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A vitalistic model to describe the thermal inactivation of *Listeria monocytogenes*

Martin B. Cole, Kenneth W. Davies, Gary Munro, Caroline D. Holyoak and Derrick C. Kilsby

Unilever Research, Colworth Laboratory, Sharnbrook, Bedford MK44 1LQ, UK

Listeria monocytogenes; Predictive model; Thermal/heat inactivation; Death kinetics; Vitalistic; Logistic

SUMMARY

Thermal inactivation of microorganisms has traditionally been described as log-linear in nature, that is the reduction in log numbers of survivors decreases in a linear manner with time. This is despite a plethora of data that shows consistent deviations from such kinetics for a wide range of organisms and conditions and that cannot be accounted for by experimental artifacts. Existing thermal death models fail to take such deviations into account and also fail to quantify the effects of heating menstruum on heat sensitivity. The thermal inactivation of *Listeria monocytogenes* ATCC 19115 has been investigated using a factorially-designed experiment comparing 45 conditions of salt concentration, pH value and temperature. Heating was carried out using a Submerged Coil heating apparatus that minimized experimental artifacts. Low pH values increased, whilst high salt concentrations decreased heat sensitivity. Results showed a significant and consistent deviation from log-linear kinetics, particularly at low temperatures. A number of distributions were tested for suitability to describe the variability of heat sensitivity within the population of heated cells (vitalistic approach). The use of the logistic function and log dose (log time) allowed the development of an accurate unifying predictive model across the whole range of heating conditions. It is proposed that this approach should be tested as a generalized modeling technique for death kinetics of vegetative bacteria.

INTRODUCTION

Thermal death kinetics is one of the few areas where mathematical models have been used traditionally to predict critical food safety margins. This has been done by plotting the logarithm of the number of survivors against the exposure time, assuming a linear relationship and calculating decimal reduction times [6,12,19]. All the early work was carried out with bacterial spores and has served the food industry and regulatory agencies well for over 50 years.

The traditional 'log-linear' model assumes that all cells (spores) in a population have equal heat sensitivity and that death of an individual is solely dependent upon the random chance that a key molecule or 'target' within it receives sufficient heat. Indeed, in situations where death is rapid, then a good fit to this hypothesis is generally observed. However, it has been consistently reported that there are significant deviations from log-linear death in other circumstances [5,15,17,18,22,24]. There have been a number of attempts to explain these deviations and to incorporate them in the overall theory. The variability in the shape of death curves and consideration of underlying causes has been excellently reviewed by Cerf [4]. There is currently no satisfactory, unifying explanation for this variability in kinetics.

Correspondence to: Dr M.B. Cole, Unilever Research, Colworth Laboratory, Sharnbrook, Bedford MK44 1LQ, UK.

Increasingly, the food industry is requiring accurate predictions for death kinetics for vegetative bacteria under a wide range of environmental conditions. Recent concern regarding the non-sporing bacterium *Listeria monocytogenes* as a source of food-borne illness has led to a number of studies on the heat resistance of this organism. Studies have been carried out on a variety of model systems and foods including milk [3] meat and vegetables [9,13] and in relation to microwave-heated foods [10]. These studies indicate that environmental conditions during heating can have sufficient effects on thermal inactivation. Whilst a number of these conditions have been studied in other bacteria, for example, the effect of reduced water activity in increasing the heat resistance of *Salmonella* spp. [11,14], they have not been quantified for *L. monocytogenes*.

In this work, the effects of salt concentration, pH value and temperature on the thermal inactivation of *L. monocytogenes* ATCC 19115 have been investigated using a factorially-designed experiment. Heating was carried out using a Submerged Coil heating apparatus [7] in order to minimize experimental artifacts, such as slow temperature equilibrium and uneven heating. Analysis of the results using the traditional log-linear death model gave a poor fit to the data. We have developed a novel model which is based on a distribution of heat sensitivity within the population of heated cells which accurately predicts the thermal inactivation of *L. monocytogenes* under a variety of experimental conditions.

METHODS

Organisms and media

L. monocytogenes ATCC 19115 was obtained from J.S. Crowther. Cultures were grown at 30 °C for 16 h in tryptic phosphate broth (TPB) at pH 7.0 [9] and maintained on slants of tryptone soy agar (TSA) (Oxoid) at 4 °C.

Experimental design

In a factorially-designed experiment the heating menstruum consisted of all combinations of five concentrations of hydrogen ions 0.1 μM (pH 7.0), 14.8 μM (pH 5.13), 29.5 μM (pH 4.83), 43.6 μM (pH 4.53) and 57.5 μM (pH 4.24) and three concentrations of NaCl 0.1 M (0% added), 0.6 M (3% added) and 1.6 M (9% added) in TPB. The heating menstruum was buffered with a combination of citric acid (0.1 M) and di-potassium hydrogen orthophosphate (0.2 M) [20]. Each combination was heated at 56, 60 and 62 °C giving a total of 45 trials. In addition, five further replicates were carried out at the mid-point of the matrix 60 °C, 29.5 μM hydrogen-ion concentration and 0.6 M NaCl.

Thermal inactivation

An overnight culture (16 h) was resuspended in 10 ml of heating menstruum at a cell concentration of approximately 10^9 ml^{-1}. After 15 min incubation at 20 °C the suspension was heated in a Submerged Coil heating apparatus [7]. Samples (0.2 ml) were removed at predetermined approximately log-increasing time intervals and rapidly cooled to 20 °C in TPB (5 ml). After 90 min resuscitation at 20 °C to allow recovery of heat-damaged cells, survivors were counted following serial dilution in TPB and plating on TSA. Only counts represented by between 20 and 300 colonies per plate were used in the assessment of heat inactivation. In addition to this, in a small number of experiments, heating was carried out using the method of Coote et al. [10]. 1 ml aliquots of concentrated cell suspension (10^9 ml^{-1}) were sealed in plastic lay flat tubing and fully submerged in a water bath at the required heating temperature, and removed at predetermined time intervals. The numbers of survivors was determined as described earlier and the results compared to those obtained using the Submerged Coil heating apparatus.

Statistical analysis of data

Probit analysis. Percentage survivors against log time (dose) for each heating condition were converted to probit values [24] and a regression line fitted to the data using the 'PROBIT' routine of SAS statistical package (SAS Software Ltd) on a VAX mini computer. In addition to this, a more general class of distributions allowing different degrees of skew (asymmetry) and kurtosis (peakiness) was also fitted to the data by the method of Prentice [21] (Fig. 1).

Curve fitting. When using Probit analysis the tacit assumption is made that all microbes will die if treated for long enough. An alternative approach is to fit a logistic function of log survivors to log time which allows not only for this possibility but also that some may survive (see Fig. 2). A symmetric four-parameter equation was used as follows:

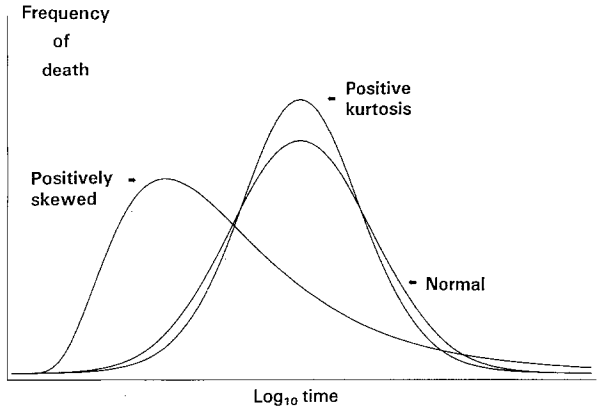

Fig. 1. Diagram to illustrate the effect of positive kurtosis (peakiness) and skew (asymmetry) on an idealized normal distribution.

$$y = \alpha + \frac{\beta}{1 + e^{\lambda - \delta x}} \quad (1)$$

where $y = \log_{10}$ (viable count), $x = \log_{10}$ (time).

The equation represents a curve with symmetry about the point $x = \lambda/\delta$ where the slope has a maximum $\beta\delta/4$ and two horizontal asymptotes given by $x = \alpha$ and $x = \alpha + \beta$. The equation was rewritten in the following form.

$$y = \alpha + \frac{\omega - \alpha}{1 + e^{4\sigma(\tau - x)/(\omega - \alpha)}} \quad (2)$$

where Alpha, α = upper asymptote
Omega, ω = lower asymptote
Tau, τ = position of maximum slope
Sigma, σ = maximum slope (see Fig. 2).

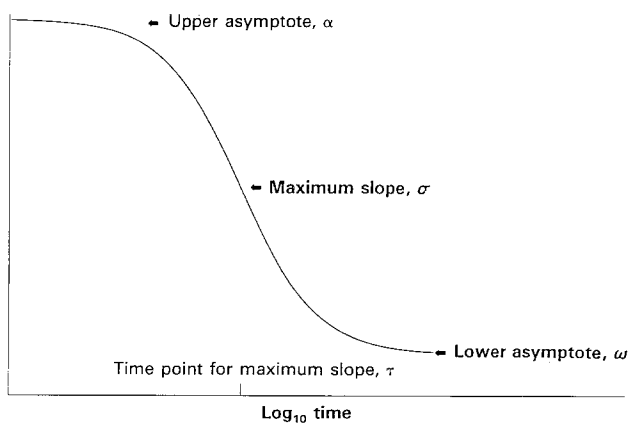

Fig. 2. Diagram to illustrate the four parameters α (upper asymptote), ω (lower asymptote), δ (maximum slope) and τ (time to maximum slope) of a logistic curve for thermal death.

For the purpose of curve fitting, when time = 0 then log time was taken as −1. The discrepancy between −1 and −infinity was considered to have little effect on the resulting fit.

Analysis of variance of fitted parameters. An analysis of variance of each of the fitted parameters was carried out on the 45 data sets under different conditions of heating. The residual variance from this analysis was compared statistically to the variance within six replicates obtained under one heating condition (60 °C, 29.5 μM hydrogen-ion concentration and 0.6 M NaCl). Curves were then refitted using the logistic equation but with the parameters at α, ω and σ set at their mean values.

Modeling of curve position. The effect of pH, salt concentration and temperature on the position of the heating curve (τ) on a log time scale was analyzed for a least squares fit to a quadratic model using SAS statistical package (SAS Software Inc.).

RESULTS

Effect of salt and pH on thermal inactivation

High concentrations of NaCl (1.6 M) significantly increased the heat resistance of *L. monocytogenes* at temperatures between 56 °C and 62 °C and between pH values of 4.24 and 7.0. For example, after 30 min at 56 °C in TPB (contains 0.1 M NaCl) the number of surviving cells had been reduced by a factor of greater than 10^4, whereas in the presence of salt (to give 1.6 M NaCl) in TPB after the same time period the number of viable cells had only been reduced by a factor of 10. The pH value of the heating menstruum also affected thermal inactivation, with low pH values generally decreasing heat resistance.

Log-linear model

There was a significant deviation from log-linear death kinetics (Fig. 3(A)), particularly under conditions where the rate of inactivation was slower e.g. at a low temperature (56 °C) and at a high salt concentration (1.6 M NaCl). Similar kinetics were obtained when cells were heated in lay flat tubing. Linear regression of all the data points for each of the 45 conditions gave a poor fit to the data (rms error = 0.72). The same 45 curves were also plotted using log heating time as the x axis (Fig. 3(B)).

Probit analysis

Predicted values obtained following a probit analysis of the data gave a closer fit to the actual data when compared to a simple log-linear model (rms error = 0.57). Despite the improvement in fit, there was a small but systematic bias in the model as illustrated by the example plot of the mid-point of the experimental matrix (29.5 μM hydrogen-ion concentration and 0.6 M NaCl) in Fig. 4.

A more generalized class of distributions allowing different degrees of skew (asymmetry) and kurtosis (peakiness) (see Fig. 1) was fitted. Allowing the distribution to be

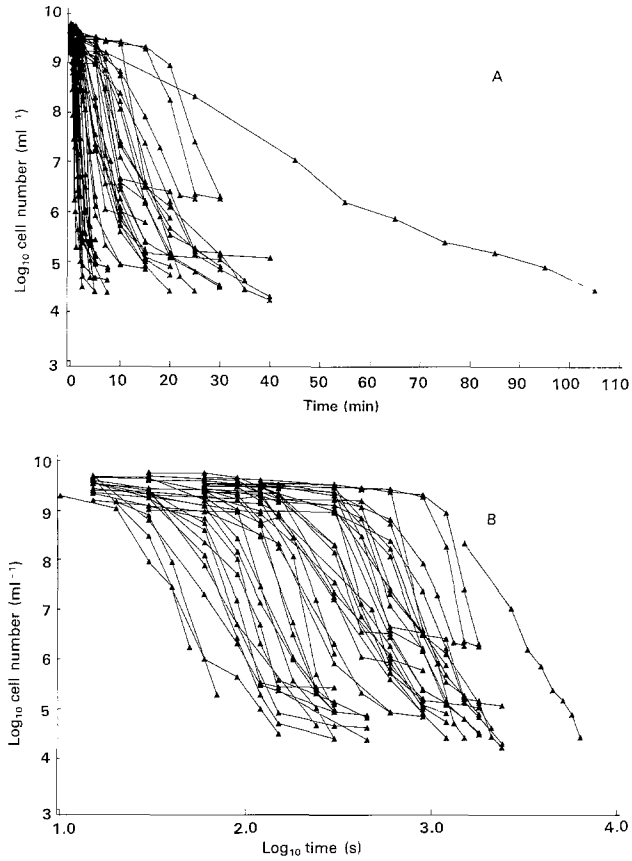

Fig. 3. Thermal inactivation of *L. monocytogenes* ATCC 19115. Cells were heated at all combinations of temperatures of 56 °C, 60 °C, 62 °C, salt concentrations of 0%, 3% and 9% added NaCl (giving 0.1 M, 0.6 M and 1.6 M in TPB) and hydrogen-ion concentrations of 0.1 μM (pH 7.0), 14.79 μM (pH 4.83), 29.51 μM (pH 4.53), 43.65 μM (pH 4.36) and 57.54 μM (pH 4.24) in TPB giving a total of 45 conditions. The logarithm of survivors is plotted against heating time (A) and log heating time (B).

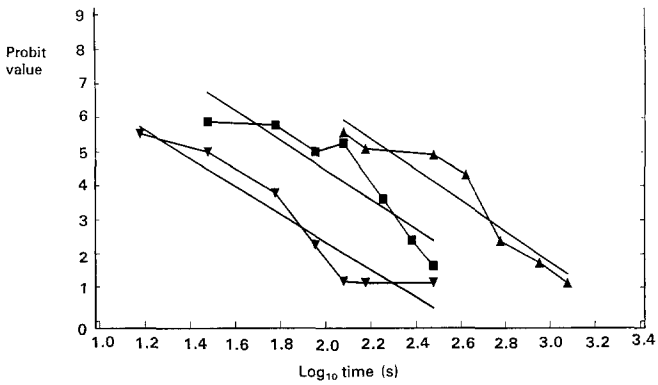

Fig. 4. Example probit plots showing the effect of temperature 56 (▲), 60 (■) and 62 (▼) °C on the thermal inactivation of *L. monocytogenes* for the mid-pont of the matrix of experimental conditions (hydrogen-ion concentration, 29.51 μM, pH 4.53 and salt concentration, 3% added NaCl, 0.6 M in TPB). Straight solid lines indicate best lines of fit following a probit analysis carried out using SAS statistical package (SAS Software, Inc.).

asymmetrical did not improve the fit to the data. Allowing peakiness to vary did eliminate the apparent bias in the model and also improved the fit (rms error = 0.337).

Logistic, logarithmic dose model

Curve fitting. Another way of describing an essentially peaky distribution with long tails is by using the logistic function. On examination of Fig. 3(B), the possibility that the data could be described by a single logistic curve, shifted on a log time axis was investigated. Of the 45 data sets obtained in the experiment it was possible to fit logistic curves to 43 of them. Insufficient points were obtained in the remaining 2 data sets (Temperature = 56 °C, H^+ = 14.79 μM, NaCl = 0.1 M, 0% added and Temperature = 62 °C, H^+ = 57.54 μM, NaCl = 0.1 M, 0% added) to allow convergence in the iterative fitting procedure. The resulting estimates for the four parameters of the logistic curve (see Fig. 2) fitted to these 43 curves along with replicate curves obtained at the mid-point of the experimental matrix (60 °C, 29.5 μM hydrogen-ion concentration and 0.6 M NaCl (3% added) are given in Table 1.

Analysis of variance of fitted parameters. Although it was impossible to test the full temperature, hydrogen-ion interaction due to omitted data sets the main effects and all other 2-factor interactions were tested. The analysis showed that τ is significantly influenced by hydrogen-ion concentration ($P>0.0001$) and the salt-temperature interaction ($P<0.02$) but that α, ω and σ were not significantly influenced by any effect. The relevant means are shown in Table 2.

The coefficient of variation (percentage variability) although not directly related to the significance level, does give some information on the general level of variability. The value for α and τ was less than 5%, but exceeded 20% and 40% respectively for ω and σ.

Modeling of curve position. Curves were refitted with α, ω and σ set at their mean values of 9.49, 4.47 and −9.74 respectively (Fig. 5). This allowed the inclusion of two data sets previously omitted (rms error = 0.125). The analysis of variance of the resulting τ values shows a significant effect of hydrogen-ion concentration ($P<0.0001$) and an even more significant interaction of salt and temperature. The means from the second variance analysis (Table 3) varied slightly from the previous (Table 2) due mainly to the inclusion of the two additional data sets (rms error = 0.305).

The effect of temperature (°C) [T], salt concentration (% NaCl added) [S] and hydrogen-ion concentration (μM) [H] on the position of the curve τ (log heating time, (s)) is shown graphically in Fig. 6 and can be represented by the following equation which has an rms error of 0.11.

$$\begin{aligned}\tau = &-26.73 + 1.132[T] - 5.407 \times 10^{-3}[H] + 8.186[S] \\ &- 1.080 \times 10^{-2}[T]^2 - 1.046 \times 10^{-1}[S]^2 \\ &- 2.688 \times 10^{-1}[T][S] + 1.885 \times 10^{-3}[T][S]^2 \\ &+ 2.206 \times 10^{-3}[S][T]^2\end{aligned} \quad (3)$$

TABLE 1

Table to show the resulting estimates for the four parameters of the logistic curve (see Eqn 2, in text) fitted to 43 data sets along with replicate curves at the mid-point of the experimental matrix (60 °C, 29.5 μM hydrogen-ion concentration and 0.6 M (3% added NaCl)). Logistic curves were fitted to the logarithm of survivors against log time using SAS statistical package (SAS Software Ltd)

Obs	Rep	Temp	Salt	Hyd	Alpha	Sigma	Omega	Tau
1	1	56	0	0.10	9.50	−6.11	2.50	3.07
2	1	56	0	14.79	9.42	−10.79	4.88	2.66
3	1	56	0	29.51	9.32	−7.11	4.78	2.51
4	1	56	0	43.65	9.55	−7.67	5.43	2.55
5	1	56	0	57.54	9.48	−7.85	4.70	2.36
6	1	56	3	0.10	9.21	−9.30	3.79	3.04
7	1	56	3	14.79	9.58	−8.13	5.28	2.82
8	1	56	3	29.51	9.41	−8.22	4.74	2.81
9	1	56	3	43.65	9.41	−6.85	5.86	2.71
10	1	56	3	57.54	9.51	−7.79	5.58	2.55
11	1	56	9	0.10	9.55	−6.92	2.82	3.54
12	1	56	9	29.51	9.45	−29.36	6.22	3.09
13	1	56	9	43.65	9.29	−8.52	4.58	3.07
14	1	56	9	57.54	9.38	−13.97	1.67	3.07
15	1	60	0	0.10	9.29	−6.24	3.71	2.66
16	1	60	0	14.79	9.49	−11.77	3.86	2.27
17	1	60	0	29.51	9.42	−10.72	4.31	2.24
18	1	60	0	43.65	9.58	−8.08	4.62	2.09
19	1	60	0	57.54	9.67	−8.03	4.42	1.90
20	1	60	3	0.10	9.13	−8.28	4.77	2.35
21	1	60	3	14.79	9.63	−15.29	2.13	2.40
22	1	60	3	29.51	9.63	−4.06	3.37	1.82
23	1	60	3	43.65	9.68	−11.16	4.49	2.12
24	1	60	3	57.54	9.70	−8.58	4.86	2.10
25	1	60	9	0.10	9.58	−10.58	3.81	3.01
26	1	60	9	14.79	9.54	−9.20	4.60	2.90
27	1	60	9	29.51	9.59	−10.11	4.18	2.89
28	1	60	9	43.65	9.73	−7.09	4.95	2.82
29	1	60	9	57.54	9.59	−8.99	4.74	2.61
30	1	62	0	0.10	9.62	−10.27	4.25	1.90
31	1	62	0	14.79	9.43	−10.68	4.64	1.93
32	1	62	0	29.51	9.77	−6.55	4.80	1.77
33	1	62	0	43.65	9.48	−9.67	3.60	1.67
34	1	62	3	0.10	9.41	−8.79	4.23	2.04
35	1	62	3	14.79	9.42	−9.78	4.97	2.00
36	1	62	3	29.51	9.56	−9.49	5.35	1.89
37	1	62	3	43.65	9.77	−7.89	4.53	1.66
38	1	62	3	57.54	9.34	−12.35	4.05	1.72
39	1	62	9	0.10	9.37	−8.80	4.79	2.72
40	1	62	9	14.79	9.01	−8.38	4.68	2.68
41	1	62	9	29.51	9.70	−6.41	6.43	2.63
42	1	62	9	43.65	9.66	−7.57	4.76	2.49
43	1	62	9	57.54	9.76	−6.83	4.74	2.53
44	2	60	3	29.51	8.56	−13.22	5.78	1.95
45	3	60	3	29.51	8.68	−4.71	5.48	2.04
46	4	60	3	29.51	9.39	−4.16	4.81	2.17
47	5	60	3	29.51	9.03	−4.02	5.17	2.21
48	6	60	3	29.51	9.42	−3.82	5.57	2.09

TABLE 2

Table of means for the four parameters of the logistic curve following analysis of variance. Only main effects and 2-factor interactions between temperature and salt concentration were calculated due to omitted data sets (see text for details)

		α	σ	ω	τ	Salt		
						0	3	9
Temperature	56	9.4	−10.1	4.4	2.9	2.6	2.8	3.2
	60	9.5	−9.3	4.2	2.4	2.2	2.2	2.9
	62	9.5	−9.4	4.7	2.1	1.8	1.9	2.7
Salt	0	9.5	−8.6	4.3	2.2			
Hydrogen ion concentration	0.1	9.4	−8.4	3.9	2.7			
	14.8	9.4	−10.7	4.3	2.6			
	29.5	9.5	−10.4	5.0	2.4			
	43.7	9.6	−9.4	4.7	2.4			
	57.5	9.6	−9.1	4.5	2.2			

Example prediction. To predict log reduction in viable numbers after 100 s at:
Temperature [T] = 62 °C
Added salt concentration [S] = 0% (0.1 M NaCl)
Hydrogen-ion concentration [H] = 0.1 μM (pH 7.0)
Substituting into Eqn 3:

$$\tau = -26.73 + 1.132[62] - 5.407 \times 10^{-3}[0.1] + 8.186[0] - 1.080 \times 10^{-2}[62]^2 - 1.046 \times 10^{-1}[0]^2 - 2.688 \times 10^{-1}[62][0] + 1.885 \times 10^{-3}[62][0]^2 + 2.206 \times 10^{-3}[0][62]^2$$

$$\tau = 1.94$$

Substituting into Eqn 2:

$$y = \alpha + \frac{\omega - \alpha}{1 + e^{4\sigma(\tau - x)/(\omega - \alpha)}}$$

where $\alpha = 9.49$
$\omega = 4.47$
$\tau = 1.94$
$\sigma = -9.74$
x = log heating time = 2(100 s)
$y = 6.41$

Predicted Log reduction = α(Initial inoculum) − y
= 9.49 − 6.41
= 3.08
(Observed Log reduction = 2.89)

DISCUSSION

Increasingly, the food industry requires accurate predictions of the death of vegetative bacteria under mild heating conditions and under a wide range of environmental conditions where it is not possible to build in the relatively large safety margins traditionally used in thermal processing for ambient stability, e.g. canning. Deviations from the log-linear death model under mild heating regimes mean that it cannot be relied upon for predicting product safety.

The theory underlying most log-linear death models assumes that inactivation results from random 'single hits' of key targets within microbial cells. There are several alternative theories to explain microbial death kinetics. One assumes that there is a distribution of heat sensitivities within a population of cells and that all cells receive the same amount of heat. This leads us to produce a dose response curve where time at a given temperature is the measure of dose. This theory has been described by Withell [24] where it was proposed that log dose is the appropriate parameter to normalize the observed response. The probit transformation does this by linearizing the normal probability function thus producing a straight line [2]. This approach was evaluated and a very much better fit to the data was obtained than the log-linear model. However, on careful examination of data we could detect a systematic bias in the probit vs log time plots. One reason for failure to fit the probit plot is deviation from normality. In the first instance we investigated lack of normality by the use of 'skew' but it soon became obvious that this did not account for the observed bias. When the use of kurtosis (peaky distributions) was investigated, a closer fit to the data was observed and this convinced us that we had a symmetrical distribution albeit not entirely normal. There are other distributions that are symmetrical but not normal that have been used to investigate microbial kinetics [16], one we have particular experience with is the logistic curve which gave a good fit to all the available data.

Using the logistic curve and a log dose model the only significant correlation that was observed was between environmental conditions and the position of the curve on the log time axis, or τ (that is α, ω and σ did not vary significantly as conditions varied). This enabled us to build a generalized model where α, ω and σ could be calculated as the mean of 48 observations. This further increased the goodness of fit and provided a generalized model for predicting the death of *L. monocytogenes* under a wide variety of environmental conditions. The resulting model is relatively simple, based as it is on a single variable τ and is easy to use. There were insufficient data points around the lower asymptote to be able to be sure that it remains constant under different conditions. Therefore, while setting ω to a mean value did allow a good fit to the experimental data, we cannot be completely confident that the model describes survival after very long heating periods.

Until now it has been difficult to obtain a model which can be used to make predictions as environmental parameters other than heat are varied [1,14,15,23]. This approach enables us to move from one set of environment conditions to another with confidence and accuracy. Although this model only goes as high as 62 °C other work within this laboratory indicates that the time for a six-log reduction

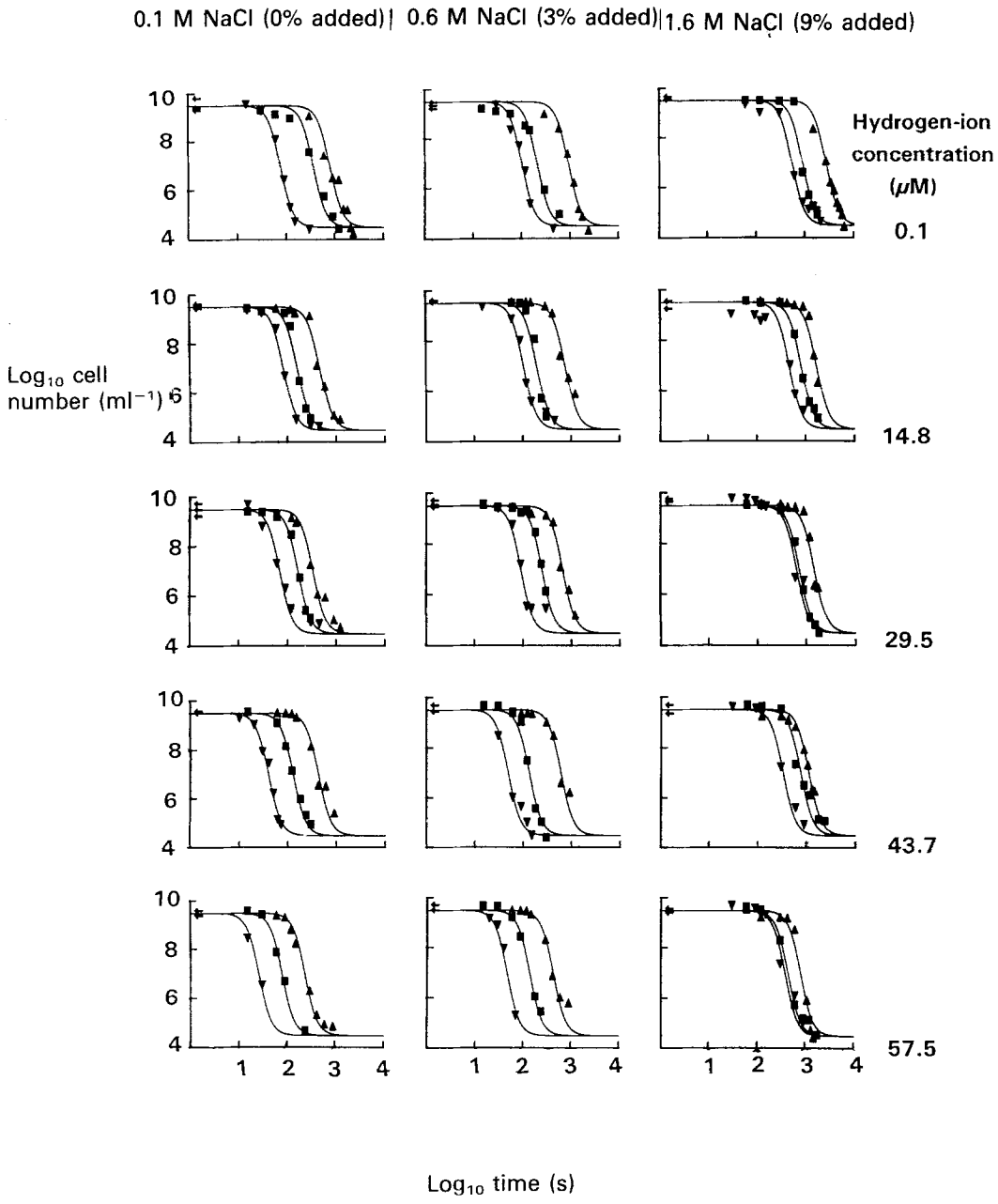

Fig. 5. Effect of salt concentration (0–9% added NaCl, 0.1–1.6 M in TPB), hydrogen-ion concentration (0.1 μM (pH 7.0)–57.54 μM (pH 4.24) and temperature (56(▲), 60(■) and 62(▼) °C) on the thermal inactivation of *L. monocytogenes* ATCC 19115. Actual experimental points are plotted together with the best fit to a logistic equation (see Fig. 2) with α, ω and σ fixed at their mean values of 9.49, 4.47 and −9.74 respectively (see Methods for details).

predicted at 70 °C is very close to other published data [10, 13].

The environmental parameters used to build this model were hydrogen-ion concentration and salt concentration. The model not only illustrates the protective effect of salt but also for the first time quantifies it. As pH was decreased from 7.0 there was a small but significant increase in heat sensitivity of *L. monocytogenes*. It will be observed that hydrogen-ion concentration and not pH was used as the other variable. In our experience [8] the observed response of bacterial kinetics to hydrogen-ion concentration is almost linear which both simplifies and increases the power of the resulting predicting model. Measurements of approximately equal log times and mild temperatures were used to develop this modeling technique. The use of linear time intervals or high temperatures would have meant that the results would have lacked the necessary discrimination to effectively compare different approaches.

In order to produce a predictive model for the death of *L. monocytogenes* we recognized the need to use very well

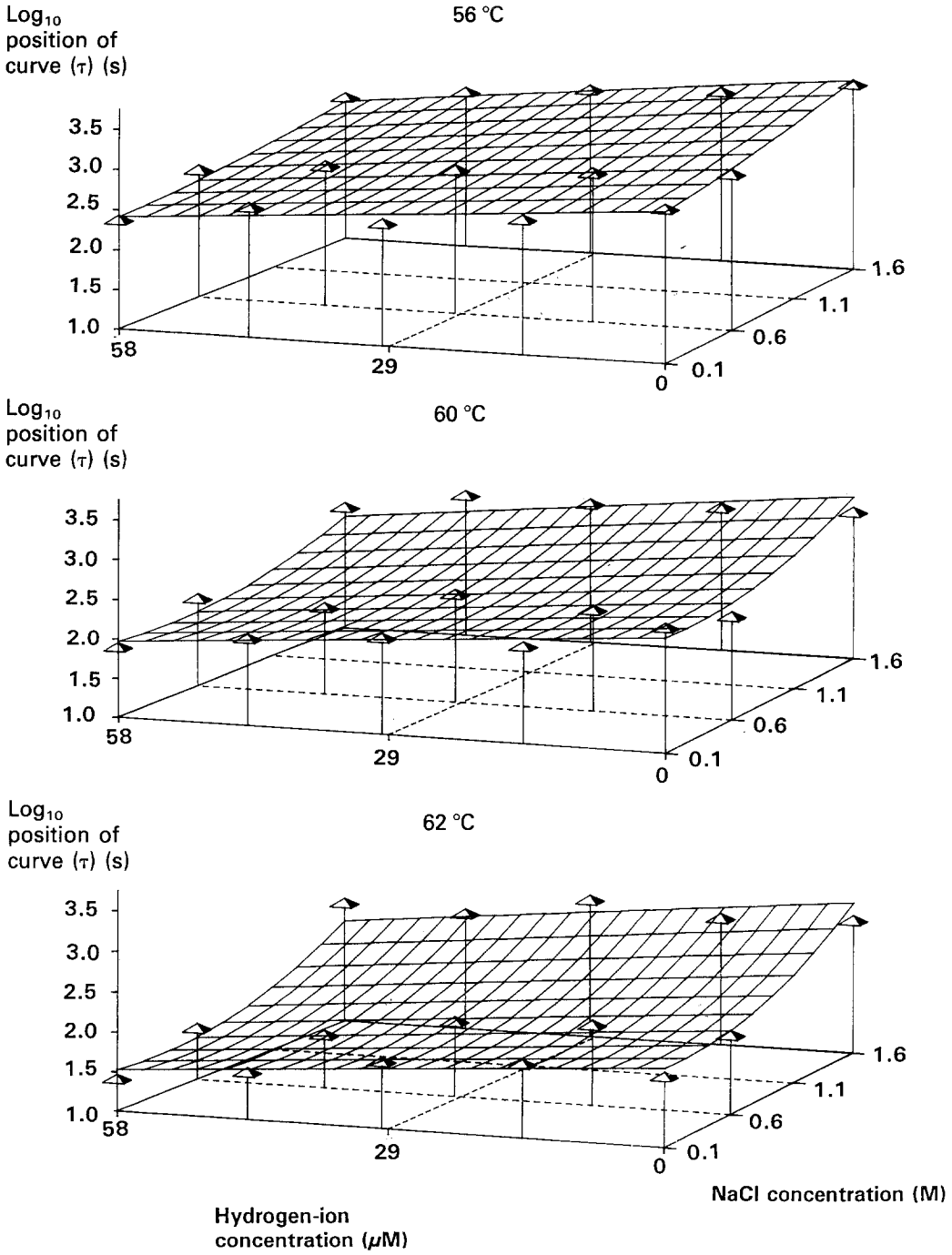

Fig. 6. Three-dimensional response surfaces showing the influence of added salt concentration and hydrogen-ion concentration on the position of maximum slope τ (log time, s) of a logistic curve describing the thermal inactivation of *L. monocytogenes* ATCC 19115 at 56 °C, 60 °C and 62 °C. The effects of salt concentration of hydrogen-ion concentration on τ were quantified by fitting to a polynomial equation (Eqn 1 in Results) using SAS statistical package (SAS Software, Inc.).

controlled heating times and temperatures by using the Submerged Coil heating apparatus and to employ an experimental design enabling us to achieve the necessary discrimination between test conditions. Our first approach was to use classical (mechanistic) log-linear death curves for modeling but the very high variability observed convinced us that this was not a robust model. The use of the log dose (vitalistic approach) resulted in a large increase in confidence but it was only when the logistic curve was used with the log dose model that an accurate unifying predictive model could be developed across the whole range of conditions tested. We propose that this approach should be tested as a generalized modeling technique for the death kinetics of vegetative bacteria.

TABLE 3

Table of means following analysis of variance when the parameters α (upper asymptote), ω (lower asymptote) and σ (maximum slope) of a logistic curve were set at their mean values of 9.49, 4.47 and −9.74 respectively

		τ	Salt		
			0	3	9
Temperature	56	2.9	2.6	2.8	3.2
	60	2.4	2.2	2.2	2.9
	62	2.2	1.8	1.9	2.8
Salt	0	2.2			
	3	2.3			
	9	2.9			
Hydrogen ion concentration	0.1	2.6			
	14.8	2.5			
	29.5	2.5			
	43.7	2.4			
	57.5	2.3			

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