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Stochastic evaluation of *Salmonella enterica* lethality during thermal inactivation

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ABSTRACT

Stochastic models take into account the uncertainty and variability of predictions in quantitative microbial risk assessment. However, a model that considers thermal inactivation conditions can better predict whether or not bacteria in food are alive. To this end, we describe a novel probabilistic modelling procedure for accurately predicting thermal end point, in contrast to conventional kinetic models that are based on extrapolation of the D value. We used this new model to investigate changes in the survival probability of *Salmonella enterica* serotype Oranienburg during thermal processing. These changes were accurately described by a cumulative gamma distribution. The predicted total bacterial reduction time with a survival probability of 10^{-6}—the commercial standard for sterility—was significantly shorter than that predicted by the conventional deterministic kinetic model. Thus, the survival probability distribution can explain the heterogeneity in total reduction time for a bacterial population. Furthermore, whereas kinetic methodologies may overestimate the time required for inactivation, our method for determining survival probability distribution can provide an accurate estimate of thermal inactivation and is therefore an important tool for quantitative microbial risk assessment of foods.

1. Introduction

Thermal inactivation processes for assuring microbiological food safety must meet certain criteria. To this end, the food industry relies on kinetic or deterministic models to describe temporal changes in the number of surviving bacteria in foods (e.g. log-linear model, Weibull model and other semi-logarithmic survival curves). The traditional concept of microbial inactivation kinetics is based on a study dating back nearly a century that estimated the thermal end point (Bigelow and Esty, 1920), which has long been the standard for safe food production.

Although it is necessary to correctly describe thermal death behaviour to establish the appropriate heating temperatures and times for microbial inactivation, there are some problems with the deterministic approach. One is that it relies on point estimates or single values such as the mean or average of a dataset to generate a single risk estimate value (Cassin and Paoli, 1998) that disregards the variability and uncertainty of biological 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. 'Variability', on the other hand, represents a true heterogeneity of the population that is a consequence of the physical system and irreducible by additional measurements (Nauta, 2000). The former reflects the true heterogeneity of a population, which is a characteristic of the physical system and is irreducible, while the latter arises from the lack of perfect knowledge of a parameter value and can be minimised by additional measurements (Nauta, 2002). Thermal death in a bacterial population is a phenomenon that exhibits greater heterogeneity for lower bacterial counts (Aspridou and Koutsoumanis, 2015). Another problem with deterministic models is that they estimate survival probability from decimal reduction kinetics of bacterial counts. Conventional thermal death time calculations assume that once a certain level of reduction has been achieved, the probability of actual survival is so low that it can be discounted. However, the deterministic methodology does not accurately describe survival probability, because the reduction levels do not reflect actual bacterial numbers obtained in an experiment. Moreover, thermal death time is typically estimated by extrapolation of a linearised isothermal semi-logarithmic survival curve, yielding an overestimate of the true time needed reach a reduction in number when isothermal survival curves have a downward concavity, and an underestimate when the curves indicate tailing (Peleg, 2006). For these reasons, deterministic methodology cannot achieve accurate

Abbreviations: PNSU, probability of a non-sterile unit; QMRA, quantitative microbial risk assessment; RMSE, root mean square error; TSA, tryptic soy agar; TSB, tryptic soy broth

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quantitative microbial risk assessment (QMRA) of contaminating bacteria in foods.

A probabilistic approach using a more highly developed mathematical methodology than point estimate-based deterministic models makes QMRA improve (Thompson and Graham, 2008). It has been suggested that probability distributions be used to describe both uncertainty and variability for quantitative risk assessment (Thompson and Graham, 2008; Vose, 1998). Recent studies support a bacterial inactivation model derived from a stochastic approach using Monte Carlo simulation (Aspridou and Koutsoumanis, 2015; Marks et al., 1998; Membré et al., 2006), while others advocate applying Monte Carlo simulation to bacterial growth (Cassin and Paoli, 1998; Nauta, 2001). Although the heterogeneity in total reduction time of bacterial populations has been reported (Aspridou and Koutsoumanis, 2015; Koyama et al., 2017), few studies (Koyama et al., 2017) have described changes in the observed survival probability of bacterial populations with a cumulative probability distribution. However, this would enable prediction of actual bacterial lethality and could explain the variability in total reduction time of bacterial populations upon thermal inactivation. The survival probability distribution could also be used in QMRA to establish appropriate thermal conditions for minimising negative impact in foods.

In this study, we developed a stochastic model for estimating the survival probability of bacterial populations by taking into account the heterogeneity in total reduction time. We investigated the relationship between initial cell counts and bacterial survival at various temperatures. Changes in total reduction probability over time were described as a probability distribution based on the reliability theory, which considers the probability of failure by describing variability with probability distribution. Based on the probabilistic evaluation, we estimated heating time for different survival probabilities ($P = 0.5$ and 10^{-6}) of a bacterial population. A probability of 0.5 is the average value described by a deterministic model (Cassin and Paoli, 1998), while 10^{-6} is the probability that the risk can be ignored (Bigelow and Esty, 1920) at a given initial cell number. We verified whether the model supported the conventional deterministic heating time of thermal inactivation, and compared the stochastically predicted heating time with the value derived from first-order kinetics of the change in the number of surviving cells.

2. Materials and methods

2.1. Bacterial strain and culture conditions

Salmonella enterica Oranienburg derived from a food-borne illness originating from smoked squid in the Aomori prefecture of Japan in 1999 was used in this study as a representative highly thermotolerant vegetative bacterial strain. The cause of the food poisoning accident is management mistake that the temperature of the drying process should have been set at 45 °C–50 °C but it was 40 °C–45 °C in which the isolates can growth (Itoh, 2001; Hiroshi et al., 2007). A stock culture of the strain was stored frozen in 10% glycerol at −80 °C; the strain was activated by incubating once at 37 °C for 24 h on tryptic soy agar (TSA; Merck, Darmstadt, Germany) and twice at 37 °C for 24 h in tryptic soy broth (TSB; Merck). The 24-h culture of *S. enterica* Oranienburg was centrifuged at 3000 ×*g* for 10 min; the cells were washed with TSB and centrifuged again under the same conditions. For the stochastic approach, harvested cells were washed with TSB and diluted in TSB to obtain cell concentrations of 10^n CFU/ml (where n = 2, 3, 4, 5, 6) by serial dilution. Diluted cultures were stored at 4 °C for the duration of the experiment. There is no concern of proliferation at 4 °C from the results of pre-experiment, but since heat resistance may change by cold insulation, there are cases where cold insulation is up to 4 h. For the deterministic approach, cells were resuspended in TSB (10^9 CFU/ml), and initial bacterial counts were determined on TSA plates after culturing at 37 °C for 24 h.

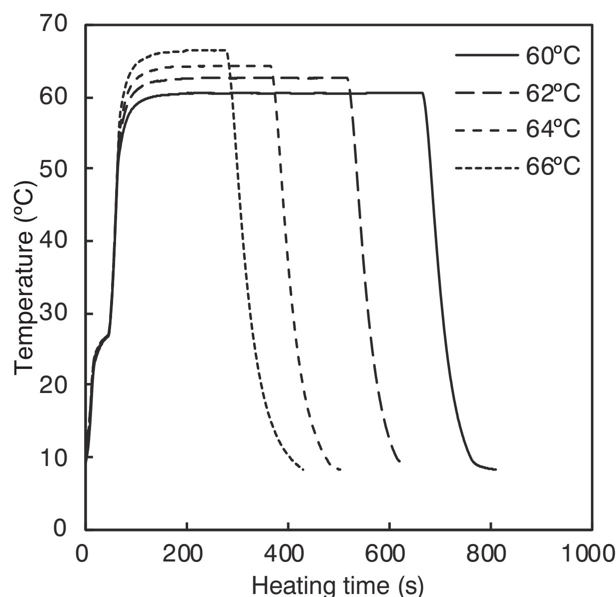

Fig. 1. An example of heating protocol even at experimental temperature: 600 s heating at 60 °C, 450 s heating at 62 °C, 300 s heating at 64 °C, and 210 s heating at 66 °C by thermal cycler. Preheating of 30 s at 25 °C to standardise the initial temperature across trials. After heating process, the 96-PCR microplates were immediately chilled at 4 °C.

2.2. Thermal treatment for analysing changes in survival probability

Aliquots of diluted culture (100 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 = 1–5). The microplates were heated at 60 °C, 62 °C, 64 °C, and 66 °C on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) after 30 s of preheating at 25 °C to standardise the initial temperature across trials as shown in Fig. 1. Because the temperature of samples was accurately and uniformly controlled in each well, this heating procedure was appropriate for evaluation of bacterial thermal inactivation. The total duration of the trials depended on the initial counts and heating temperature. In total, we analysed 15 samples at each heating temperature. The time intervals of 15 samples at each temperature were determined to reflect the rate of changes in the survival probability of *S. enterica* Oranienburg preliminary measured. The microplates were cooled by 4 °C immediately after heating and then incubated at 37 °C for 24 h to evaluate the reduction in the bacterial population based on examination of turbidity by naked eye. We assume that the recovery of all damaged bacteria was measured because there is no significant difference between 24 h incubation and over 72 h incubation as the result of pre-experiments.

2.3. Thermal treatment for analysing conventional survival kinetics

Aliquots of washed bacterial culture (100 µl) were dispensed into PCR tubes that were heated at 60 °C, 62 °C, 64 °C, and 66 °C on a thermal cycler after 30 s of preheating at 25 °C to standardise the initial temperature across trials. A total of 10 samples were obtained at each temperature at time intervals required for effective analysis of microbial inactivation kinetics. Serial 10-fold dilutions of sample in 0.1% peptone water were plated on TSA. Population survival was determined by three replicates of each plate after incubation at 37 °C for 24 h.

2.4. Modelling survival probability

The survival probability of bacterial populations was determined from 60 replicates on a microplate according to the following Eq. (1):

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