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Stochastic modeling of variability in survival behavior of *Bacillus simplex* spore population during isothermal inactivation at the single cell level using a Monte Carlo simulation

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ABSTRACT

The control of bacterial reduction is important to maintain food safety during thermal processing. The goal of this study was to illustrate and describe variability in bacterial population behavior during thermal processing as a probability distribution based on individual cell heterogeneity regarding heat resistance. Toward this end, we performed a Monte Carlo simulation via computer, and compared and validated the simulated estimations with observed values. Weibullian fitted parameters were estimated from the kinetic survival data of *Bacillus simplex* during thermal treatment at 94 °C. The variability in reductions of bacterial sporular populations was illustrated using Monte Carlo simulation based on the Weibull distribution of the parameters. In particular, variabilities in viable spore counts and survival probability of the *B. simplex* spore population were simulated in various replicates. We also experimentally determined the changes in survival probability and distributions of survival spore counts; notably, these were successfully predicted by the Monte Carlo simulation based on the kinetic parameters. The kinetic parameter-based Monte Carlo simulation could thus successfully illustrate bacterial population behavior variability during thermal processing as a probability distribution. The simulation approach may contribute to improving food quality through risk-based processing designs and enhance risk assessment model accuracy.

1. Introduction

Mathematical models have been used for predicting various bacterial behaviors in foods to ensure microbiological food safety. The first study on bacterial death kinetics during thermal inactivation was reported nearly a century ago (Bigelow and Esty, 1920). However, the generally used log-linear model, which is based on *D*-value (decimal reduction time), leads to overestimation or underestimation of thermal death time (Peleg, 2006). In addition, recent consumer demand for processed foods goes beyond the fundamental requirements of safety and longer shelf life. More emphasis is being placed on informatively labeled, high-quality, value-added foods for their convenience (Awuah et al., 2007). Conversely, thermal processing at a high temperature or heating for a long period induces chemical and physical reactions in foods that reduce product quality (Awuah et al., 2007; Fellows, 2009; Ling et al., 2015). Accordingly, the microbial inactivation process should be minimized. In order to correspond to consumer demands, mathematical models will be indispensable to provide more accurate description of the microbial behavior during inactivation.

Thermal inactivation kinetics of bacterial spores comprises a well-studied branch in predictive microbiology, having been extensively analyzed in order to ensure the safety of foods requiring sterilization for storage at ambient temperature such as canned food, retort foods, and soft drinks. Alternatively, in recent years, food products that are pasteurized and stored at chilled temperatures are increasingly produced to retain quality characteristics such as original color, scent, and taste in accordance with consumer demand. Notably, in these processed food products, microorganisms that can grow even in chilled temperatures represent a critical factor for determining product shelf life. However, there have been few reports related to psychrophilic bacterial spores control although control of such they play an important role in relatively low temperature processing conditions that enable inactivation only of vegetative cells.

Almost all mathematical models describing change in bacterial

Abbreviations: CFU, colony forming unit; IIT, individual inactivation time; PCR, polymerase chain reaction; RMSE, root mean square error; SSC, simulated survival counts; TRT, total reduction time

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counts comprise kinetic models (deterministic models), although various mathematical models (log-linear or non-log-linear models) have been used to describe the death behavior during bacterial inactivation. However, the deterministic predictive model disregards the variability and uncertainty of bacterial phenomena (Membré et al., 2006). In this context, "uncertainty" represents the lack of perfect knowledge of the parameter value, which may be reduced by further measurements. In comparison, "variability" represents the true heterogeneity of the population that is a consequence of the physical system and irreducible by additional measurements (Nauta, 2000). In the context of the guidance of quantitative microbial risk assessment applied at the European and international level for food safety management, the importance of variability of biological and natural phenomena is widely recognized (FAO/WHO, 2008). Deterministic inactivation models providing point estimation are generally recognized as being unable to achieve satisfactory food safety management (Couvert et al., 2010; Koutsoumanis and Angelidis, 2007) because these models do not take into account heterogeneity in the resistance of individual cells to a lethal stress (Casolari, 1988).

Few clues are available in the literature to tackle the individual cell heterogeneity of heat resistance. According to them, one fact is that the kinetic data models have a characteristic of providing a cumulative form of the distribution of heat resistance of individual cells (Peleg, 2006). In addition, a study has been performed to evaluate and describe the individual cell heterogeneity as a source of variability in population dynamics of microbial inactivation via Monte Carlo simulation (Aspridou and Koutsoumanis, 2015). These investigators reported the existence of variability in the total reduction times of a bacterial population during inactivation processing such that the coefficient of variation of total reduction time increases as the initial bacterial count decreases. In general, Monte Carlo simulation sets the number of iterations to a hundred or a thousand, in some cases a million in order to obtain more detailed answers. Therefore, a million experiment iterations need to be done for strict validation of this simulation. However, it is difficult to validate the result derived from the Monte Carlo simulation because the number of iterations for the validation experiment must be huge. Alternatively, if the validity of the simulation was demonstrated in relatively small iteration (e.g., 60 replicates in the present study), it would be possible to represent the variation of bacterial death behavior from conventional kinetic data-sets and describe the death behavior of bacteria in greater detail.

Accordingly, the goal of this study was to illustrate and describe the variability in bacterial population behavior during thermal processing as a probability distribution based on individual cell heterogeneity with regard to heat resistance. To achieve this goal, we aimed to calculate the survival probability of the spore population (i.e., the probability that at least one bacterial spore survives) and describe the distribution of the number of viable bacterial spores using computer simulation based on the property that the kinetic model represents the cumulative distribution of the death time of individual cells. In this study, the Weibull model is used as one of the most general kinetic models for bacterial inactivation. Furthermore, we aimed to compare and verify the computer-derived results of Monte Carlo simulation based on the kinetic model with actual experimental observed values of bacterial spore survival probability and viable numbers. Such a simulation approach may contribute to the improvement of food quality through risk-based processing designs and the enhancement of the accuracy of risk assessment models.

2. Materials and methods

2.1. Bacterial strain and sporulation conditions

The bacterium *Bacillus simplex*, a psychrophilic spore forming bacteria originating from pasteurized milk, was acquired from Hokkaido Research Organization (Japan). The stock culture was maintained at 80 °C in 10% peptone water (Merck, Darmstadt, Germany) with glycerol, consisting of peptone from casein (1 g/L), NaCl (0.5 g/L), KH_2PO_4 (0.15 g/L), $Na_2HPO_4{\cdot}12H_2O$ (0.9 g/L), and glycerol (100 g/L). The strain was activated by incubating once at 30 C for 24 h on Nutrient Agar (Eiken, Tokyo, Japan) containing peptone (10 g/L), meat extract (5 g/L), and NaCl (5 g/L), and once at 30 °C for 24 h in 200% Nutrient Broth (Merck) with NaCl containing peptone from meat (10 g/L), meat extract (6 g/L), and NaCl (5 g/L). *B. simplex* spores were produced on Spo8-agar (Helmond et al., 2017; Faille et al., 2007) comprised of Nutrient Broth (8 g/L; Merck), $MgSO_4{\cdot}7H_2O$ (0.51 g/L), KCl (0.97 g/L), $CaCl_2{\cdot}2H_2O$ (0.2 g/L), $MnCl_2{\cdot}4H_2O$ (3 mg/L), $FeSO_4{\cdot}7H_2O$ (55 mg/L), and agar (12 g/L). Activated bacterial suspension (5 mL) was centrifuged (3000 × *g*, 10 min) and condensed to 1 mL of suspension. Subsequently, 10 plates of Spo8-agar were inoculated with 100 μL of condensed suspension and incubated at 30 °C in an incubator. The spores were harvested after 6 days of incubation or when 85% of the spores were released from mother cells as observed by phase contrast microscopy. Plates were flooded with 5 mL of sterile distilled water, scraped with a bacteria spreader to loosen the spores from the agar, and spore suspensions obtained from the plates were pooled in a sterile centrifuge tube then washed and condensed by centrifugation (3000 × *g*, 10 min, 4 °C) with cold sterile distilled water. The condensed suspension was heated at 80 °C for 10 min, then immediately cooled at 4 °C with iced water. This resulted in spore crops of 1×10^{10} colony forming units (CFU)/mL of *B. simplex*; the spores were stored at 4 °C until use. The sporular suspension was used prior to heat resistance changes (within 2 months).

2.2. Thermal treatment for analyzing survival kinetics

The harvested *B. simplex* spores were heated by heating method based on previous study (Abe et al., 2018) using a thermal cycler and PCR microplate. The spores were diluted by 0.3 M phosphate buffer ($a_w = 0.98$); aliquots of diluted spore suspension (10 μL) were dispensed into three representative wells of a 96 well polymerase chain reaction microplate (PCR microplate) to obtain cell concentrations of 10^5 CFU/well in each well; aliquots of 10 μL of distilled water were dispensed into other wells. The microplates were heated at 94 °C on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) after 30 s of preheating at 25 °C to standardize the initial temperature across trials. Fig. 1 shows the thermal history of spore suspension at 94 °C for 300 s. Since the standard deviation of the temperature of 60 well is small, we can say that there is no variability in the temperature among different wells (Fig. 1). The total duration of the trials depended on each temperature at time intervals required for effective analysis of microbial inactivation kinetics. Serial 10-fold dilutions of samples in 0.3 M phosphate buffer were plated on Nutrient Agar. Survival population was determined by incubating three replicates of each microplate at 30 °C for 2 days as according to the previous study (Kobayashi et al., 2016). Furthermore, we confirmed in advance that there is no difference in the number of colonies between those cultured for 2 days and those cultured for a longer time at 30 °C.

2.3. Fitting parameters of Weibullian distributions to kinetic data

The parameters of Weibullian distribution were fitted to experimental data. The change of survival spore counts was represented in the form of a survival ratio, $S_{(t)}$, defined as the ratio between the number of survivors after an exposure time t, $N_{(t)}$, and the initial number, N_0, as $S_{(t)} = N_{(t)}/N_0$. The Weibull model considers that cells and spores in a population have different resistances, with the model just representing the cumulative form of a distribution of lethal agents (Chen, 2007). The frequency (density) form of the Weibull distribution is:

$$\frac{dS}{dt} = b' t^{n-1} \exp(-b' t^n) \quad (1)$$

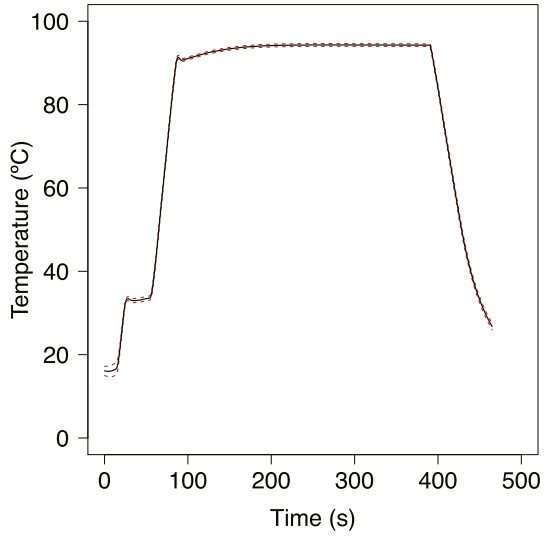

Fig. 1. An example of heating protocol: 300 s heating at 94 °C by thermal cycler. Preheating of 30 s at 25 °C to standardize the initial temperature across trials. After heating process, the 96-PCR microplates were immediately chilled at 4 °C. the red dotted lines show the standard deviation of the temperature of all 60 wells used for heating. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

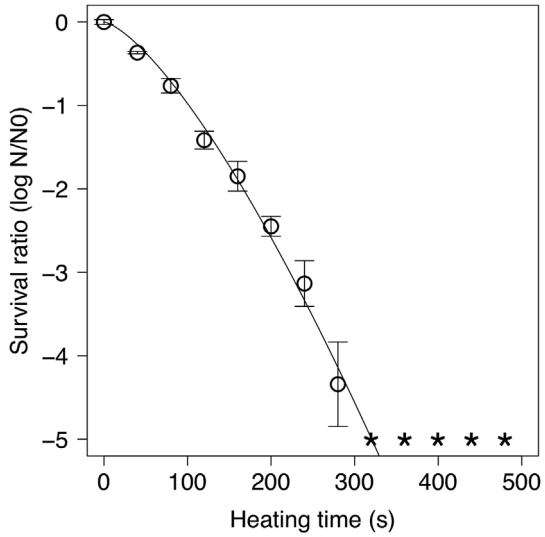

Fig. 2. Survival kinetics of *B. simplex* spore population of N_0 of 10^5 CFU heated at 94 °C in 0.3 M phosphate buffer (a_w = 0.98). Error bars indicate standard deviations. Each point with an error bar represents the mean of two or three values. No colonies were detected at the time indicated by an asterisk (*). Lines show the Fitted Weibull model of kinetic changes in surviving spore counts. Fitted parameters of b and n for the Weibullian model were 1.54×10^{-3} (p = 0.089, where p indicates the significance level of fitting) and 1.40 ($p = 4.67 \times 10^{-6}$), respectively, with an RMSE of 0.13.

and its cumulative

$$S_{(t)} = \exp(-b't^n) \tag{2}$$

or

$$\log_e S_{(t)} = -b't^n \tag{3}$$

where b' and n are parameters of the cumulative Weibull distribution. These parameters are constants from which the mode, mean, variance, and coefficient of skewness of the distribution can be calculated by standard formulas. In particular, as the 10-base logarithm is commonly used in microbiology; Eq. (3) can be written in the form:

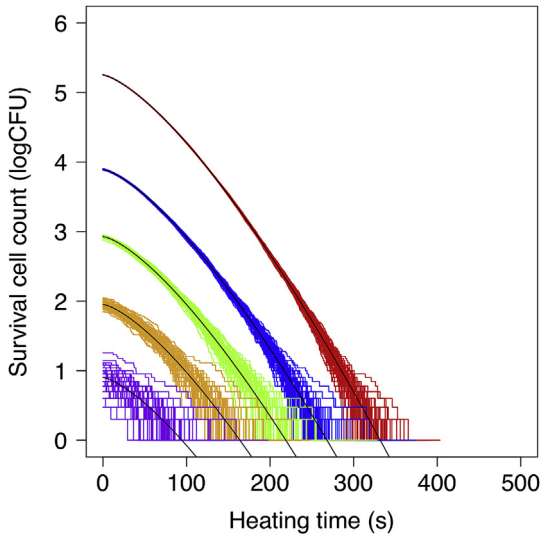

Fig. 3. Monte Carlo simulation results (100 simulations) for thermal inactivation of *B. simplex* with initial counts of 1×10^n cells, where n = 1 (purple), 2 (orange), 3 (green), 4 (blue), and 5 (red), based on fitted Weibull distributions (black lines) for individual cell inactivation times. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

$$\log_{10} S_{(t)} = -bt^n \tag{4}$$

where (Chen, 2007; Kevin et al., 2005; Mattick et al., 2001; Peleg, 2006, 2000, 1999)$b' = b(\log_e 10)$. Experimental data obtained from kinetic experiments were described by non-linear regression analysis according to Eq. (4).

2.4. Procedure for computer simulation of survival spore counts

The Weibull distribution was used to predict the inactivation of *B. simplex* spore populations of various initial spore counts N_0 using Monte Carlo simulation basically following previously reported methods (Aspridou and Koutsoumanis, 2015; Koyama et al., 2019). We added a hypothesis of variability in initial cell numbers to the computer simulation of the previous study and changed the used distribution from a gamma distribution to the Weibull distribution.

At first, the initial spore number of a simulation N_0' was generated as a random number following a Poisson distribution with an average of N_0 (Koyama et al., 2019, Aguirre et al., 2009) as follows:

$$N_0' \sim Poisson(N_0) \tag{5}$$

where N_0' is the initial spore number from the Poisson distribution with parameter N_0.

Secondly, individual sporular inactivation times of N_0' cells were estimated. Whereas a survival curve, $S_{(t)}$, describes the change in the survival ratio, a curve constitutes the cumulative form of a temporal distribution of mortality or destruction by definition.

In the present study, the Weibull model: cumulative Weibull distribution was used for the survival curve; i.e., we described the distribution of individual sporular inactivation times using the Weibull distribution. Therefore, the individual sporular inactivation times, IIT_i, could be described with random numbers following the fitted Weibull distribution as follows:

$$IIT_i \sim Weibull(b', n),\ i = 1,2,3, \ldots N_0' \tag{6}$$

where IIT_i is a randomly selected value from the fitted Weibull distribution of individual sporular inactivation times.

Finally, we estimated the survival bacterial counts in the sporular population by simulation. The simulated survival counts at an arbitrary time can be regarded as the number of the IIT_i larger than the arbitrary

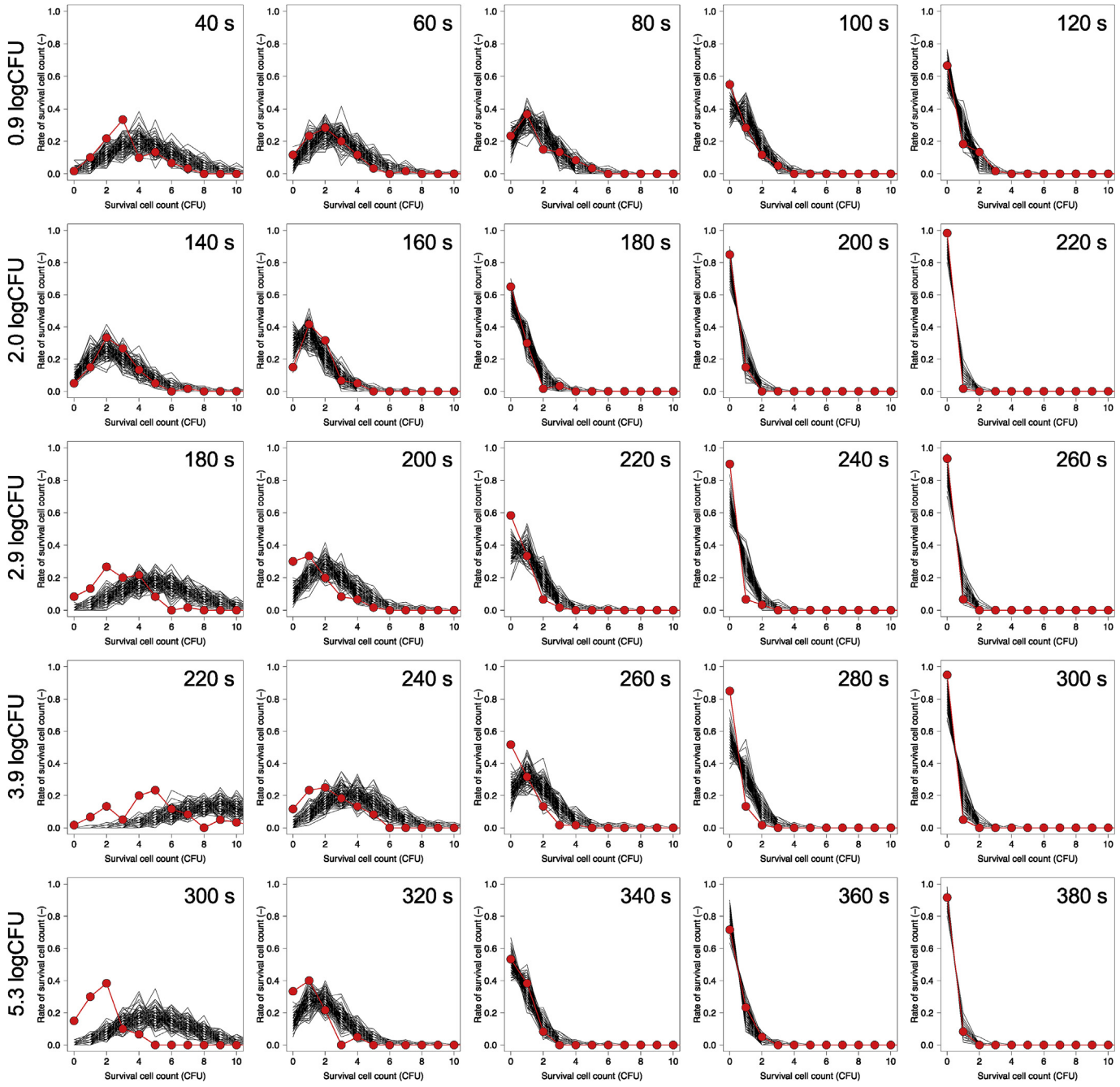

Fig. 4. Change in the observed heterogeneity of viable spore count (red points) of 60 bacterial populations each representing nearly 1×10^n cells, where n = 1, 2, 3, 4, and 5, heated at 94 °C in 0.3 M phosphate buffer (a_w = 0.98) for a particular heating time. The lines show the results of 100 Monte Carlo simulations (black lines) in each condition. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

heating time as follows:

$$SSC = \#A,\ \ A = \{x \in IIT_i | x > t\} \tag{7}$$

where *SSC* is the simulated survival cell counts at an arbitrary time *t* and *#A* is the number of elements in the individual sporular inactivation times larger than the arbitrary heating time. This procedure forms one replicate of the inactivation simulation. By repeating the inactivation simulations, the variability in the inactivation behavior of the population could be evaluated; i.e., the survival probability of the sporular population. The R script of one example simulation estimating survival cell counts and the script estimating heterogeneity in the viable spore counts were shown in Appendix 1 (Since it is a.txt file, copy and paste it to R or R studio and use it.).

2.5. Procedure for computer simulation of the survival probability of bacterial spore populations

We estimated the survival probability of sporular populations by repeating the inactivation simulations. The maximum value in the generated individual sporular inactivation times, IIT_i, is the total reduction time of the sporular population, TRT_j, with initial sporular counts of a simulation N_0' as follows:

$$TRT_j = \max(IIT_i),\ \ j = 1,2,3,\ \ ..., n_{inactivation} \tag{8}$$

where $n_{inactivation}$ is the replication number of the inactivation simulation. From the above, the simulated survival probability of the sporular population at an arbitrary heating time could be described with the

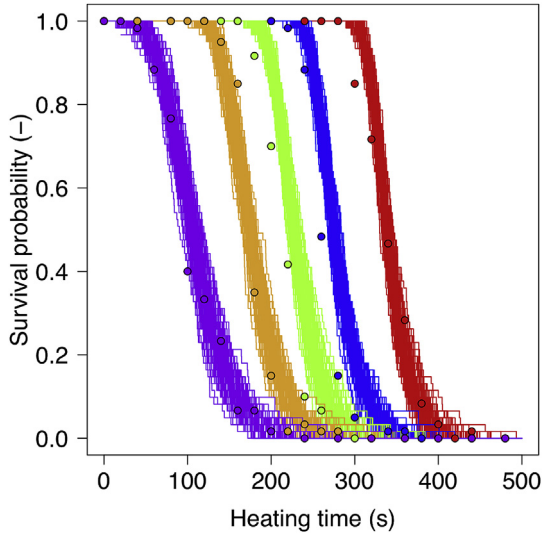

Fig. 5. Change in the observed survival probability of 60 bacterial populations (colored circles) each representing nearly 1×10^n cells, where n = 1 (purple), 2 (orange), 3 (green), 4 (blue), and 5 (red), heated at 94 °C in 0.3 M phosphate buffer (a_w = 0.98). The colored lines show Monte Carlo simulation results (100 simulations) of equivalent bacterial populations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

number of elements in the total reduction time of the sporular population larger than the arbitrary heating time as follows:

$$P_{(t)\_surivival} = \frac{\#B}{n_{inactivation}}, \quad B = \{x \in TRT_j | x > t\} \tag{9}$$

where $P_{(t)\_surivival}$ is the simulated survival probability of the sporular population at an arbitrary heating time t, and $\#B$ is the number of elements in the total reduction time of the sporular population larger than the arbitrary heating time. The R script of one example simulation estimating survival probability was shown in Appendix 1 (Since it is a.txt file, copy and paste it to R or R studio and use it.).

2.6. Thermal treatment for validation of the sporular inactivation simulation

The variability in total reduction time and the heterogeneity in viable cell counts were determined by heating method based on previous study (Abe et al., 2018) using a thermal cycler and PCR microplate. Aliquots of spore suspension diluted by 0.3 M phosphate buffer (10 μL) were dispensed into the wells of a 96 well PCR microplate to obtain cell concentrations of 10^n CFU/mL in each well (where n = 1 to 5). The microplates were heated at 94 °C on a T100 Thermal Cycler after 30 s of preheating at 25 °C to standardize the initial temperature across trials. The total duration of the trials depended on the initial counts; in total, we analyzed 18 samples at each initial count. The time intervals of 18 samples at each initial cell count were determined to reflect the rate of changes in the survival probability of *B. simplex* spores. The microplates were cooled to 4 °C immediately after heating. Subsequently, the survival of bacterial spores in each well was assessed by culturing on nutrient agar in 94 well microplates at 30 °C for 2 days. We observed the variability in total reduction time and the heterogeneity in viable counts as the number of bacterial colonies in each well after incubation.

The survival probability of sporular populations was determined from 60 replicates on a microplate according to the following equation (10):

$$\bar{P}_{(t)\_surivival} = 1 - \frac{W_t}{60} \tag{10}$$

where $\bar{P}_{(t)\_surivival}$ is the observed survival probability and W_t is the survival well count among the 60 wells of the plate in which temperatures are homogeneously distributed at heating time t. Furthermore, the viable colony number was counted in the conditions in which there was a well wherein the survival probability was not 1.0.

In the present study, the replicates number of inactivation simulation $n_{inactivation}$ was 60 in order to equal the variability of observed value and that of the simulated value. In addition, we also conducted two types of simulations with the heterogeneity in viable counts when $n_{inactivation}$ is 10^4 and the variability in total reduction time when $n_{inactivation}$ is 10^6, in order to improve the resolution of the simulated survival probability of bacterial populations. We set $n_{inactivation}$ as 10^6 for the simulated survival probability because the survival of microorganisms can be considered to be negligible when the survival probability is less than 10^{-6}. All statistical analyses were conducted with R statistical software (Version 3.5.1 for Mac OS X; http://ww.r-project.org).

3. Results

3.1. Sporular survival kinetics and fitting parameters of the Weibullian model

The sporular survival kinetics upon heating at 94 °C and the sporular survival curve indicated a downward concavity (Fig. 2). The initial cell count was 4.9 log CFU/well. Fitted parameters of b and n for the Weibullian model were 1.54×10^{-3} (p = 0.089, where p indicates the significance level of fitting) and 1.40 ($p = 4.67 \times 10^{-6}$), respectively. The root mean square error (RMSE) of the fitted model was 0.13.

3.2. Comparison of viable spore numbers between simulation and observed values

Simulation of sporular inactivation based on the estimated parameters from section 3.1 showed the same results in 60 replicates as those of the previous study (Aspridou and Koutsoumanis, 2015); i.e., when the number of bacteria was small, the variation in inactivation time until reaching an arbitrary bacterial count become large regardless of initial sporular counts (Fig. 3). In addition, our inactivation simulation showed the variability in the initial bacterial counts, resulting from the assumption that the initial sporular counts follow a Poisson distribution. The variability became relatively large in the range below 2 log CFU. Specifically, according to 60 replicates of the simulation model, the variance at the initial bacterial counts 1, 2, 3, 4 and 5 log CFU was 3.0×10^{-2}, 2.1×10^{-3}, 2.2×10^{-4}, 2.4×10^{-5} and 1.1×10^{-6} respectively.

The points in Figs. 4 and 6 mean distribution of the observed values of viable count, and the points in Figs. 5 and 7 mean observed survival probability values of bacterial spore. In addition, the lines in Figs. 4, 6 and Fig. 5, 7 mean simulated value of viable cell counts and survival probability, respectively. The variabilities in the observed survival cell counts of less than 10 CFU and those of the simulated survival sporular counts derived from simulations of 100 repetitions illustrated strongly similar behavior upon increasing the heating time within each initial sporular count (Fig. 4). Furthermore, the observed survival probability of the sporular population and the simulated survival probability derived from 100 replicate simulations also indicated comparable behavior (Fig. 5). These results demonstrated that the simulation of inactivation of the sporular population can describe the behavior along with the variability of sporular reduction during thermal inactivation.

Although the replicates of simulation ($n_{inactivation}$) value was set as 60 in order to equalize the magnitude of the variation in the experimentally observed 60 replicated values in the previous paragraph, the value for replicates of the simulation ($n_{inactivation}$) was set as more than the previous simulations (i.e. Figs. 4 and 5) to improve the resolution of the simulation. The simulated variability in survival spore counts for each condition (initial count: 15 log CFU) by 10^4 replicates of simulation is shown in Fig. 6 and the accuracy of simulated variability in survival

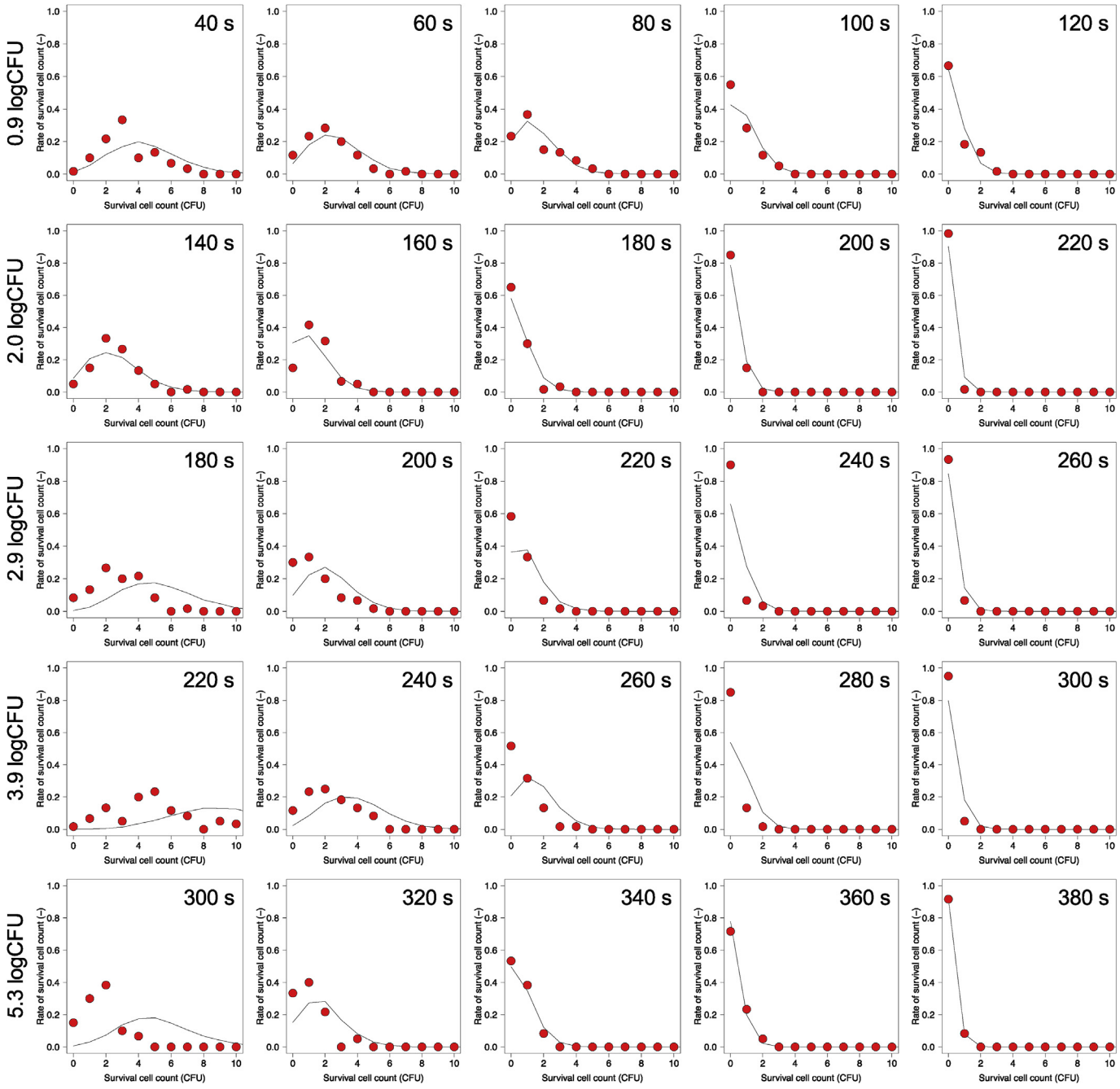

Fig. 6. Change in the simulated heterogeneity of viable spore count of 10^6 simulations each representing nearly 1×10^n cells, where n = 1, 2, 3, 4, and 5, heated at 94 °C in 0.3 M phosphate buffer (a_w = 0.98) for a particular heating time. The red points show the observed results of viable spore count of 60 bacterial populations in each condition. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

spore counts compared with observed survival probabilities is summarized in Table 1. The RMSEs were very small (0.0080.08) and we confirmed a strong similarity between the observed value and the predicted value by 10^4 replicates of simulation. Furthermore, the estimated survival probability for each initial count (15 log CFU) by 10^6 replicates of simulation is shown in Fig. 7 and the accuracy of simulated survival probabilities of the spore population compared to observed survival probabilities is shown in Table 2. The validity of the prediction by 10^6 replicates of simulation was illustrated as small RMSEs (0.0080.029). Notably, it is possible to predict survival probability almost continuously without using an approximation as shown in Fig. 7 by the simulation of numerous $n_{inactivation}$ values. For example, this method can be used for risk assessment, such as estimating how many defective products will be produced in one year by setting $n_{inactivation}$ to be the number of products produced per year, because $n_{inactivation}$ can be arbitrarily set.

Comparing the viable counts between observation and the high-resolution-simulation, we obtained a high accuracy of the Monte Carlo simulation with low RMSE as shown in Table 1. However, as shown Fig. 6, a difference occurred between the estimated distribution and the observed distribution when the initial bacterial count was large (3 log CFU or more). This represents the same trend as observed in Fig. 4. As mentioned above, the present Monte Carlo simulation describes only the variability among two heterogeneities; uncertainty cannot be described by the present Monte Carlo simulation. As shown by the error bar in Fig. 2, the measurement data underlying the Weibull model had a

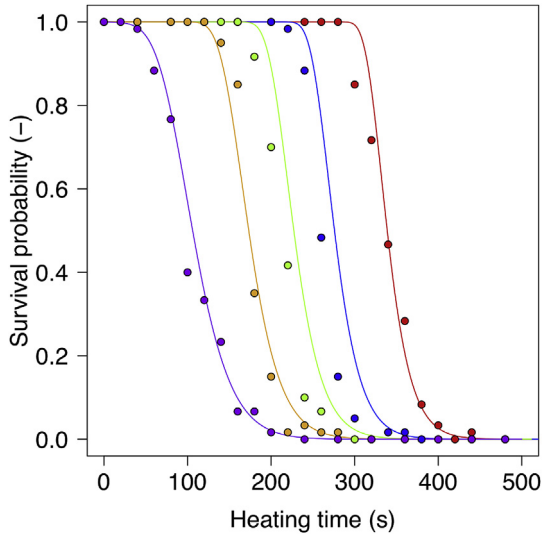

Fig. 7. Change in the simulated survival probability of 10^6 simulations (colored lines) each representing nearly 1×10^n cells, where n = 1 (purple), 2 (orange), 3 (green), 4 (blue), and 5 (red), heated at 94 °C in 0.3 M phosphate buffer (a_w = 0.98). The colored circles show the observed results of 60 bacterial populations of equivalent cell number. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Accuracy of simulated variabilities in survival spore counts when $n_{inactivation}$ is 10^4 replicates.

Initial counts (logCFU)	Heating time (s)	RMSE (-)
0.9	40	0.018
	60	0.008
	80	0.009
	100	0.012
	120	0.010
2.0	140	0.009
	160	0.015
	180	0.008
	200	0.006
	220	0.009
2.9	180	0.026
	200	0.021
	220	0.019
	240	0.024
	260	0.008
3.9	220	0.080
	240	0.081
	260	0.023
	280	0.021
	300	0.020
5.3	300	0.018
	320	0.008
	340	0.009
	360	0.012
	380	0.010

large standard deviation when the number of bacteria decreased (3 log CFU or less). In addition, when the initial number of bacteria increased (≥3 log CFU), distribution shifts occurred. As shown in Fig. 3, simulation with an initial bacterial count of ≥3 log CFU had a strong influence from measured data below 3 log CFU. These findings indicated that the magnitude of the error between the estimated value and the observed value likely comprised the cause of the standard deviation of the observed values of kinetic data. As the number of bacteria decreases, the variability and the uncertainty of observed data increases. It likely causes large uncertainty in kinetic model. For these reasons, we considered this difference in Fig. 4 as originating from the uncertainty of the fitted Weibull model.

Table 2
Accuracy of simulated survival probabilities of spore population when $n_{inactivation}$ is 10^6 replicates.

Initial counts (logCFU)	R^2 value (-)	RMSE (-)
0.9	0.99	0.008
2.0	0.99	0.011
2.9	0.96	0.029
3.9	0.95	0.024
5.3	0.99	0.011

4. Discussion

The validity of the estimation by the present Monte Carlo simulation was demonstrated as shown in Figs. 4–7. The simulation approach was effective to estimate bacterial reduction when bacteria are sterilized or when the number of bacteria is reduced by 100 or less, because small bacterial counts induce greater heterogeneity of inactivation time until an arbitrary number of survivors is reached, as shown Fig. 3.

Although some researchers have developed stochastic models until now, novel points of the present study compared with all of the previous studies are enabling to simulate the bacterial behavior in the form of probability distributions and to verify the distributions of experimentally observed distributions. In the previous simulation studies (Aspridou and Koutsoumanis, 2015; Corradini et al., 2010; Corradini and Peleg, 2009; Horowitz et al., 2010; Koyama et al., 2019), they focused on the variability of single cells. However, the previous studies did not pay attention to the distributions in the survival cell counts and the change in the survival probability of bacterial populations which are the most important measurement of the present study. Especially, Aspridou and Koutsoumanis (2015) expressed the death time of individual bacterial cell as a gamma distribution, which is not a general model as representing the bacterial reduction behavior. Therefore, the previous studies have not been able to estimate the probability of occurrence of contaminated food by arbitrary heat treatment condition from general kinetic model. The significant progress of this study is that distribution describing bacterial survival behavior can be derived from a Weibull model which is generally representing bacterial reduction kinetics. Although the survival probabilities of the bacterial population have been estimated by fitting with an approximate probability distribution (Abe et al., 2018), the conventional kinetic data in literature cannot be used in the previous reported procedure. In contrast, the present study has realized to simulate appropriate probabilistic bacterial survival behaviors such as the distributions in the survival cell counts or the change in the survival probability of bacterial populations based on the estimated parameters of conventional survival kinetic model.

However, this Monte Carlo simulation enabled description of only the variability among the two heterogeneities, because the simulation does not take into account heterogeneity in parameters (uncertainty). For example, when the initial bacterial count was large (≥3 log CFU), a difference occurred between the estimated value and the observed value under the condition in which the bacteria survive, which likely arose from the uncertainty of the Weibull model as the source of the simulation. In order to carry out a simulation taking uncertainty into consideration, Bayesian statistics or bootstrapping methods should be applied to clarify the distribution that parameters follow; i.e., the parameters of distribution describing single cell inactivation times should be determined by the random number of the distribution that parameters follow. However, the Monte Carlo simulation, which takes only variability but not uncertainty into consideration, successfully

Table 3
Statics of goodness-of-fit test of fitted Poisson distributions to variability in survival spore counts.

Initial counts (logCFU)	Heating time (s)	Parameter of Poisson distribution	Chi-squared-statistic	Degree of freedom	Significance probability	Rejection of the null hypothesis
0.9	40	3.2	9.2	6	0.16	
	60	2.1	3.6	5	0.61	
	80	1.6	5.9	4	0.20	
	100	0.7	2.5	2	0.29	
	120	0.5	6.1	2	0.05	*
2.0	140	2.5	8.2	5	0.15	
	160	1.4	7.8	3	0.05	*
	180	0.4	5.3	2	0.07	
	200	0.2	1.4	0	0.00	**
	220	0.0	0.0	0	0.00	**
2.9	180	2.7	8.1	5	0.15	
	200	1.3	2.9	4	0.57	
	220	0.5	0.5	2	0.76	
	240	0.1	4.4	1	0.04	*
	260	0.1	0.3	0	0.00	**
3.9	220	4.7	18.8	9	0.03	*
	240	2.2	4.8	4	0.31	
	260	0.7	1.7	3	0.63	
	280	0.2	0.2	1	0.64	
	300	0.0	0.1	0	0.00	**
5.3	300	1.6	7.8	3	0.05	
	320	1.0	11.4	2	0.00	**
	340	0.5	3.2	1	0.07	
	360	0.3	0.7	1	0.39	
	380	0.1	0.4	0	0.00	**

described the heterogeneity of bacterial reduction in the present study. This indicates that the degree of uncertainty of the Weibull curve derived in this study is relatively small compared to the variability of bacterial reduction.

It is expected that this simulation will yield similar phenomena with other bacterial reductions and other inactivation methods because *B. simplex* was only used as a model microorganism to clarify the mathematical characteristics of natural randomness (variability); thus, verification using other conditions and organisms is necessary. If validated, the simulation may enable quantitative microbial risk assessment at a single cell level in various bactericidal methods of various bacteria. In particular, this simulation should be valid for risk assessments of specific bacteria that may cause infection or problems upon ingestion of even a small number of cells, such as enterohemorrhagic *Escherichia coli* and *Salmonella* spp., in addition to its efficacy for assessing the deterioration probability of canned or retort food caused by e.g., *Bacillus* spp. or *Clostridium* spp. It may also be used to predict the survival probability of psychrophilic spore-forming bacteria in long-life chilled food. Moreover, although the probability of survival of a bacterial population has been approximately expressed using a gamma distribution in a few prior studies (Abe et al., 2018; Koyama et al., 2017), in the simulation method from the present study, it was possible to predict survival probability without using approximation.

It has been claimed that the final numbers of survivors after heat treatment exhibit Poisson distributions. Using the maximum likelihood estimation method and goodness-of-fit test, we determined whether the heterogeneity of the observed bacterial numbers in the present study also followed the Poisson distribution. As a result, the null hypothesis was not rejected in almost all of the conditions wherein the observed heterogeneity of survivor counts followed Poisson distribution, although the null hypothesis was significantly rejected in some conditions (Table 3). Overall, considering the goodness-of-fit test, the final numbers of survivors after heat treatment approximately followed the Poisson distribution.

In the case of adopting conditions in which several bacteria are predicted to survive using a simulation with a priority on food quality, it is necessary to set conditions wherein surviving cells do not proliferate even if they survive. Even if bacteria enter the body, it is critical to establish conditions that will not cause infection or disease onset. In order to realize such outcomes, we argue that the simulation from the present study should be used in combination with a growth model and a dose-response model. Furthermore, if these latter models are expressed within a stochastic process model, it should be possible to perform comprehensive quantitative microbial risk assessment from production to digestion that predicts whether food poisoning will occur in the consumer throughout the process by which raw materials become food products and are distributed.

Notably, the simulation approach will enable calculation of the survival probability and heterogeneity in the viable cell count from the kinetic parameters estimated from conventional experimental data. In other words, there are possibilities that the results of 60 iterations of experiments can be derived from only three iteration experiments using Monte Carlo simulation. If the developed simulation can be applied to other bacteria as well as *B. simplex*, the numerous existing thermal inactivation data in databases such as ComBase (Baranyi and Tamplin, 2004) will enable simulation of the respective bacterial survival probability and heterogeneity in the viable cell counts although the problem of uncertainty remains. Furthermore, if this simulation procedure can also be applied to thermal in addition to other non-thermal inactivation processes, we should be able to obtain stochastic results from conventional kinetic data in the literature and/or databases. As the developed simulation would realize numerical “visualization” of the microbial risk in foods, modifying the process/inactivation conditions to improve food quality could then be determined by risk-based evaluation.

Although the developed simulation could predict survival probability in the present study, problems still exist for practical use, such as the unevenness of damage to bacteria caused from temperature unevenness. As the bacterial solution used in the present study was very small (as low as 10 μL), temperature unevenness in the bacterial solution was considered to be almost negligible. However, in actual thermal

inactivation of food, temperature unevenness owing to heating obviously occurs. Therefore, when simulation prediction is performed, a temperature-predictable physical model established via heat transfer with a fine mesh will be required. Concurrently, a secondary model of the relationship between temperature and the parameters of the Weibull model will be necessary to predict the bacterial death associated with each mesh.

In conclusion, a Monte Carlo simulation based on kinetic data that explains individual cell heterogeneity could describe the variability of the total reduction time and the change in the survival probability of bacterial populations during thermal inactivation. It was also found that the simulation could describe the heterogeneity in the bacterial survival numbers. The Monte Carlo simulation thus exhibits potential to be applied for quantitative microbial risk assessment, risk management, and risk-based process design of mainly thermal inactivation along with other inactivation methods for various bacteria and foods to enhance food safety.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.03.005.

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