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Effect of storage temperature on the lag time of *Geobacillus stearothermophilus* individual spores

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ABSTRACT

The lag times (λ) of *Geobacillus stearothermophilus* single spores were studied at different storage temperatures ranging from 45 to 59 °C using the Bioscreen C method. A significant variability of λ was observed among individual spores at all temperatures tested. The storage temperature affected both the position and the spread of the λ distributions. The minimum mean value of λ (i.e. 10.87 h) was observed at 55 °C, while moving away from this temperature resulted in an increase for both the mean and standard deviation of λ. A Cardinal Model with Inflection (CMI) was fitted to the reverse mean λ, and the estimated values for the cardinal parameters T_{min}, T_{max}, T_{opt} and the optimum mean λ of *G. stearothermophilus* were found to be 38.1, 64.2, 53.6 °C and 10.3 h, respectively. To interpret the observations, a probabilistic growth model for *G. stearothermophilus* individual spores, taking into account λ variability, was developed. The model describes the growth of a population, initially consisting of N_0 spores, over time as the sum of cells in each of the N_0 imminent subpopulations originating from a single spore. Growth simulations for different initial contamination levels showed that for low N_0 the number of cells in the population at any time is highly variable. An increase in N_0 to levels exceeding 100 spores results in a significant decrease of the above variability and a shorter λ of the population. Considering that the number of *G. stearothermophilus* surviving spores in the final product is usually very low, the data provided in this work can be used to evaluate the probability distribution of the time-to-spoilage and enable decision-making based on the "acceptable level of risk".

1. Introduction

The presence of *Geobacillus stearothermophilus* spores in thermally processed foods constitutes one of the most important quality problems for the food industry. The high prevalence and concentration of spores in the raw materials, the adhesive characteristics of spores that enhance their persistence in industrial environments and, most importantly, the high heat resistance of the spores are among the major factors explaining the importance of this endospore-former (Yoo et al., 2006; Postollec et al., 2012; André et al., 2013). Thus, the estimation of the risk of *G. stearothermophilus* growth to spoilage levels constitutes a major target of quality managers.

Recently, Kakagianni et al. (2016) developed a predictive model for the effect of storage temperature on the growth of *G. stearothermophilus*, which can be used to predict the time-to-spoilage as the time required by the contaminating spores to germinate and multiply from the initial to a spoilage level. The model was developed and successfully validated for evaporated milk based on data from experiments with high initial contamination levels. In practice, however, the number of spores surviving the heat treatment is usually very low. Depending on the use of the model, predictions based on high initial contamination levels can be on the "fail-safe" or the "fail-dangerous" side. For example, when the model is used to predict the time-to-spoilage, the above predictions represent a worst-case scenario. This is because the early

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germinated and outgrown spores within the population begin to multiply up to a microbial concentration sufficient to spoil the food leading to shorter estimations of the time-to-spoilage compared to low initial contamination levels (Baranyi, 1998, 2002; Pin and Baranyi, 2006; Baranyi et al., 2009). In contrast, when the objective is to evaluate the duration of a quality control test in which products are stored at the optimum temperature for growth for a certain time and the percentage of spoiled items is evaluated, the above predictions can lead to "fail-dangerous" estimates. Thus, for an effective risk assessment of spoilage the growth model needs to be combined with quantitative data on the variability of individual spore behavior.

Lag time is much more uncertain and difficult to predict compared to growth rate (Baranyi, 2002). However, the credibility of a mathematical model in predicting the conditions that lead to microbiological spoilage depends highly on its ability to describe the effect of the environment on lag time. This implies that a better understanding of the lag phase is of great importance for the effective application of predictive models in food quality management. For a complete understanding of lag time, the behaviour of single cells or spores has to be taken into account through the development of stochastic models which are able to deal with more "realistic" low contamination events (Koutsoumanis and Lianou, 2013).

Available studies on single spore lag time focus on either *Bacillus* (Aguirre et al., 2012; Pandey et al., 2013; Zhou et al., 2013; van Melis et al., 2014; Aguirre et al., 2015; Pandey et al., 2015; Warda et al., 2015) or *Clostridium* (Stringer et al., 2005, 2009; Webb et al., 2007; Wang et al., 2012; Smelt et al., 2013; Lenz et al., 2014) species, while data on *G. stearothermophilus* are very limited. Zhou et al. (2013) studied the kinetics of germination of individual spores of *G. stearothermophilus* measured by Raman spectroscopy and differential interference contrast microscopy. Previous studies, however, demonstrated that germination and lag time are independent (Stringer et al., 2005). The variability of post-germination stages such as outgrowth and doubling is relatively large, and as a result, the germination time cannot be used to predict the overall lag time.

The objective of the present work was to assess the impact of different storage temperature conditions on the lag time of individual *G. stearothermophilus* spores, and to demonstrate the role of individual spore heterogeneity in growth predictions using a stochastic modeling approach.

2. Materials and methods

2.1. Bacterial strain and inoculum preparation

The type strain *G. stearothermophilus* ATCC 7953 was used for all experiments in the present study. The stock culture of the strain was stored frozen (−70 °C) onto Microbank™ porous beads (Pro-Lab Diagnostics, Ontario, Canada). The working culture was stored refrigerated (5 °C) on nutrient agar (NA; Lab M Limited, Lancashire, United Kingdom) slants and was renewed bimonthly. Prior to each experiment, the microorganism was activated by transferring a loopful from the NA slants into 10 ml nutrient broth (NB; Lab M Limited) and incubating at 55 °C for 24 h. The 24-h *G. stearothermophilus* cultures were subjected to a heat shock treatment in order to eliminate vegetative cells and enhance the homogeneous activation and germination of dormant endospores of the organism (Antolinos et al., 2012; Yuan et al., 2012). Then, the heat-shocked cultures were centrifuged (6000 rpm for 20 min) in a refrigerated centrifuge (4 °C) (model PK120R, ThermoElectron Corporation, Waltham, MA). The harvested cells were washed with 20 ml of quarter-strength Ringer's solution (Lab M Limited) through centrifugation under the same conditions. The pellet was finally resuspended in 10 ml of tryptone soy broth (TSB; Lab M Limited) in order to obtain an initial concentration of *ca.* 10^6 spores/ml.

2.2. Growth kinetic experiments

2.2.1. Maximum specific growth rate

The maximum specific growth rate (μ_{max}) of *G. stearothermophilus* was evaluated in TSB at 45, 47.5, 50, 55 and 59 °C. The μ_{max} values corresponding to each storage temperature were estimated by means of absorbance detection times of serially decimally diluted cultures using the automated turbidimetric system Bioscreen C (Oy Growth Curves Ab Ltd., Raisio, Finland) as described previously (Lianou and Koutsoumanis, 2011; Kakagianni et al., 2016). In this study, the range of initial concentrations obtained in the microtiter plates was approximately 10^6–10^2 cfu/well. Optical density (OD) measurements in the Bioscreen C were measured at 15-min intervals using the wideband filter (420–580 nm) for a total time period such that a considerable OD change was observed, if possible, for all five decimally diluted cultures. The microtiter plates were agitated for 15 s at medium amplitude prior to the OD measurements. The absorbance time to detection (T_{det}) was defined as the time required for the OD to reach 0.2 units. Specifically, proper dilutions of the content of each well of a microtiter plate (100 wells per plate) were surface plated onto tryptone soy agar (TSA; Lab M Limited) plates during the exponential growth phase of the cultures at T_{det} (OD_{det} = 0.2 $A_{420\text{-}580}$). Colonies were enumerated after incubation of the plates at 55 °C for 24 h allowing the calculation of the bacterial concentration at the chosen T_{det} (N_{det}). The detection times (h) of five serial decimal dilutions of the bacterial culture were plotted against the corresponding natural logarithm of their initial concentrations, and μ_{max} values were determined by linear regression as the negative reciprocal of the slope of the regression line (Dalgaard and Koutsoumanis, 2001). For each temperature, five samples (i.e. quintuple wells of five serially diluted cultures) were analysed ($n = 5$).

2.2.2. Lag time of individual spores

Assuming an exponential bacterial growth at a constant μ_{max}, after the lag period and until the T_{det}, the single-spore lag time (λ) values of the tested microorganism were estimated based on the generated turbidity growth curves and according to the Bioscreen C methodology (Baranyi and Pin, 1999; Baranyi et al., 2009; Aguirre et al., 2011). The culture of the tested strain was decimally and binary diluted in TSB to obtain a target concentration of *ca.* 1 spore/well. Two microtiter plates (100 wells per plate) were inoculated with 350 μl of the aforementioned diluted organism's culture in each well, incubated in the Bioscreen C at different temperatures (45, 47.5, 50, 55 and 59 °C), and the OD was recorded at 420–580 nm at 15-min intervals. Microtiter plates were shaken at medium amplitude for 15 s before measuring OD. The average initial number of spores per well (m) was estimated by assuming that the probability of having one spore per well is described by the Poisson distribution (Baranyi et al., 2009), as a result of the dilution process, using the following equation:

$$m = -\mathrm{Ln}P_0 \quad (1)$$

where P_0 is the probability of having no growth in a well (estimated as the percentage of wells without detected growth). To ensure that most of the positive wells contained on average one spore, when microtiter plates showed a growth/no growth ratio less than 0.2 or higher than 0.7, were discarded and the experiment was repeated.

The individual spore λ values were calculated using the

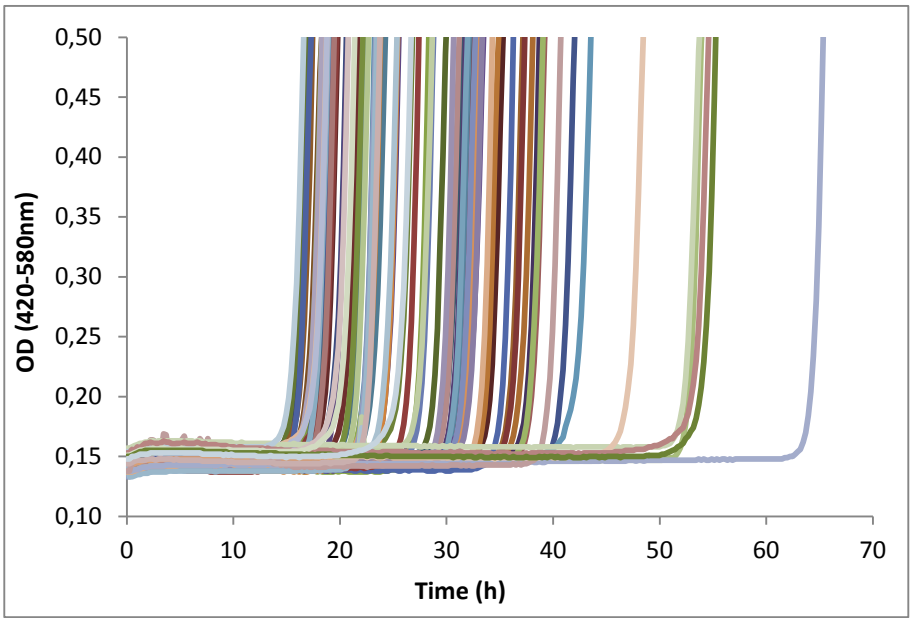

Fig. 1. OD growth curves during growth of 199 single spores of *Geobacillus stearothermophilus* in tryptone soy broth at 45 °C.

following equation proposed by Baranyi and Pin (1999):

$$\lambda = T_{det} - \frac{\mathrm{Ln}(N_{det}) - \mathrm{Ln}(N_0)}{\mu_{max}} \quad (2)$$

where T_{det} is the time (h) required for the OD at 420–580 nm to reach 0.2 units, μ_{max} is the maximum specific growth rate (1/h) determined under the experimental conditions as described in Section 2.2.1, $\mathrm{Ln}N_{det}$ is the natural logarithm of the cell population (ln cfu/well) at T_{det}, $\mathrm{Ln}N_0$ is the natural logarithm of the average initial number of spores initiating growth (ln cfu/well) in the considered well estimated according to Eq. (1). For each tested condition, the experiments were repeated as many times needed to obtain about 200 values of λ.

2.2.3. Statistical analysis and growth simulation

Various theoretical distributions were fitted to the cumulative distributions of individual spore λ values using @RISK 6.0 Professional Edition (Palisade Corporation, Newfield, New York, USA). The goodness of fit was evaluated using the Root Mean Square Error (RMSE) values. Monte Carlo simulation was performed to predict growth for different N_0 (1, 10 and 100 spores) taking into account the variability in individual spore λ using the best fitted distribution. The simulation was performed using 10,000 iterations.

3. Results

The differences in the T_{det} from OD curves of 199 individual spores growth at 45 °C are illustrated in Fig. 1. The observed T_{det}, μ_{max} and N_{det} values were used to evaluate the variability of single *G. stearothermophilus* spores λ based on equation (2). The estimated μ_{max} values (mean ± standard deviation) were 0.452 ± 0.022, 0.563 ± 0.051, 0.883 ± 0.047, 1.666 ± 0.047 and 1.955 ± 0.091 h^{-1} for 45, 47.5, 50, 55 and 59 °C, respectively. The bacterial concentration parameter N_{det} was found to be unaffected by temperature ($P \geq 0.05$) with a mean (±standard deviation) value of 6.99 (±0.36) log cfu/well.

Fig. 2 and Table 1 present the cumulative probability distributions and the statistics of the individual spore λ at the different tested temperatures. As shown, λ varied significantly among individual spores at all temperatures tested. The minimum mean value of λ was 10.87 h and it was observed at 55 °C. Moving away from the latter temperature resulted in an increase for both the mean and the standard deviation of λ. The Gamma distribution was further fitted to the λ data, following the theory of Baranyi and Pin (2001). The estimated parameters and the RMSE values of the fitting are presented in Table 2. A comparison between the observed and fitted quantiles (Fig. 3) clearly revealed that the Gamma distribution is suitable for describing the individual spore λ for all the tested temperatures.

In order to quantify the temperature dependence of individual spore λ, the following Cardinal Model with Inflection (CMI) (Rosso et al., 1993) was fitted to the reverse mean λ:

$$\frac{1}{\lambda}(T) = \frac{1}{\lambda_{opt}} \cdot \rho(T) \quad (3)$$

$$\rho(T) = \begin{cases} 0 & , \quad T \leq T_{min} \\ \dfrac{(T - T_{max})(T - T_{min})^2}{(T_{opt} - T_{min})\left[(T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})(T_{opt} + T_{min} - 2T)\right]} & , \quad T_{min} < T < T_{max} \\ 0 & , \quad T \geq T_{max} \end{cases} \quad (4)$$

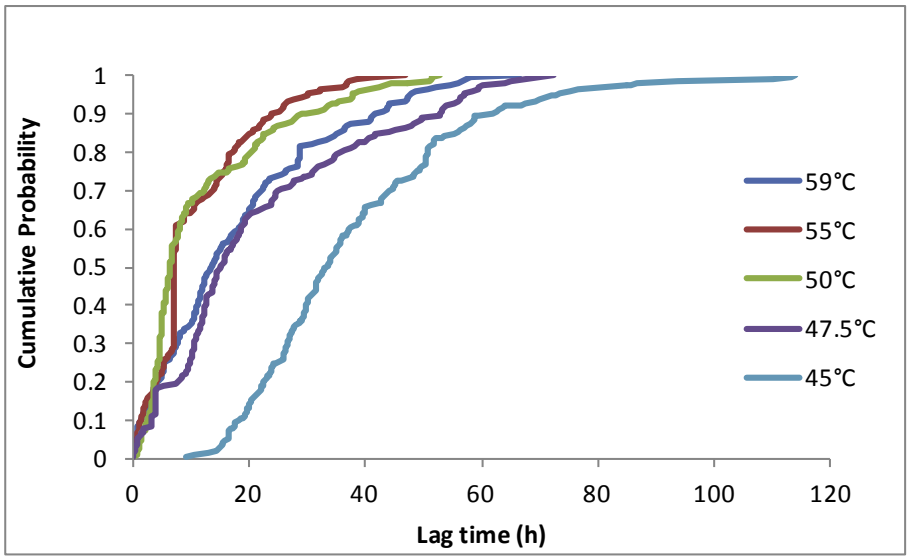

Fig. 2. Cumulative distributions of *Geobacillus stearothermophilus* single spore lag times (λ) at different growth temperature conditions. The number of data were 224, 199, 227, 199 and 193 for 45, 47.5, 50, 55 and 59 °C, respectively.

Table 1
Statistics of the individual spore lag time (λ) of *Geobacillus stearothermophilus*.

Storage temperature (°C)	N[b]	λ^{α} (h)						
		Mean	St. Deviation	Median	5th Percentile	95th Percentile	Skewness	Kurtosis
45	224	37.9	18.6	33.3	16.4	72.6	1.39	2.62
47.5	199	21.4	17.7	15.3	1.26	56.9	1.03	0.102
50	227	11.7	11.8	6.46	1.50	37.8	1.66	2.06
55	199	10.9	9.20	7.16	0.705	29.9	1.42	1.85
59	193	17.9	15.0	13.6	0.760	47.7	1.00	0.305

[a] Individual spore lag time.
[b] Number of spores tested.

Table 2
Parameter estimation of the Gamma distribution fitted to the individual spore lag times of *Geobacillus stearothermophilus*. The probability density function of the Gamma distribution in the shape-rate parametrization is: $f(x,\alpha,\beta)=\frac{\beta^{\alpha}x^{\alpha-1}e^{-x\beta}}{\Gamma(\alpha)}$ *for* $x>0$ *and* $\alpha,\beta>0$.

Temperature (°C)	a	β	*RMSE*[a]
45	4.69	7.84	0.0186
47.5	1.30	16.5	0.0316
50	1.28	7.81	0.0576
55	1.40	4.72	0.0603
59	1.10	17.1	0.0200

[a] Root Mean Square Error.

where T_{min} and T_{max} are the theoretical minimum and maximum temperature for growth (λ becomes infinite), respectively, T_{opt} is the theoretical optimum temperature for growth (λ reaches a minimum value), and λ_{opt} is the value of lag at $T = T_{opt}$. In order to stabilise the variance, a square root transformation was used. Both the R^2 (Table 3) and the graphical evaluation of the fitting (Fig. 4) indicated a satisfactory performance of the CMI in describing the effect of temperature on the mean λ of *G. stearothermophilus* individual spores. The estimated values for the cardinal parameters T_{min}, T_{max} and T_{opt} were 38.2, 64.2 and 53.6 °C, respectively, while the mean λ_{opt} of *G. stearothermophilus* was estimated 10.3 h (Table 3).

To interpret the observations, a stochastic model for *G. stearothermophilus* spores' growth was developed by introducing the distribution of individual spores λ values into a simple exponential growth with lag model (Koutsoumanis and Lianou, 2013) as follows:

$$N_t = \sum_{1}^{N_0} \begin{cases} 1 & for \quad t \le \lambda_i \\ e^{\mu_{max}(t-\lambda_i)} & for \quad t > \lambda_i \end{cases} \tag{5}$$

where N_t is the total number of cells or spores in a population at time t, N_0 is the initial number of spores at $t = 0$, μ_{max} is the maximum specific growth rate and λ_i is the lag time of individual spores following the gamma distribution. The model describes the growth of a bacterial population, initially consisting of N_0 spores, over time as the sum of cells in each of the N_0 imminent subpopulations originating from a single spore. The above approach allows for taking into account the heterogeneity in the growth dynamics of single spores by introducing λ variability in the model using Monte Carlo simulation.

Fig. 5 presents the output of the model (Eq. (5)) for spore levels N_0 equal to 1 (Fig. 5a), 10 (Fig. 5b) and 100 spores (Fig. 5c) at 45 °C based on a Monte Carlo simulation with 10,000 iterations and with a Uniform distribution for t [t ~ Uniform (0, 80)]. As shown, the output of the model for $N_0 = 1$ spore is a stochastic growth curve in which the number of cells in the population at any time is a distribution. An increase in N_0 to 100 spores resulted in a significant decrease of the above variability as well as in a

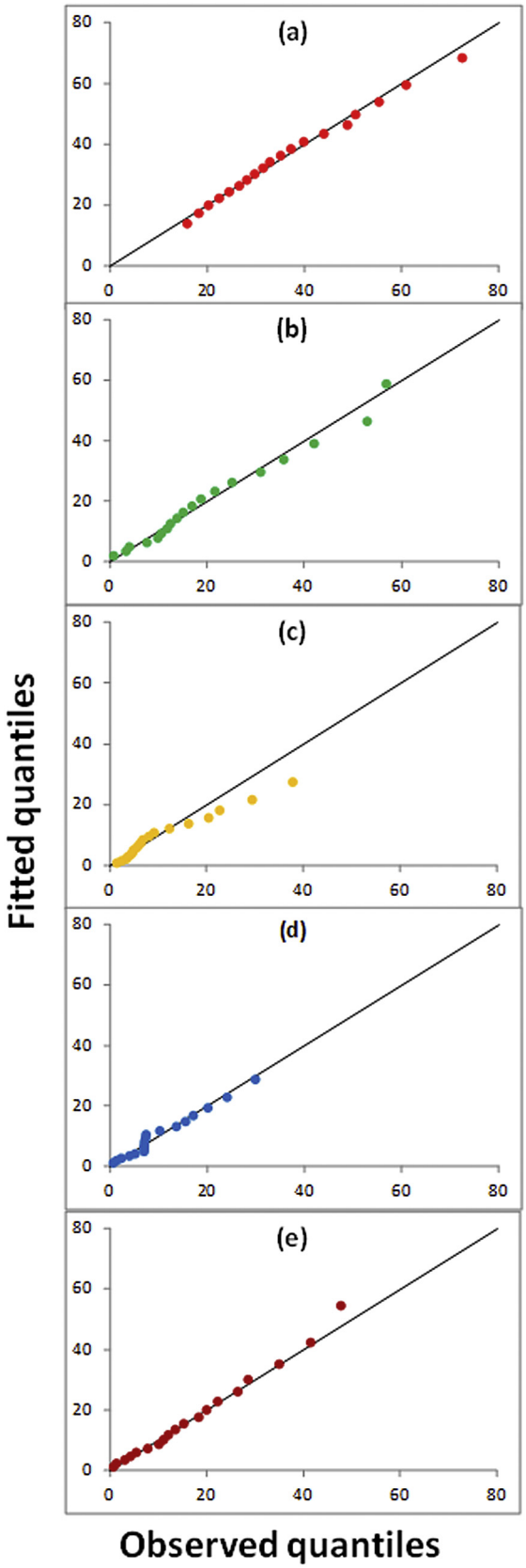

Fig. 3. Comparison between observed and fitted quantiles of the Gamma distribution for *Geobacillus stearothermophilus* single spore lag times (λ) at 45 °C (a), 47.5 °C (b), 50 °C (c), 55 °C (d) and 59 °C (e).

Table 3
Estimated values and statistics for the parameters of the Cardinal Model with Inflection (Equations (3) and (4)) describing the effect of temperature on the reciprocal of the mean lag time (λ) of individual spores of *Geobacillus stearothermophilus*.

Parameter	Estimated Value	Standard Error	R^2[a]
λ_{opt} (h)	10.3	0.5	0.977
T_{max} (°C)	64.2	2.3	
T_{min} (°C)	38.2	5.1	
T_{opt} (°C)	53.6	0.8	

[a] R^2: coefficient of determination.

shorter lag phase of the population. The model can be used to evaluate the probability distribution of the time-to-spoilage, with the latter being defined as the time required by the initial contaminating spores to germinate and multiply to a spoilage level (Kakagianni et al., 2016).

4. Discussion

The Bioscreen C method, applied in the present work to study the λ of single *G. stearothermophilus* spores, has been extensively used for monitoring single cell and spore kinetics (George et al., 2008; Métris et al., 2008; Dupont and Augustin, 2009; Miled et al., 2011; Aguirre et al., 2015; Blana et al., 2015). One of the main assumptions of the method is that growth is initiated from a single spore in a well. The number of spores in each well can be estimated based on the ratio between positive wells (wells showing growth) and negative wells (wells showing no growth) according to the Poisson distribution. In all experiments carried out in this study, the percentage of positive wells was always between 20 and 70% providing an average number of spores in a well between 0.22 and 1.2. According to the Poisson distribution, the above values correspond to a percentage of wells with one spore ranging from 51.4% to 98.1%. Previous studies have shown that the above range allows for the estimation of the distributions of single cell parameters (Métris et al., 2003; Stringer et al., 2005). Indeed, Stringer et al. (2005) studied the lag times of *Clostridium botulinum* individual spores utilizing both the Bioscreen C and a microscopic method, and reported that the two methodologies produced distributions with very similar estimates of both mean lag value and variability.

The findings of this study highlight a considerable heterogeneity in the λ of *G. stearothermophilus* single spores. In the case of spores, the lag time is mainly determined by the germination and outgrowth processes (Smelt et al., 2002; Stringer et al., 2005). Thus, the observed λ variability can be considered as a reflection of the differences in the rates of the above processes among spores. The stochastic fluctuations in the number of the nutrient germinant receptors (nGRs) per spore due to epigenetic variations among individual spores, appears to be one of the main reasons of the significant heterogeneity in spore germination capacity at the population level (Elowitz et al., 2002; Stringer et al., 2005; Chen et al., 2006; Webb et al., 2007; Ghosh and Setlow, 2009; Peng et al., 2009; Kong et al., 2010; Zhang et al., 2010). Moreover, the stochastic inactivation of nGRs during heat treatment can contribute to the differences in the germination rates among individual spores (Smelt et al., 2008). Finally the differences in the levels of specific lytic enzymes such as CwlJ and SleB, which initiate the spores' peptidoglycan cortex degradation during germination can be an additional variability source (Setlow, 2003; Moir, 2006; Ghosh and Setlow, 2009; Peng et al., 2009).

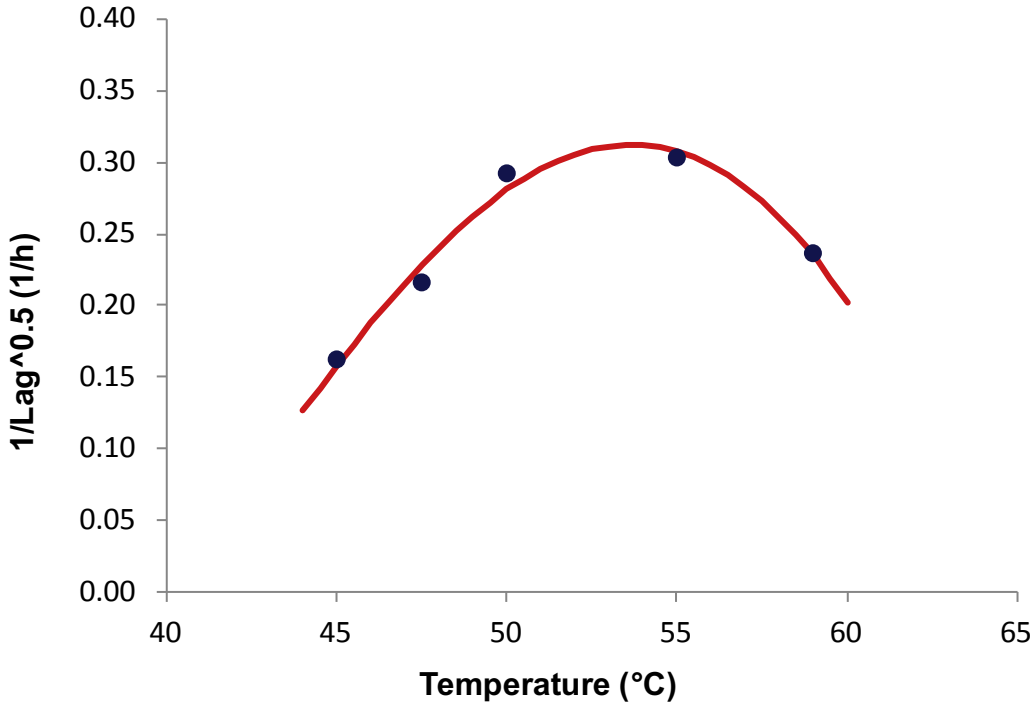

Fig. 4. Effect of temperature on the reciprocal of the mean lag times of *Geobacillus stearothermophilus* single spores fitted to the Cardinal Model with Inflection (solid line) (Equations (3) and (4)). Points (○) represent observed values.

The storage temperature affected both the position and the spread of the λ distributions of *G. stearothermophilus* spores. In agreement to previous single cell studies (Métris et al., 2003; Guillier et al., 2005; 2006), the mean and the standard deviation of the λ values estimated in this study followed a similar trend in relation to temperature, with longer and more scattered λ as conditions move away from the optimum temperature. Moreover, at all tested temperatures, the λ distributions of *G. stearothermophilus* spores showed a positive skewness (Table 1) indicating an asymmetry with a tail extending towards longer times. This confirmed available microscopic and/or turbidimetric findings on other bacilli (Leuschner and Lillford, 1999; Smelt et al., 2008; Pandey et al., 2013) and clostridia (Billon et al., 1997; Stringer et al., 2009, 2011), but also on non-sporeforming bacteria such as *Listeria* spp. and *Escherichia coli* O157:H7 (Métris et al., 2003; McKellar and Lu, 2005; Guillier and Augustin, 2006; Li et al., 2006; McKellar and Hawke, 2006). The above skewed shape was successfully described with a Gamma distribution which has been previously used for individual cell λ values (Métris et al., 2003). Information about the shape of λ distributions are of great importance in predicting microbial growth at low contamination levels due to the stochastic nature of microbial lag phase (Baranyi, 1998, 2002).

The temperature dependence of the mean *G. stearothermophilus* λ was considerably different from that reported for the growth rate of the same strain by Kakagianni et al. (2016). In the latter study, the authors reported an optimum temperature (mean value ± standard deviation) for growth $T_{opt} = 61.82 \pm 0.20$ °C, which is about 9 °C higher than the corresponding parameter for the mean lag time estimated in the present study (Table 3). This difference was confirmed by the μ_{max} values estimated in this study which showed a continuous increase as temperature increased from 47.5 to 59.0 °C, in contrast to what was the case for the mean lag time which showed a minimum value at 55.0 °C. These results indicate that the physiological state theorem (Baranyi, 1998) applied for vegetative cells, considering the lag as inversely proportional to the maximum specific growth rate, is not necessarily valid in the case of spores. This can be attributed to the different processes during the lag time of spores and vegetative cells. As described previously, in the case of spores the lag time is mainly determined by the germination and outgrowth process, the rate of which can be considerable different than the growth rate of vegetative cells. Indeed, Stringer et al. (2005) reported that the time intervals for germination, emergence, outgrowth and doubling of *C. botulinum* spores were all independent of each other. Consequently, although in the case of vegetative cells with known physiological state, growth at different temperatures can be predicted based on a model for the growth rate (Koutsoumanis et al., 2010), predicting growth of spores may require models for both lag time and growth rate since the effect of temperature on these two growth kinetic parameters can be substantially different.

G. stearothermophilus is an important spoiler in various heat treated foods. Considering that the number of surviving spores in the final product is usually very low, knowledge of their lag time variability is important for predicting growth and spoilage in a probabilistic way (Fig. 5). Probabilistic modeling approaches that take into account the variability of the factors affecting microbial behavior can provide more realistic estimation of food quality. Most importantly, probabilistic models enable decision-making based on the “acceptable level of risk”, and can provide structured information allowing decision-makers to compare various actions and identify those that can lead to effective and economic reduction of quality risks.

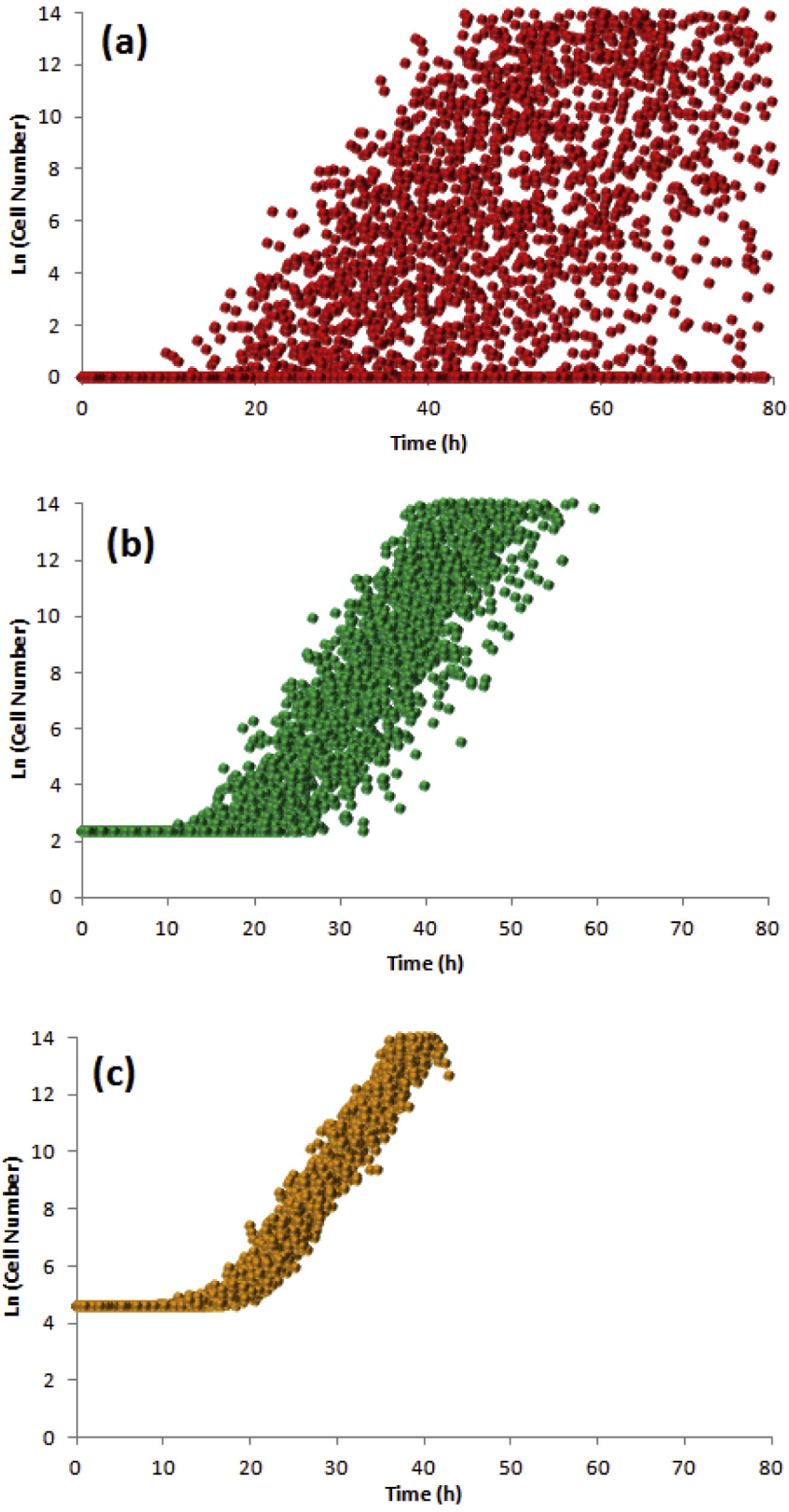

Fig. 5. Simulations of *Geobacillus stearothermophilus* growth at 45 °C for various initial contamination levels: 1 spore (a), 10 spores (b) and 100 spores (c). Growth is predicted by the stochastic model (Equation (5)) using Monte Carlo simulation with 10,000 iterations and a Uniform distribution for time t [t ~ Uniform (0, 80)].

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