Journal of Applied Microbiology 1998, **84**, 185–191

Mathematical modelling of the heat resistance of *Listeria monocytogenes*

J.C. Augustin, V. Carlier and J. Rozier

Service d'Hygiène et Industrie des Denrées Animales et d'Origine Animale, Ecole Nationale Vétérinaire d'Alfort, Maisons Alfort, Cedex, France

6093/02/97: Received 10 February 1997, revised 16 April 1997 and accepted 29 April 1997

J.C. AUGUSTIN, V. CARLIER AND J. ROZIER. 1998. The heat resistance of *Listeria monocytogenes* phagovar 2389/2425/3274/2671/47/108/340 (1992 French outbreak strain) in broth was studied at 55, 60 and 65 °C. Experiments were carried out on bacterial cultures in three different physiological states: cultures at the end of the log phase, cultures heat-shocked at 42 °C for 1 h, and subcultures of cells resistant to prolonged heating. Survivor curves were better fitted using a sigmoidal equation than the classical log-linear model. This approach was justified by the existence of heat resistance distributions within the bacterial populations. Peaks ($\log_{10}$ of heating time) of heat resistance distributions of untreated, heat-shocked, and selected cultures at 55, 60 and 65 °C were 0·34, −0·90 and −1·84 min, 0·74, −0·51 and −1·24 min, and 0·17, −0·94 and −1·45 min, respectively. The widths of the distributions are proportional to 0·29, 0·36 and 0·41 $min^{0.5}$, 0·26, 0·36 and 0·41 $min^{0.5}$, and 0·34, 0·44 and 0·41 $min^{0.5}$. An increase in the thermal tolerance could then be induced by sublethal heat shock or by selection of heat resistant cells.

INTRODUCTION

Listeria monocytogenes has been known to be a foodborne pathogen for the last 15 years. The organism has been implicated as a causative agent in several recent outbreaks of foodborne listeriosis worldwide, in particular in France in 1992 (Goulet *et al.* 1993; Jacquet *et al.* 1994). This outbreak of 279 cases was caused by a serovar 4b (phagovar 2389/2425/3274/2671/47/108/340) and implicated contaminated pork tongue in jelly as the major vehicle of the outbreak (Goulet *et al.* 1993; Jacquet *et al.* 1995).

Listeria monocytogenes is an unusually thermotolerant Gram-positive, non-spore-forming bacterium. Consequently, the 1992 French outbreak led to a re-examination of the effectiveness of heat treatments of processed foods. A detailed study of the heat resistance of that particular strain of *L. monocytogenes* was needed.

Numerous studies have shown that it was possible to increase the heat resistance of *L. monocytogenes* by applying a prior sublethal heat shock (Farber 1989; Fedio and Jackson 1989; Bunning *et al.* 1990; Farber and Brown 1990; Knabel *et al.* 1990; Linton *et al.* 1990, 1992). This increase in heat resistance is also observed when the temperature of the medium increases very slowly (Quintavalla and Campanini 1991; Stephens *et al.* 1994). In practice, such conditions can occur during the heat treatment of bulk foods. The heat resistance of cultures at the end of the log phase has been compared to that of cultures which have sustained a sublethal heat shock to determine the heat-shock response of the 1992 French epidemic strain of *L. monocytogenes*.

Despite widely differing results reported in published studies of the heat resistance of *L. monocytogenes*, a D value of 2 min at 60 °C and an average z value of 6 °C seem to be typical (Farber 1989; Mackey and Bratchell 1989; Augustin 1996). These D and z values are based on classical thermobacteriology which assumes that the thermal inactivation kinetics of micro-organisms are logarithmic. For many years, however, non-logarithmic survivor curves with characteristic shoulders and tails have been described (Tomlins and Ordal 1976). Such sigmoidal curves have recently been described with *L. monocytogenes*; and a logistic equation (Cole *et al.* 1993), or modified Gompertz equations (Bunning *et al.* 1990; Linton *et al.* 1995; Linton *et al.* 1996) have been proposed to model these survivor curves. Several mechanisms have been proposed to explain this phenomenon but the most satisfying

Correspondence to: Dr J.C. Augustin, Service d'Hygiène et Industrie des Denrées Animales et d'Origine Animale, Ecole Nationale Vétérinaire d'Alfort, 7 avenue du Général de Gaulle, F94704 Maisons Alfort Cedex, France.

is a distribution of heat resistance within the bacterial population (Tomlins and Ordal 1976). In this study, sigmoidal survivor curves have also been observed and a new function to fit these curves has been proposed. This function can be justified by the existence of a distribution of heat resistance within the *L. monocytogenes* population. The distributions of heat resistance of cultures at the end of the log phase, of heat-shocked cultures, and of subcultures of cells resistant to prolonged heating, have been determined in order to evaluate whether an increased thermal tolerance is transmissable to subcultures.

MATERIALS AND METHODS

Bacterial strains and culture

The strain of *Listeria monocytogenes* 4b phagovar 2389/2425/3274/2671/47/108/340 was supplied by Dr J. Rocourt (Institut Pasteur, Paris, France) and maintained at 4 °C on plate count agar (Difco Laboratories, Detroit, MI, USA). Cultures were grown in tryptone soya (Oxoid, Unipath Ltd, Basingstoke, UK) plus 0·6% yeast extract (AES, Combourg, France) (TSYE) broth at 30 °C for 24 h. The pH of the broth was 7·0 and its a_w was 0·99.

Physiological states

Experiments to characterize the thermal tolerance of the cells were carried out on bacterial cultures in the following three physiological states: (i) untreated state, cultures grown in TSYE broth at 30 °C for 24 h; (ii) heat-shocked state, normal cultures heat-shocked at 42 °C for 1 h (Bunning *et al.* 1990); (iii) selected state, subcultures of cells from a normal culture which survived a heat treatment of 60 °C for 20 min, subsequently grown in TSYE broth at 30 °C for 24 h. The concentration of the cultures was approximately 10^9 cells ml^{-1}.

Preparation of the samples

The cultures were vortexed and 3 ml samples were placed in sterile glass 10 × 100 mm thermal death time (TDT) tubes (Seval, Paris, France).

Heating and cooling of the samples

For each study, the TDT tubes were sealed and first, completely immersed in a Polystat constant temperature water bath (Bioblock, Illkirch, France) for different lengths of time at 55, 60 and 65 °C. They were then immersed in cold water (12 °C). Surviving bacteria from two tubes were enumerated at each time; two and three trials were conducted at 55 and 65 °C, and 60 °C, respectively.

Temperature of TSYE broth during heating and cooling was determined using a temperature recorder probe (Microlide S.A., St Léonard de Noblat, France) inserted into a TDT tube. The following equations were used to describe the rise and fall of the temperature:

$$T(t) = (T_i - T_h) \cdot e^{-kt} + T_h \text{ (heating);}$$

$$T'(t) = (T_m - T_i) \cdot e^{-kt} + T_i \text{ (cooling, where zero time is taken to be the beginning of the cooling phase);}$$

where t is time (min); T(t) and T′(t), respective heating and cooling temperature profiles of the medium (°C); T_i, initial temperature of the medium (22 °C); T_h, heating temperature (°C); T_m, maximum temperature attained by the medium (°C); and k is 2·16 (min^{-1}). The *k* value was determined by linear regression analysis after log transformation.

Enumeration of surviving bacteria

After cooling, the contents of the TDT tubes were serially diluted as required in bacto-tryptone (Difco) plus 0·85% sodium chloride (Prolabo, Paris, France). The appropriate dilutions were then plated on tryptic soya (Oxoid) plus 0·6% yeast extract (AES) agar, either with a model DS spiral plater (Interscience, St Nom la Bretèche, France), or by surface plating 0·2 ml. Plates were incubated at 37 °C for 24 and 48 h before enumeration. The $\log_{10}$ of survivors per gram were then plotted as a function of the duration of heating.

Identification of survivors

A biochemical confirmation of survivors of the prolonged heat treatments was carried out using the CAMP reaction (Christie, Atkins and Munch-Pedersen) and carbohydrate (rhamnose and xylose) fermentation tests, followed by serotyping into serogroups 2 or 4, using sera made in this laboratory.

Determining actual heating durations from the applied heating durations

The duration of the applied heat treatment was corrected for the lengths of the rising and falling phases of the temperature of the sample. The corrected duration was calculated to be the length of heat treatment at the given temperature which would have the same effect on the organisms as the actual heat treatment applied, assuming instantaneous heating and cooling of the sample.

With reference to the F concept, the following expression for the corrected duration of the heat treatment, t_c, was obtained:

$$t_c = \int_{t^{45}}^{t_h} 10^{(T(t)-T_h)/z}\,dt + \int_0^{t'_{45}} 10^{(T'(t)-T_h)/z}\,dt$$

where t is the time of the applied heat treatment (min); T(t), temperature of the sample during heating (°C); t_h, end time of the heat treatment; T_h, heating temperature (°C); T′(t), temperature of the sample during cooling (°C); t_{45}, time needed for the sample to reach a temperature of 45 °C during heating (min) (heat injury begins at temperatures above 45 °C (Zaika *et al.* 1990)); t'_{45}, time needed for the sample to cool to 45 °C (min); and the z value was taken to be 6 °C, which is a typical value for *L. monocytogenes* (Augustin 1996).

This corrected heating time was used to fit the destruction model to the data.

Models for the survivor curves and the heat resistance distributions

Survivor curves were fitted with a sigmoidal function expressing a logistic decay of the survivor concentration with the $\log_{10}$ of the corrected heating time. This model is justified by a distribution of heat resistance within the bacterial population close to the log-normal distribution.

Assuming that the destruction of micro-organisms can be described by the following sigmoidal equation:

$$\text{cfu}(t) = \text{cfu}(0) \cdot (1 + e^{(x-m)/s^2})^{-1}$$

the probability distribution of heat resistance within the microbial population can be derived as:

$$f(x) = \frac{e^{(x-m)/s^2}}{s^2(1+e^{(x-m)/s^2})^2}$$

where cfu (t) is the concentration of survivors; cfu (0), initial concentration of the population; x, $\log_{10}$ of the heating time t (min); m, peak of the heat resistance (min); s, parameter proportional to the standard deviation of the heat resistance ($\text{min}^{0.5}$); f, probability density function of heat resistance.

The heat resistance of bacterial populations is then quantified by two parameters: m, which is the $\log_{10}$ of the heating time necessary to destroy 50% of the population; s, which represents the width of the heat resistance distribution.

Model fit, parameter confidence limits, and parameter comparisons

Values of m and s parameters were determined by fitting the new destruction model to the data using the ordinary least-squares criterion. Confidence limits ($\alpha = 0{\cdot}05$) for parameter values were determined from the asymptotic standard errors. These values were computed by the NONLIN subroutine of SYSTAT 5 software (Systat Inc., Evanston, IL, USA).

Comparisons of parameter-values were done according to Huet *et al.* (1987, 1992). The statistic $S = n\ (\log\ (\sigma^2\ (°)) - \log\ (\sigma^2))$ was calculated, where n is the total number of observations; σ^2 (°), the residual variance for the model under the null hypothesis; σ^2 the residual variance for the model without equality constraints on the parameters. The residual variance is RSS/(n-N), where RSS is the residual sum of squares and N is the total number of parameters. This statistic, S, was compared to the χ^2 quantile with q degrees of freedom, where q is the number of relations defining the null hypothesis. Difference between the parameters was considered significant when S was greater than the χ^2 quantile with a significance level $\alpha = 0{\cdot}05$.

RESULTS

Survivor curves obtained in this study were well fitted with the new sigmoidal function (Figs 1, 2 and 3).

The correlation exhibited between observed and predicted values was $R^2 = 0{\cdot}87$ (Fig. 4). The classical log-linear model gives an R^2 coefficient of 0·59.

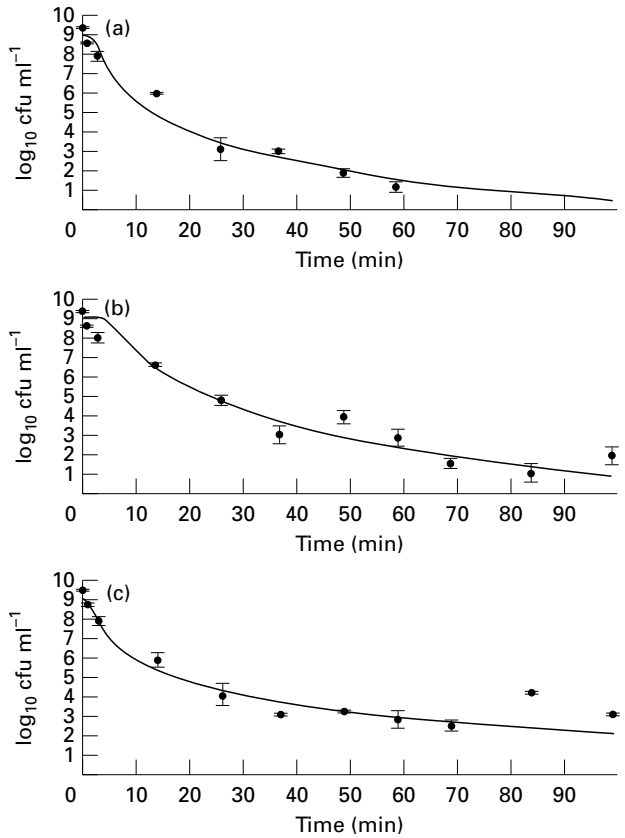

Fig. 1 Survivor curves of the (a) untreated, (b) heat-shocked and (c) selected cultures of *Listeria monocytogenes* at 55 °C. Plotted points are the means of the observed values. The lines are the predicted survivor curves. Vertical bars indicate one standard deviation

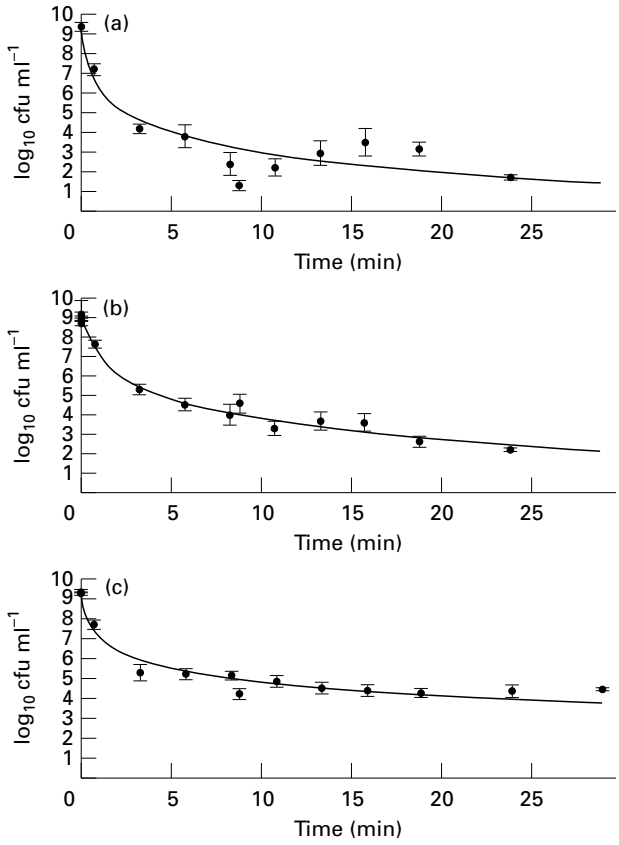

Fig. 2 Survivor curves of the (a) untreated, (b) heat-shocked and (c) selected cultures of *Listeria monocytogenes* at 60 °C. Plotted points are the means of the observed values. The lines are the predicted survivor curves. Vertical bars indicate one standard deviation

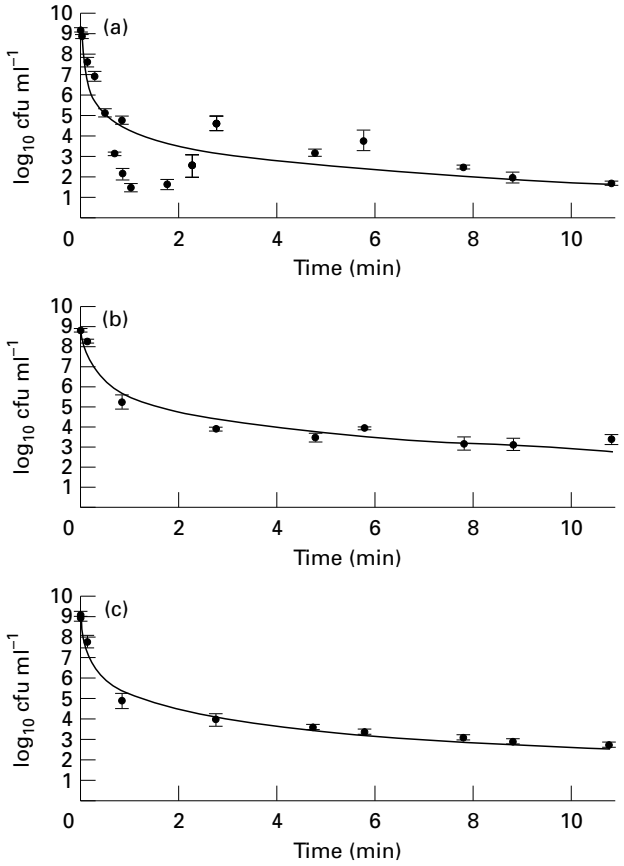

Fig. 3 Survivor curves of the (a) untreated, (b) heat-shocked and (c) selected cultures of *Listeria monocytogenes* at 65 °C. Plotted points are the means of the observed values. The lines are the predicted survivor curves. Vertical bars indicate one standard deviation

Heat resistance distributions were plotted (Fig. 5) using m and s values obtained for each culture at each temperature (Table 1).

Evolution of heat resistance with the physiological state

The m values of the heat resistance distribution for the heat-shocked culture at 55, 60 and 65 °C were higher than the m values of the untreated one (Fig. 5), but these differences were only significant at 55 °C ($P < 0{\cdot}05$). On the other hand, there was no significant difference between the s values of the two cultures at 55, 60 and 65 °C ($P > 0{\cdot}05$).

There was no significant difference between the m values of the untreated culture and the selected one at 55, 60 and 65 °C ($P > 0{\cdot}05$). The selected culture had s values significantly higher than those of the untreated one at 55 and 60 °C ($P < 0{\cdot}05$) but not at 65 °C ($P > 0{\cdot}05$).

Evolution of heat resistance with the heating temperature

The m values of the heat resistance of the three cultures decreased significantly with the temperature ($P < 0{\cdot}05$) and although only three temperatures had been tested, this decrease seemed linear. This linear variation of the $\log_{10}$ of a time of destruction squared the z concept. The z values for the three cultures were obtained by linear regression of the peak with the temperature (Table 1). The z value of the selected culture was higher than the z value of the heat-shocked culture, which was higher than the z value of the untreated one, but these differences were not significant ($P > 0{\cdot}05$).

The s values of the heat resistance of the untreated culture increased with the temperature but this increase was not significant between 60 and 65 °C ($P > 0{\cdot}05$). Similarly, the s value of the heat-shocked culture increased significantly with the temperature ($P < 0{\cdot}05$). With the selected culture, the s value at 60 °C was significantly higher ($P < 0{\cdot}05$) than the

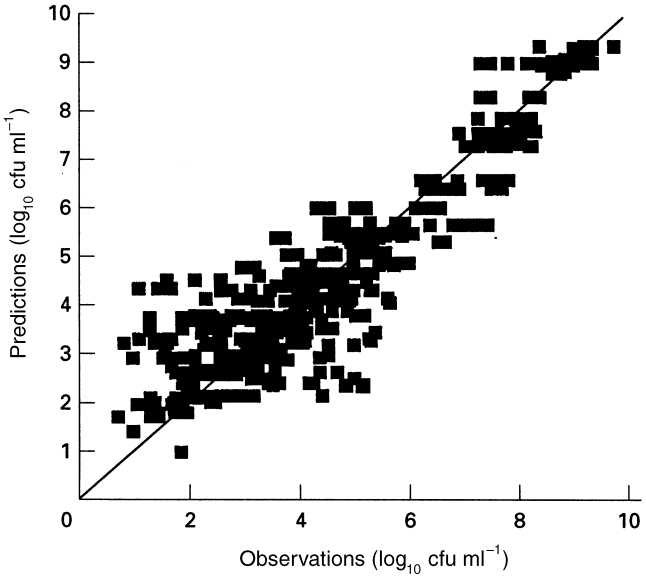

Fig. 4 Correlation of predicted $\log_{10}$ of survivor concentrations *vs* the observed concentrations. R^2 adjusted 0·874; observations, 640

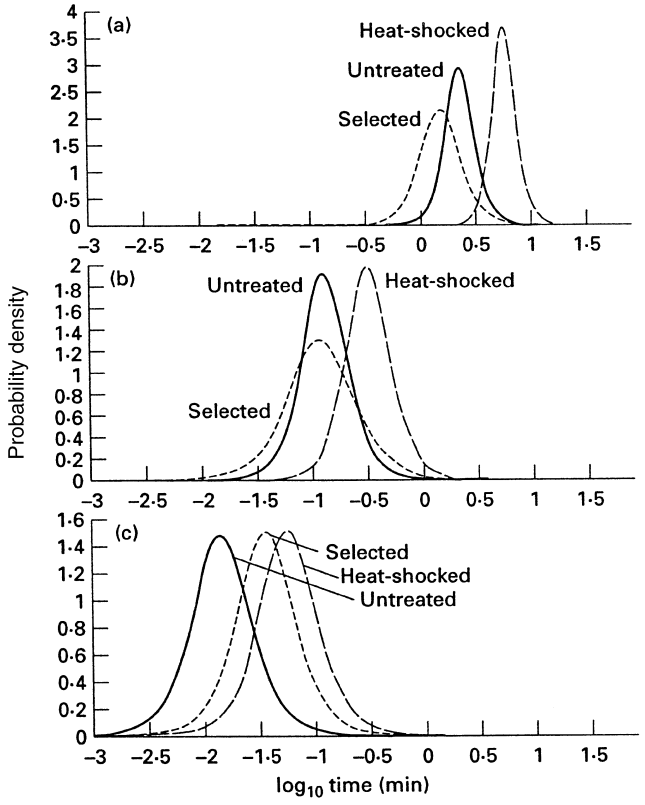

Fig. 5 Probability distributions of heat resistance of the untreated, heat-shocked and selected cultures of *Listeria monocyotgenes* at (a) 55 °C, (b) 60 °C and (c) 65 °C

55 °C one, but the *s* value at 65 °C was significantly lower ($P > 0{\cdot}05$) than the 60 °C one. These opposite results and the small number of temperatures tested did not allow any conclusion to be drawn about the law of variation of the *s* value with the temperature.

DISCUSSION

Survivor curves obtained in this study consistently exhibited tails. One of the theories explaining this tailing phenomenon is the existence of a heat distribution within the bacterial population. This theory was first expressed by Withell (1942) who proposed a log-normal distribution. As the normal distribution is difficult to use, the use of a logistic function was preferred in this study. This function gave a good fit of the observed survivor curves. The hypothesis of a heat resistance distribution appears to be confirmed without knowing whether it is an adaptive response of cells during heating or the result of an innate variation. However, this theory allowed a better fit of the survivor curves than the classical log-linear model. In a related study in ham (Carlier *et al.* 1996), the survivor curves were shown to fit best with the log-linear model. This discrepancy could be due to the use of selective (Palcam) enumeration agar in the study in ham. Indeed, the more heat-resistant bacteria subjected to prolonged heating are unable to repair the heat damage sustained when placed in selective media containing inhibitors, giving linear survivor curves. In spite of this, in the present study these bacteria were able to repair the damage with non-selective (TSYE) enumeration agar, giving survivor curves with tails. If the repair of heat-damaged cells can occur in foods, these tailing phenomena must be taken into account to evaluate the efficiency of the food cooking process.

It has been shown, in harmony with others studies (Farber 1989; Fedio and Jackson 1989; Bunning *et al.* 1990; Farber and Brown 1990; Knabel *et al.* 1990; Linton *et al.* 1990; Linton *et al.* 1992), that there was a significant increase of the heat resistance of *L. monocytogenes* when the cultures were heat-shocked at a sublethal temperature. The time required to kill 50% of the microbial population is about 2·5 and 4 times higher for the heat-shocked culture than for the untreated one at 55 and 60 °C, and 65 °C, respectively. These results must also be taken into account when cooking bulk foods as the rate of heating is very low and an adaptive response similar to heat shock can occur during it.

If the heterogeneity of heat resistance was innate, it would be possible to obtain subcultures more heat resistant than the original one. This concept is very controversial; some authors have emphasized such a phenomenon (Corry and Roberts 1970; Duitschaever and Jordan 1974), whereas Vas and Proszt (1957) and Moats *et al.* (1971) were unable to obtain subcultures more heat resistant than the original. In this study, the time required to kill 50% of the microbial population at

Table 1 *m*- and *s*-values of the probability densities of heat resistance of *Listeria monocytogenes* at 55, 60 and 65 °C

Culture	Temperature (°C)	*m* (min)	*s* ($min^{0.5}$)	*z* (°C)
Untreated	55	0·34 (0·20, 0·48)*	0·29 (0·27, 0·31)	4·6 (0·5, 8·7)
	60	−0·90 (−1·17, −0·64)	0·36 (0·34, 0·38)	
	65	−1·84 (−2·18, −1·50)	0·41 (0·39, 0·44)	
Heat-shocked	55	0·74 (0·62, 0·85)	0·26 (0·24, 0·28)	5·0 (0·1, 10·0)
	60	−0·51 (−0·75, −0·26)	0·36 (0·34, 0·37)	
	65	−1·24 (−1·55, −0·94)	0·41 (0·38, 0·43)	
Selected	55	0·17 (0·01, 0·33)	0·34 (0·32, 0·36)	6·2 (0, 13·7)
	60	−0·94 (−1·34, −0·54)	0·44 (0·41, 0·47)	
	65	−1·45 (−1·69, −1·20)	0·41 (0·38, 0·43)	

* The values in parentheses are the 95% confidence limits.

55 and 60 °C was 1·1–1·5 times lower for the selected culture than for the untreated one. This result agrees with the result of the previous study in ham were the *D* values of the selected culture were lower than these of the normal one. This result is, however, to blend because the difference is not significant and at 65 °C, the selected culture had a higher *m* value than the untreated one. The width of the heat resistance of the selected culture is, on the other hand, significantly higher than that of the untreated culture. This implies the presence of extremely heat-resistant cells in higher concentration. These results appear to show that on average, it is possible to select subcultures less resistant but containing more very heat resistant cells than the mother culture.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Jocelyne Rocourt who kindly provided the *L. monocytogenes* strains, and the Fédération Française des Industries Charcutières which supported this study.

REFERENCES

Augustin, J.C. (1996) Résistance de *Listeria monocytogenes* aux traitements physiques. *Pathologie Biologie* **44,** 790–807.

Bunning, V.K., Crawford, R.G., Tierney, J.T. and Peeler, J.T. (1990) Thermoresistance of *Listeria monocytogenes* and *Salmonella typhimurium* after sublethal heat-shock. *Applied and Environmental Microbiology* **56,** 3216–3219.

Carlier, V., Augustin, J.C. and Rozier, J. (1996) Heat resistance of *Listeria monocytogenes* (Phagovar 2389/2425/3274/2671/47/108/340): D- and z-values in ham. *Journal of Food Protection* **59,** 588–591.

Cole, M.B., Davies, K.W., Munro, G., Holyoak, C.D. and Kilsby, D.C. (1993) A vitalistic model to describe thermal inactivation of *Listeria monocytogenes*. *Journal of Industrial Microbiology* **12,** 232–239.

Corry, J.E.L. and Roberts, T.A. (1970) A note on the development of resistance to heat and gamma radiation in *Salmonella*. *Journal of Applied Bacteriology* **33,** 733–737.

Duitschaever, C.L. and Jordan, D.C. (1974) Development of resistance to heat and sodium chloride in *Streptococcus faecium* recovering from thermal injury. *Journal of Milk Food Technology* **37,** 382–386.

Farber, J.M. (1989) Thermal resistance of *Listeria monocytogenes* in foods. *International Journal of Food Microbiology* **8,** 285–291.

Farber, J.M. and Brown, B.E. (1990) Effect of prior heat shock on heat resistance of *Listeria monocytogenes* in meat. *Applied and Environmental Microbiology* **56,** 1584–1587.

Fedio, W.M. and Jackson, H. (1989) Effect of tempering on the heat resistance of *Listeria monocytogenes*. *Letters in Applied Microbiology* **89,** 157–160.

Goulet, V., Lepoutre, A., Rocourt, J., Courtieu, A.-L., Dehaumont, P. and Veit, P. (1993) Epidémie de listériose en France. Bilan final et résultats de l'enquête épidémiologique. *Bulletin Epidémiologique Hebdomadaire* **4,** 13–14.

Huet, S., Jolivet, E. and Messéan, A. (1992) *La régression non-linéaire, méthodes et applications en biologie.* Paris: INRA éditions.

Huet, S., Laporte, J. and Vautherot, J.F. (1987) Statistical methods for the comparison of antibody levels in serums assayed by enzyme linked immuno sorbent assay. *Biométrie Praximétrie* **28,** 61–80 .

Jacquet, C., Catimel, B., Brosch, R. *et al.* (1995) Investigations related to the epidemic strain involved in the French listeriosis outbreak in 1992. *Applied and Environmental Microbiology* **61,** 2242–2246.

Jacquet, C., Miegeville, A.-F., Catimel, B., Huynh, G., Courtieu, A.L. and Rocourt, J. (1994) La listériose humaine en France en 1991, 1992 et 1993. Bilan à partir des souches adressées aux centres nationaux de référence. *Bulletin Epidémiologique Hebdomadaire* **28,** 123–125.

Knabel, S.J., Walker, H.W., Hartman, P.A. and Mendonca, F. (1990) Effects of growth temperature and strictly anaerobic recov-

ery on the survival of *Listeria monocytogenes* during pasteurization. *Applied and Environmental Microbiology* **56,** 370–376.

Linton, R.H., Carter, W.H., Pierson, M.D. and Hackney, C.R. (1995) Use of a modified Gompertz equation to model nonlinear survival curves for *Listeria monocytogenes* Scott A. *Journal of Food Protection* **58,** 946–954.

Linton, R.H., Carter, W.H., Pierson, M.D., Hackney, C.R. and Eifert, J.D. (1996) Use of a modified Gompertz equation to predict the effects of temperature, pH, and NaCl on the inactivation of *Listeria monocytogenes* Scott A heated in infant formula. *Journal of Food Protection* **59,** 16–23.

Linton, R.H., Pierson, M.D. and Bishop, J.R. (1990) Increase in heat resistance of *Listeria monocytogenes* Scott A by sublethal heat shock. *Journal of Food Protection* **53,** 924–927.

Linton, R.H., Webster, J.B., Pierson, M.D., Bishop, J.R. and Hackney, C.R. (1992) The effect of sublethal heat shock and growth atmosphere on the heat resistance of *Listeria monocytogenes* Scott A. *Journal of Food Protection* **55,** 84–87.

Mackey, B.M. and Bratchell, N. (1989) The heat resistance of *Listeria monocytogenes. Letters in Applied Microbiology* **9,** 89–94.

Moats, W.A., Dabbah, R. and Edwards, V.M. (1971) Interpretation of nonlogarithmic survivor curves of heated bacteria. *Journal of Food Science* **36,** 523–526.

Quintavalla, S. and Campanini, M. (1991) Effect of rising temperature on the heat resistance of *Listeria monocytogenes* in meat emulsion. *Letters in Applied Microbiology* **12,** 184–187.

Stephens, P.J., Cole, M.B. and Jones, M.V. (1994) Effect of heating rate on thermal inactivation of *Listeria monocytogenes*. *Journal of Applied Bacteriology* **77,** 702–708.

Tomlins, R.L. and Ordal, Z.J. (1976) Thermal injury and inactivation in vegetative bacteria. In *Inhibition and Inactivation of Vegetative Microbes* ed. Skinner, F.A. and Hugo, W.B. pp. 153–190. New York: Academic Press.

Vas, K. and Proszt, G. (1957) Observations on the heat destruction of spores of *Bacillus cereus. Journal of Applied Bacteriology* **21,** 431–441.

Whitell, E.R. (1942) The significance of the variation on shape of time-survivor curves. *Journal of Hygiene* **42,** 124–183.

Zaika, L.L., Palumbo, S.A., Smith, J.L. *et al.* (1990) Destruction of *Listeria monocytogenes* during frankfurter processing. *Journal of Food Protection* **53,** 18–21.