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Analysis of the Influence of Environmental Parameters on *Clostridium botulinum* Time-to-Toxicity by Using Three Modeling Approaches†

DONALD W. SCHAFFNER,[1]* WILLIAM H. ROSS,[2] AND THOMAS J. MONTVILLE[1]

Department of Food Science, Rutgers—The State University of New Jersey, New Brunswick, New Jersey 08901-8520,[1] and Health Canada, Food Directorate, Banting Research Center, Ottawa, Ontario K1A 0L2, Canada[2]

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This study used the technique of waiting time modeling to analyze the combined effects of temperature, pH, carbohydrate, protein, and lipid on the time-to-toxicity of *Clostridium botulinum* 56A. Waiting time models can be used whenever the time to the occurrence of some event is the variable of interest. In the case of the time-to-toxicity data, the variable is the time from the beginning of an experiment until a tube is identified as positive. The statistical analysis used the SAS procedure LIFEREG and included determination of the form of the response surface, identification of the error distribution, and simplification of the response surface. We found that increasing the macromolecule concentration decreased the probability of toxin formation. The probability of toxin formation also decreased at lower temperatures and at pHs further from the optimum. The waiting time modeling approach to developing models for botulinal toxin formation compared favorably with other approaches but had one specific advantage. Waiting time models have the inherent advantage that safety concerns regarding predictions are automatically quantified in the analysis by formally identifying a distribution of times-to-toxicity. The use of this time-to-toxicity distribution permits a customizable margin of safety (e.g., one in a million) not possible with other approaches.

Predictive food microbiology has gained favor in recent years as a means of conducting initial microbial safety estimates for food products. Many different approaches for modeling the behavior of the important human pathogen *Clostridium botulinum* have been used, but all of the approaches can be grouped into three basic types: probability, primary-secondary (7), and other approaches.

Probability models are used to estimate the probability of toxin formation directly, using a polynomial expression incorporating the environmental variables. The first model used to predict the expected fraction of toxic samples was developed by Roberts et al. (27). Those authors used a logistic model in which the probability of toxin production was inversely related to a polynomial expression describing the effect of the experimental variables. Lindroth and Genigeorgis (23) modeled the probability that a single spore would initiate growth and produce toxin, using a similar expression. Those authors have also expanded this general model type for a variety of other systems and experimental variables (2, 12, 13, 15, 19, 21, 24). Data presented by Tanaka et al. (33) were used to construct a probability model based on the quadratic equation (unpublished report) which predicts the time-to-toxin production by *C. botulinum* in processed cheese as a function of percent moisture, sodium chloride, and emulsifying salts. This model was later published (3) and corrected (34). Dodds (9) modeled the probability of toxin production and time-to-toxicity as a function of quadratic polynomial expressions with interactions using time, pH, and water activity.

Primary-secondary models use (i) either a kinetic model to describe lag time and growth of the organism or a probability model to predict the chance of toxin formation over time and then (ii) another model to predict the effect of environmental factors on the parameters of the first model. Gibson et al. (16) developed a kinetics model for the growth of *C. botulinum* type A in pasteurized pork slurry by using logistic and Gompertz functions. The relationship between the time to reach the maximum rate of growth and incubation temperature and sodium chloride concentration was described graphically. Whiting and Call (36) used nonlinear regression to estimate the parameters of a primary model for probability of growth at a given time and then used polynomial expressions containing experimental variables to predict the parameters of the primary model. This approach was expanded to develop a model for nonproteolytic type B *C. botulinum*, where inoculum size and time-to-toxicity confidence intervals were also included in the model (37). Most recently Graham et al. (17) developed a kinetic model for the growth of nonproteolytic *C. botulinum* from spores by using the Gompertz (16) and Baranyi-Roberts (5) models. Parameters of these sigmoid functions were in turn modeled as a function of temperature, pH, and sodium chloride concentration by using a quadratic polynomial.

The link between probability models (9, 23, 27) and kinetic models (16, 17) was recognized implicitly by Baker et al. (4) and discussed by Ross and McMeekin (30), and the distinction between them may in many cases be an artificial one. If *C. botulinum* can grow, it will probably produce toxin, so extent and rate of growth and time-to-toxicity are closely linked.

Other approaches used to model *C. botulinum* behavior include that of ter Steeg and Cuppers (34), who used a waiting time modeling approach to develop expressions for the effect of environmental parameters on time for 100-fold multiplication of *C. botulinum* in a model processed cheese system.

Waiting time models can be used whenever the time to the

* Corresponding author. Mailing address: Department of Food Science, Rutgers—The State University of New Jersey, 65 Dudley Rd., New Brunswick, NJ 08901-8520. Phone: (732) 932-9611, ext. 214. Fax: (732) 932-6776. E-mail: schaffner@aesop.rutgers.edu.

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occurrence of some event is the variable of interest. In the case of the time-to-toxicity data, this is the time from the beginning of an experiment until a tube is identified as positive. The analysis of time-to-toxicity data requires identification of the appropriate error distribution. The error distribution describes the variability of the waiting times in the experiment. The importance of proper error distributions in predictive food microbiology has been noted by several researchers (1), and proper distributions are essential if models are to be used in quantitative risk analyses. Waiting time modeling techniques are easily implemented by using most popular statistical analysis software, are flexible, and are simple to interpret, and the resulting models provide the user with a powerful tool for prediction and risk assessment.

Rogers and Montville (29) used linear regression to model the factors that influence the ability of nisin to inhibit *C. botulinum* in a model food system. The difference in time-to-toxin production between the nisin-containing and control (nisin-free) conditions was modeled. The data for the influence of the environmental factors on the time-to-toxin production in the absence of nisin from these experiments were never analyzed independently. This study used the technique of waiting time modeling to analyze the combined effects of temperature, pH, carbohydrate, protein, and lipid on the time-to-toxicity of *C. botulinum* for the Rogers-Montville (29) data set.

MATERIALS AND METHODS

Microbiology. The methods and techniques used to obtain the data used in our analysis are summarized below and were fully detailed by Rogers and Montville (29) and Rogers (28). *C. botulinum* 56A spores were cultured in a basal medium consisting of 1.0% (wt/vol) glucose (Sigma Chemical), 0.5% (wt/vol) peptone (Difco), 0.5% (wt/vol) yeast extract (Difco), and 0.01% (wt/vol) resazurin (Sigma). Protein, phospholipid, and soluble starch were added to the medium according to a fractional factorial design for a model food system containing three levels of five variables (temperature, pH, protein, phospholipid, and carbohydrate) that was generated by using ECHIP (Hockessin, Del.) statistical software. In addition to the primary experiments (done in triplicate), the program designated several conditions to be repeated (independently in triplicate) so that the reproducibility and variability of the results could be calculated. Each combination of food components for each experiment was prepared separately, pH adjusted separately, and inoculated separately. The macromolecule variables were protein (chicken egg albumin at 0.075, 0.75, and 7.5% [wt/vol]), lipid (lecithin at 0.075, 0.75, and 7.5% [wt/vol]), and carbohydrate (cornstarch at 5, 17.5, and 30% [wt/vol]). The other variables were pH (adjusted with HCl to 5.5, 6.0, or 6.5) and incubation temperature (15, 25, or 35°C).

C. botulinum 56A spores were heat shocked at 80°C for 10 min and inoculated into each sample to give a final concentration of 10^4 spores/ml. Cultures were removed from incubation and refrigerated when gas was observed. Botulinal counts on modified McClung agar were determined by manual spread plating. Botulinal toxin was determined by the U.S. Department of Agriculture enzyme-linked immunosorbent assay method, based on that described by Dezfulian and Bartlett (8). The experiment was halted after 60 days of observation.

Modeling. Waiting time analysis was conducted by using the SAS procedure LIFEREG. The process of selecting an appropriate model and estimating its parameters requires three steps: determination of the form of the response surface, identification of the error distribution, and simplification of the response surface.

(i) Response surface. The form of the response surface reflects the potential effects that the experimental variables may have on the time-to-toxicity. Both cubic (18) and quadratic (26) polynomial equations have been used to fit predictive food microbiology data sets. If too many parameters are used to fit the data, the risk of random fluctuations producing accidental and erroneous effects is increased. Gauch (14) showed that overfitting tends to increase the variance of the predicted values. Based on this reasoning, we consider a quadratic response surface sufficient to account for the effect of these variables on time-to-toxicity, with all cross products included accounting for potential interactions. The full model is as follows:

$$\theta = \beta_0 + \beta_1 P + \beta_2 C + \beta_3 L + \beta_4 \mathrm{pH} + \beta_5 T + \beta_6 P^2 + \beta_7 C^2 + \beta_8 L^2 + \beta_9 \mathrm{pH}^2 + \beta_{10} T^2 + \beta_{11} PC + \beta_{12} PL + \beta_{13} P\mathrm{pH} + \beta_{14} PT + \beta_{15} CL + \beta_{16} C\mathrm{pH} + \beta_{17} CT + \beta_{17} L\mathrm{pH} + \beta_{19} LT + \beta_{20} \mathrm{pH}T \quad (1)$$

where θ is the value of the response surface, P is percent protein (chicken egg albumin), C is the percent carbohydrate (cornstarch), L is the percent lipid (lecithin), pH is the initial pH of the medium, and T is the incubation temperature. The subscripted β values represent regression constants.

The quadratic term for carbohydrate concentration ($\beta_7 C^2$) was deleted from the model, since the design for this experiment makes it a redundant term. This term is redundant because it can be written exactly as a fixed, linear combination of the other components of the model. This redundancy is simply a result of the particular subset of the total number of possible experimental conditions that were chosen by Rogers and Montville (29). The redundant term provides no additional information beyond that which is given by the other terms.

(ii) Error distributions. To identify an appropriate error distribution, the full quadratic response surface model was fit twice to the data, specifying first the generalized gamma distribution and then the log-logistic distribution. The generalized gamma model is a three-parameter distribution containing many of the common choices of waiting time distributions as special cases (including the lognormal, Weibull, and gamma). The log-logistic distribution is an alternative two-parameter distribution with properties similar to the lognormal. The choice of error distributions was based on maximum-likelihood estimation techniques.

There are several reasons why the log-logistic distribution may be a suitable choice for time-to-toxicity data. Each tube will be inoculated with many viable spores. If the spores act independently, the time it takes for a tube to become toxic is related to the shortest time that it takes an individual spore in that tube to initiate growth and toxin production. A mathematical model for the distribution of shortest times is the Weibull distribution. However, if the ecology of each tube was somewhat different (different numbers of spores and variation in physiology, etc.), then a slightly different Weibull model may be appropriate for each tube. The log-logistic model incorporates the Weibull distribution for individual tubes together with between-tube variation.

For the log-logistic model, the probability, $P(t)$, that the time-to-toxicity of any tube is less than or equal to t is given by the following formula:

$$P(t) = \frac{1}{1 + e^{-(y - \theta)/\kappa})} \quad (2)$$

where $y = \ln(t)$, θ is the response surface, and κ is a scale parameter for the error. The scale parameter is proportional to but not equal to the standard deviation. Note that the response surface calculates the natural logarithm of the time at which the probability of toxicity is 1/2 (the median of the distribution). The log-logistic model can also be written as follows:

$$P(t) = \frac{t^b}{a^b + t^b} \quad (3)$$

where $\theta = \ln(a)$ and $\kappa = l/b$.

(iii) Response surface simplification. The response surface can often be simplified by removing individual terms without changing the statistical significance of the fitted model. A backward selection method was used to eliminate terms from the response surface in this analysis. The final submodel must be consistent with the underlying microbiology (i.e., it should not predict infinite rate increases with increasing temperatures or increasing growth rates above the maximum growth temperatures) (39).

The full response surface was fit to the data. The log-likelihood ratio statistic for the full model, L_F, was recorded, and the individual terms are then ranked according to their reported chi-squared statistics, with larger values (hence, smaller P values) ranked highest. The term ranked lowest was removed from the response surface equation, and the new model was fit to the data. The log-likelihood ratio for this submodel, L_S, was recorded, and the remaining terms were ranked as before. The log-likelihood ratio statistic, $\lambda = 2(L_F - L_S)$, was calculated and compared with the percentiles of the chi-squared distribution with degrees of freedom equal to the number of terms removed from the response surface.

If the log-likelihood ratio statistic was not significant, then the last two steps (term removal and λ calculation) were repeated until statistical significance was achieved. The resulting submodel was the one with the fewest remaining terms that were not statistically significantly different from the full model. A 5% level of significance was used in this analysis.

Comparison with other approaches. Dodds (9) defined lag time (LT) as the time until first toxin production for each experimental setting, while Baker and Genigeorgis (2) defined LT as the sampling period prior to toxin detection. In either analysis, multiple linear regression models were fit to log(LT). The use of the time to first toxin production or the lower censoring value to define LT adds an element of safety to the analysis. The degree of safety afforded by these LT definitions depends on the intensity of the inspection protocol and the underlying variability of the times-to-toxicity. Both of these approaches were applied to these data and compared with the waiting time model.

An analysis using nonlinear regression to fit a modified logistic cumulative distribution function to the data (36) was attempted, but this analysis was numerically unstable and failed to provide satisfactory results for many treatment combinations.

TABLE 1. *P* values for variable removal with the backward selection procedure of model parameters for the waiting time model

Model parameter[a]	*P* for removal of the following variable:								
	None	*L*	*PT*	*LT*	*PC*	*T*pH	*C*	*C*pH	*L*pH
λ	—[b]	0.999[c]	0.979[c]	0.972[c]	0.943[c]	0.896[c]	0.773[c]	0.752[c]	0.222[c]
Constant	*[d]	*	*	*	*	*	*	*	*
pH	*	*	*	*	*	*	*	*	*
T	*	*	*	*	*	*	*	*	*
P	*	*	*	*	*	*	*	*	*
pH^2	*	*	*	*	*	*	*	*	*
T^2	*	*	*	*	*	*	*	*	*
P^2	*	*	*	*	*	*	*	*	*
L^2	0.38	0.01	0.01	*	*	*	*	*	*
*P*pH	*	*	*	*	*	*	*	*	*
PL	*	*	*	*	*	*	*	*	*
CT	0.10	0.09	0.09	0.10	0.11	0.11	0.02	*	*
CL	*	*	*	*	*	*	*	*	*
*L*pH	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	—
*C*pH	0.27	0.23	0.19	0.14	0.12	0.14	0.32	—	—
C	0.31	0.27	0.23	0.18	0.16	0.20	—	—	—
*T*pH	0.27	0.26	0.26	0.27	0.35	—	—	—	—
PC	0.48	0.46	0.47	0.47	0.47	—	—	—	—
LT	0.66	0.66	0.66	—	—	—	—	—	—
PT	0.84	0.84	—	—	—	—	—	—	—
L	1.00	—	—	—	—	—	—	—	—

[a] *P*, protein term; *C*, carbohydrate term; *L*, lipid term; pH, pH term; *T*, temperature term; λ, log-likelihood ratio statistic.
[b] —, Term not present in model.
[c] *P* value for the likelihood ratio statistic.
[d] *, *P* value of <0.01.

RESULTS

For this data set, the log-logistic distribution yielded a larger log-likelihood ratio statistic than other distributions and was therefore considered to be the appropriate distribution. A record of the steps in the backward selection procedure is given in Table 1. Note that the final term removed from the model (*L*pH) contributed significantly to the model ($P < 0.05$); however, the overall change in the submodel from the full quadratic model was not significant, and so the term was removed. Parameter estimates for the final model are listed in Table 2.

Explanation of the parameters and interactions. The parameters from equations 1 and 2 and their statistical significance (Tables 1 and 2) can be used to gain some insight into possible mechanisms influencing *C. botulinum* toxin formation and their suitability and importance for controlling the growth of this food pathogen. All of the environmental factors studied here (pH, temperature, and concentrations of protein, carbohydrate, and lipid) influenced *C. botulinum* toxin formation to a statistically significant extent, because every factor is represented by at least one parameter in the final model for equation 4 (see below) (Table 2).

The easiest way to consider the net results of a series of complex interactions (e.g., the lipid concentration, present in three model terms [L^2, *PL*, and *CL*] [Table 2]) is to present the results graphically. Figure 1 shows the effect of varying each term in the model under an otherwise constant set of conditions ($C = 30\%$, $L = 7.5\%$, $P = 0.075\%$, pH = 5.5, and $T = 15°C$; 10 days). The *y* axis in each plot is the probability of toxin formation under the conditions specified. Families of plots for other conditions look slightly different but indicate the same trends (data not shown). The effect of pH on probability of toxin formation is shown in Fig. 1A, with minimum probabilities at pHs of 5.5 and 6.5 and an optimum occurring between pH 6.00 and 6.25. Figure 1B shows the effect of temperature on toxin formation. Under the conditions selected, the model predicts the lowest probability of toxin formation at a temperature of 15°C and a maximum probability of toxin formation above 30°C.

The relationships between the lipid, carbohydrate, and protein macromolecule concentrations and probability of toxin formation all show a similar trend (Fig. 1C, D, and E), with the highest probability of toxin formation at the lowest macromolecule concentration and the lowest probability of toxin formation at the highest macromolecule concentration.

Comparison of predictions to observations. For the log-logistic model, the response surface defines the median (50th percentile) of the time-to-toxicity distribution corresponding to each combination of explanatory variables. Observed toxicity times are compared to response surface estimates in Fig. 2. The 99.8th percentile (adjusted for censoring and estimation

TABLE 2. Parameter estimates and standard errors for the final waiting time model

Variable[a]	Estimate	SE
Constant	241.39	53.16
P	1.446	0.428
pH	−76.98	17.91
T	−0.4856	0.0496
P^2	−0.282	0.0528
L^2	−0.0113	0.0021
pH^2	6.3227	1.493
T^2	0.00759	0.001
*P*pH	0.1033	0.0203
PL	0.01389	0.0029
CT	−0.000559	0.000148
CL	0.00516	0.000792
κ	0.299	0.0178

[a] *P*, protein term; *C*, carbohydrate term; *L*, lipid term; pH, pH term; *T*, temperature term; κ, scale parameter (proportional to but not equal to the standard deviation).

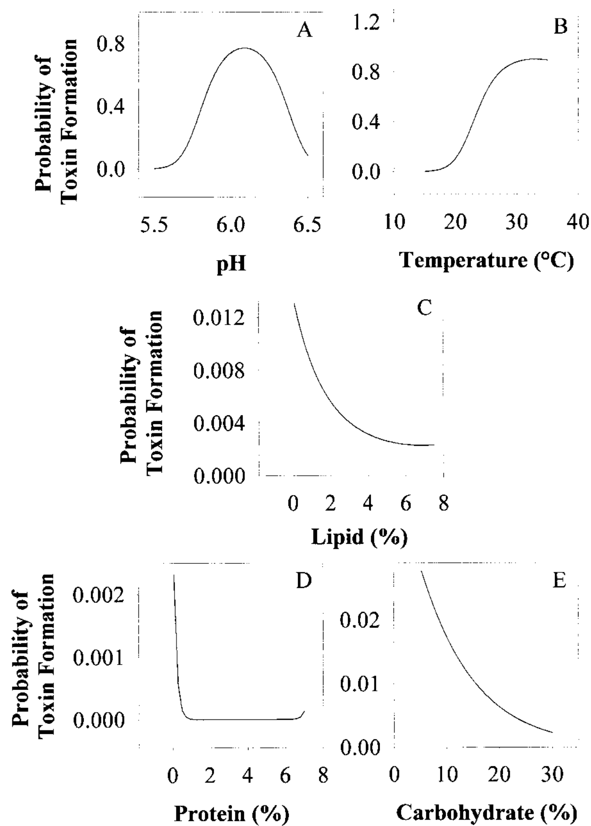

FIG. 1. Influence of intrinsic and extrinsic factors on the waiting time model predicted probability of toxin formation within 10 days by *C. botulinum* in a model system (7.5% lipid, 0.075% protein, and 30% carbohydrate) at pH 5.5 and 15°C.

error) is also plotted, as a dashed line. Three of the 227 observations exceed this percentile. This would be expected only 1% of the time, suggesting that one or more of these three observations might be outliers. Refitting the model after removal of these three data points had no effect on fitting the response surface and only slightly affected the estimate of the scale parameter.

The probability of toxicity can be viewed as the probability of a single package in one batch being toxic. For example, a probability of toxicity equal to 0.000001 (one in a million) can also be thought of as one package in one million being toxic. The time at which the probability of toxicity is any value of interest (such as one in a million) is easily calculated by using this model. Letting P_0 denote the probability level of interest, then the corresponding time, t_0, is calculated by using the following formula:

$$\ln(t_0) = \theta + \kappa \ln\left(\frac{P_0}{1 - P_0}\right) \quad (4)$$

Contour lines in Fig. 3 indicate the time (in days) at which the probability of toxicity is 0.000001 (one in a million) for various experimental conditions.

Comparison with other approaches. Figure 4 displays a scatter plot comparing log(LT) as predicted by the approach of Dodds (9) with the median value predicted by the waiting time model. There is close agreement between the median from the waiting time distribution and most of the Dodds log(LT) predictions. If there were perfect agreement, all of the data would fall along the (solid) line of equivalence. However, for toxicity times close to 1 day, the log(LT) predicted by the Dodds method exceeds the median of the data. This means that actual times-to-toxicity would be less than predicted more than 50% of the time. A similar point can be noted by examining the short-dashed line (a linear regression of the two predictions). This regression line lies above the line of equivalence at times less that 5 days but below the line of equivalence at times greater than 5 days. All log(LT) predictions are on or above the 10th percentile of the toxicity time distribution (long-dashed line). We expect that future LT values would be less than the predicted time-to-toxicity at least 10% of the time.

When LT is defined as by Baker and Genigeorgis (2), there is little difference between the median of the waiting time-to-toxicity distribution and the predicted Baker-Genigeorgis LT (Fig. 5). As with the previous case, the Baker-Genigeorgis predicted log(LT) exceeds the waiting time distribution median for predictions close to 1 day, but in this case the short-dashed (regression) line more closely approximates the line of equivalence. All predictions are well above the 10th percentile of the time-to-toxicity distribution.

DISCUSSION

Explanation of the parameters and interactions. As one of us has noted elsewhere (25), extreme care should be used in interpretation of the statistical and practical significance of interaction effects, with an emphasis on avoiding overinterpretation. This is especially important when the simple linear terms (in this case *P*, *C*, *L*, pH, or *T*) do not appear in the final model. For example, since the lipid term is the first to be dropped from the model, a simplistic conclusion would be that lipid concentration does not have a significant effect on *C. botulinum* toxin formation. This is untrue, because the effects of lipid concentration on toxin formation are incorporated into the model by the L^2, *PL*, and *CL* terms.

The effect of pH on microbial growth is known to show optimum, minimum, and maximum values (20). Our results show the same trends as those observed by others modeling the effect of pH on growth of or toxin production by *C. botulinum* (9, 17, 31, 36) or on growth of other sporeformers (25). Enzymes are known to have pH optima similar to those shown by whole cells. pH is also known to affect transport of nutrients into the cell. Our model, and the predictions shown in Fig. 1A,

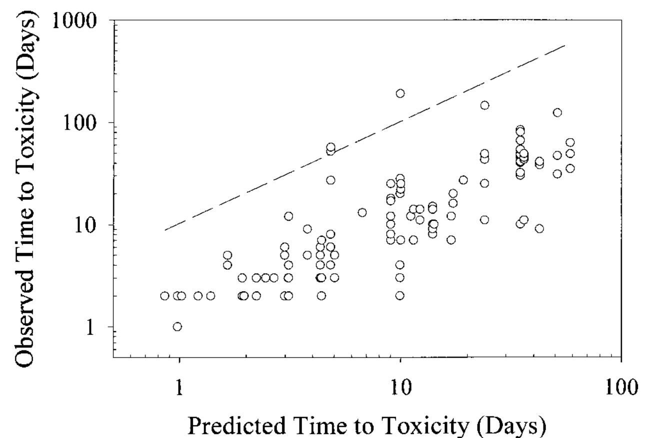

FIG. 2. Observed time-to-toxicity of *C. botulinum* in a model system versus median time-to-toxicity predicted by using a waiting time model. The 99.8th percentile (adjusted for censoring and estimation error) is plotted as a dashed line.

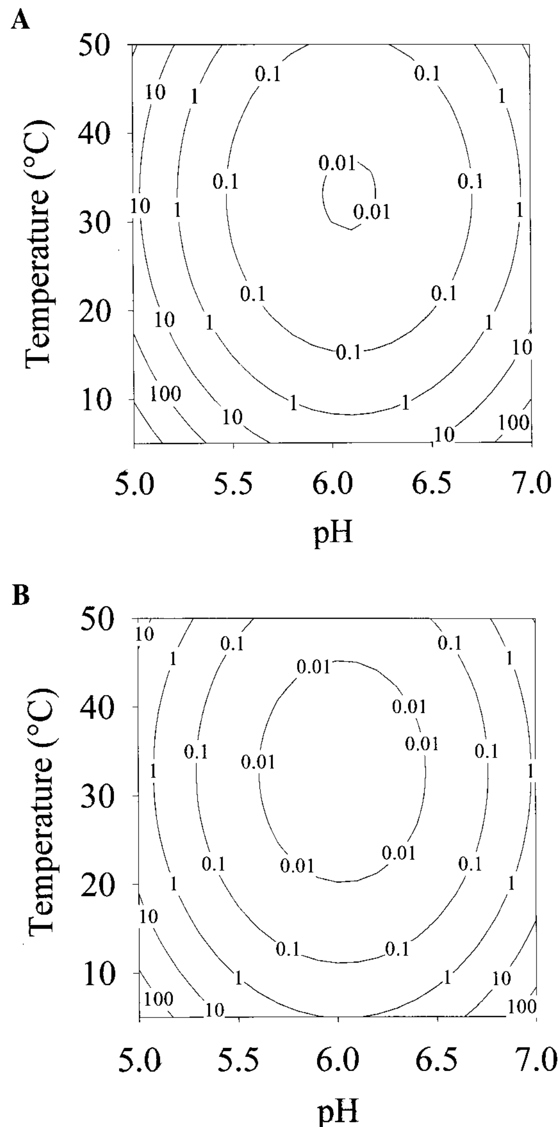

FIG. 3. Predicted time-to-toxicity (in days) of *C. botulinum* in a model system as influenced by macronutrients, incubation temperature, and pH. Contour lines represent the predicted time at which the probability of toxicity is one in a million. (A) Predictions where the model system contains 0.075% (wt/vol) chicken egg albumen, 30% (wt/vol) cornstarch, and 7.5% (wt/vol) lecithin. (B) Predictions where the model system contains 7.5% (wt/vol) chicken egg albumen, 30% (wt/vol) cornstarch, and 0.075% (wt/vol) lecithin.

suggests that either of these two mechanisms may be operating here.

It is known that most simple chemical and biochemical reactions show a dependence on temperature governed by Arrhenius rate kinetics (38), which do not describe multienzyme microbial growth very well. A complex version of the simple Arrhenius equation, which appears to describe the effect of temperature on microbial growth fairly well, has been developed (32) and applied to food-borne pathogens (1). This model supposes one key enzyme (reversibly inactivated at low and high temperatures) which determines growth rate as a function of temperature for a given microbe. Because of the nonlinearity shown in Fig. 1B, a similar sort of key-enzyme dependence may be governing the relationship between the probability of *C. botulinum* toxin formation and temperature. As with the pH results, our results generally correspond to those of others

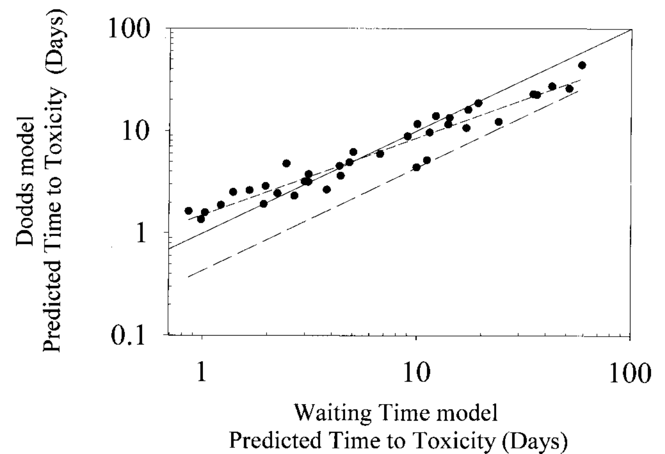

FIG. 4. Comparison of predicted times-to-toxicity of *C. botulinum* obtained by using the model of Dodds (9) and the waiting time model median values. The solid line is the line of equivalence (perfect agreement). The short-dashed line is the linear regression of the two predictions. The long-dashed line is the 10th percentile of the toxicity time distribution.

modeling the effect of temperature on growth of or toxin production by *C. botulinum* (13, 16, 21, 24, 36).

The relationship between macromolecule concentrations and probability of toxin formation (Fig. 1C, D, and E) might at first seem counterintuitive, because higher nutrient concentrations should lead to better conditions for spore germination, more rapid cell growth, and a greater (not lesser) chance of toxin formation. The results for lipid concentration (Fig. 1C) are most easily explained. Lecithin (phosphatidylcholine) was used to change the lipid concentration in the model system. The purity of the lecithin preparation (Sigma type XV-E, from fresh frozen egg yolk; phosphatidylcholine content, ~60%) was such that free fatty acids (FFAs) were likely present in the mixture. FFAs are well-known germination inhibitors (22), so as the concentration of lipid (lecithin) increased, so did the concentration of inhibitory FFAs.

The results for protein (Fig. 1D) were initially surprising, because all clostridia are nutritionally demanding (11) and the

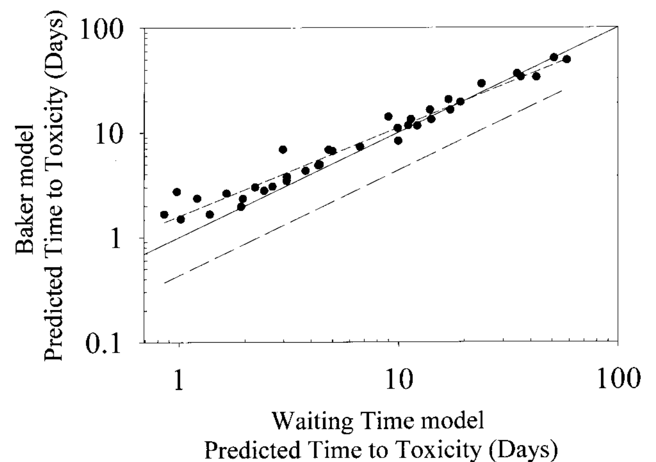

FIG. 5. Comparison of predicted times-to-toxicity of *C. botulinum* obtained by using the model of Baker and Genigeorgis (2) and the waiting time model median values. The solid line is the line of equivalence (perfect agreement). The short-dashed line is the linear regression of the two predictions. The long-dashed line is the 10th percentile of the toxicity time distribution.

proteolytic strain of *C. botulinum* used here would proteolyse proteins required to supply amino acids. However, a literature search revealed that egg white proteins (as were used in this study) inhibit *C. botulinum* proteases (35) and would thus decrease the supply of amino acids available to support growth.

Limitations of previous modeling approaches. One potential difficulty in applying the linear regression approach is the handling of left- and right-censored waiting times. A left-censored waiting time is created in these experiments when a sample contains toxin the first time it is observed. A right-censored waiting time is created when an experiment is concluded but a sample has yet to show toxin production. For left-censored values, LT = 0, for which log(LT) is not defined. As a result, these values must be removed from the analysis or replaced by some arbitrary value. Since these values represent an extreme for the response in the linear regression analysis, they may have a substantial effect on model selection and parameter estimation. This effect is demonstrated in Fig. 4 and 5, where short time-to-toxicity values are generally overestimated by other approaches.

Similarly, when experiments are terminated after a fixed period of time, the resulting LT values are restricted. The result is to artificially reduce the effect of important factors in the linear regression. This will again affect model selection and parameter estimation.

A further difficulty with the application of the linear regression approach is the identification of the error distribution. In effect, linear regression analysis assumes that waiting times satisfy a lognormal probability law. For the analysis presented here, the model fit was improved by considering alternative error distributions. In addition, it should be noted that the inspection protocol restricts the possible values of LT. For example, Dodds (9) used six inspection times over a period of 60 days, thereby restricting LT to be one of only six possible values. Experimental settings for which no tubes turned positive within 60 days were excluded from the analysis.

Whiting and Call (36) used nonlinear regression to apply a modified logistic cumulative distribution function (CDF) as the primary model for waiting times. This is a two-step procedure that first fits a parametric CDF to the proportion of positive tubes at each inspection time separately for each treatment. For each experimental setting, the response variable of the nonlinear regression analysis (the cumulative proportion of positive tubes) is correlated. As a result, the reported standard errors of the parameter estimates are incorrect. This may also introduce additional bias in parameter estimation. The overall effect on model selection is unknown.

The waiting time model alternative. Waiting time models provide a flexible approach to analyzing the effects of various treatments on time-to-toxicity while identifying an appropriate error distribution and accounting for any censoring introduced by the inspection protocol. The ease of conducting a statistical analysis by using this approach compares favorably with that for linear regression methods, because most popular statistical software packages contain methods for analyzing for waiting time. The results of such an analysis are directly applicable to prediction and to the assessment of the risk of toxicity occurring in a particular batch. Safety concerns regarding predictions are automatically introduced into the analysis by formally identifying a distribution of times-to-toxicity. This allows the analyst to choose the margin of safety (for example, one in a million). Unlike linear regression based on LT, this approach directly quantifies the level of safety in the analysis.

The waiting time approach does have several important limitations that should be noted. First, additional laboratory work may be required to collect the data used for modeling. In particular, the nonlinear regression step requires a sufficient number of tubes at each treatment level indicating toxicity at different inspection times. Second, the statistical phase of the analysis may also be more labor-intensive, as each error distribution determines a different CDF, which requires a separate nonlinear regression for each treatment combination. Finally, in fitting several different models, numerical stability and failure of the fitting algorithm to converge are also an issue.

It is also important to note that other factors not present in the Rogers-Montville (29) data set may influence the variability of the results. For example, it has been shown previously that the germination times of individual spores of *C. botulinum* may differ significantly (6). This germination time difference means that larger inoculum sizes (e.g., as used by Rogers and Montville [29]) can result in a smaller variability of time-to-toxicity (31) than for food samples, which typically contain smaller numbers of spores (10). Thus, the models presented here may predict less variability in time-to-toxicity than expected in naturally contaminated food samples.

Conclusions. We have provided preliminary evidence that higher concentrations of macromolecules (protein, lipid, and carbohydrate) may reduce the probability of toxin formation by *C. botulinum*. This research has also confirmed that lower temperatures and pHs farther from the optimum reduce the probability that *C. botulinum* will form toxin within the time period studied. We have also demonstrated that the waiting time modeling approach to developing models for toxin formation by *C. botulinum* is possible and compares favorably with approaches used by others.

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