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Modeling the *Listeria innocua* micropopulation lag phase and its variability

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ABSTRACT

Listeria innocua micropopulation lag phase and its variability have been modeled as a function of growth temperature, intensity of heat stress, and the number of surviving cells initiating growth. Micropopulation lag phases were found to correlate negatively with inoculum size and growth temperature and positively with heat shock intensity. Validation of the models using experimental milk samples indicated that the average lag phase duration predicted is shorter and more variable than the observed, meaning that they should be considered safe for risk assessment. Our results suggest that the effect of inoculum size on the population lag phase has both stochastic and physiological components.

1. Introduction

The microbial lag phase reflects the time required for cells to adapt to a new environment and start duplicating. Models of microbial growth give accurate estimates of specific growth rate but usually inaccurate estimates of lag time, probably because numerous factors influence lag time and controlling them all is not feasible.

The lag phase depends on many factors, including potentially all physical or chemical conditions of the growth environment. In addition, cell injuries caused by heating, freezing, drying (Mackey and Kerridge, 1988), irradiation (Aguirre et al., 2011) and other preservation treatments (Robinson et al., 2001) considerably extend the lag phase and increase its variability. Many microbiologists have studied the effect of inoculum size on bacterial lag time (Augustin et al., 2000; Duffy et al., 1994; Gay et al., 1996; Mackey and Kerridge, 1988; Pascual et al., 2001; Pin and Baranyi, 2006; Pin et al., 1999; Robinson et al., 2001; Stephens et al., 1997), and their studies have come to contradictory conclusions. Some studies have suggested that inoculum size has no effect on lag phase duration, but these studies were carried out under optimal or near-optimal microbial growth conditions in broth culture (Brouillaud-Delattre et al., 1997; Duffy et al., 1994; Jason, 1983) and food (Mackey and Kerridge, 1988). Conversely, studies conducted under suboptimal growth conditions have reported that inoculum size influences lag phase (Augustin et al., 2000; Gnanou Besse et al., 2006; Pin and Baranyi, 2006; Robinson et al., 2001). This influence on lag phase has been reported either when the inoculum was very small (Augustin et al., 2000) or when only a small proportion of the population was able to grow as a result of an applied stress (Augustin et al., 2000; Pascual et al., 2001). Robinson et al. (2001) observed that, in media containing inhibitory concentrations of NaCl, both the mean lag time and variations between replicate inocula increased as the inoculum size became smaller. Those authors identified two types of inoculum size effects on population lag: a cooperative or inhibitory effect of high cell concentrations, and a statistical effect at low cell concentrations arising from the variability in individual lag times. There is little information about the possible effects of cell–cell interactions on lag time, although cell signaling has been shown to affect the emergence of cells from dormancy (Mukamolova et al., 1998), and some evidence suggests that pheromone-like substances may promote bacterial growth (Kaprelyants et al., 1999).

Baranyi and his team (Baranyi, 1998; Baranyi and Pin, 1999; Pin and Baranyi, 2006; Baranyi et al., 2009) analyzed the effects of inoculum size on population lag and showed that as the size of the inoculum decreases, the population lag time increases by an amount that depends on the distribution of individual lag times and the maximum specific growth rate. They suggested that population lag time must be less than the average lag time of the individual cells in the population, since cells with short lags begin to multiply at once and their descendants dominate the population. They also observed that the smaller the inoculum is, the more variable is the lag phase. How exactly does inoculum size affect lag phase variability? There is no completely satisfactory answer to

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this question. Although McKellar and Knight (2000) modeled the lag phase of *Listeria monocytogenes* at the nearly optimal growth temperature of 30 °C by taking into account the variability of individual cells, this kind of model is unlikely to be reliable under suboptimal or stress conditions, when the lag phase becomes longer and more variable (Baranyi, 2002; Gnanou Besse et al., 2006; Metris et al., 2003; Robinson et al., 2001).

Predictive microbiology modelers have developed systems that accurately describe microbial behavior. Growth kinetics (Juneja and Marks, 2006; Pin and Baranyi, 2006), growth/no growth boundaries (Koutsoumanis, 2008; Mejlholm et al., 2010), and growth effects of different kinds of stress (e.g. irradiation, high pressure, heat) (Aguirre et al., 2011; Black et al., 2008; Smelt et al., 2008; Zhu et al., 2008) have been studied to improve the accuracy of growth models. This research has increased our understanding of the variability in microbial response to the environment (Aguirre et al., 2009; Francois et al., 2006; Koutsoumanis, 2008; Metris et al., 2005), and it shows that lag phase varies broadly in many situations (Aguirre et al., 2011; Lianou and Koutsoumanis, 2011; Stringer et al., 2011). Thus, in order to make accurate predictions about small populations of cells, it is particularly important to take into account this variability, particularly when the cells have been exposed to stress (Li et al., 2006).

Since natural contamination of foods may occur with very few cells, and these cells are frequently stressed by food processing conditions, it is essential to generate improved models that take into account what injuries cells have suffered before they contaminate food and that use a stochastic approach to deal with variability in the behavior of individual cells and micropopulations (Guillier et al., 2005). The objective of this study was to model the lag time of micropopulations and individual cells, as well as the variability in that lag time, following different heat shocks and subsequent growth at different refrigeration temperatures.

2. Material and methods

2.1. Bacterial strain and inoculum preparation

Listeria innocua (CECT 910, NCTC 11288, ATCC 33090) was kept frozen at −20 °C in tryptic soy broth (TSB; Pronadisa, Madrid, Spain) supplemented with 20% glycerol. The strain was subcultured twice in sterile TSB at 37 °C for 24 h to reach the stationary phase, with a concentration of *ca.* 10^9 cfu/ml. Cell suspensions were then used to inoculate sterile TSB solution or commercial sterile whole milk (Asturiana, Asturias, Spain).

2.2. L. innocua growth and inactivation parameters

The decimal reduction time (D value) in whole milk was taken from Aguirre et al. (2009) and the D value in TSB was calculated by log linear regression.

The maximum specific growth rate (μ_{max}) was estimated in TSB at 7, 11 and 16 °C from turbidity growth curves in three replicate experiments using an automated spectrophotometer (Bioscreen C, Labsystems, Helsinki, Finland) kept in a cold room at 5 ± 1 °C. Serial five-fold dilutions of each 24-h culture were prepared in fresh TSB to obtain dilutions from approximately 10^7 to 10^0 cfu ml^{-1}. Twenty replicate samples (350 μl) from each dilution were inoculated into wells of Bioscreen microplates. The plates were loaded into the Bioscreen C at an incubation temperature of 7, 11 or 16 °C. After shaking at medium intensity for 10 s, turbidity measurements were determined using a wide band filter at 420 to 580 nm at 30-min intervals. Plates were incubated for enough time to reach stationary phase in the most dilute samples. Dilutions of each initial inoculum were plated by spreading onto TSA, then they were incubated at 37 °C and finally colonies were counted. Using the Bioscreen device, the time to detection (T_d), defined as the time required to reach an absorbance of 0.20, was obtained from each well, and a mean value was calculated for each dilution. The μ_{max} was estimated from the reciprocal of the absolute value of the regression slope of the *Td* versus ln(N).

The μ_{max} in whole milk was estimated at 7 and 12 °C in a cooled incubator (model FOC225I, Velp Scientifica, Usmate, Italy) (Aguirre et al., 2012b).

2.3. Heat shock, inoculum size and growth temperature

Heat treatment at 54 °C was applied to obtain 0, 3 and 5 logarithmic reductions (D) in TSB ($D_{54\,°C}$ = 6.6 min for TSB) and 1, 3 and 5 logarithmic reductions in whole milk [$D_{54\,°C}$ = 13.7 min (Aguirre et al., 2009)] in a temperature-controlled water bath (model TFB, Bunsen S.A., Marid, Spain). Temperature was monitored with a thermocouple (Testo AG 720, Kirchzarten, Germany). Tubes containing 9 ml of TSB or whole milk were kept in the water bath and, once at 54 °C, were inoculated with 1 ml of the abovementioned *L. innocua* suspension to yield the samples that would be treated for 5 log reductions (5*D* samples). 5*D* samples were diluted to a concentration around 10^7 cfu/ml to obtain 3*D* samples. Unheated samples were inoculated with a suspension of 10^4 cfu/ml. All tests were performed 3 times. When the heat shock was completed, the contents of the TSB tubes were mixed with 90 ml of cold TSB in a flask immersed in an ice water bath, where the flask was kept for 1 min in order to end the heat shock but minimize the possible adverse effects of cold. From these mother solutions, half-dilutions were prepared in order to obtain several inoculum sizes ranging from around 200 cells/ml down to 1 cell/ml (Table 3). The same procedure was used to obtain different inocula to analyze the lag phase in TSB at 7, 11 and 16 °C.

For experiments with milk, 100 μl of heat-shocked *L. innocua* suspensions in whole milk (see Table 1 for inoculum sizes) were mixed with 900 μl of cold, sterile whole milk in an Eppendorf tube and incubated at 7 or 12 °C. Each batch was composed of at least 150 samples. To estimate the average number of cells per well, 1 ml from each dilution were mixed with melted tryptic soy agar (TSA; Pronadisa) in plates and incubated at 37 °C for 48 ± 2 h. Approximately 20 plates were counted for each dilution and treatment.

2.4. Lag time in TSB (Bioscreen)

Once the heat shock was completed, samples were half-diluted down to 1 cell/ml. Then micropopulation and individual cell lag times were estimated in TSB from turbidity growth curves generated using the Bioscreen C, as described in several studies (Aguirre et al., 2009; Guillier et al., 2005; Metris et al., 2003). To do this, 300 μl

Table 1
Average number of *Listeria innocua* cells per sample (inoculum) cultured in whole milk at 7 or 12 °C. These cells are the survivors after a heat treatment at 54 °C applied with the purpose to achieve 1, 3 or 5 log reductions in the population size. Data are the average of direct counts of *ca.* 20 plates.

Log reduction	Growth temperature (°C)			
	7		12	
	Av	Sd	Av	Sd
1	1.3	1.3	1.2	1.2
	5.7	5.2	2.6	2.4
	34.5	8.7	30.4	6.3
	55.7	9.3	55.7	9.3
	117.8	17.5	122.5	18.5
3	0.7	0.6	0.9	0.7
	2.1	1.6	2.4	2.7
	16.7	5.6	18.4	5.6
	71.4	12.2	63.4	10.9
	126.1	18.6	111.3	16.8
5	0.9	0.9	0.7	0.8
	11.5	5.1	7.5	3.4
	27.1	7.9	20.1	6.3
	42.5	9.0	39.3	8.1
	89.3	15.0	76.3	12.3
	131.3	21.2	121.5	17.8

Av. Average number of cells per sample (inoculum size). *Sd*. standard deviation.

from the diluted samples (50 samples per dilution) were transferred to the two microplates of the Bioscreen (50 wells per dilution). To estimate the initial average number of cells per well, 300 μl from each dilution were mixed with molten TSA in plates and incubated at 37 °C for 48 ± 2 h. Approximately 20 plates were counted for each dilution and treatment.

Cultures were grown at 7, 11 and 16 °C. The increase in optical density (OD) was tracked by measuring absorbance in the wavelength range from 420 to 580 nm using the Bioscreen C every 30 min for up to 4 weeks at 7 °C, 15 days at 11 °C and 8 at 16 °C. Cultures were shaken for 10 s at medium intensity before OD was measured.

Lag times were estimated using the detection time, defined as the time required for the OD in the wavelength range between 420 and 580 nm to reach 0.2 units, which corresponds to a concentration of *ca.* 10^7 cfu/well. This value was checked by preparing a dilution series from a culture grown in TSB at 37 °C for 24 h. ODs of the dilutions were measured using the Bioscreen, bacterial counts were estimated by plating on TSA, and lag times were estimated based on the following equation (Baranyi and Pin, 1999):

$$\mathrm{Lag} = T_d - \left(\frac{\ln(Nd) - \ln(N_0)}{\mu}\right) \quad (1)$$

where Nd is the bacterial number (cfu) at T_d, N_0 the number of cells (cfu) initiating growth in the well under consideration, and μ (h^{-1}) is the specific growth rate determined from the growth curve obtained under the experimental conditions described above.

2.5. Modeling the effect of heat shock, inoculum size and growth temperature on lag phase

Two multiple regression models were fitted to the data (Eq. (2)) using the software Statistical Analysis System (SAS® version 9.2, SAS Institute Inc., Cary, NC, USA) based on a stepwise-regression algorithm to determine which effects and variables affect the response variable and to form a model that fits the data well without incurring the negative effects of overfitting the model. All variables left in the model are significant at the 0.050 level. No other variable met the 0.050 significance level for entry into the model.

One model quantifies the effect of heat shock, inoculum size and growth temperature on the average lag phase of *L. innocua* in TSB. The second quantifies the effect of the same factors on the standard deviation (Sd) of the lag phase.

The general polynomial model developed in the present study takes the form

$$\ln(y(\text{or } z)) = a_0 + a_1T + a_2D + a_3C + a_{12}TD + a_{13}TC + a_{23}DC + a_{11}T^2 + a_{22}D^2 + a_{33}C^2 \quad (2)$$

where y is the average lag phase; z, the Sd of lag phase; T, the growth temperature (°C); D, the average number of logarithmic reductions achieved in the population; C, the decimal logarithm of the average number of cells per well (inoculum size); and $a_0 \ldots a_{33}$, the coefficients to be estimated.

2.6. Model validation in whole milk

To validate the models generated, lag phase data were obtained in milk and compared to the model predictions.

To estimate the average number of cells per sample, the entire contents of the Eppendorf tubes immediately after the heat shock (see Section 2.3) were mixed with molten TSA in plates and kept at 37 °C for 48 ± 2 h. Approximately 20 samples were counted for each treatment.

After the third day of incubation, growth was checked daily by plating 100 μl of 5 samples into molten TSA and incubating at 37 °C for 32 ± 2 h. When the cell number in any sample was found to be higher than *ca.* 10^4 cfu, 100 μl of all remaining samples were plated into TSA using a spiral platter system (Eddy Jet, IUL Instruments, Barcelona, Spain) and the sampling times were recorded. Plates were then incubated at 37 °C for 48 ± 2 h and colonies were counted using an image analyzer (Countermat Flash, IUL Instruments, Barcelona, Spain). To ensure an adequate number of colonies, two dilutions per sample were prepared.

Lag time was calculated from the plate counts using Eq. (1) and replacing T_d with T_{count}, defined as the time when the sample was plated out. In this case, $\ln(Nd)$ was the natural logarithm of the cell number detected at T_{count}, and $\ln(N_0)$ the natural logarithm of the average initial number of bacteria after heat shock, as determined by direct counting of 20 samples (see Section 2.6).

Table 1 summarizes the conditions chosen for validation (inoculum sizes, logarithmic reduction and growth temperature).

In order to compare data from the model system in TSB with data from milk and thereby validate the model predictions, the accuracy factor (Af) and bias factor (Bf) were estimated according to the equations of Ross (1996):

$$A_f = 10^{\left(\sum |\log(X\text{pred}/X\text{obs})|/n\right)} \quad (3)$$

$$B_f = 10^{\left(\sum \log(X\text{pred}/X\text{obs})\right)/n}. \quad (4)$$

2.7. Other statistical analysis

To find the best fit, a family of distributions was fitted to the experimental data using EasyFit 5.5 software (Mathware Techonologies). The chi-square test was applied to find the goodness of the fit, being gamma distribution the best, followed by lognormal and normal distributions.

Variances in the distributions of lag phases were compared using a permutation test, as described by Aguirre et al. (2011, 2012a).

Coefficient of variance (CV) was calculated to quantify the dispersion of the data. Since CV is the standard deviation divided by the mean ($CV = 100 * Sd/\text{mean}$), this scaled measure compares the degree of variation between populations with different means (Jongenburger et al., 2010).

The probability density function [$f(x)$] of a gamma-distribution estimated with Microsoft Excel was used to calculate the predicted lag phase distributions:

$$f(x) = \frac{\lambda e^{-\lambda x}(\lambda x)^{\kappa-1}}{\Gamma(\kappa)} \quad (5)$$

where x is a lag phase; $f(x)$, its relative frequency; λ rate; κ shape; e, the base of the natural logarithm; and $\Gamma(\kappa)$, the gamma function, which was calculated using the Microsoft Excel function tool. The rate and shape were used to characterize the gamma distribution and were calculated by simple calculus according to the following equations, using the mean and Sd predicted by the models:

$$\lambda = \text{mean}/\text{variance} \quad (6)$$

$$\kappa = \lambda * \text{mean}. \quad (7)$$

The Eq. (5) can be used replacing the rate by the scale (θ), being:

$$\theta = 1/\lambda. \quad (8)$$

2.8. Simulation of population growth on the basis of lag phases of individual cells

Simulations were performed to analyze whether the effect of inoculation size on the lag phase duration of a population reflects only a stochastic effect or includes a physiological effect as well, such as a

quorum-sensing process or other type of intercellular communication. The growth of populations of individual cells with a previously determined lag phase (see Section 2.4) and constant μ_{max} for a given growth temperature (see Table 2) was simulated using Microsoft Excel.

3. Results

3.1. Specific growth rate

Table 2 shows the maximum specific growth rates (μ_{max}) of *L. innocua* on TSB and milk. The highest rate was 0.31 h^{-1} on TSB at 16 °C, reflecting the well-known mesophilic character of this species. Growth was slightly slower in milk than in TSB, although the differences were negligible.

3.2. Effect of heat shock, inoculum size and growth temperature on the lag phase

Average lag phase and *Sd* of micropopulations were calculated from *ca.* 100 samples and are shown in Table 3. In all cases, the lag phase of micropopulations correlated negatively with inoculum size and growth temperature and positively with heat shock intensity. The longest lag phase was detected in the micropopulations very likely to be composed of one cell that survived the most severe heat shock and then were incubated at the lowest temperature. Conversely, untreated micropopulations showed shorter lag phases than heat-shocked ones, and the higher the growth temperature was, the shorter was the lag phase. The lag phase variability (measured as *Sd*) also correlated negatively with inoculum size and growth temperature and positively with heat shock intensity. On the other hand, the *CV*s were similar at all incubation temperatures (Table 3), although the differences between lag phases were remarkable. For example, average lag phases of the smallest inocula surviving a 5*D* heat treatment were 304, 212 and 92 h at 7, 11 and 16 °C, while the corresponding *CV*s were 41, 38 and 34% (Table 3).

Fig. 1 shows the lag phase frequency distributions for different inoculum sizes of *L. innocua* grown on TSB at different temperatures after heat treatment at 54 °C to achieve 0, 3 and 5 logarithmic reductions. Histogram analysis shows that the frequency of shorter lag phase bins increased as inoculum size increased. In other words, the average lag phase of small inocula was longer and more variable than that of an inoculum of a hundred cells. In general, the lag phase distributions showed no tails, especially when inocula were large; in these cases, the shape was approximately symmetrical. However, with greater heat stress and smaller inoculum, the shape of the distributions lost symmetry and tails appeared, particularly at lower growth temperatures. The gamma distribution showed the best fit in almost all distributions, followed by lognormal and normal distributions. For this reason, the gamma distribution was chosen. Furthermore, the skewness of the distributions were generally positive (Table 3), indicating that the tail on the right side is longer than that of the left side and the bulk of the values lie to the left of the mean, a typical shape of gamma distributions, characterized by κ values > 1.

Table 3 shows significant differences ($\alpha < 0.05$) among the lag phase distributions of cells that had been heat-treated in the same way and incubated at the same temperature; these differences are due to different numbers of cells initiating growth, as calculated using the permutation test. The greater the difference is between the average number of cells in the inoculum, the more probable it is that the lag phase distributions differ significantly.

To integrate the effects of heat shock intensity, growth temperature and inoculum size on the population lag phase and on its variability, polynomial models were generated (Eq. (2)). The coefficients of significant variables according to the stepwise-regression algorithm are shown in Table 4.

3.3. Model validation

To validate the models generated, their predictions were compared with experimental lag phase data of *L. innocua* in milk. Comparison of the observed and predicted average lag phases (Fig. 2A) and *Sd*s (Fig. 2B) under the conditions described in Table 1 shows that the predictions of both models showed good agreement with observations, although the predicted lag phase was generally slightly shorter than observed. *Af* was 1.106 for the average lag phase and 1.203 for *Sd*; *Bf* was 0.990 for the average lag phase and 1.035 for *Sd*.

3.4. Application of the models

The models predict the lag phase and *Sd* (Table 4). Both parameters were estimated for each heat treatment, growth temperature and inoculum size, then these parameters were used to simulate the lag phase distributions after assuming that lag phases fit a gamma distribution (Baranyi and Pin, 2001). Simulations were performed by applying Eq. (5) to several inoculum sizes containing *L. innocua* micropopulations that survived 5 logarithmic reductions and were grown at 7 °C. Predicted rates were 0.023, 0.062 and 0.212 for 1, 10 and 100 cells, respectively, while the corresponding shapes were 7.39, 13.59 and 31.28. The larger the inoculum is, the larger are the rate and shape and, as expected, the narrower is the lag phase distribution (Fig. 3). The difference between the broad dispersion in the predicted lag phases of individual cells and the relatively narrow distribution of lag phases for a population of 100 cells is remarkable.

Our model may also be applied to predict lag phase distributions for a given number of survivors after different heat treatments. Fig. 4 is an example of such predictions and shows the frequency distribution of lag phases of 10 viable cells after heat treatments inactivating 0, 90, 99.9 and 99.999% of the initial population. The corresponding rates are 0.271, 0.211, 0.119 and 0.062; the shapes are 31.68, 26.83, 19.16 and 13.59. The more intense the inactivating treatment is, the smaller are the rate and shape, yielding a wider distribution.

4. Discussion

In this work, models have been developed to describe the effect of inoculum size, heat shock intensity and growth temperature on the lag phase of *L. innocua* and its variability.

The data in Table 3 provide insight into which parameters exert greater influence on the micropopulation lag phase and its variability. As expected, the higher the incubation temperature is, the shorter and less variable is the lag phase. The more intense the heat treatment is, the longer and more variable is the survivor lag phase, since repair of sublethal injury requires biosynthesis to restore lost components, which introduces a delay before cell division is possible and leads to different recovery times depending on how damaged the cells are. Similar to the effect of heat treatment, the smaller the inoculum is, the longer and more variable is the lag phase. Close analysis of the data reveals that these three variables interact to influence the lag phase and its variability. Nevertheless, the parameter with the greatest effect on the lag phase duration is the growth temperature, since in the narrow interval from 7 to 16 °C examined here, the lag phase of untreated cells grown at 16 °C was less than 25% of the lag

Table 2
Maximum specific growth rates (μ_{max}) of *Listeria innocua* on different substrates. Data are the average of three replicates.

Substrate	Temperature (°C)	μ_{max} (h^{-1})	*SE*[a]
TSB	7	0.09	0.016
TSB	11	0.14	0.001
TSB	16	0.31	0.007
Whole milk	7	0.08	0.009
Whole milk	12	0.14	0.006

[a] Standard error of μ_{max}.

Table 3
Effect of inoculum size, heat shock intensity and growth temperature on lag phase duration and variability of *Listeria innocua* in TSB. Lag phase durations marked with different letters in the same square show significant differences ($\alpha < 0.05$) in their distributions, based on the permutation test.

No. of log reduction	Growth temperature																	
	7 °C						11 °C						16 °C					
	Average cell/sample	*Sd*[a]	Average lag phase (h)	*Sd*	*CV*[b] (%)	Skewness	Average cell/sample	*Sd*	Average lag phase (h)	*Sd* (h)	*CV* (%)	Skewness	Average cell/sample	*Sd*	Average lag phase (h)	*Sd* (h)	*CV* (%)	Skewness
0	212.8	27.4	64.9 a	6.1	9.3	−0.580	258.9	48.0	33.8 a	2.9	8.6	0.123	222.6	26.0	13.7 a	0.8	5.8	−0.361
	151.5	19.5	80.3 ab	8.1	10.1	−0.364	174.3	26.0	36.0 ab	3.2	8.8	0.580	141.5	15.8	13.9 a	0.8	6.0	0.619
	83.2	10.2	84.3 b	10.4	12.3	0.320	123.5	50.5	38.6 abc	3.5	9.1	0.627	83.5	9.5	14.0 a	0.9	6.3	0.555
	44.7	6.1	89.4 c	12.3	13.8	0.677	89.4	9.2	40.5 abc	3.8	9.3	0.461	44.0	4.4	14.6 ab	1.0	6.6	0.094
	23.3	3.1	108.9 cd	15.6	14.3	−0.219	64.3	6.6	41.5 bcd	5.1	12.2	0.947	21.8	4.3	15.3 abc	1.3	8.6	0.945
	12.8	2.5	124.2 de	20.4	16.4	0.186	33.8	9.0	45.7 cde	5.6	12.1	1.349	12.0	1.9	17.5 cbd	1.6	9.3	0.964
	7.8	2.3	130.2 df	22.4	17.2	0.852	23.1	4.1	49.7 ef	6.7	13.5	0.206	9.0	1.4	21.1 cde	2.8	13.3	−0.229
	6.6	1.3	135.6 dg	24.8	18.3	1.064	7.5	2.7	54.6 efg	9.3	17.0	1.107	5.0	1.4	21.3 def	3.1	14.5	−0.301
	3.8	1.8	143.5 efgh	31.6	22.0	1.096	3.3	2.2	60.2 fg	12.7	21.1	0.980	3.5	1.2	23.7 defg	4.3	18.2	−0.568
	2.8	1.6	152.7 efgh	36.0	23.6	0.831	2.1	1.2	65.3 gh	14.9	22.8	0.996	2.4	1.3	24.3 efg	4.9	20.1	1.855
	2.1	1.1	160.6 fgh	40.2	25.0	0.677	0.7	0.6	84.2 h	20.7	24.6	0.631	2.0	0.9	25.6 efg	5.3	20.8	−0.637
	1.1	1.0	175.2 h	47.7	27.2	0.504							1.5	1.0	27.8 fg	6.0	21.5	1.330
	0.7	0.7	187.4 h	52.9	28.2	0.268							0.7	0.7	31.8 g	7.2	22.6	1.665
3	232.2	27.2	91.7 a	11.6	12.7	0.381	242.1	30.8	54.3 a	5.5	10.1	−0.724	250.4	45.5	26.1 a	1.6	6.1	0.111
	146.9	18.2	112.5 ab	16.1	14.3	0.418	162.4	18.8	60.4 ab	7.0	11.7	0.250	150.5	19.6	26.8 a	1.7	6.3	0.353
	91.3	10.2	112.8 abc	17.1	15.1	0.347	98.0	13.5	62.2 abc	7.7	12.4	0.246	101.2	14.5	27.5 ab	1.9	6.8	−0.135
	52.3	7.5	119.6 abcd	21.2	17.7	0.865	76.5	8.6	62.2 abc	7.9	12.7	0.299	74.5	10.9	28.6 abc	2.0	7.0	0.173
	25.6	5.2	150.6 bcde	29.6	19.7	0.399	60.4	10.0	64.1 abcd	10.0	15.7	0.316	56.8	9.5	29.4 abc	2.6	8.8	0.527
	15.7	4.2	172.9 cde	36.9	21.4	0.248	50.9	7.8	69.9 abcd	11.2	16.1	0.301	23.0	4.5	32.7 bcd	3.8	11.5	0.827
	7.1	3.2	180.4 de	42.9	23.8	0.437	38.6	2.5	71.5 abcde	14.4	20.1	0.815	15.0	2.3	33.5 cde	5.4	16.1	0.991
	3.6	2.3	190.6 e	49.7	26.1	0.468	22.1	5.2	82.6 bcde	17.5	21.2	0.841	8.4	3.1	35.8 def	6.9	19.3	−0.035
	2.7	2.1	202.7 e	60.7	29.9	0.493	8.5	2.7	84.1 cde	19.7	23.5	0.250	4.9	1.2	40.3 ef	8.9	22.1	0.390
	1.1	1.6	219.9 e	73.1	33.2	0.833	5.5	2.0	88.4 def	22.1	25.1	0.321	1.2	0.8	47.7 ef	12.3	25.7	0.306
	0.7	0.8	234.3 e	88.6	37.8	0.662	2.7	1.7	96.0 def	26.6	27.7	0.338	0.6		53.3 f	16.0	30.0	0.396
							1.3	0.9	123.1 ef	37.6	30.6	0.343						
							0.7	0.7	155.5 f	53.8	34.6	0.311						
5	245.4	31.3	134.9 a	20.6	15.3	0.224	235.5	31.3	80.5 a	11.4	14.2	−0.703	234.0	47.4	36.5 a	2.5	6.9	−0.355
	152.7	22.4	156.9 ab	26.5	16.9	0.133	165.3	19.2	90.4 ab	13.8	15.2	0.430	160.3	8.3	38.3 a	2.8	7.2	0.250
	87.2	12.1	165.8 abc	30.6	18.4	0.229	95.3	11.2	92.9 ab	15.7	16.9	0.252	131.9	12.0	39.3 ab	3.1	7.9	1.471
	48.2	7.0	185.2 bcd	41.3	22.3	0.002	69.4	7.3	100.8 abc	19.6	19.5	0.789	80.3	80.3	42.0 ab	3.3	7.9	0.357
	25.4	5.2	200.0 bcde	49.0	24.5	0.004	51.5	5.2	111.9 abcd	24.3	21.7	1.083	71.9	8.8	45.0 abc	3.9	8.6	1.982
	14.6	4.1	214.4 cde	56.9	26.6	0.595	25.3	4.8	131.6 bcde	31.0	23.6	0.726	43.9	43.9	45.4 abcd	5.3	11.7	2.052
	6.5	3.2	228.7 de	65.9	28.8	0.499	13.1	2.3	142.7 cdef	36.1	25.3	0.718	30.3	5.4	48.7bcde	7.1	14.6	1.422
	4.1	2.6	245.7 de	78.2	31.8	0.911	7.4	1.8	153.0 defg	42.5	27.8	0.367	21.3	4.3	55.9 cde	11.0	19.6	0.891
	2.8	2.2	265.0 e	89.9	33.9	0.454	3.1	1.4	160.9 efg	50.4	31.3	0.413	9.1	2.3	59.7 def	13.8	23.1	0.715
	1.4	1.9	293.8 e	109.2	37.2	0.545	1.5	1.0	176.9 fg	59.9	33.9	0.671	4.4	1.8	68.7 ef	17.6	25.6	0.795
	0.7	0.6	304.3 e	124.3	40.9	0.812	0.8	0.7	212.5 g	80.1	37.7	0.900	1.2	1.0	84.6 f	24.1	28.5	0.888
													0.8	0.6	91.6 f	31.1	34.0	0.989

Average cell per sample come from 20 direct plate counts. Average lag phases come from at least 100 data.
[a] Standard deviation.
[b] Lag phase coefficient of variation ($Sd * 100$/mean).

at 7 °C (Table 3). In fact, the higher the growth temperature is, the more the lag phase lengthens as a consequence of heat treatment (Fig. 5). These findings indicate that the lag phases of organisms that survive heat treatments and are then maintained under refrigeration, which is common in the industrial preparation of many foods, are actually quite long. Even so, a drop in the storage temperature will not affect the lag phase duration as dramatically as it does the specific growth rate: the value of μ_{max} falls by approximately 50% when the growth temperature falls by 3–4 °C in the interval between 0 and 10 °C, as reported by studies of *L. monocytogenes* and *innocua* growth in broth and foods (Combase, 2011).

In contrast to the situation with lag phase duration, the parameter with the greatest effect on lag phase variability is inoculum size (Table 3). Pin and Baranyi (2006) simulated the growth of populations containing 1, 2, 4, 8, 16 and 100 cells based on the lag phases of individual cells observed by Elfwing et al. (2004) and concluded that growth curves initiated with few cells showed longer lag times than those initiated with more cells. They also found that lag time variances were greater at small inoculum sizes. This has been called the stochastic effect (Baranyi, 2002), since it is due to a statistical effect arising from the lag phase variability of individual cells. Our experimental data confirm these simulations of the stochastic effect and the statements of Pin and Baranyi (2006), who observed that the effect of inoculum size on lag time can be observed with small inocula, although this assertion should be treated with caution, because the threshold number of cells needed to detect an effect of inoculum size on lag phase duration depends on the distribution of single-cell lag times and any factor that affects this distribution. Indeed, differences in lag time duration have been reported for relatively large inocula when cells were severely stressed by starvation (Augustin et al., 2000; Gay et al., 1996) or heating (Stephens et al., 1997), or when the growth conditions were very close to the growth/no growth boundary (Pascual et al., 2001; Robinson et al., 2001), since the lag phase distribution of individual cells shows considerable spread under these conditions. In contrast to these findings, Robinson et al. (2001) observed that under optimal conditions, lag times of uninjured *L. monocytogenes* grown at 37 °C were little affected by inoculum size and there was little variation between replicate inocula even at very low cell numbers, but the inclusion of 1.2 M NaCl in the growth medium triggered an influence of inoculum size on lag phase duration. Our data with unstressed cells

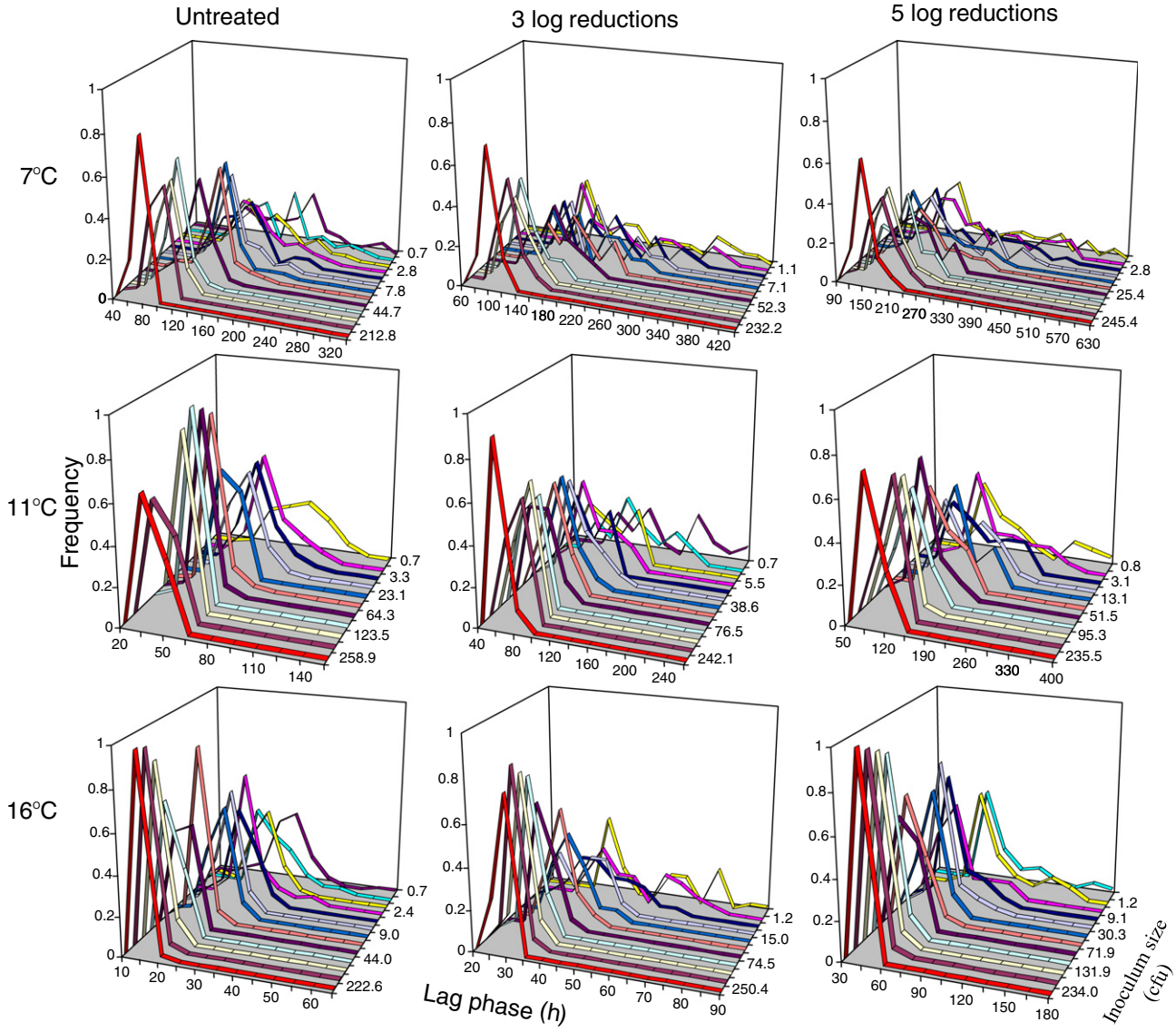

Fig. 1. Lag phase distributions of micropopulations of *Listeria innocua* grown in TSB at different temperatures after heat treatment at 54 °C to give 0, 3 and 5 log reductions.

Table 4
Polynomial coefficients to model the lag phase duration and its *Sd* for *Listeria innocua* in TSB, based on the Eq. (2). The corresponding coefficients of determination (R^2) and root mean square error are shown.

Polynomial coefficients	Average lag phase	*SE*	*Pr > F*	*Sd*	*SE*	*Pr > F*
a_0	6.2442	0.0828	<0.0001	4.1840	0.0519	<0.0001
a_1	−0.1408	0.0154	<0.0001			
a_2				0.1030	0.0285	0.0005
a_3	−0.3500	0.0076	<0.0001	−0.4155	0.0709	<0.0001
a_{12}	0.0111	0.0006	<0.0001	0.0096	0.0016	<0.0001
a_{13}				−0.0284	0.0042	<0.0001
a_{23}						
a_{11}	−0.0026	0.0006	0.0001	−0.0093	0.0003	<0.0001
a_{22}	0.0100	0.0015	<0.0001	0.0105	0.0044	0.0199
a_{33}				−0.0559	0.0223	0.0137
R^2		0.994			0.989	
RMSE		7.25			3.10	

SE. Standard error, *Sd*. Standard deviation. RMSE. Root Mean Square Error. All variables left in the model are significant at the 0.050 level. No other variable met the 0.050 significance level for entry into the model.

showed an effect of inoculum size on lag phase at a growth temperature of 16 °C. These differences indicate that any deviation from optimal growth conditions may affect lag phase duration and its variability.

The effect of inoculum size on lag phase duration and its variability may be a purely stochastic process. However, it may also have a physiological basis, for example in cell–cell interactions that affect bacterial growth, as proposed by Kaprelyants and Kell (1996). That study found that prokaryotes communicate with each other using signaling molecules, many of which were found to stimulate bacterial growth. According to Mukamolova et al. (2002), the social behavior of cells is important for many different cellular processes associated with multiplication, differentiation, survival in changing environments and death. For example, acylated homoserine lactones have been shown to shorten the lag phase of *Nitrosomonas europea* in biofilms in a concentration-dependent manner (Batchelor et al., 1997), suggesting that these signaling molecules are responsible for a cooperative effect among cells. In order to determine whether the effect of inoculum size on lag phase in our study has only a stochastic component or also includes a physiological one, we simulated the growth of a population of cells with known lag phases (average number of cells per sample < 1,

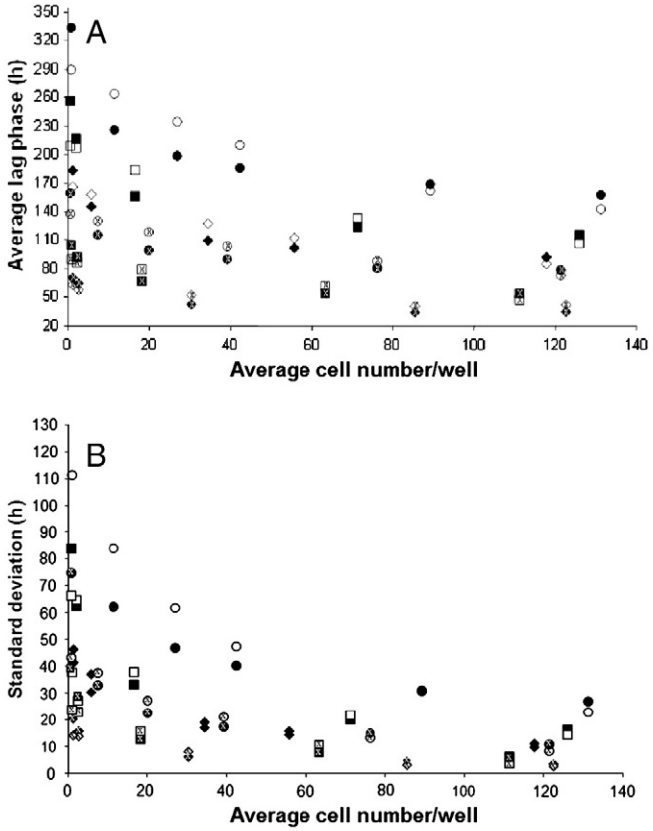

Fig. 2. Validation of a model to predict the average lag phase and its variability in *Listeria innocua* growing in whole milk. Predicted lag phase (solid symbols) and observed lag phase (empty symbols) are given for different inoculum sizes (A), together with the standard deviation of the lag phase (B). Cultures were treated at 54 °C to give decimal reductions of 1 (diamonds), 3 (squares) or 5 (circles), and then incubated at 7 °C (symbols without x) or 12 °C (symbols with x).

Table 3) and a constant μ_{max} from Table 2. Likewise, the growth of micropopulations with inocula > 1 cell was simulated based on the lag phases in Table 3 and the same μ_{max}. The predicted times for micropopulations of all inoculum sizes to reach 10^6 cells were longer than the observed times (Fig. 6). This suggests that the effect of inoculum size on the lag phase cannot be attributed solely to a stochastic process; instead, the presence of other cells, even in very low numbers, seems to shorten the micropopulation lag phase.

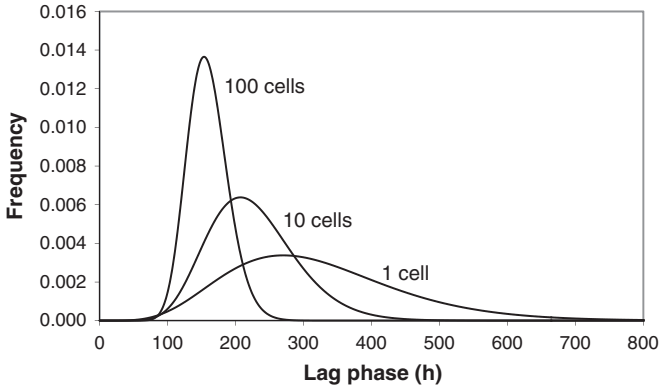

Fig. 3. Frequency distribution of lag phases simulated by applying Eq. (5). Rate and shape were calculated with Eqs. (6) and (7), where mean and Sd were predicted by the models described in Table 4. In this simulation, *Listeria innocua* cultures of different inoculum sizes (1, 10, 100 cells) were subjected to 5 logarithmic reductions using heat treatment, then incubated at 7 °C.

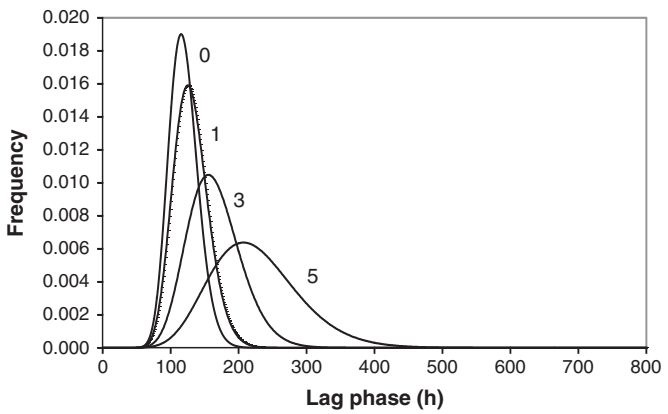

Fig. 4. Frequency distribution of lag phases simulated by applying Eq. (5). Rate and shape were calculated with Eqs. (6) and (7), where mean and Sd were predicted by the models described in Table 4. Effect of heat stress (numbers beside the curves show the decimal reduction achieved) on the lag time distribution of *Listeria innocua* (10 cells) at 7 °C.

In our study, micropopulation lag phases were found to correlate negatively with inoculum size and growth temperature and positively with heat shock intensity. This behavior is consistent with the idea that cell–cell interactions influence bacterial multiplication. Based on the scenario suggested by Kaprelyants and Kell (1996), the larger the cell population is, the more signal substance will be released and one cell will quickly receive enough signaling molecules to initiate growth. If, however, there are very few cells, they will need more time to synthesize and release an adequate number of signaling molecules to initiate multiplication. Furthermore, the ability of cell–cell interaction to reduce lag time will depend on cell concentration and the proximity of cells to one another. If a solid food contains a relatively small number of cells per gram, these cells may be completely isolated from each other, without any chance of communication. As a consequence, their lag times may be much longer than those predicted by models based on inocula of "only" hundreds or thousands of cells per ml.

Thus, stochastic and cell–cell communication processes are not mutually exclusive but instead may work together to explain the effect of inoculum size on lag phase duration and variability. Future work is needed to explore this interplay since communication between bacterial cells is not well documented, and the complex intracellular processes that occur during the lag phase are poorly understood. In addition, any physiological explanation of lag phase duration and variability will need to take into account that a quorum-sensing effect seems unlikely at very low inoculum densities (Gnanou Besse et al., 2006). It may be that the relative contribution of stochastic and physiological processes

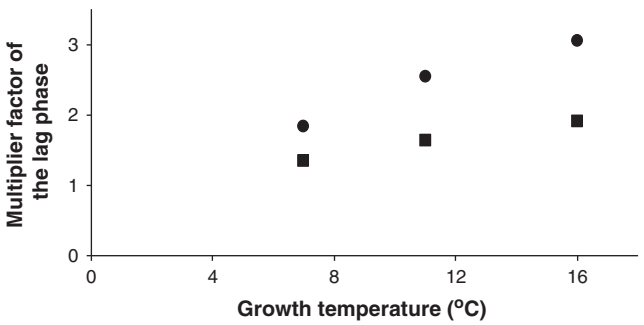

Fig. 5. Effect of heat stress intensity on the lag phase of surviving *Listeria innocua* grown in TSB. Cultures subjected to a 3*D* treatment are shown as squares; cultures subjected to 5*D* treatment, as circles. Comparisons are shown between heat-treated and untreated cultures of the same inoculum size grown at the same temperature.

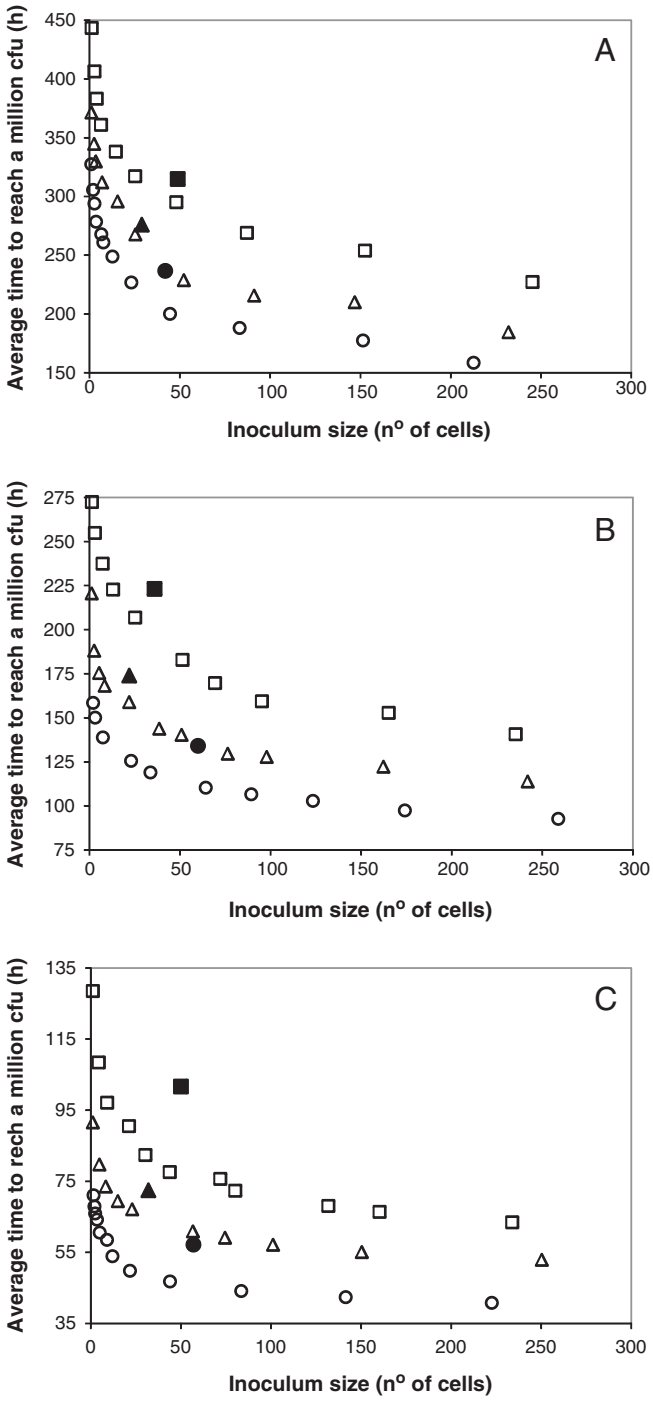

Fig. 6. Comparison of times required for micropopulations with different inocula (empty symbols) to reach 10^6 cells with those times predicted by a simulation (solid symbols) of a population formed by cells, which lag phases have been used to build individual cell distributions (average number of cells per sample less than 1, Table 3) at 7 °C (A), 11 °C (B) and 16 °C (C) for untreated cells (squares), heat treated to decline the population in 3 log reductions (triangles) and 5 log reductions (circles). Growth specific rates are assumed to be constant (0.09, 0.14 and 0.31 h^{-1} for 7, 11 and 16 °C, respectively).

to the lag phase changes with cell number, such that the stochastic effect predominates at low inoculum sizes.

Stephens et al. (1997) suggested another factor to explain lag phase variability. They showed that the variability in lag times of heat-injured cells is likely to be due to the fact that the cells were in different stages of the cell cycle when they were exposed to heat. Large cells at the end of the cell cycle are more heat-resistant than small cells that have just formed. This results in different lag phases for the cells surviving the heat treatment.

Baranyi and Roberts (1994) suggested that lag should be considered a consequence of both the present and past environments of the cells. They defined a parameter called the initial physiological state of the cells (α_0), a dimensionless number between 0 and 1 that quantifies the suitability of the current environment for the population (i.e. the history effect) (Baranyi and Pin, 2001).

$$\ln(\alpha_0) = -\mathrm{Lag}/\mu_{max}. \quad (9)$$

Rescaling this parameter to obtain the inverse of the physiological state led to a new parameter h_0, or the work to be done by the population to adapt to the new environment (Robinson et al., 1998):

$$h_0 = -\ln(\alpha_0). \quad (10)$$

This is equal to the product of the lag and the μ_{max}. Several authors (Robinson et al., 1998; McKellar et al., 2002; Pin et al., 2002) have shown that h_0 is constant for the same organism grown under the same conditions but at different temperatures, which reflects the proportionality between the growth rate and the time spent by cells to adapt. As temperature decreases, a longer adaptation time is needed and the duplication is faster.

Eqs. (9) and (10) do not consider the inoculum size, which our results show to affect lag phase and consequently h_0. Fig. 7 shows the effect of the inoculum size on the work to be done under our experimental conditions. The larger the inoculum is, the smaller is h_0, which is consistent with the idea that a physiological process contributes to lag phase duration and variability. Cells may interact each other and shorten the adaptation time to the new growth conditions. From the data of D'Arrigo et al. (2006) and Metris et al. (2008), the work to be done by *L. innocua* that survived heat treatments of different intensity was calculated and compared with data in Fig. 7. In all cases, the same tendency was observed: the work to be done increases with the degree of the stress applied to cells. Surprisingly, however, the h_0 of unheated cells depends on inoculum size and growth temperature (Fig. 6A), whereas that of the most stressed cells depends only on inoculum size (Fig. 6C).

The polynomial models in Table 4 estimate the lag phase and its variability as a function of growth temperature, the stress intensity (in terms of the average number of log reductions in the population), and the number of surviving cells initiating growth. The model predictions were compared with experimental lag phase data of *L. innocua* in milk (Fig. 2). The *Af* [Eq. (3)] indicates the spread of the observations around the model predictions: ideally the *Af* should be 1, but *Af* typically increases by 0.10–0.15 for every variable in the model (Ross et al., 2000). Therefore, a model that satisfactorily predicts the effect of three variables (growth temperature, heat treatment and inoculum size) on the microbial lag phase could be expected to have an *Af* of 1.3–1.5. Our validation trials produced an *Af* of 1.106 for lag duration and 1.203 for *Sd*, both of which are well below these limits.

A Bf [Eq. (4)] for lag phase greater than 1 indicates that the model over-estimates lag phase values and is fail-dangerous, whereas a value smaller than 1 indicates that the model is fail-safe (Jeyamkonda et al., 2001). A *Bf* greater than 1 for *Sd* indicates that the model predictions are more variable than the observations, i.e. it is unlikely that lag phases observed fell out of the interval predicted. The models described in the present study gave a *Bf* of 0.990 for lag duration and 1.035 for *Sd*, indicating that the average lag phase duration predicted will be shorter and more variable than the real one, meaning that they should be considered safe for risk assessment. Since our modeling approach shows good fit to reality, it may significantly improve predictive tools for quantitative risk assessment.

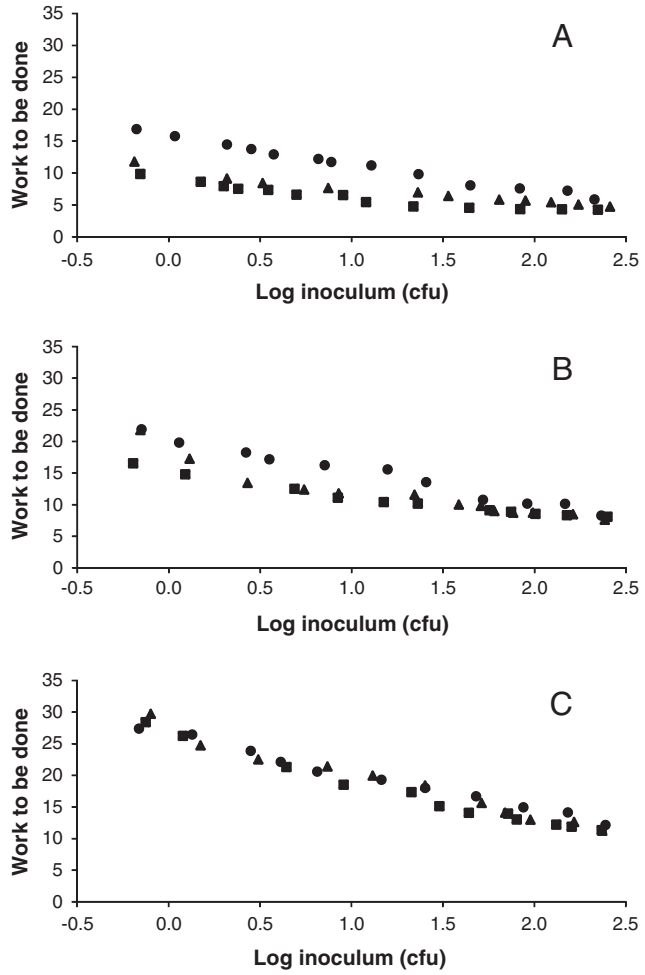

Fig. 7. Effect of inoculum size on the work to be done by cells to adapt to the present environment. Cells were left untreated (A) or heat-treated to produce a 3-log reduction (B) or 5-log reduction (C) at 7 °C (circles), 11 °C (triangles) and 16 °C (squares).

This sensitivity of lag phase to environmental factors highlights the usefulness of the models described in the present study. The data in Table 3 and the distributions in Fig. 3 show that for cells that survive 5 log reductions due to heat treatment and are then incubated at 7 °C, the expected average lag phase for 1 cell is twice that of 100 cells, and the difference in the spread of the expected lag phase distribution is even more remarkable (Fig. 3). These predictions may be useful for quantitative microbial risk assessment, when considered together with other sources of randomness, such as uncertainty, variability, consumption data, and dose–response. In this regard, the relative importance of the variability of inputs has recently been studied by Ellouze et al. (2011) and Busschaert et al. (2011).

In conclusion, the effect of inoculum size, stress and growth conditions of survivors on the lag phase of *L. innocua* and its variability depend on a complex set of interactions. Factors that appear to modulate this effect include the physiological state of the cell, level of stress applied, and growth temperature. It is important to understand how these interactions affect the lag phase in order to predict and control microbial growth in food. The population size of a pathogen in food depends on its initial concentration, as well as on many other factors. Consequently, when performing quantitative microbial risk assessment studies, it is important to take into account the initial contamination level, not only because this number is important in itself, but because it significantly affects the lag phase duration and its variability.

Acknowledgments

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References

Aguirre, J.S., Pin, C., Rodriguez, M.R., Garcia de Fernando, G.D., 2009. Analysis of the variability in the number of viable bacteria after mild heat treatment of food. Applied and Environmental Microbiology 75, 6992–6997.
Aguirre, J.S., Rodriguez, M.R., Garcia de Fernando, G.D., 2011. Effects of electron beam irradiation on the variability in survivor number and duration of lag phase of four food-borne organisms. International Journal of Food Microbiology 149, 236–246.
Aguirre, J.S., Bravo, M.C., Ordoñez, J.A., Garcia de Fernando, G.D., 2012a. The Poisson distribution is applied to improve the estimation of individual cell and micropopulation lag phases. Advances in Microbiology 2, 146–161.
Aguirre, J.S., Ordoñez, J.A., Garcia de Fernando, G.D., 2012b. A comparison of the effects of E-beam irradiation and heat treatment on the variability of *Bacillus cereus* inactivation and lag phase duration of surviving cells. International Journal of Food Microbiology 153, 444–452.
Augustin, J.C., Brouillaud-Delattre, A., Rosso, L., Carlier, V., 2000. Significance of inoculum size in the lag time of *Listeria monocytogenes*. Applied and Environmental Microbiology 66, 1706–1710.
Baranyi, J., 1998. Comparison of stochastic and deterministic concepts of bacterial lag. Journal of Theoretical Biology 192, 403–408.
Baranyi, J., 2002. Stochastic modelling of bacterial lag phase. International Journal of Food Microbiology 73, 203–206.
Baranyi, J., Pin, C., 1999. Estimating bacterial growth parameters by means of detection times. Applied and Environmental Microbiology 65, 732–736.
Baranyi, J., Pin, C., 2001. A parallel study on bacterial growth and inactivation. Journal of Theoretical Biology 210, 327–336.
Baranyi, J., Roberts, T.A., 1994. A dynamic approach to predicting bacterial growth in food. International Journal of Food Microbiology 23 (3–4), 277–294.
Baranyi, J., George, S.M., Kutalik, Z., 2009. Parameter estimation for the distribution of single cell lag times. Journal of Theoretical Biology 259 (1), 24–30.
Batchelor, S.E., Cooper, M., Chhabra, S.R., Glover, L.A., Stewart, G.S.A.B., Williams, P., Prosser, J.I., 1997. Cell density-regulated recovery of starved biofilm populations of ammonia-oxidizing bacteria. Applied and Environmental Microbiology 63, 2281–2286.
Black, E.P., Linton, M., McCall, R.D., Curran, W., Fitzgerald, G.F., Kelly, A.L., Patterson, M.F., 2008. The combined effects of high pressure and nisin on germination and inactivation of *Bacillus* spores in milk. Journal of Applied Microbiology 105, 78–87.
Brouillaud-Delattre, A., Maire, M., Collette, C., Mattei, C., Lahellec, C., 1997. Predictive microbiology of dairy products: influence of biological factors affecting growth of *Listeria monocytogenes*. Journal of AOAC International 80, 913–919.
Busschaert, P., Geeraerd, A.H., Uyttendaele, M., Van Impe, J.F., 2011. Sensitivity analysis of a two-dimensional quantitative microbiological risk assessment: keeping variability and uncertainty separated. Risk Analysis 31 (8), 1295–1307.
Combase, 2011. http://www.combase.cc/index.php/en/combase-browser.
D'Arrigo, M., Garcia de Fernando, G.D., Velasco de Diego, R., Ordoñez, J.A., George, S.M., Pin, C., 2006. Indirect measurement of the lag time distribution of single cells of *Listeria innocua* in food. Applied and Environmental Microbiology 72 (4), 2533–2538.
Duffy, G., Sheridan, J.J., Buchanan, R.L., McDowell, D.A., Blair, I.S., 1994. The effect of aeration, initial inoculum and meat microflora on the growth kinetics of *Listeria monocytogenes* in selective enrichment broths. Food Microbiology 11, 429–438.
Elfwing, A., LeMarc, Y., Baranyi, J., Ballagi, A., 2004. Observing growth and division of large numbers of individual bacteria by image analysis. Applied and Environmental Microbiology 70, 675–678.
Ellouze, M., Gauchi, J.P., Augustin, J.C., 2011. Use of global sensitivity analysis in quantitative microbial risk assessment: application to the evaluation of a biological time temperature integrator as a quality and safety indicator for cold smoked salmon. Food Microbiology 28 (4), 755–769.
Francois, K., Devlieghere, F., Uyttendaele, M., Standaert, A.R., Geeraerd, A.H., Nadal, P., Van Impe, J.F., Debevere, J., 2006. Single cell variability of *Listeria monocytogenes* grown on liver pate and cooked ham at 7 degrees C: comparing challenge test data to predictive simulations. Journal of Applied Microbiology 100, 800–812.
Gay, M., Cerf, O., Davey, K.R., 1996. Significance of pre-incubation temperature and inoculum concentration on subsequent growth of *Listeria monocytogenes* at 14 degrees C. Journal of Applied Bacteriology 81, 433–438.
Gnanou Besse, N., Audinet, N., Barre, L., Cauquil, A., Cornu, M., Colin, P., 2006. Effect of the inoculum size on *Listeria monocytogenes* growth in structured media. International Journal of Food Microbiology 110, 43–51.
Guillier, L., Pardon, P., Augustin, J.C., 2005. Influence of stress on individual lag time distributions of *Listeria monocytogenes*. Applied and Environmental Microbiology 71, 2940–2948.
Jason, A.C., 1983. A deterministic model for monophasic growth of batch cultures of bacteria. Antonie Van Leeuwenhoek 49, 513–536.

Jeyamkonda, S., Jayas, D.S., Holley, R.A., 2001. Microbial growth modelling with artificial neural networks. International Journal of Food Microbiology 64, 343–354.
Jongenburger, I., Reij, M.W., Boer, E.P.J., Gorris, L.G.M., Zwietering, M.H., 2010. Factors influencing the accuracy of the plating method used to enumerate low numbers of viable micro-organisms in food. International Journal of Food Microbiology 143, 32–40.
Juneja, V.K., Marks, H.M., 2006. Growth kinetics of *Salmonella* spp. pre- and post-thermal treatment. International Journal of Food Microbiology 109, 54–59.
Kaprelyants, A.S., Kell, D.B., 1996. Do bacteria need to communicate with each other for growth? Trends in Microbiology 4 (6), 237–242.
Kaprelyants, A.S., Mukamolova, G.V., Kormer, S.S., Weichart, D.H., Young, M., Kell, D.B., 1999. Intracellular signalling and the multiplication of prokaryotes: bacterial cytokines. Symposia of the Society for General Microbiology 57, 33–69.
Koutsoumanis, K., 2008. A study on the variability in the growth limits of individual cells and its effect on the behavior of microbial populations. International Journal of Food Microbiology 128, 116–121.
Li, Y., Odumeru, J.A., Griffiths, M., McKellar, R.C., 2006. Effect of environmental stresses on the mean and distribution of individual cell lag times of *Escherichia coli* O157: H7. International Journal of Food Microbiology 110, 278–285.
Lianou, A., Koutsoumanis, K.P., 2011. Effect of the growth environment on the strain variability of *Salmonella enterica* kinetic behavior. Food Microbiology 28, 828–837.
Mackey, B.M., Kerridge, A.L., 1988. The effect of incubation temperature and inoculum size on growth of salmonellae in minced beef. International Journal of Food Microbiology 6, 57–65.
McKellar, R.C., Knight, K., 2000. A combined discrete-continuous model describing the lag phase of *Listeria monocytogenes*. International Journal of Food Microbiology 54, 171–180.
McKellar, R.C., Lu, X., Knight, K.P., 2002. Growth pH does not affect the initial physiological state parameter (p_o) of *Listeria monocytogenes* cells. International Journal of Food Microbiology 73 (2–3), 137–144.
Mejlholm, O., Gunvig, A., Borggaard, C., Blom-Hanssen, J., Mellefont, L., Ross, T., Leroi, F., Else, T., Visser, D., Dalgaard, P., 2010. Predicting growth rates and growth boundary of *Listeria monocytogenes* — an international validation study with focus on processed and ready-to-eat meat and seafood. International Journal of Food Microbiology 141, 137–150.
Metris, A., George, S.M., Peck, M.W., Baranyi, J., 2003. Distribution of turbidity detection times produced by single cell-generated bacterial populations. Journal of Microbiological Methods 55, 821–827.
Metris, A., Le Marc, Y., Elfwing, A., Ballagi, A., Baranyi, J., 2005. Modelling the variability of lag times and the first generation times of single cells of *Escherichia coli*. International Journal of Food Microbiology 100, 13–19.
Metris, A., George, S.M., Mackey, B.M., Baranyi, J., 2008. Modeling the variability of single-cell lag times for *Listeria innocua* populations after sublethal and lethal heat treatments. Applied and Environmental Microbiology 74 (22), 6949–6955.
Mukamolova, G.V., Kaprelyants, A.S., Young, D.I., Young, M., Kell, D.B., 1998. A bacterial cytokine. Proceedings of the National Academy of Sciences of the United States of America 95, 8916–8921.
Mukamolova, G.V., Turapov, O.A., Kazarian, K., Telkov, M., Kaprelyants, A.S., Kell, D.B., Young, M., 2002. The rpf gene of *Micrococcus luteus* encodes an essential secreted growth factor. Molecular Microbiology 46, 611–621.
Pascual, C., Robinson, T.P., Ocio, M.J., Aboaba, O.O., Mackey, B.M., 2001. The effect of inoculum size and sublethal injury on the ability of *Listeria monocytogenes* to initiate growth under suboptimal conditions. Letters in Applied Microbiology 33, 357–361.
Pin, C., Baranyi, J., 2006. Kinetics of single cells: observation and modeling of a stochastic process. Applied and Environmental Microbiology 72, 2163–2169.
Pin, C., Sutherland, J.P., Baranyi, J., 1999. Validating predictive models of food spoilage organisms. Journal of Applied Microbiology 87, 491–499.
Pin, C., Garcia de Fernando, G., Ordonez, J.A., Baranyi, J., 2002. Analysing the lag-growth rate relationship of *Yersinia enterocolitica*. International Journal of Food Microbiology 73 (2–3), 197–201.
Robinson, T.P., Ocio, M.J., Kaloti, A., Mackey, B.M., 1998. The effect of the growth environment on the lag phase of *Listeria monocytogenes*. International Journal of Food Microbiology 44 (1–2), 83–92.
Robinson, T.P., Aboaba, O.O., Kaloti, A., Ocio, M.J., Baranyi, J., Mackey, B.M., 2001. The effect of inoculum size on the lag phase of *Listeria monocytogenes*. International Journal of Food Microbiology 70, 163–173.
Ross, T., 1996. Indices for performance evaluation of predictive models in food microbiology. Journal of Applied Bacteriology 81, 501–508.
Ross, T., Dalgaard, P., Tienungoon, S., 2000. Predictive modelling of the growth and survival of *Listeria* in fishery products. International Journal of Food Microbiology 62, 231–245.
Smelt, J.P.P.M., Bos, A.P., Kort, R., Brul, S., 2008. Modelling the effect of sub(lethal) heat treatment of *Bacillus subtilis* spores on germination rate and outgrowth to exponentially growing vegetative cells. International Journal of Food Microbiology 128, 34–40.
Stephens, P.J., Joynson, J.A., Davies, K.W., Holbrook, R., Lappin-Scott, H.M., Humphrey, T.J., 1997. The use of an automated growth analyser to measure recovery times of single heat-injured *Salmonella* cells. Journal of Applied Microbiology 83, 445–455.
Stringer, S.C., Webb, M.D., Peck, M.W., 2011. Lag time variability in individual spores of *Clostridium botulinum*. Food Microbiology 28, 228–235.
Zhu, M.J., Mendonca, A., Ismail, H.A., Ahn, D.U., 2008. Effects of irradiation on survival and growth of *Listeria monocytogenes* and natural microflora in vacuum-packaged turkey hams and breast rolls. Poultry Science 87, 2140–2145.