Model of the influence of time and mild temperature on *Listeria monocytogenes* nonlinear survival curves

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Abstract

Heat treatment has long been regarded as one of the most widely used and most effective means of destroying pathogens in food. Up to now the linear relationship between the death rate and the temperature has been used when choosing the best heat treatment to apply. However, the information given by this linear relationship is no longer sufficient when nonlinear survival curves are observed. Consequently, the agri-food industry needs a tool to choose the best mild heat treatment to apply in the case of nonlinear survival curves. This study deals with the temperature-induced death of *Listeria monocytogenes* CIP 7831 in the stationary phase of growth. Eleven temperatures were tested. With the proposed primary and secondary models good fits of our data were obtained. A model describing both the effect of the duration of treatment and the temperature on the logarithm of the number of survivors was then built. A clear increase in the precision of the estimation of the parameters was obtained with this model. Moreover, with this model a new graphical strategy to choose a mild heat increase regarding a maximal survivor number has been proposed. © 1998 Elsevier Science B.V.

Keywords: *Listeria monocytogenes*; Heat treatment

1. Introduction

When the environmental temperature of a bacterial population increases up to a nonviable temperature higher than $T_{\text{max}}$, i.e. the maximum temperature for which the growth of a bacterial population can be observed, bacterial death is observed. The survival curve of the population is then obtained by plotting the logarithm of the number of surviving microorganisms against the time of exposure to the nonviable temperature.

The linear relationship observed (Bigelow, 1920; Watkins and Winslow, 1932; Charm, 1958) between
the logarithm of the number of microorganisms and time, has led authors to summarize information about population death by the death rate estimated on the survival curve. In the case of linear survival curves, the death rate is independent of time and inoculum (Casolari, 1981). In 1920, Bigelow observed a linear relationship between the logarithm of the death rate and the temperature inducing death. This observation led him to define the Z value as the reciprocal of the slope of this straight line. The Z value represents the required increase in temperature to increase the death rate ten-fold.

Significant deviations from linear survival curves have been consistently reported (Withell, 1942; Whiting, 1993; Linton et al., 1995; Pflug and Holcomb, 1983; Jordan et al., 1948). Three kinds of deviations have been observed: (a) a shoulder or initial lag in cell inactivation, followed by linear or nearly linear behaviour (b) a concave curve and (c) a sigmoid curve (Casolari, 1981). In most cases, deviations from linear survival curves were observed when death was induced by a mild increase in temperature (Cole et al., 1993; Pruitt and Kamau, 1993; Little et al., 1994). When nonlinear survival curves are observed, the death rate and the Z value are no longer appropriate to summarize the population death (Moats, 1971), and calculations from these nonlinear survival curves have proven to be complicated (King et al., 1979; Rahn, 1930; Withell, 1942; Jordan et al., 1948; Charm, 1958; Hansen and Rieman, 1963).

Heat treatment has long been one of the most widely used and most effective means of preventing spoilage and pathogenic microorganisms in food (Linton et al., 1995). After heat treatment, the number of surviving pathogens in the foodstuff has to be under an acceptable threshold. So far, the definition of the heat treatment required to reach this goal, is based on the death rate and the Z value (Casolari, 1981; Cole et al., 1993; Linton et al., 1995). Nevertheless, it has been reported that after heat treatment the acceptable threshold could be exceeded (Black et al., 1978; Schiemann, 1978; Hugues, 1980; Lovett et al., 1982). The capacity of certain bacteria to resist heat treatment is a cause of concern, for example to the milk industry (Sanz Perez et al., 1982; Bradshaw et al., 1987; Golden et al., 1988): microorganisms which have withstood the heat treatment will grow during storage (Carpenter and Harrison, 1989; Whiting and Masana, 1994). The ability to withstand a treatment is partly linked to the growth stage of the bacterial population before treatment. According to the literature, a bacterial population in the stationary phase of growth is more heat resistant than an exponentially growing one (Hurst et al., 1974; Jenkins et al., 1990; Knochel and Gould, 1995; Smith, 1995). Moreover, the stationary phase of growth is the state most often encountered in a natural environment (Rees et al., 1995; Jiang and Chai, 1996).

Colony forming units (cfu) values higher than the acceptable threshold could be explained by a deviation from linearity in the survival curve. This deviation from linearity should be increasingly observed because of the consumer demand for ‘fresh’ and ready-to-eat products. Processing of such foodstuffs implies a mild increase in temperature (O’Connor-Cox et al., 1991; Baranyi et al., 1996; Roberts, 1995; Hansen and Knochel, 1996), for which nonlinear survival curves are frequently observed (Stiles and Witter, 1965). Some concern has thus been expressed about the microbiological risk involved in processing these new products (Hansen and Knochel, 1996; Baird-Parker, 1994). Consequently the agri-food industry requires a new mathematical tool in order to describe death induced by mild heat.

More precise descriptions of survival curves require experimental data which are not easily available. Let us consider the case of Listeria monocytogenes. Although unusual heat resistance of this pathogen has been reported (Coote et al., 1991) in, for example, meat (Mackey and Bratchell, 1989) and milk (Bradshaw et al., 1985), data on its thermal inactivation are limited (Bradshaw et al., 1985).

The aim of the study presented in this paper, is to propose a way of building a new system for determining mild heat treatment, with regard to an acceptable survival level. The use of empirical modelling allows us to propose a strategy to cope with nonlinear survival curves such as those observed with Listeria monocytogenes in our laboratory. This study deals with heat induced death of Listeria monocytogenes in the stationary phase of growth since it represents the most realistic and pessimistic situation from an agri-food viewpoint.
2. Materials and methods

2.1. Bacterial strain and media

The reference strain *Listeria monocytogenes* CIP 7831 (ATCC 35152) was used in this study. Stock culture was stored at −196°C in brain heart infusion broth (BioMérieux, Marcy l’Etoile, France) and subcultured twice at 35°C on Columbia sheep blood agar (Diagnostics Pasteur, Marne la Coquette, France). Before the temperature increase the bacterial population was cultured on Columbia sheep blood agar at 35°C for 24 h in order to obtain cells in the stationary phase of growth. All the experiments were conducted with the same batch of trycase soy broth (BioMérieux).

2.2. Temperature change

2.2.1. Definition

The temperature increase studied was defined as follows: an abrupt increase from a before-stress temperature, \( T_{bs} \) fixed between \( T_{min} \) and \( T_{max} \) to a stress temperature \( T_s \) higher than \( T_{max} \), for a given stress duration \( d_s \).

Since we were interested in the response of a bacterial population to a mild temperature increase the stress temperatures chosen were a little higher than \( T_{max} \). The maximal growth temperature of the studied *L. monocytogenes* is roughly 48–49°C, thus the studied stress temperatures \( T_s \) varied between 53 and 60°C; eleven experiments were tested: 53, 54, 55, 56, 56.5, 57, 57.5, 58, 58.5, 59 and 60°C. Stress durations varied between 0 and 120 min for stress temperatures of 56°C and 60°C and between 0 and 250 min for the other temperatures.

2.2.2. Experimental design

Two 250-ml special Erlenmeyer flasks, containing 225 ml of trycase soy broth were used for these experiments: they were equipped with a system to check temperature inside the flask. Their caps received spinal needles (Becton Dickinson, San Jose, USA) each one of which was used only once for inoculation and retrieving of samples. The two flasks were incubated in thermostated water baths. The medium was aerated by shaking with a magnetic stirrer.

One of the flasks was maintained at \( T_{bs} = 35°C \) and inoculated with 25 ml of a bacterial suspension to yield an initial count of \( 3 \times 10^6 \) cfu/ml. After shaking for 5 min, 25 ml of the suspension maintained at \( T_{bs} \) were taken and injected into the second Erlenmeyer maintained at \( T_s \). Regularly, 4 ml of the bacterial suspension maintained at \( T_s \) were removed.

Sampling dates corresponding to different stress durations, were chosen in agreement with studied \( T_s \), i.e. the sampling frequency increased with the increase in \( T_s \) as we presumed the decrease of \( D \) values.

2.3. Plate counts

At each sampling time, the viable number of cells was determined by plating 0.1 ml of appropriate dilutions of the sample twice. After 48 h and 72 h of incubation at 35°C, the colonies were counted using a specific apparatus (Bioblock Scientific 88752, Illkirch, France).

2.4. Data analysis

2.4.1. Modelling of survival curves

Different rules were postulated in order to build the model:

1. simplicity of the model
2. limiting the number of parameters of the mathematical model
3. parameters of the model having a graphic or biological meaning in order to facilitate the choice of their initial values and to allow an assessment of the reliability of estimations by the biologists.

Model Eq. (1) was used in this study to describe the survival curves observed:\(^1\)

\[
\begin{align*}
\text{If } d_s < \text{lag, } & \log_{10}N(d_s) = \log_{10}N_0; \\
\text{if } d_s \geq \text{lag, } & \log_{10}N(d_s) = \log_{10}N_0 - k(d_s - \text{lag})
\end{align*}
\]

\(N_0\) is the inoculum, \(k\) is the death rate, \(\text{lag}\) is the lag time before death.

\(^1\)Equations Eqs. (1)−(4) are statistical models, i.e. an error term should be added on the right side of these equations.
The model in Eq. (1) was chosen by comparing the fitting of different models to our experimental data. Two logistic models with delay (lag) were considered: one with an adaptation rate, between the lag phase and the decrease phase, equal to the death rate and one with the adaptation rate equal to infinity — instantaneous transition between lag and decrease. Moreover, during the lag phase a death rate noted \( k_0 \) was estimated. Since the number of parameters of the two models was the same, the residual sum of square (RSS) obtained after fitting was used to choose between these two models. The model with an instantaneous adaptation was thus chosen since globally, better fits were obtained with it.

In order to cope with one of our guidelines — limiting the number of parameters, the necessity of \( k_0 \) in the model was tested by the nested models test (Bates and Watts, 1988). Given our experimental data, with a level of significance, \( \alpha \), of 5%, in most cases — i.e. ten out of eleven — \( k_0 \) did not induce sufficient decrease in the RSS to be considered in the final model. Consequently, model Eq. (1) with instantaneous transition between lag and decrease and with no decrease during lag, was chosen.

Due to estimations of survival curves characteristics realized with Eq. (1), secondary models Eqs. (2) and (3) were built:

\[
k = -a10^{-\frac{T_s - 53}{Z}}
\]

where \( Z \) is the classical \( Z_{10} \) value — i.e. the required increase in temperature to multiply the death rate ten-fold,

\[
lag = c - d(T_s - 53)
\]

\( T_s \) varying between 53 and 60°C.

Models Eqs. (2) and (3) allowed us to build a mathematical model describing the evolution of \( \log_{10}(\text{cfu/ml}) \), when both \( d_s \) and \( T_s \) vary. This model is expressed as Eq. (4):

\[
\log_{10}N(d_s, T_s^*) = \begin{cases} 
\log_{10}N_0 + a10^{-\frac{T^*}{d}} (d_s - (c - dT_s^*)), & \text{if } d_s \geq c - dT_s^* \\
\log_{10}N_0, & \text{otherwise} 
\end{cases}
\]

with \( T_s^* = T_s - 53 \)

When a new mathematical model is built it is interesting to assess in which way this new model improves the description of the data. Consequently the evolution of \( \log_{10}(\text{cfu/ml}) \) when both \( d_s \) and \( T_s \) vary was also described by using the model classically used in agri-food, built with Eq. (2) and the assumption of linear survival curves, it was expressed as Eq. (5):

\[
\log_{10}N(d_s, T_s^*) = \log_{10}N_0 + a10^{-\frac{T^*}{d}} d_s,
\]

with \( T_s^* = T_s - 53 \)

After fitting model Eq. (4) or Eq. (5) to our data, 5000 theoretical points were computed with the parameters fixed to their estimated values. In order to facilitate the representation of the fit, the surface defined by these points was smoothed using distance weighted least squares routine of SYSTAT 5.2 (Systat, Evanston, Illinois, USA).

2.4.2. Parameters estimation

All fits were performed by nonlinear regression using the ordinary least square criterion.

2.4.3. Residuals

The residual analysis for each fit was performed thanks to the graphs of residuals vs. the control variable and vs. the predicted variable.

2.4.4. Jackknife estimations — confidence intervals

In this study, confidence intervals were computed with the jackknife theory. The jackknife (Quenouille, 1956) is an iterative method which consists of estimating Eq. (1) parameters on a new data set obtained by deleting one observation from the initial one. This is repeated until all observations of the initial data set have been omitted once. By combining all these estimations, new and more robust estimations are obtained (Simonoff and Thai, 1986). Moreover, this theory allows estimation of symmetric confidence intervals without linearization of model Eq. (1) which is nonlinear with regard to its parameters.

3. Results

Curves of \( \log_{10}(\text{cfu/ml}) \) versus stress duration \( (d_s) \) are plotted in Fig. 1. From \( T_s \) to 60°C, two phase survival curves were observed. The first phase consisted of rough constancy in \( \log_{10}(\text{cfu/ml}) \) num-
Fig. 1. Survival curves observed when a population of *L. monocytogenes* in stationary phase of growth has undergone an increase in temperature up to the stress temperature indicated on the plots. Survival curves were fitted with model Eq. (1) and with the linear model (dotted line).
ber; whereas the second phase consisted of a sharper decrease in \( \log_{10}(\text{cfu/ml}) \) value. As indicated in Fig. 1, duration of the first phase decreased with stress temperature. This figure also points out the good quality of fit of model Eq. (1), whatever the stress temperature. The part of total variation of the data explained by Eq. (1) was roughly 98% except for 56°C for which Eq. (1) explained 80% of the total variation. Globally, according to the residual distributions, no deviation from the nonlinear regression hypotheses was detected. This was not the case when the survival curves were fitted with a linear model, as indicated by Fig. 1.

The relationship between \( k \) and \( T_s \) presented an exponential trend (Fig. 2). This trend was correctly described by Eq. (2) which explained 94% of the total variation of the data. Estimation of parameters \( a \) and \( (1/Z) \) obtained by fitting Eq. (2) to our data are presented in Table 1. According to Fig. 2 we can assume that like for higher stress temperatures (Bigelow, 1920), \( k \) decreases exponentially on increasing stress temperatures from 53 to 60°C.

The relationship between lag and \( T_s \) was correctly described by Eq. (3) (Fig. 3) which explained 90% of the total variation of the data. Estimation of parameters \( c \) and \( d \) obtained by fitting model Eq. (3) to our data are presented in Table 1.

The linear relationship observed between lag and \( T_s \) allows the discrimination between an increase in temperature — \( (d, T_s) \) couples — for which death will be observed and one for which death will not be observed (Fig. 4). With this linear relationship, it is possible to predict the lowest stress duration necessary for death to be observed, given the stress temperature; or the lowest stress temperature necessary, given the stress duration.

### Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Model Eq. (2)</th>
<th>Model Eq. (3)</th>
<th>Model Eq. (4)</th>
<th>Model Eq. (5)</th>
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<tbody>
<tr>
<td>( a )</td>
<td>0.0132±0.0041</td>
<td>0.0193±0.0020</td>
<td>0.0119±0.001248</td>
<td></td>
</tr>
<tr>
<td>1/Z</td>
<td>0.1446±0.0207</td>
<td>0.1249±0.0088</td>
<td></td>
<td>0.3197±0.0183</td>
</tr>
<tr>
<td>( c )</td>
<td>106±13.68</td>
<td>102±10.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( d )</td>
<td>14±2.88</td>
<td>14±1.48</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Estimation±standard error.
Fig. 4. Representation in a $d_s$ vs. $T_s$ frame of the theoretical linear relationship between lag and $T_s$ estimated with Eq. (3). For an increase in temperature such that the point $(d_s, T_s)$ is below this straight line (expressing lag vs. $T_s$) no death is observed; for an increase in temperature such that the point $(d_s, T_s)$ is above this straight line death is observed. On both sides of this straight line, 95% confidence bands for predicted lag are plotted.

Eq. (4), the control variables of which were $d_s$ and $T_s$, was built by using Eqs. (2) and (3); $k$ and lag were replaced in Eq. (1) by their secondary models.

Estimations of parameters $a$, $1/Z$, $c$ and $d$ obtained when Eq. (4) was fitted to our data are presented in Table 1. Similar estimations of these parameters were obtained with Eq. (4) or with Eqs. (2) and (3), nevertheless estimations obtained with model Eq. (4) were more precise (Table 1). Moreover, $1/Z$ was estimated with a greater precision with Eq. (4) than with Eq. (5) (Table 1). The corrected $R$-squared (Tomassone et al., 1993), which takes into account the degrees of freedom of the model, was 0.83 with Eq. (4), whereas with Eq. (5) it was 0.71.

The theoretical surface obtained by fitting all our data with Eq. (4), is presented in Fig. 5a. This figure reveals the existence of a plateau in $\log_{10}(\text{CFU/ml})$ evolution, the border of which was determined by linear relationship Eq. (3) observed between lag and $T_s$ (Fig. 5a). On this plateau no death was observed (Fig. 5a). When all our data were fitted with Eq. (5) (Fig. 5b), the observations were randomly arranged around the theoretical surface, this is in disagreement with the background of the regression.

From the theoretical surface built, sets of points $(d_s, T_s)$ defining equal heat treatments regarding the number of survivors, could be defined by cutting the theoretical surface according to a plane perpendicular to the vertical axis — i.e. axis representing $\log_{10}(\text{CFU/ml})$. This could be made for different
inducing death was also valid for mild temperatures. This result leads us to assume validity of this law first discovered by Bigelow (1920) for \textit{L. monocytogenes} and all the temperatures higher than \( T_{\text{max}} \). Studies with other strains could be realized in order to assess the ability of this law to describe the relationship between the death rate, estimated in the linear part of the survival curve, and the stress temperature whatever the shape of the survival curve.

A linear relationship between the lag time preceding the decrease in log(cfu/ml) and the stress temperature was observed. This result has already been observed but in the case of acidity induced death. Parish and Higgins (1989) studying a strain of \textit{L. monocytogenes} have observed nonlinear survival curves with a lag time preceding decrease in log(cfu/ml). When lag times were plotted against the stress pH, a linear trend was observed. These two results reveal that simple mathematical models are satisfying to describe the death even in the case of nonlinear survival curves. If this is valid for other strains and other products, building of a new indicator of death in the case of nonlinear survival curves would be facilitated which would be a great advantage for agri-food industry.

With the simple secondary models describing the death rate and the lag time against the stress temperature used in this study, a mathematical model values of log\(_{10}\) (cfu/ml). Curves thus obtained are presented in Fig. 6, all \((d_s, T_s)\) points on a given curve, labelled by log\(_{10}\) (cfu/ml) value at which the surface has been cut (Fig. 6), will induce the same survival number, given the inoculum.

4. Discussion

As in many other cases (Withell, 1942; Linton et al., 1995), this study has shown that the linear model, for which the death rate is constant whatever the stress duration, is not always the most appropriate to describe the log\(_{10}\) (cfu/ml) evolution. Consequently in many cases no tool is available to choose the best mild heat treatment to apply.

Modelling of survival curves obtained in our laboratory with \textit{L. monocytogenes} in TSB, has shown that the linear relationship between the logarithm of the death rate and the temperature...
with our data concerning *L. monocytogenes* has allowed us to define a strategy to choose appropriate couples \((d_s, T_s)\) in regard to an acceptable threshold for the survivors. As indicated in Section 3, sets of points \((d_s, T_s)\) inducing the same survival number for a fixed inoculum can be defined (Fig. 6) with the surface representing the evolution of log(cfu/ml) against time and mild temperature (Fig. 5). For example it could be considered to define this set of points for the latter acceptable threshold: all the \((d_s, T_s)\) couples \((d_s, T_s)\) points placed on the \(\log_{10}\) (cfu/ml) = legal or acceptable threshold curve would define appropriate heat treatments. Finally the choice of one couple \((d_s, T_s)\) could be made by comparing how functional qualities of the product are altered with short stress duration and relatively high stress temperature, or with long stress duration and relatively low stress temperature treatment.

The same work was made with Eq. (5) (Fig. 6). The superimposition of the curves obtained with Eq. (4) or Eq. (5) built with our data, pointed out that milder treatments could be defined if the fact the death rate was not constant with \(d_s\) was integrated in especially pathogenic ones. This proposition seems reasonable especially as simple mathematical models could be applied to other strains in stationary or exponential phase of growth, whatever the shape of the survival curves. This facility is in fact a great advantage since the death depends upon a number of factors. The quantitative characteristics and the shape of survival curves vary a lot with the protocol, the medium, the physiological stage of the inoculum (Watkins and Winslow, 1932) and the rate of increase in temperature (Mackey and Derrick, 1987b; Tsuchido, 1982). Moreover, the heat resistance is dependent upon the environmental conditions prior to the heat treatment. Authors have observed the increase in heat resistance with the increase of the viable temperature before heat treatment (Mackey and Derrick, 1987a, 1986). This great variability implies that for each new foodstuff, survival of pathogens or microorganisms responsible for spoilage, this new product has to be studied (Garibaldi et al., 1969).

These latter remarks point out the necessity for agri-food institutions to build a knowledge database on survival curves of different microorganisms, especially pathogenic ones. This proposition seems reasonable especially as simple mathematical models could describe these data.

Since the demand of consumers for fresh and natural foodstuffs implies mild heat treatment, it seems more and more difficult to completely eradicate a bacterial population contaminating a foodstuff. Consequently, the study of death of microorganisms has to include a study of the regrowth of the survivors, in order to predict the shelf life of a foodstuff more precisely. This is currently done, for the strain studied herein, in our laboratory (Bréand et al., 1997).

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**References**


