



ORIGINAL ARTICLE

Simulation and modelling of the effect of small inoculum size on time to spoilage by *Bacillus stearothermophilus*

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*Mathematical models developed in predictive food microbiology typically use large (approx. 10^5 cfu ml⁻¹) inoculum sizes. Real food systems may contain low microbial loads (1–10 cfu ml⁻¹) introducing an additional uncertainty unaccounted for by model predictions. This research investigated the effects of very low inoculum sizes on the time to spoilage of *Bacillus stearothermophilus* ATCC 12980 spores. Microtiter plates (96-well) were filled with tryptic soy broth and inoculated with various concentrations of *B. stearothermophilus* spores. Time to spoilage was defined as colour change of the medium containing bromocresol purple (corresponding to c. 10^8 cfu ml⁻¹) from purple to yellow. A Poisson distribution best described the number of spores in a well. Spoilage times showed maximum variability (7.25–17 h) at 1 spore well⁻¹, and negligible variability (c. 6.5 h) at 500 spores well⁻¹. A simplified Gompertz function described spoilage kinetics. Mathematical modelling and simulation approaches were used to study spoilage times. The modelling approach had a fail-safe bias in its predictions and provided greater accuracy than the simulation, which was fail-dangerous. The simulation approach provided potentially greater mechanistic insight into the causes of spoilage time variability, and supported the notion that the effects of biovariability and interactions among individual spores manifest at very low inoculum sizes.*

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Introduction

Bacillus stearothermophilus is a Gram-positive, non-pathogenic, spore-forming bacterium that thrives in high temperature environments. It typically survives canning and sterilization procedures of food products due to its heat-resistant spores. Feeherry et al. (1987) observed flat-sour spoilage of properly processed low-

acid canned foods by *B. stearothermophilus* when those foods were stored at elevated temperatures. This organism is of special interest to the US Army, which must store thermally-processed military rations in tropical climates where ambient temperatures may allow *B. stearothermophilus* growth.

Traditional predictive food microbiology studies use large inocula for convenience and to reduce variability. For example, Sutherland et al. (1995) modelled the growth of *Escherichia coli* using an inoculum size of 10^5 cfu ml⁻¹. Conversely, studies of food contamination by *Clostridium botulinum* spores report an actual incidence as low as 1 spore kg⁻¹ fish, 0.1 spore

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kg⁻¹ in meat and 0.6 spore kg⁻¹ in vegetables (Dodds 1993).

The importance of inoculum size on the lag phase has been reported for Gram-negative bacteria (Baranyi and Pin 1999) and non-spore-forming Gram-positive bacteria (Augustin et al. 2000). This same effect was reported by Graham et al. (1996), who noted a lack of consistency on time to toxicity of *C. botulinum* spores at low inoculum sizes. Similarly, Whiting and Oriente (1997) observed an increased variability in time to turbidity experiments with *C. botulinum* when inoculating broth medium with varying number of spores (10¹–10⁴ spores ml⁻¹). However, the effect of very low inoculum sizes on the germination and growth of spore-forming bacteria has not been reported.

The objective of the present study was to quantify and model the effect of very low inoculum size (1–10 spores well⁻¹) on time to spoilage (TTS) by *B. stearothersophilus* in a model system where spoilage is represented by a colour change.

Materials and Methods

Data collection

Spore preparation. *B. stearothersophilus* ATCC 12980 cells were grown on tryptic soy agar (TSA) (Difco Laboratories, Detroit, Michigan, USA) and incubated at 55°C for 20 h. A single, white, smooth colony was transferred to tryptic soy broth (TSB) (Difco) and incubated at 55°C for 24 h. One hundred microlitres of the culture broth was plated on TSA and incubated at 55°C for 16 h, to obtain a white lawn of cells. These cells were dissolved in 0.05 M sodium phosphate buffer (pH 7.0) by shaking in the presence of small glass beads (5.0 mm diameter). Three ml of the resulting suspension was placed in Fernbach flasks containing 250 ml sporulation agar (SA) and incubated for 5 days at 55°C. SA was the medium of Cook and Brown as described by Feeherry et al. (1987), which was composed of 1.5 g Bacto-Peptone (Difco), 0.75 g Bacto-Tryptone (Difco), 0.75 g yeast extract (Difco), 0.37 g Lab Lemco beef extract (Oxoid, Ogdensburg, New York, USA), 0.25 mg MnSO₄ (Sigma,

St. Louis, Missouri, USA). In addition, the 250 ml SA also contained 6.25 g TSA (Sigma), 40 mg CaCl₂ (Sigma), and 17.5 mg MgSO₄ (Sigma).

The lawn of growth on SA was removed with 10 ml of distilled water and small glass beads as above. Aliquots (25 ml) were centrifuged, the supernatant discarded, and 25 ml of lysozyme solution (10 mg chicken white enzyme (Sigma) in 100 ml phosphate buffer) added to each pellet. The pellet–lysozyme solution was stirred at 37°C for 1 h. The resulting spore suspension was centrifuged for 15 min at 8000 g, and the process repeated until the supernatant was clear and a uniformly white spore pellet was obtained. The pellet and the supernatant were refrigerated and kept as the stock solution.

Inoculum distribution. Aliquots from the stock solution of *B. stearothersophilus* spores were used in all the experiments. Aliquots were diluted to final desired concentrations. Spore preparations were heat activated for 20 min at 100°C and 20-µl samples were dispensed on TSA using an eight-channel pipette dispenser (Gilson, France) and incubated for 24 h at 55°C. Spore number distributions were determined for 22 experiments with a target inoculum size between 1–10 spores ml⁻¹. At least 30 replicates were plated for each inoculum size.

Colonies were counted and population frequency plot for a given target inoculum size was constructed for the determination of inoculum distribution. The spore number frequency distributions were fitted to three discrete distributions (Poisson, geometric and binomial) using BestFit (Palisade Corp., Newfield, New York, USA) and χ^2 test was used to estimate the goodness of fit.

Mathematical modelling

Spoilage experiments and primary model. The same activated spore solution used in the inoculum distribution experiment was also pipetted using an eight-channel pipette dispenser to 96-round bottom well Elisa plates (Corning Glass Works, Corning, New York, USA) containing 180 µl of TSB (Difco) with 8 mg l⁻¹

bromocresol purple (BCP) (Sigma). BCP causes colour change of the medium from purple to yellow when the growth of the organisms in the medium reduces the TSB pH to a certain level. The spoilage of an inoculated well was defined as the colour change of the well from purple to yellow. Target inoculum sizes ranged from 1–10 spores well⁻¹. Several experiments with higher inocula (100, 500 spores well⁻¹) were also performed. Eight wells on each plate were left un-inoculated as control. The plates were sealed with PCR tape (Corning Glass Works) and incubated at 55°C in an incubator (Model 146 A, Fischer Scientific Marvel Industries, Richmond, Indiana, USA). The number of purple and yellow wells in each plate was recorded at regular time intervals. The percentage of yellow wells from each experiment was fitted to a simplified Gompertz function (Gibson and Roberts 1986):

$$[W(t)] = e^{-e^{-B'(t-M')}} \quad (1)$$

where $W(t)$ is the fraction of yellow microtiter plate wells among all inoculated wells at time t , M' is the time at which the rate of change in the fraction of yellow wells is maximum and B' is related to the slope of the curve at M' such that B'/e is the slope of the tangent at M' . The C parameter present in a typical Gompertz function was dropped from the equation since all wells eventually turned yellow under all conditions studied, implying a constant C that equals 1.

Secondary model. Linear models were found to be sufficient to describe the transformed variables $\log(B')$ and $\log(M')$ as a function of the mean inoculum λ , obtained from inoculum distribution experiment. Higher order models and models with different transformations were also tested, but no significant improvement was found.

The models developed for B' and M' had the form:

$$\log(Y) = a_0 + a_1 * \lambda \quad (2)$$

where Y was B' or M' .

The fitted model was used to predict B' and M' as a function of inoculum size. Predicted spoilage curves at certain inoculum levels were generated using Equation 1 with B' and M' parameters obtained from Equation 2.

Simulation

Number of cells causing spoilage. The number of cells required to cause spoilage (N_s) was determined from growth curves obtained as follows: activated (20 min, 100°C) spores of *B. stearothermophilus* from the stock solution were diluted to a final concentration of 10^3 cfu ml⁻¹ in TSB containing BCP (8 mg l⁻¹). Samples were incubated at 55°C in a digital water bath (Fisher Scientific, Pittsburgh, Pennsylvania, USA), removed, and plated on TSA at regular time intervals using a spiral plater (Autoplate 4000, Spiral Biotech Inc, Bethesda, Maryland, USA). Plates were incubated at 55°C for 24 h and counted using a laser colony counter (Model 500A, Spiral Biotech Inc). Time to spoilage (TTS) was defined as the time when the color of the growth medium changed from purple to yellow. The number of cells required to cause spoilage (N_s) was defined as the bacterial population at TTS.

Germination, outgrowth and lag time distribution estimation

Number of cells required to cause spoilage (N_s) was determined as above. Doubling time was assumed to be constant for all cells following germination (Zhao et al. 2000, Mackey and Kerridge 1988, Duffy et al. 1994). The data from spoilage curves for five experiments where wells contained approximately one spore were pooled and fit to a Poisson distribution using BestFit (Palisade Corp., Newfield, New York, USA). The TTS for these five experiments were pooled (excluding wells which showed no growth) and fit to an exponential distribution with BestFit. The exponential distribution was used to describe the T_{gol} distribution for each spore in the simulation, shown in Equation 3, since the distribution of TTS and T_{gol} are directly correlated.

$$f(T_{gol}) = \frac{e^{-\frac{T_{gol}}{\beta}}}{\beta} \quad (3)$$

The β parameter is the mean germination, outgrowth and lag time as estimated by exponential distribution. The exponential distribution parameter and doubling time were adjusted until the simulated TTS distribution matched the TTS data from the five pooled experiments.

Simulation scenario. The computer simulation was developed in Excel (Microsoft Corporation, Redmond, Washington, USA) using the add-in simulation software @Risk (Palisade Corp.). The initial number of spores in a well was generated from the Poisson distribution as determined above. A T_{gol} time from the exponential distribution was also generated for each spore. The simulated time was incremented until the T_{gol} for a given spore was reached and cell doubling began. The simulation stopped when N_t , the number of cells at time t , was equal to or greater than N_s , and the simulated time was recorded as TTS for that well. The simulations were run for each inoculum size with Latin Hypercube sampling until convergence was reached (500–2000 iterations). The cumulative distribution of TTS was plotted to obtain the simulated spoilage curve.

Evaluation of mathematical modelling and simulation

The performance of the modelling and simulation approaches was evaluated using bias and accuracy methods developed by Ross (1996). These two performance indices were expressed in the following two equations:

$$Bias = 10 \frac{\sum \log \left(\frac{TTS_{pred}}{TTS_{obs}} \right)}{n} \quad (4)$$

$$Accuracy = 10 \frac{\sum \left| \log \left(\frac{TTS_{pred}}{TTS_{obs}} \right) \right|}{n} \quad (5)$$

where TTS_{pred} was the predicted time to spoilage, TTS_{obs} was the observed time to spoilage, and n was the number of prediction/observation pairs used to calculate the index. Statistical correlation using linear regression was used to determine if the bias or accuracy of the modelling or simulation approaches varied with inoculum size.

Results

Inoculum size distribution

Poisson, geometric and binomial distribution parameter were fit to *B. stearothermophilus* spore inoculum size histograms. While no sin-

gle distribution was always better than the others, the Poisson distribution gave acceptable fits under all conditions tested and always ranked first or second and was therefore selected as the appropriate probability distribution for inoculum size. The Poisson distribution is commonly used to describe the number of bacterial cells in liquids (Haas and Heller 1988, Jarvis 1989).

Modelling

Table 1 shows the Gompertz parameters obtained from the fits of each spoilage experiment. The B' parameter tended to increase with increasing inoculum size. The M' parameter showed a progressive decrease from low to high inoculum size with values ranging from 10.91 to 6.00 h.

The models obtained for B' and M' were:

$$\log(B') = -0.6512 + 0.1157 * \lambda \quad (6)$$

and

$$\log(M') = 1.0335 - 0.0227 * \lambda \quad (7)$$

Figure 1 shows the observed values for B' and M' at different inoculum levels and the fitted linear models. Fig. 1(a) shows that the value for $\log(B')$ increased as inoculum size increased. Results for M' (Fig. 1(b)) show a progressive decrease for $\log(M')$ with increased spore concentration, which is as expected since spoilage occurs earlier with increased inoculum size. The r^2 are 0.77 and 0.76 for the B' model and M' model, respectively.

Simulation

The fixed parameters used as inputs in the simulation were the cell doubling time ($T_d = 0.30$ h) and the number of cells needed to cause colour change ($c. 10^8$ cfu ml⁻¹). A decreasing exponential function ($\beta = 8.12$) was used for T_{gol} . Convergence was reached under all conditions (and always within 2000 iterations) with little effect of inoculum size on the number of simulations needed for convergence.

Evaluation of modelling and simulation

The simulation TTS relied on the exponential distribution of TTS from five experiments with

Table 1. Gompertz parameters for time to spoilage curves of *Bacillus stearothermophilus* at various inoculum sizes

Mean inoculum size ^a (spores well ⁻¹)	Gompertz parameters	
	<i>B'</i> (h ⁻¹)	<i>M'</i> (h)
0.74	0.584	10.91
0.90	0.145	10.14
1.10	0.306	9.84
1.15	0.574	10.73
1.30	0.317	10.83
1.45	0.250	8.77
1.50	0.305	8.91
1.60	0.325	9.27
1.70	0.354	9.10
2.15	0.315	11.09
2.21	1.086	10.24
2.24	0.398	10.76
2.72	0.420	10.38
3.70	0.370	7.50
3.75	0.454	8.71
4.37	0.404	8.22
4.38	0.552	9.31
6.25	1.380	7.93
7.18	2.000	7.14
8.50	2.180	7.61
9.11	1.830	6.64
10.37	5.555	6.00

^aDetermined by fitting spore distribution histograms with Poisson distribution.

approximately 1 spore well⁻¹. These data were pooled and fit to a Poisson distribution to verify the assumption that they were representative of 1 spore well⁻¹. The mean of the fit Poisson was 1.03, validating this assumption, and the use of this data in creating distributions for the simulation.

A summary of bias and accuracy factors for time to spoilage is shown in Table 2. Bias and accuracy had overall values of 0.90 and 1.15 for the modelling approach. Overall bias and accuracy for the simulation were 1.05 and 1.18, respectively. The modelling approach was slightly more accurate (1.15 vs 1.18) and showed a greater (and fail-safe) bias (0.90 vs 1.05). The accuracy of the simulation decreased (positive correlation of 0.78) and the bias increased (positive correlation of 0.56) with increasing inoculum size, while the modelling approach showed no such trend (correlation for accuracy and bias were both positive but less than 0.1).

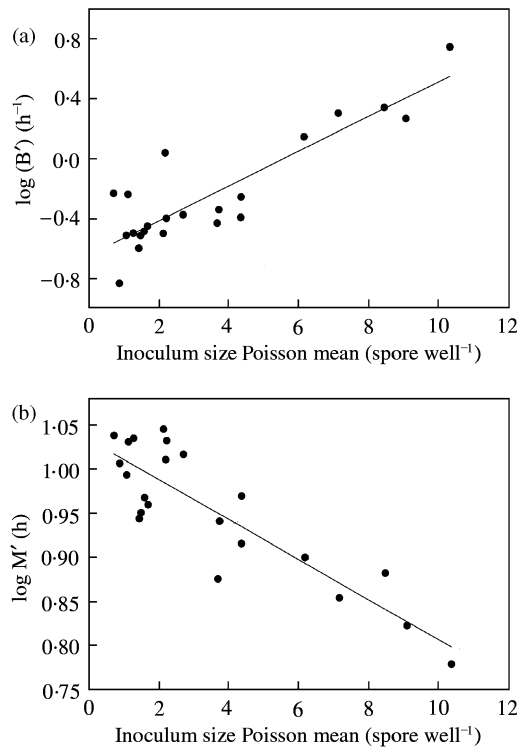


Figure 1. *B'* and *M'* as a function of inoculum size. Solid circles represent experimental values obtained from the simplified Gompertz equation. Inoculum sizes were obtained as the mean of Poisson distributions fit to the spore enumeration experiments. The solid line represents linear models for *B'* and *M'* in (a) and (b) respectively.

Representative experiments are shown in Fig. 2(a), (b) and (c). These figures show the observed values for the fraction of microtiter wells spoiled over time and the predictions generated by the modelling and simulation approaches. In (a) (Poisson $\lambda = 1.50$ spores well⁻¹) the modelling approach underestimates the percent spoiled at early time intervals, but the overall predictions are very close to what were observed. The simulation approach consistently underestimated the amount of spoilage. In (b) (Poisson $\lambda = 3.70$ spores well⁻¹), both the modelling and spoilage predictions are generally in good agreement with the observed data. The mathematical model predicted greater amounts of spoilage in the middle range of the experiment (10–25 h). The simulation more accurately reflected the data, though both predictive models

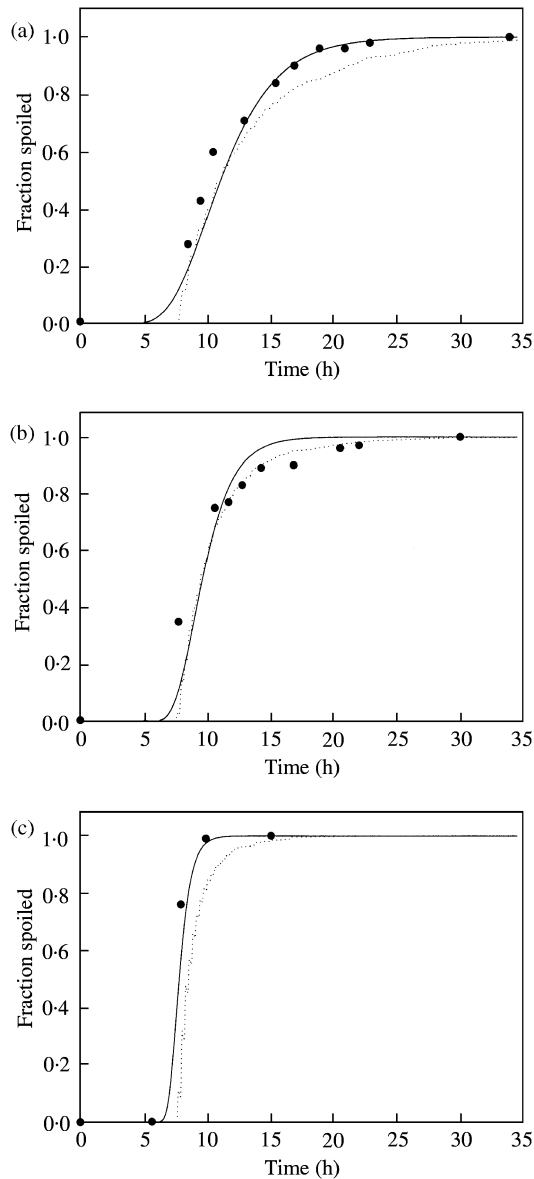


Figure 2. Comparison of spoilage curves by *B. stearothermophilus* at three different inoculum sizes. Closed circle represents experimental results; solid line represents predictions from the modelling approach; and dotted line represents predictions from simulation approach. (a) 1.50 spores well⁻¹, (b) 3.70 spores well⁻¹, (c) 7.18 spores well⁻¹.

underestimated early spoilage and overestimated late spoilage. In (c) (Poisson $\lambda = 7.18$ spores well⁻¹) the simulation values underpredict spoilage while the modelling approach predictions are close to experimental data. A trend of increasing steepness of the

curves when inoculum sizes increase can be observed from Fig. 2(a–c). At 10 h, for example, about 40% spoiled with inoculum 1.5, while this increased to 70% for inoculum 3.7 and 100% for inoculum 7.18. This indicates that at low inoculum sizes, the spoilage time varies significantly, while at higher inoculum sizes the spoilage times are more homogeneous from experiment to experiment. This trend is affirmed by isolated experiments at higher inocula: at 500 spores well⁻¹ 100% of wells spoiled after 6.4 h of incubation with negligible well-to-well variability in spoilage time (data not shown).

This trend can be shown in another way using the predictions from modelling and simulation. T_{diff} is defined as the difference in hours between when 90% of the microtiter wells spoiled and 10% of the wells spoiled. T_{diff} at three putative inoculum sizes is shown in Table 3 for the two approaches. The same trend was observed: lower inoculum sizes tended to show greater variability. When inoculum sizes increase, the value of T_{diff} ranges from more than 10 h to 1 h for the modelling approach, while the value of T_{diff} varies from almost 6 h to just over 1 h for the simulation.

Discussion

Simulation: determination of appropriate distributions and doubling time

The Poisson distribution was found to provide the best description of the number of spores in a well. The Poisson distribution is commonly used to describe the sample-to-sample variation of microbes in foods (Jarvis 1989). This distribution has also been used to model other microbial population distributions (Haas 1983) including *E. coli* (Hildebrandt et al. 1986), and plankton in water (Edgar and Laird 1993).

The T_{gol} was modelled using the exponential distribution (Equation 3). This distribution is commonly used to model the time between events and the lifetime of a device with a constant probability of failure (Anonymous 1996). This distribution has also been used in microbiology to estimate the presigmoidal delay seen in time to turbidity experiments where samples contained a varying concentration of

Table 2. Bias and accuracy for the modelling and the simulation approach for the time to spoilage by *Bacillus stearothermophilus*.

Mean inoculum size (spores well ⁻¹)	Simulation		Modelling	
	Bias	Accuracy	Bias	Accuracy
0.74	1.02	1.09	0.90	1.12
0.90	1.06	1.14	0.96	1.10
1.10	1.06	1.08	0.94	1.08
1.15	1.00	1.09	0.86	1.16
1.30	1.14	1.22	0.95	1.06
1.45	1.07	1.08	0.97	1.11
1.50	1.22	1.22	1.02	1.06
1.60	1.22	1.23	0.96	1.11
1.70	1.15	1.15	0.99	1.09
2.15	0.93	1.14	0.83	1.21
2.21	1.12	1.17	0.97	1.09
2.24	1.00	1.17	0.88	1.13
2.72	1.03	1.21	0.85	1.18
3.70	0.98	1.08	0.92	1.15
3.75	0.88	1.24	0.81	1.26
4.37	0.81	1.31	0.74	1.37
4.38	0.81	1.23	0.74	1.36
6.25	1.24	1.24	1.01	1.04
7.18	1.43	1.43	1.02	1.06
8.50	1.24	1.24	0.86	1.17
9.11	1.29	1.29	0.95	1.10
10.37	1.53	1.53	1.02	1.09
Overall	1.05	1.18	0.90	1.15

Table 3. Comparison of the modelling and simulation approaches in the prediction of spoilage time by *Bacillus stearothermophilus* at different inoculum sizes

Mean inoculum size (spores well ⁻¹)	Time to spoilage (h)							
	Modelling				Simulation			
	10% ^a	50%	90%	<i>T_{diff}</i> ^b	10%	50%	90%	<i>T_{diff}</i>
1	7.4	11.5	18.0	10.6	5.7	7.3	11.4	5.7
2	7.5	10.7	15.7	8.1	5.7	6.8	10.3	4.6
5	7.3	8.8	11.0	3.7	5.7	6.2	7.7	2.0
10	6.1	6.5	7.1	1.0	5.5	5.7	6.6	1.1

^aTime to spoilage for the selected percent fraction spoiled microtiter wells.

^bTime difference between 90% wells spoiled and 10% wells spoiled.

organisms (Cuppers and Smelt 1993, Chea et al. 2000). Other, more complicated distributions have been used to describe germination, out-growth and lag, such as the log normal distribution used by Riemann (1967). The log normal was a slightly better fit to the five pooled experiments' TTS data set than the exponential (RMS errors of 0.00067 vs 0.0010, respectively), but the exponential is a much simpler (one vs

two parameters) distribution so it was chosen for its parsimony (Gauch 1993).

The population of *B. stearothermophilus* cells was assumed to double in the stimulation every 0.3 h (*T_d*). We made the simplifying assumption that cell replication occurred at a constant rate, and thus assumed a pure exponential growth model in the simulation. These assumptions are supported by several studies. Growth

rate is generally unaffected at fixed environmental conditions with inoculum size the only varying parameter (Cuppers and Smelt 1993, Zhao et al. 2000). Powell (1955) argued that even if different cells in the same generation have different doubling times, the overall effect observed after several generations is a mean doubling time because the effects of fast and slow growing cells tend to cancel each other out. The pure exponential model has also been shown to be as adequate and more robust in describing growth than more complex models, such as the Baranyi (Buchanan et al. 1997).

Including a variability component for T_d could enhance the simulation. While the methodology used in this study did not permit estimation of growth rate variability, other advanced techniques recently used in our lab (Caipo 1999) do allow the incorporation of this factor.

The modelling approach

Whiting and Buchanan (1994) proposed a model classification scheme for predictive food microbiology that has become popular. This empirical primary–secondary modelling approach has a long history of use in predictive food microbiology (Gibson et al. 1988). In addition to the classic examples mentioned above, the primary–secondary modelling approach has also been used to model non-thermal inactivation (Whiting et al. 1996) and time to turbidity (Whiting and Oriente 1997).

Given the general suitability, simplicity and flexibility of the primary–secondary modelling approach, it was used to model the effect of inoculum size on the TTS of our model system. In our case, the primary model describes the change in the number of yellow-coloured microtiter plate wells ($W(t)$) with time, and the secondary model describes the influence of inoculum size on the parameters of the primary model (B' and M').

As the inoculum size increased from 1 to 10 spores well⁻¹, the parameter B' also increased (Fig. 1(a)). The parameter B' is correlated with the rate of change in the fraction of spoiled wells with time at the inflection point (M'). This means that as inoculum size in-

creased, the difference in time from the first to last well's spoilage decreased. Thus, higher inoculum sizes had less observed variability in TTS. This effect is reflected in the increasing steepness of the slope through Fig. 2(a–c). This observation can be explained by considering that as the number of spores in a well increased, the chance of a well containing a rapidly germinating spore also increased. As more wells contained at least one rapidly germinating spore, which is a determining factor for TTS, the overall variability of the spoilage time for a given set of wells decreased. This trend is taken to its logical extreme in the results from separate experiments using higher inoculum sizes (100 and 500 spores-well⁻¹). In these experiments, all the wells spoiled at almost exactly the same time (data not shown).

An analogous but inverse relationship was observed for M' (Fig. 1(b)). The time at which the inflection point (maximum rate of change in fraction of yellow wells with time) occurred tended to decrease as inoculum size increased. A similar explanation to that proposed for B' also can account for these results. The first spore to germinate can be assumed to produce descendants that ultimately cause spoilage, so the germination time of the first spore becomes the rate-limiting factor in determining TTS. As the number of spores in a well is increased, the chance of a well containing a rapidly germinating spore is also increased, and thus the overall time when the inflection point is reached becomes shorter.

Spoilage as a function of inoculum size

Similar to the differences in TTS we observed, Barayni and Pin (1999) reported higher variability of time-to-detection of *Pseudomonas* growth at lower inoculum sizes and Graham et al. (1996) noted an apparent lack of consistency in observed time to toxicity when working with low number of spores. This led these authors to suggest additional research on the spore germination process, and to note the potentially marked influence that spore numbers might have on microbial behaviour in foods. These observations can be explained using the concept of 'biovariability'. Billon et al. (1997)

use this term to describe the differences in germination time between spores in a population and noted that biovariability effects are masked by large inocula. Billon et al. (1997) also noted that the mechanistic models for bacterial spore germination proposed by Woese et al. (1968) offer an explanation to this phenomenon. According to Woese's model, every spore has a variable number of germinative molecules in its spore coat. Germination time is proportional to the number of activated molecules, so a spore-to-spore difference in the number of germinative molecules could explain spore-to-spore biovariability. If these germinative molecules can diffuse into the medium and reach other spores, this could support the idea of a quorum-sensing mechanism for germination. Quorum sensing would affect biovariability, as each spore would not act independently of other spores. This would further support the need to research smaller inoculum sizes of spoilage or pathogenic spore-forming bacteria in foods, as studies with large inoculum sizes will already have suitable quora, and will germinate much more quickly than spores that are fewer in number and more spatially separated in a food.

Billon et al. (1997) also made the important observation that the effects of biovariability may manifest at higher inoculum sizes where environmental conditions are less than optimum. This observation is supported by Tompkin et al. (1978) who obtained similar (albeit more qualitative) results studying the effect of nitrite inhibition in canned meat samples containing approx. 100 spores g^{-1} of *Clostridium botulinum*. The Tompkin et al. (1978) study showed that under identical inhibitory conditions (50 $\mu g mol^{-1}$ sodium nitrate and 0.2% sodium isoascorbate), time-to-swelling ranged from 20–40 days when the inoculum size was 78 spores can^{-1} , but in experiments where the inoculum size was only 14 spores can^{-1} , time-to-swelling ranged from 10–80 days. Whiting and Oriente (1997) observed similar results in time to turbidity experiments using *C. botulinum* spores. Time to turbidity was observed after 1–2 days at high inocula (10^{4-5} spores ml^{-1}) with very low variance and 7 days for low inocula (10^2 spores ml^{-1}) with a large variance. The authors attributed these differ-

ences to an existent distribution of germination times that made individual effects apparent at low inocula. The simulation results obtained in our study add additional support to the biovariability hypothesis as a possible explanation for the variability seen in all the studies mentioned above (Whiting and Oriente 1997; Tompkin et al. 1978; Billon et al. 1997).

Bias and accuracy

Indices of performance as proposed by Ross (1996) were calculated for both the modelling and the simulation approaches. The accuracy of both approaches is similar (Table 2), with modelling being slightly better. The bias and accuracy of both approaches are within the range of other published predictive models. Bias and accuracy ranges reported by Ross (1996) were 1.00–1.02 and 1.17–1.53 respectively. Neumeyer et al. (1997) found values for bias to fall in the range of 0.52–1.34, and accuracy to fall in the range of 1.06–1.81. In similar studies, Dalgaard and Jorgensen (1998) found bias values from 0.19–1.10, and accuracy values from 1.50–4.00. Our values for bias and accuracy were computed for the data used to develop the models, and not for independent validation data. Nevertheless it is reassuring to note that our values for bias and accuracy are generally in the same range as those found by the other researchers.

The modelling approach shows a bias of less than 1, indicating it is fail-safe, while the simulation approach has a fail-dangerous bias (Table 2). More interestingly, while the modelling approach was relatively unaffected by increasing inoculum size, both the accuracy and bias of the simulation increased at higher (closer to 10 spore $well^{-1}$) inoculum sizes. The distributions used in the simulation are empirically derived from experiments with approximately one spore $well^{-1}$, and so it simulated 10 spores $well^{-1}$ as 10 independent single spores, uninfluenced by the others. The mathematical model, however, is empirically derived from experiments with approximately 1–10 spores $well^{-1}$, and thus incorporates any interaction and effect the spores have on each other. The increasing failure of the simulation approach as inoculum size increases could mean

that the assumption of independence of spores is inaccurate during germination, outgrowth and lag. The phenomenon of quorum sensing has been linked to bacterial luminescence (Engbrecht et al. 1983), virulence determination (Passador et al. 1993) and antibiotic biosynthesis (Bainton et al. 1992), however it has not previously been associated with spore germination. While the present study is insufficient to conclude a quorum sensing mechanism contributes to the lessening of biovariability at very small inoculum sizes, interaction between spores would be a logical reason why the simulation approach would become less accurate as the inoculum size increased. This quantitative work suggests that a more physiological study is necessary to further explore quorum sensing in *B. stearothersophilus* germination, and to identify the signalling molecules involved.

Conclusion

This research has demonstrated that inoculum size is an important component of the variability observed in the TTS of a model system by *B. stearothersophilus*. A traditional and strictly empirical modelling approach provided greater accuracy, and a conservative fail-safe bias, while a Monte Carlo-based simulation approach provided potentially greater mechanistic insight into the causes of spoilage time variability. This research provides an important step in the development of models that include very low inocula variability effects in their predictions to describe spoilage in a quantitative manner.

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