Journal of Industrial Microbiology, 12 (1993) 139–143

Published by The Macmillan Press Ltd

An introduction to predictive microbiology and the development and use of probability models with *Clostridium botulinum*

Karen L. Dodds

Health Protection Branch, Health & Welfare Canada, Ottawa, Canada

(Received 29 September 1992)

Key words: Probability models; *Clostridium botulinum*; Irradiation; Polynomial equations

SUMMARY

Traditionally, food microbiologists have relied on empirical studies to assess the microbiological safety of a particular food. However, these studies are time-consuming and, because only one or two inhibitory factors are usually dealt with, they are often of limited value. Today, the food industry is constantly developing new products with new formulations and alternative packaging strategies, resulting in a wide diversity of factors to be studied. It is therefore advantageous to develop mathematical models describing microbial growth which may be used to predict how changes in formulations or storage conditions may affect microbial growth. A brief overview of the basic concepts and steps of modeling procedures will be presented, along with some of the difficulties encountered therein. The safety of foods with respect to *Clostridium botulinum* depends on the probability (P) of growth or of toxigenesis, and P has been the dependent variable in several models. The development of these probability models will be discussed.

INTRODUCTION

Traditionally, food microbiologists have relied on empirical studies to assess the microbiological safety of a particular food [2]. In these studies, the important parameters governing microbial growth such as temperature, pH, salt level, and water activity (a_w), were defined, and maximum and minimum limits permitting growth, such as shown in Table 1, were established. To industry, there is little value in studies dealing with only one variable at a time. Most of these limits were determined with all other parameters optimal, which is very rarely the case with food products.

TABLE 1

Empirically defined limits for microbial growth

	Minimum temperature (°C)	Minimum pH	Maximum brine (%)	Minimum a_w
Clostridium botulinum				
Group I	10	4.6	10	0.94
Group II	3.3	5.0	5	0.97
Salmonella	5.2	4.0	8	0.95
Listeria monocytogenes	3	5.6	10	–

Correspondence to: K.L. Dodds, Health Protection Branch, Health Canada, Ottawa, Canada.

Mention of brand or firm names does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

There is also increased awareness that preservative factors often act in combination, giving food products microbiological protection at levels which individually would not be inhibitory. There are many different factors which might influence microbial growth in a food product. For example, temperature is a very important factor. pH is also very important, but so is the acidulant used to establish the pH. Similarly, a_w is important, as is the humectant used. The presence of different preservatives, the use of different atmospheres and the oxidation-reduction potential (Eh) of the food all affect microbial growth. Today the food industry is constantly developing new products with new formulations and alternative packaging strategies, resulting in a wide diversity of factors to be studied. Therefore, it is to industry's advantage to develop mathematical models describing microbial growth which may be used to predict how changes in formulations or storage conditions may affect microbial growth.

Introduction to modeling

The steps involved in modeling seem very simple and straightforward. The first step, as for any experiment, is the planning. The next step is data collection. After collecting the data, different models are fitted, and the model which best describes the data is selected. Lastly, the model should be validated using data not used to fit the model.

All experiments should be carefully planned, and this is particularly true when modeling is the end-point. The first requirement is a very clear statement of the problem. For example, what is the probability of toxin production by *Clostridium botulinum* in a certain product. Next, factors which are critical to the food product must be identified. These are the characteristics of a product which can be varied, and the variation in them can be controlled. For

example, in the case of shelf-stable cooked potatoes [1], both pH and a_w were critical. These are the independent or predictor variables, and the response or predicted variable would be the probability of toxin production or the time until toxin production. The levels of the independent variables to be studied must be carefully determined. These levels are usually restricted by the food product being studied, and may only affect the food within a certain range. There would be no sense in studying potatoes with 10% salt because consumers would find this too salty. Choosing the experimental design and establishing the specific samples to be tested are often synonymous. Now the limitations are not only the food, but also available resources, especially personnel and facilities. If only two levels of a factor are studied, only a linear effect can be determined. If a response surface is desired, three or more levels are required. The predictive ability of any model is best within the range of conditions tested, and prediction outside the range is not advisable. In the example of the shelf-stable cooked potato, a combination of MPN methodology and a full factorial experiment [1] was used to study four levels of a_w and five levels of pH. Samples were analyzed in triplicate at seven intervals. This meant a total of 1260 samples per experimental run.

Fitting the experimental results to a model is where modeling really becomes interesting. Regression is the technique used to quantify the relationship between variables when the value of one variable is affected by changes in the values of other variables. In its simplest form, the relationship is linear and there is one dependent variable and one independent variable. This takes us back to high school math and the equation $y = mx + b$. If there is more than one independent variable, multiple linear regression can be used. In modeling microbial growth in a closed system, we all know we are modeling a sigmoidal curve, the growth curve. There are several choices of equations for sigmoidal curves [12]. Logistic regression gives a symmetrical sigmoidal curve, so it is best for organisms growing under optimal conditions. The Stannard equation is also for a sigmoidal curve. The Gompertz curve is used for a situation where the relative growth rate is believed to decrease exponentially with time. The Ratkowsky [9] equation is predictive for growth dependent on temperature.

Modeling work with C. botulinum – *historical*

Modeling work with *C. botulinum* presents difficulties not seen in many other situations. Often the detection of toxin is more important than growth and modeling microbial growth is much more straightforward. The response, growth, is fairly easily determined, and is continuous. However, in detecting toxin, there is an all or none response. There are problems with reproducibility, with variation in spore inoculum due to the medium in which the spores are produced, stored or heated, and their age, and whether or not to heat activate the spores, what inoculum level to use, how to inoculate, etc. This is why many investigators studying toxin production have determined the probability of toxin production, or have used logistic regression as the model.

The safety of foods with respect to *C. botulinum* depends on the probability (P) of growth or of toxigenesis [4]. This approach was first used in the early 1920s to develop a food safety standard for low-acid canned foods. A mathematical relationship was developed between the thermal process and the probability of spore survival. Using a thermal process equivalent to a 12D cook would result in only a single can of 10^{12} cans with one spore per can containing a viable spore. Or, it would result in a one in 10^{12} chance for the survival of a single, initial spore. As Riemann [10] expressed it in 1966, the success of a preservation method with regard to *C. botulinum* depends on the probability that one spore will germinate, grow out, and give rise to toxin production in the preserved product. Assessment of the safety of shelf stable canned cured meats requires the incorporation of several factors into the risk analysis of *C. botulinum* growth and toxigenesis. These products receive a relatively mild thermal process but are nonetheless stable due to other factors, principally the addition of nitrite, salt and other curing agents which inhibit surviving spores, and the low initial concentration of *C. botulinum* spores in the product. The desire to quantify these effects was expressed as early as 1973 by Pivnick and Petrasovits [8]. They presented an equation: preservation = destruction plus inhibition, and expressed each element on the basis of 1 $\log_{10}$.

Roberts and his group summarized a vast amount of experimental work [11] and estimated the probability of botulinum toxin production in a model pork system as a function of salt, nitrite, isoascorbate, thermoprocess, pH and other factors. The probability (P) of toxigenesis was expressed in a single regression equation, $P(\%) = 1/(1 + e^{-\mu})$, where μ is the predictor. The predictor is expressed by an equation which includes the individual terms of regression on factors, and their interactions which vary over the experimental range of each factor. Levels of factors and their interactions which minimize the probability of toxigenesis reduce the value of μ.

Hauschild [3] assessed the botulism hazard from cured meat products by estimating the probability of a single spore to initiate growth and toxigenesis using the equation P = MPN spore outgrowing/MPN of spores inoculated. If inoculation was at a single level, the Halvorson and Ziegler equation, MPN = $\ln(n/q)$, where n = the number of inoculated packs and q = the number of nontoxic packs after incubation was used. The value log $1/P$ represents the number of log units of spores required for one spore to grow out and give rise to toxin under given conditions. Log $1/P$ thus expresses decimal inhibition of spores, analogous to the D-value used in thermal processing.

Modeling the effects of pH and a_w on toxin production by C. botulinum

Vacuum-packed cooked potatoes have been commercially available in Europe for several years, however potatoes provide a good substrate for botulinal growth and have been implicated in botulism outbreaks. A combination of factorially designed experiments and MPN methodology was used [1] to quantify individual and combined effects of a_w,

pH and storage time on toxin production. A preliminary experiment was done to determine critical levels of a_w and pH, followed by a large experiment to develop the model and yet another experiment to validate the model. Different a_w levels were obtained in cooked, mashed potatoes by adding solid NaCl. The different pH levels were obtained by adding ascorbic acid. The average difference between actual and target values was 0.001 for a_w and 0.08 for pH, indicating that these variables were under tight control.

For the factorial experiment, four levels of a_w and five levels of pH with three inoculation levels were sampled over a period of 60 days. Analysis of variance showed that storage time, a_w and pH all had highly significant effects on the probability of toxigenesis. Interaction effects between a_w and pH, and a_w and time were also highly significant, as was a quadratic term for a_w. We used multiple linear regression performed by the general linear models procedure of SAS release 5.04 to develop models (Table 2). Only data for treatments showing at least one positive sample were included in the analysis and only terms which were statistically significant by the ANOVA were included. In terms of explaining variation, the best model is for the lag time before toxin is detected. Contour plots of the effect of a_w and pH on log P and on lag time graphically illustrated the results (results not shown). A second fully factorial experiment was carried out to verify and test the predictive ability of the models. Only one treatment, at a_w = 0.965 and pH = 5.0, had an observed lag time shorter than the predicted lag time. These experiments demonstrated that both decreased a_w and pH inhibited *C. botulinum*, something we knew qualitatively before, but had not quantified.

Modeling the effects of MAP and irradiation on C. botulinum *toxin production*

Toxin production in modified atmosphere packaged, irradiated fresh pork loin chops inoculated with *C. botulinum* was studied [5–7]. Initially, the effects of three factors at three levels each were examined. One of the steps undertaken was to code the data. This has the advantage that the scale of units is then negated. For example, the scale of irradiation was only from 0 to 1, whereas for temperature it was from 5 to 25. Without coding, the factor coefficients would reflect this difference, but with coding, the coefficients reflect absolute differences. Chops were put in high barrier bags, inoculated with spores and modified atmosphere packaged, then irradiated. During these studies, we looked at different atmospheres. Initially, we looked at O_2 seeing it as a possible inhibitor of *C. botulinum*. Initial O_2 was 0, 10 or 20%, balance N_2. Samples were stored at 5, 15 or 25 °C and analyzed over 42 days (d).

The gas composition of samples was monitored by headspace analysis. In all cases, where O_2 was initially present, it decreased with time and CO_2 increased. If samples were irradiated, both the rate and extent of the decrease in O_2 and increase in CO_2 were decreased. As well, as the storage temperature decreased, the rates decreased. However, even at 5 °C, the O_2 in samples initially packaged with 20% O_2 decreased to less than 2% by 28 days and the CO_2 increased to 18%. One thing these results demonstrated is that because the composition of the atmosphere in MAP pork changes, it is an independent variable over which we had poor control.

As Table 3 shows, samples incubated at 25 °C were toxic within 2 days, irrespective of atmosphere composition or irradiation dose. At 15 °C, treatments packaged with either 10 or 20% O_2 were toxic after 14 days, whether or not they had been irradiated. Treatments packaged in the absence of O_2 showed an increased time until toxin production, to 21 days if they were not irradiated, and to 43 days if they were irradiated at 1.0 kGy. From this table, it is obvious that temperature is the overriding independent variable affecting toxin production. From the statistical analysis, a stepwise regression, temperature is highly significant, P = 0.0001, and it explains most of the variation in results, ~74%. However, initial O_2 is also highly significant, and irradiation and final CO_2 and several cross-products are also significant.

TABLE 2

Regression equations relating storage time in days (d), lag time, a_w, pH, and the probability (P) of toxigenesis of *C. botulinum* in cooked, vacuum-packed potatoes stored at 25 °C [1]

Parameter	Equation	r^2
Log P	$= -577.62 - 6.96d + 1399.49\ a_w - 41.97\ pH - 840.64\ a_w^2 + 44.64\ a_w{}^*\ pH + 7.23\ d{}^*a_w$	0.71
Lag time	$= -358455 + 738260\ a_w + 2421\ pH - 380770\ a_w^2 - 32.81\ pH^2 - 2196\ a_w{}^*\ pH$	0.85
Log_{10} lag time	$= 17870 - 35570\ a_w - 232.4\ pH + 17712\ a_w^2 + 227.5\ a_w{}^*\ pH + 1.1\ pH^2$	0.98

TABLE 3

Effect of storage temperature, modified atmosphere and irradiation dose on toxin production by inoculated *C. botulinum* in fresh pork chops [7]

Storage temperature (°C)	Initial oxygen (%)	Irradiation dose (kGy)	Earliest toxin detection (d)
25	20	0.5	2
	10	0.0	2
	10	1.0	2
	0	0.5	2
15	20	0.0	14
	20	1.0	14
	10	0.5	14
	0	0.0	21
	0	1.0	43
5	20	0.5	> 44
	10	0.5	> 44
	10	1.0	> 44
	0	0.5	> 44

The second experiment was designed to try and separate the effects of initial O_2 and final CO_2. These different treatments were used to give a continuum of final levels of CO_2. Two kinds of gas absorbents, one which absorbed only CO_2 and one which absorbs CO_2 and O_2, were used. Again, irradiation was included as a factor.

The absorbents created a difference in the atmospheres. The levels of CO_2 reached in packages without an absorbent were much higher than in those treatments with an absorbent. Table 4 shows the results for toxin detection. Surprisingly, the first samples to become toxic were those packed with 20% O_2 with a CO_2 absorbent. The next samples to become toxic were those packed with 20% O_2, with or without 20% CO_2, without an absorbent and non-irradiated.

Statistical analysis showed that O_2, CO_2 and irradiation all contributed significantly to the model. Much to our surprise, the presence of the absorbents was also significant. As with the last model, final O_2 was not significant but final CO_2 was. These factors explained 83% of the variation seen.

In a third experiment, we looked at five levels of CO_2 – 15, 30, 45, 60 and 75%, balance N_2 and irradiation. At all levels, CO_2 increased, but the increase was decreased at higher initial levels. Results of the toxin assay are in Table 5. The first treatments to become toxic were those with low levels of CO_2 which were not irradiated. The time until toxin detection increased as the irradiation dose increased and as the level of initial CO_2 increased. Statistical analysis showed the initial CO_2 to be very significant, but it did not explain very much of the variation.

Data from the preliminary challenge study were analyzed by regression using the RSREG procedure of SAS release 5.18. We used the second order polynomials thus generated to derive predictive models (Table 6). Using a model relating time until toxin production to the initial O_2 level, irradiation dose and storage temperature, surface response graphs were developed to graphically illustrate the effect of O_2 level and irradiation dose on the time until toxin production at 15 °C (results not shown). In all cases, the time until toxin production increased as the headspace O_2 level decreased and the irradiation dose increased. As the temperature increased, the time until toxin production, even for the least favorable conditions (0% O_2/1 kGy), decreased from 49 to 27 days, indicating the importance of temperature. A second model was generated using only results at 15 °C to relate time until toxin production to initial O_2 and irradiation dose. It is interesting to note that irradiation had a much greater effect at low levels of O_2. The earliest time predicted by the model is 12.3 days and corresponds to an atmosphere containing 14% O_2 without irradiation.

TABLE 4

Effect of modified atmosphere, absorbent type and irradiation dose on toxin production by inoculated *C. botulinum* in fresh pork chops stored at 15 °C [6]

Initial O_2 (%)	Initial CO_2 (%)	Gas absorbed	Irradiation dose (kGy)	Earliest toxin detection (d)
20	0	CO_2	0.0	14
20	0	CO_2	1.0	19
20	0	none	0.0	19
20	20	none	0.0	21
20	20	none	1.0	28
0	0	none	0.0	28
0	0	CO_2 + O_2	0.0	28
20	0	none	1.0	28
0	20	none	1.0	28
0	0	none	1.0	28
0	0	CO_2 + O_2	1.0	28
0	20	none	0.0	35

TABLE 5

Effect of irradiation dose and initial CO_2 on toxin production by inoculated *C. botulinum* in fresh pork chops stored at 15 °C [5]

Irradiation dose (kGy)	Initial CO_2 (%)	Earliest toxin detection (d)
0.0	15	14
	30	14
	45	28
	60	28
	75	21
0.5	15	28
	30	14
	45	35
	60	28
	75	21
1.0	15	21
	30	28
	45	28
	60	28
	75	35

TABLE 6

Regression equations relating initial O_2, irradiation dose (Ir), storage temperature (T), and storage time (d), to the time until toxin production (TTP) and the probability (P) of toxigenesis of *C. botulinum* in inoculated fresh pork chops

Parameter	Equation	r^2
TTP	$= 117.31 - 0.78\ O_2 - 2.75\ Ir - 8.95\ T + 0.04\ O_2^2 - 0.92\ O_2{*}Ir + 16.0\ Ir^2 + 0.18\ T^2$	0.98
TTP[a]	$= 25.67 - 2.05\ O_2 + 17.33\ Ir + 0.08\ O_2^2 - 0.92\ O_2{*}Ir$	0.92
Log P	$= -7.6 + 0.0024\ d + 0.00099\ O_2 + 0.523\ Ir + 0.299\ T - 0.0002\ d + 0.00036\ d{*}O_2 - 0.00013\ O_2^2 - 0.014\ d{*}Ir - 0.0028\ O_2{*}Ir - 0.197\ Ir^2 + 0.0025\ d{*}T + 0.00063\ O_2{*}T - 0.016\ Ir{*}T - 0.005\ T^2$	0.95

[a]Effect of temperature removed.

Another model was derived using the probability of toxigenesis as the response. Storage time and temperature were the most significant factors ($P < 0.0001$) affecting probability. Three-dimensional graphs were also developed using this model to illustrate the effects of initial O_2 and irradiation dose on the probability of toxigenesis by a single spore at specific temperatures (results not shown). The probability of toxigenesis increased as the O_2 concentration increased and as irradiation decreased.

Three-dimensional graphs also illustrated the effects of storage time and temperature on the probability of toxigenesis in non-irradiated samples packaged without O_2 (results not shown). The probability increased gradually as the storage time increased. However, there was a pronounced increase in probability as temperature increased, again indicating the importance of this factor.

CONCLUSIONS

Modeling is proving to be a very valuable technique from several points of view. The effects of different parameters on inhibition of microbial growth or toxin production can be quantified, giving much useful information. For example, results of the modeling done on the shelf stable potatoes [1] showed that there was a stronger inhibitory effect if a_w was decreased as compared to pH. Modeling permits the interactive effects between different inhibitory parameters to be quantified. Finally, modeling gives the ability to change a formulation but still predict shelf life. Presently, the approach is somewhat limited because researchers normally deal with a specific food product or a specific model system and the applicability of models developed this way to other products is uncertain. However, as more data is generated and more models developed, we should be able to develop more general, robust models.

REFERENCES

1 Dodds, K.L. 1989. Combined effect of water activity and pH on inhibition of toxin production by *Clostridium botulinum* in cooked, vacuum-packed potatoes. Appl. Environ. Microbiol. 55: 656–660.

2 Farber, J.M. 1986. Predictive modeling of food deterioration and safety. In: Foodborne Microorganisms and Their Toxins: Developing Methodology (Pierson, M.D. and N.J. Stern, eds), pp. 57–90, Marcel Dekker, New York.

3 Hauschild, A.H. 1982. Assessment of botulism hazards from cured meat products. Food Technol. 36(12): 95–104.

4 Hauschild, A.H. 1989. *Clostridium botulinum*. In: Foodborne Bacterial Pathogens (Doyle, M.P., ed.), pp. 111–189, Marcel Dekker, New York.

5 Lambert, A.D., J.P. Smith and K.L. Dodds. 1991a. Effect of headspace CO_2 concentration on toxin production by *Clostridium botulinum* in MAP, irradiated fresh pork. J. Food Protect. 54: 588–592.

6 Lambert, A.D., J.P. Smith and K.L. Dodds. 1991b. Effect of initial O_2 and CO_2 and low-dose irradiation on toxin production by *Clostridium botulinum* in MAP fresh pork. J. Food Protect. 54: 939–944.

7 Lambert, A.D., J.P. Smith and K.L. Dodds. 1991c. Combined effect of modified atmosphere packaging and low-dose irradiation on toxin production by *Clostridium botulinum* in fresh pork. J. Food Protect. 54: 94.

8 Pivnick, H. and A. Petrasovits. 1973. A rationale for the safety of canned shelf-stable cured meat. Protection = Destruction + Inhibition. In: 19th Meeting European Meat Researchers, Sept. 1973, p. 1086, Paris.

9 Ratkowsky, D.A., J. Olley, T.A. McMeekin and A. Ball. 1982. Relationship between temperature and growth rate of bacterial cultures. J. Bacteriol. 149: 1–5.

10 Riemann, H. 1967. The effect of the number of spores on growth and toxin formation by *C. botulinum* type E in inhibitory environments. In: Botulism 1966 (Ingram, M. and T.A. Roberts, eds), pp. 150–168, Chapman & Hall, London.

11 Roberts, T.A., A.M. Gibson and A. Robinson. 1981. Prediction of toxin production by *Clostridium botulinum* in pasteurized pork slurry. J. Food Technol. 16: 337–355.

12 Zwietering, M.H., I. Jongenburger, F.M. Rombouts and K. van't Riet. 1990. Modeling of the bacterial growth curve. Appl. Environ. Microbiol. 56: 1875–1881.