Times (h) and Exponential Growth Rate, EGR, (log_{10}(CFU/ml)/h) 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
				Clostridium perfringens			
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
37.0	3.37	3.56	0.40	1.43	1.37	0.15	-0.41
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
				Clostridium botulinum			
12.0	69.29	133.48	39.24	0.06	0.04	0.01	-0.78
15.0	38.91	57.30	10.34	0.08	0.08	0.02	-0.49
25.0	19.19	12.41	2.09	0.67	0.38	0.07	-0.50
37.0	6.12	4.65	0.84	0.50	0.92	0.15	-0.54
40.0	6.45	3.93	0.66	1.36	1.01	0.17	-0.47
46.0	3.52	3.60	0.80	0.50	0.85	0.22	-0.51
48.0	3.17	4.84	1.66	0.65	0.55	0.18	-0.54

[a] Germination, outgrowth and lag.

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 modeling the growth characteristics, GOL and EGR, there are two equations with 6 unknown parameters, a_l, b_l, a_g, b_g, T_{min}, and T_{max}.

$$\begin{aligned} egr &= B(P - A)/4 \\ GOL &= M + (4h(0) - 2)/B \end{aligned} \qquad (5)$$

For each temperature, the means of the estimated GOL times and exponential growth rates were calculated and used to determine unknown parameters (Table 3 & 4). The regression analyses were performed on the means which could reasonable 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. We found that some of the experimentally determined values were outside of the computed 95% confidence interval. While a "reasonable" agreement between the predicted and observed transformed values was noticed, 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 safety, upper confidence limits of growth should be used.

In summary, this paper presents Ratkowsky type equations (models) for predicting the effect of temperature on GOL and EGR of *C. perfringens* and *C. botulinum* during cooling of certain cooked meat products. From these equations and assumptions, the expected growth that would occur with the changing temperatures during cooling of meat products can be calculated. Research is being planned to validate assumptions and equations presented in this paper.

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