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Different model hypotheses are needed to account for qualitative variability in the response of two strains of *Salmonella* spp. under dynamic conditions

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ABSTRACT

In this article, the thermal inactivation of two *Salmonella* strains (*Salmonella* Enteritidis CECT4300 and *Salmonella* Senftenberg CECT4565) was studied under both isothermal and dynamic conditions. We observed large differences between these two strains, with *S. Senftenberg* being much more resistant than *S.* Enteritidis. Under isothermal conditions, *S.* Senftenberg had non-linear survivor curves, whereas the response of *S.* Enteritidis was log-linear. Therefore, weibullian inactivation models were used to describe the response of *S. Senftenberg*, with the Mafart model being the more suitable one. For *S.* Enteritidis, the Bigelow (log-linear) inactivation model was successful at describing the isothermal response. Under dynamic conditions, a combination of the Peleg and Mafart models (secondary model of Mafart; t* of Peleg) fitted to the isothermal data could predict the response of *S. Senftenberg* to the dynamic treatments tested (heating rates between 0.5 and 10 °C/min). This was not the case for *S. Enteritidis*, where the model predictions based on isothermal data underestimated the microbial concentrations. Therefore, a dynamic model that considers stress acclimation to one of the dynamic profiles was fitted, using the remaining profiles as validation. In light of this, besides its quantitative impact, variability between strains of bacterial species can also cause qualitative differences in microbial inactivation. This is demonstrated by *S.* Enteritidis being able to develop stress acclimation where *S. Senftenbenberg* could not. This has important implications for the development of microbial inactivation models to support process design, as every industrial treatment is dynamic. Consequently, it is crucial to consider different model hypotheses, and how they affect the model predictions both under isothermal and dynamic conditions.

1. Introduction

Access to safe food is a basic requirement for human health, while at the same time, food safety and security are becoming increasingly difficult. Although anyone may contract a foodborne disease, populations such as small children, elderly people, pregnant women, immunocompromised people and those living in poverty or who are food insecure are particularly vulnerable (FAO, 2021). According to the European Food Safety Authority (EFSA) One Health 2020 Zoonoses report, salmonellosis is the second most commonly reported gastrointestinal infection in humans and the main cause of food-borne outbreaks in the EU/EEA in 2020. In total, 52,702 confirmed cases of salmonellosis in humans were reported with an EU notification rate of 13.7 cases per 100,000 population. *Salmonella* caused 22.5% of all food-borne outbreaks and the vast majority (57.9%) of the salmonellosis food-borne outbreaks were caused by *S.* Enteritidis (Authority & European Centre for Disease Prevention and Control, 2021). Therefore, *Salmonella* spp. are one of the most relevant hazards to the food industry and are often a main aspect of quality control systems.

Over the past decades, food safety management has switched to a more risk-based approach to achieve food safety control (Koutsoumanis & Aspridou, 2016). In this sense, Quantitative Microbial Risk Assessment (QMRA), is currently the reference approach to ensure food safety and also the basis for decision-making (WHO, 2021). The risk associated

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with a given hazard is described using quantitative indicators (e.g. expected number of cases or probability of illness per serving). Therefore, the reliability of a QMRA is strongly dependent on the availability and quality of mathematical models able to describe the microbial response to the conditions encountered within the food chain.

In this study, mathematical models were selected to describe the inactivation of *Salmonella* spp. Although different technologies can be used for microbial inactivation (Mañas & Pagán, 2005), thermal treatment is still one of the most effective and easiest applied techniques to most food products (Peng et al., 2017). One of the main limitations of the predictive models currently available is that, due to experimental limitations, they were developed based on data gathered under isothermal conditions. Several scientific studies have questioned the validity of these models for dynamic treatments (i.e. with non-constant temperature), showing that models fitted to isothermal data often failed to predict the microbial response under dynamic conditions (Clemente-Carazo et al., 2020; Garre, Huertas, et al., 2018; Hassani et al., 2006; Stasiewicz et al., 2008; Valdramidis et al., 2007). One hypothesis to describe this deviation is stress acclimation, which is based on the concept that microbial cells respond to sublethal stresses by increasing their thermal resistance (Khan et al., 2022; Richter et al., 2010). Therefore, if the heating phase is sufficiently long, it would increase the stress resistance in the microbial cells, thus increasing their chance to survive the treatment (Garre, Huertas, et al., 2018).

A second main limitation of currently available microbial inactivation models is the impact of variability. In the context of microbial inactivation models, variability includes differences in the observed microbial response due to genetic and physiological differences between the cells. It is thus different to uncertainty, which includes experimental error and other sources of misinformation that can be reduced by gathering additional data with increased quality (Nauta, 2000). Several recent studies have attempted to assess the variability of microbial inactivation at different levels and to quantify its relevance (Aspridou & Koutsoumanis, 2015; den Besten et al., 2018; Harrand et al., 2021). However, to the best of our knowledge, a single study has evaluated the relevance of strain variability under dynamic conditions (Clemente-Carazo et al., 2020). Consequently, in this study, we advance in the understanding of variability in microbial inactivation under dynamic conditions by comparing the responses of two strains of *Salmonella* spp. (S. Enteritidis CECT 4300 and S. Senftenberg 4564). Two strains were considered in our study to evaluate the implications of variability in heat resistance in biological safety management. Strain selection was motivated by one being a reference strain commonly used in thermal resistance studies, while the other being a variant of remarkably high heat resistance. This approach will inform whether results obtained for reference strains are extrapolable for strains with extreme phenotypes, a question of high relevance for the study of variability in predictive microbiology.

2. Materials and methods

2.1. Bacterial culture and media

Experiments were performed using *Salmonella enterica* serovar Enteritidis CECT 4300 and *Salmonella enterica* serovar Senftenberg CECT 4565. Both strains were provided by the Spanish Type Culture Collection (CECT, Valencia, Spain). They were selected due to their unique characteristics. *S.* Enteritidis is usually considered as a reference strain for this species, while *S.* Senftenberg is a well-known heat-resistant strain (Clemente-Carazo et al., 2021; Guillén, Marcén, Mañas, et al., 2020).

The bacterial strains were stored at – 80 ± 2 °C (20% glycerol) until use. To perform experiments, fresh cultured plates were grown weekly in trypticase soy agar (TSA, Scharlau Chemie, Barcelona, Spain) for each strain. The fresh cultures were incubated for 24 h at 37 ± 1 °C in an incubator. Then, a single colony from the fresh culture plate was transferred to 10 mL of trypticase soy broth (TSB; Scharlau Chemie) and incubated at 37 ± 1 °C for 24 h. At this time, the cultures had already attained the stationary growth phase, with a concentration of approx. 10^9 CFU/mL.

2.2. Thermal treatments

Thermal treatments were carried out using a Mastia thermoresistometer (Conesa et al., 2009). Before starting the treatment, the vessel was filled with 400 mL of peptone water (10 g/L peptone from casein (Scharlau Chemie) and 5 g/L NaCl (Scharlau Chemie) as the standard heating medium, to avoid other effects, e.g. complex matrixes such as food items. In order to achieve a homogeneous temperature distribution, the vessel of the thermoresistometer was constantly stirred during the treatment. The heating medium was inoculated with 0.2 mL of the bacterial suspension in order to achieve approximately 10^6 CFU/mL.

Isothermal experiments were performed at different sampling times and temperatures for the two strains. Experiments with *S.* Enteritidis were carried out at 55, 57.5, and 60 °C. On the other hand, for *S.* Senftenberg, thermal treatment was performed at higher temperatures (60, 62.5, 65 and 67.5 °C) due to the heat resistance of the strain. Once the temperature in the vessel was stable, the bacterial suspension was inoculated. The heating medium was adjusted to pH 7.0 for both strains during treatments.

For dynamic conditions, five different temperature profiles were tested for each strain with varying heating rates (supp. Table 1). In each of them, the thermoresistometer was set to the initial temperature of the treatment. Once the temperature of the medium stabilised, it was inoculated with the cell suspension and the selected heating ramp was initiated.

The same procedure for both isothermal and dynamic profiles was followed for determining the viable cell count. Sterile test tubes were used to collect a sample of 3 mL at pre-set intervals and after appropriate serial dilutions in sterile 0.1% peptone water, they were plated in TSA and incubated at 37 °C for 48 h. A minimum of two experiments were performed per condition, with freshly prepared cultures.

2.3. Modelling microbial inactivation under isothermal conditions

For the analysis of isothermal inactivation, the log-linear Bigelow model and the Mafart and Peleg inactivation models from the Weibull family were chosen.

The Bigelow model assumes a log-linear relationship between the fraction of survivors (S) and the treatment time (t), as shown in Equation (1).

$$log_{10}S = -\frac{t}{D(T)} \quad (1)$$

The slope of the inactivation curve is quantified by parameter $D(T)$; also known as the D-value, which is equal to the time required to reduce the microbial population tenfold. Its relationship with temperature (T) is supposed to be log-linear (Equation (2)).

$$log_{10}D(T) = log_{10}D_{ref} - \frac{T - T_{ref}}{z} \quad (2)$$

The sensitivity of the D-value to temperature changes is quantified by the z-value (z), equivalent to the temperature increase required to reduce the D-value by 90%. This model introduces a reference temperature (T_{ref}) without a biological interpretation but with an impact on parameter identifiability. In this equation, the value of D calculated at T_{ref} is represented by D_{ref}.

Under isothermal conditions, the primary model of the Mafart model (Mafart et al., 2002) is expressed as shown in Equation (3) where (T), usually called the δ-value at temperature T, can be interpreted as the time required for the first log-reduction of the microbial density for a treatment at temperature T. The p value corresponds to the shape factor of the Weibull distribution and describes the concavity direction of the

isothermal inactivation survivor curve. If $p = 1$, the shape of the isothermal survivor curve is log-linear and the results are equivalent to those obtained using the Bigelow model. When p is larger than one, the curve has a downward concavity, whereas when it is lower than one, there is a tail.

$$log_{10}S = -\left(\frac{t}{\delta(T)}\right)^{p} \tag{3}$$

Regarding the secondary model, the Mafart model hypothesises that the inactivation rate follows an exponential relationship with temperature (Equation (4)), similar to the Bigelow model. The z-value (z) is the temperature change that is required to achieve a tenfold reduction in the δ-value. The parameter δ_{ref} represents the value of (T) estimated at the reference temperature.

$$log_{10}\delta(T) = log_{10}\delta_{ref} - \frac{T - T_{ref}}{Z} \tag{4}$$

The Peleg model (Peleg & Cole, 1998) uses a different, equivalent parameterization of the primary model based of $b(T)$ instead of $\delta(T)$ (Equation (5)). Furthermore, the shape factor is represented by n instead of p. Nonetheless, under isothermal conditions, both models are equivalent via the identity $(T) = (1/(T))^{p}$.

$$log_{10}S = -b(T)\cdot t^{n} \tag{5}$$

On the other hand, the Peleg model uses a different secondary model than Mafart's. As shown in Equation (6), this model assumes a log-logistic relationship between $b(T)$ and temperature. If the temperature is much lower than the critical temperature (Tc), then $b(T)$ equals zero and no inactivation takes place. When the temperature exceeds Tc, $b(T)$ has a linear relationship with temperature with slope k. In this model, there is a super-linear transition between both regimes.

$$b(T) = \ln\left(1 + e^{k\cdot(T - T_c)}\right) \tag{6}$$

2.4. Modelling microbial inactivation under dynamic conditions

In this work, we used five different models to describe microbial inactivation under dynamic conditions: the Bigelow model, three Weibullian models and the acclimation model proposed by Garre et al. (Garre, Huertas, et al., 2018).

The Bigelow model can be extrapolated to dynamic conditions by calculating first derivatives with respect to time, assuming that the coefficients are constant (Equation (7)).

$$\frac{dlog_{10}S}{dt} = -\frac{1}{D(T)} \tag{7}$$

where the value of $D(T)$ at any time point is defined by the secondary model (Equation (2)).

According to van Zuijlen et al. (2010), the Mafart model can be used for dynamic conditions by calculating first derivatives of its primary model (Equation (3)) considering that the coefficients remain constant. This results in the differential equation shown in Equation (8), where the symbols have the same interpretation as in Equation (3).

$$\frac{dlog_{10}S}{dt} = -p\cdot\left(\frac{1}{\delta(T)}\right)^{p}\cdot t^{p-1} \tag{8}$$

In this model, the value of $\delta(T)$ at any time point is given by the secondary model of the Mafart model (Equation (4)).

The Peleg model adds an additional step to account for the fraction of the population that is already inactivated at any given time (Peleg & Penchina, 2000). This is accomplished by using an equivalent time $t^{\star} = -\left(\frac{log_{10}S(t)}{b(T)}\right)^{1/n}$ instead of the treatment time, t. This results in differential Equation (9), where the symbols have the same interpretation as for the Peleg model under isothermal conditions (Equation (5)).

$$\frac{dlog_{10}S}{dt} = -b(T)\cdot n\cdot\left(\frac{-log_{10}S(t)}{b(T)}\right)^{(n-1)/n} \tag{9}$$

In a similar way as for the Mafart model, the value of $b(T)$ at any time point is given by the secondary model (Equation (6)).

Therefore, whereas for isothermal conditions the only difference between the Peleg and Mafart models is the secondary model, under dynamic conditions there is an additional difference: the use of t* in the Peleg model. Consequently, in this article, we also used an additional model (the Mafart/Peleg model in the rest of the manuscript) that introduces t^{*} into the Mafart model.

In the Mafart model, the equivalent time, t^{*}, can be calculated as.

$$t^{\star} = \left(-\delta(T)^{p}\cdot log_{10}S\right)^{1/p} \tag{10}$$

Then, substituting in (8), the differential equation of the Mafart/Peleg model would be.

$$\frac{dlog_{10}S}{dt} = -p\cdot\left(\frac{1}{\delta(T)}\right)^{p}\cdot\left(-\delta(T)^{p}\cdot log_{10}S\right)^{(p-1)/p} \tag{11}$$

Garre et al. (2018) followed a different modelling approach to describe microbial inactivation under dynamic conditions. Their model is based on the hypothesis that sublethal stress during the heating phase of a dynamic treatment induces a physiological response of the microbial cells, increasing their resistance to the latter part of the heat treatment (stress acclimation). They proposed an extension of the Bigelow model, where the inactivation rate (k) of the microbial concentration (N (t)) is the product of two terms: $k = k_1\cdot k_2$ (Equation (12)).

$$\frac{dN}{dt} = -k_1\cdot k_2\cdot N(t) \tag{12}$$

The first term, k_1, represents the effect of the instantaneous temperature on the inactivation rate under the same assumptions as the Bigelow model as shown in Equation (13), where z is the z-value, T_{ref} is a reference temperature without biological interpretation and D_{ref} is the D-value (time to reduce the microbial count in 1 decimal logarithm during an isothermal treatment) at the reference temperature.

$$k_1 = \frac{ln10}{D(T)} = \frac{ln10}{D(T_{ref})\,10^{-(T - T_{ref})/z}} \tag{13}$$

Stress acclimation is introduced in this model through the term k_2. This model uses the hypothesis that the physiological state of the cell can be described by a theoretical variable, $p(t)$. At the beginning of the experiment, $p(t) = 0$ indicating the lack of any stress acclimation. When the treatment temperature exceeds a stress-inducing temperature (T_{si}) the value of this variable changes through the treatment up to $p(t) = 1$, indicating the maximum acclimation the microbial cell can develop. These hypotheses are included in the model through the empirical equation (14), where a and E are two rate parameters (Garre, Egea, et al., 2018).

$$\frac{dP}{dt} = \begin{cases} 0 & ;\ T < T_{si} \\ a(1-p)e^{E/(T - T_{si})} & ;\ T \geq T_{si} \end{cases} \tag{14}$$

Then, in this model, the effect of the acclimation on the inactivation rate is described by an empirical equation (Equation (15)), where c is a model parameter that quantifies the relevance of the acclimation on the inactivation rate. In this model, the maximum acclimation results in an increase of the D-value by a factor of $1 + c$.

$$k_2 = \frac{1}{1 + C\cdot P(t)} \tag{15}$$

2.5. Numerical methods for model fitting and calculation of predictions

The models were fitted to the data obtained under isothermal conditions using the one-step approach with the *bioinactivation* package for

R (Garre et al., 2017). We used the *fit_isothermal_inactivation* function, which uses nonlinear regression through the Newton-Raphson algorithm. For the models that use a reference temperature, this value was fixed to the medium of the temperature range as recommended by Peñalver-Soto et al. (2019).

The acclimation model was fitted in two steps using the same approach as in Garre et al. (2018). First, the Bigelow model was fitted to the data under isothermal conditions using the one-step approach with *bioinactivation*. Then, the parameters of the acclimation model (*c, a, e*) were estimated from one dynamic experiment (with a heating rate of 0.5 °C/min) using the adaptive Monte Carlo algorithm (Haario et al., 2006) included in the *FME* package for R (Soetaert & Petzoldt, 2010). The convergence of the algorithm was assessed following the usual conventions (Brooks, 2011), needing 5,000 iterations with a burning length of 1,000 iterations and a covariance update every 500 iterations. For the fits, the value of *Tsi* was set to 37 °C. The data obtained for the other four dynamic profiles was used for model validation.

Predictions under dynamic conditions were estimated by numerical integration. For the Mafart and Peleg models, we used the *predict_dynamic_inactivation* function of *bioinactivation*, which uses the Livermore Solver for Ordinary Differential Equations. (LSODA) algorithm (Hindmarsh, 1983). LSODA is a state-of-the-art numerical algorithm for solving ordinary differential equations (ODEs). Among other advancements with respect to older methods (e.g. Runge-Kutta), it includes adaptive stepsize or an automatic switch between a solver for stiff or non-stiff ODEs. The predictions for the Mafart/Peleg and acclimation models were estimated using the LSODA algorithm through the *deSolve* R package (Soetaert et al., 2010).

The goodness of the model fits and the predictions was evaluated based on the n residuals (e) using the Mean Error ($ME = \frac{1}{n}\sum_{i=1}^{n} e$) and Root Mean Squared Error ($RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n} e^2}$). The RMSE quantifies the magnitude of the noise of the residuals, being defined between 0 and + infinite. On the other hand, the ME describes if there is a consistent bias between the model predictions and the observations, with negative values of ME indicating that the model predictions lay below the observations.

All the calculations were implemented in R version 3.5.3. The code is available in the GitHub page of one of the co-authors (https://github.com/albgarre/acclimation-Salmonella).

3. Results

3.1. Inactivation of Salmonella Senftenberg under isothermal and dynamic conditions

The inactivation of *S. Senftenberg* observed under isothermal conditions is depicted in Fig. 1. The data points have a clear curvature, so the Weibull model is more suitable than the Bigelow one for describing the survivor curves. The plot shows the fit of both the Peleg (blue) and Mafart (red) models to the data, showing that the Mafart model describes the data better under isothermal conditions (RMSE = 0.35 log CFU/ml for Mafart; RMSE = 0.51 log CFU/ml for Peleg). This can be attributed to the different secondary models used in both modelling approaches. Although both models use an equivalent primary model, the Mafart model assumes a log-linear relationship between δ and temperature, whereas the Peleg model assumes a log-logistic one. In view of the results illustrated in Fig. 1, this assumption of the Peleg model is less suitable than the assumptions of the Mafart model for our data on the inactivation of *S. Senftenberg*, especially at low temperatures.

Consequently, the Mafart model with $\delta_{60} = 2.04 \pm 0.42 min; p = 0.$

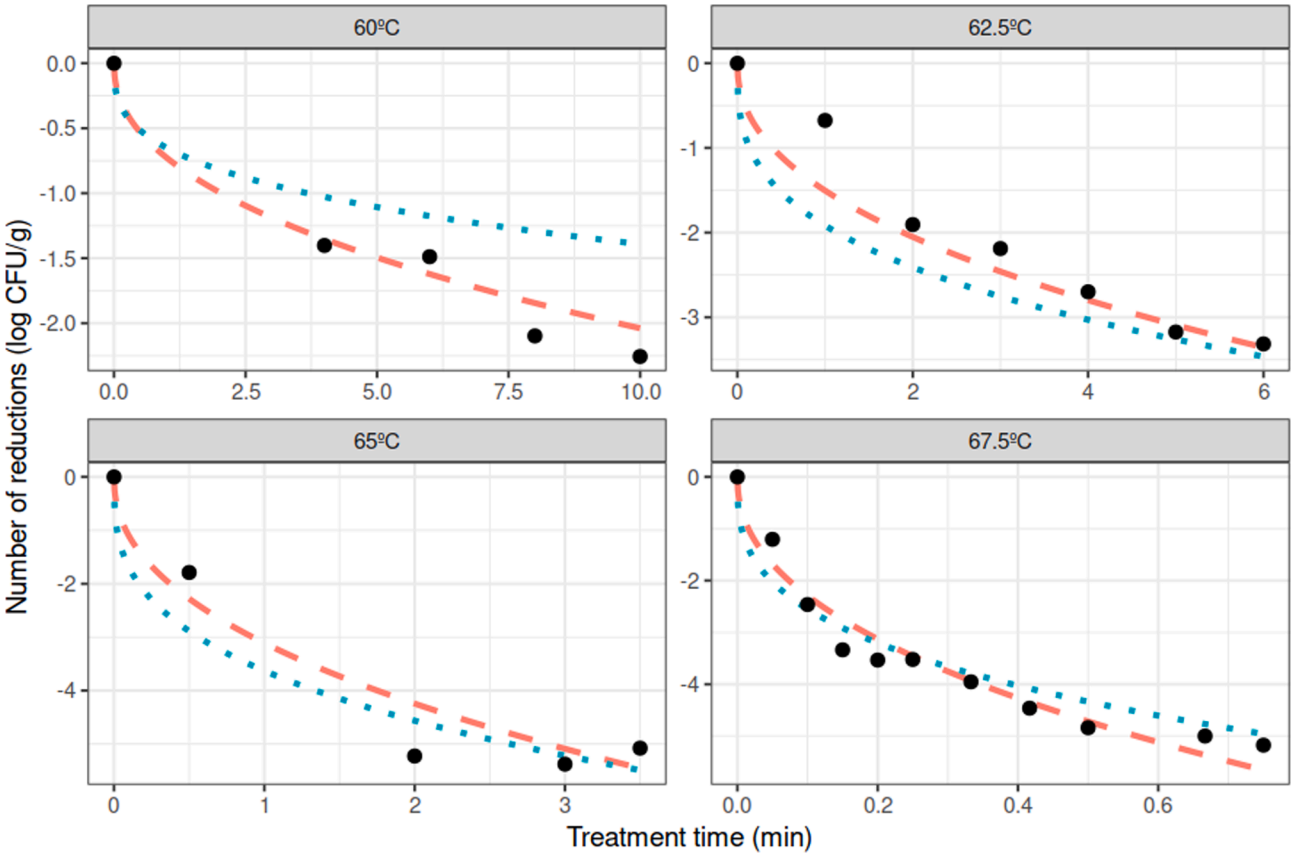

Fig. 1. Isothermal inactivation of *S.* Senftenberg at 60, 62.5, 65 & 67.5 °C. The black dots represent the experimental data; the red dashed line is the fit of the Mafart model; the blue dotted line is the fitting of the Peleg model. Models were fitted using the one-step approach. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$45 \pm 0.03; z = 3.55 \pm 0.15°C$ was used to predict the response of this strain under dynamic conditions. Fig. 2 shows that these predictions have a clear bias with respect to the experimental data for every dynamic profile tested. This plot also shows that, in spite of its poor fit to the data gathered under isothermal conditions, the Peleg model fitted to isothermal data ($T_c = 60.12 \pm 0.37°C; n = 0.33 \pm 0.04; k = 0.74 \pm 0.061°C^{-1}$) is able to describe the overall trend of the observations. This observation is further confirmed in Table 1, where the ME of each prediction is reported, showing ME for the Mafart model close to 1 log CFU/ml for every dynamic profile and ME close to 0 log CFU/ml for the Peleg model.

The poor predictive power of the Mafart model under dynamic conditions can be attributed to the second difference between the Mafart and Peleg models: the introduction of t^* in the latter. The Peleg model is based on the hypothesis that the curvature of the survivor curves is due to a heterogeneous distribution of stress resistance within the population. Then, t* accounts for the fraction of the population that had already been inactivated, defining an "equivalent time" at the instantaneous temperature. Therefore, for treatments with constant temperature, t* has no effect on the model predictions. However, for dynamic profiles, this introduces an additional difference between the Peleg and Mafart models besides their different secondary models.

In order to obtain a unique model able to describe the inactivation of *S. Senftenberg* under both isothermal and dynamic conditions, we defined a "Mafart/Peleg model" that uses both a log-linear secondary model and introduces t* (Equation (10)). Note that, because t* has no influence under isothermal conditions, this model has the same

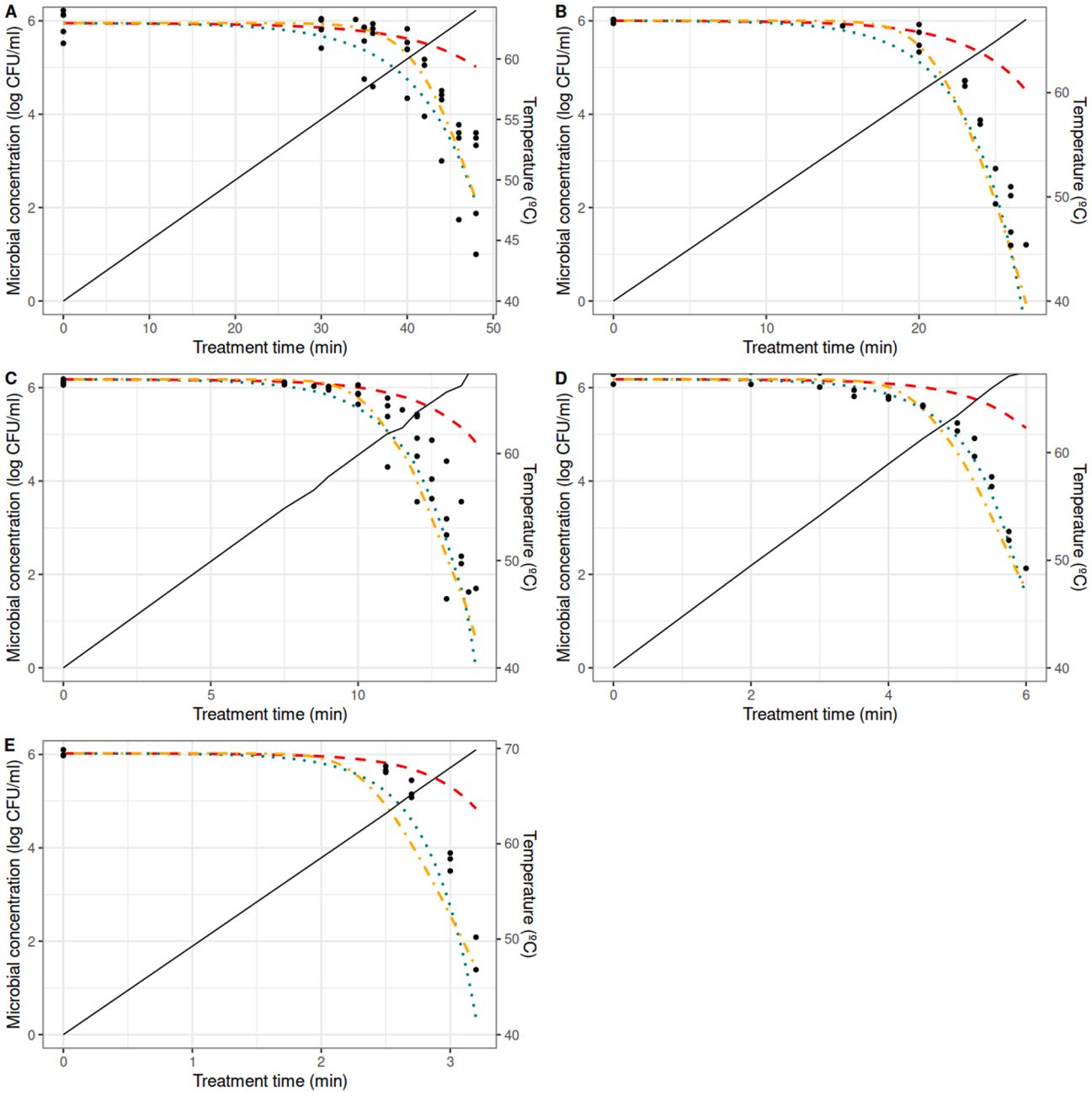

Fig. 2. Comparison between predictions based on isothermal experiments and observed inactivation under dynamic conditions for S. Senftenberg. The temperature profiles included different heating rates: A) 0.5 °C/min, B) 1 °C/min, C) 2 °C/min, D) 5 °C/min, E) 10 °C/min. The black dots represent the experimental data; (-) prediction of the Mafart model based on isothermal data; (··) prediction of the Peleg model based on isothermal data; (-·-) prediction of the Mafart/Peleg model based on isothermal data; (-) temperature profile.

Table 1
Statistical indexes evaluating the precision of the model predictions for *S. Senftenberg* for dynamic conditions based on the models fitted to data gathered under isothermal conditions.

	Mafart model		Peleg model		Mafart/Peleg model	
Heating rate (°C/min)	ME (log CFU/ml)	RMSE (log CFU/ml)	ME (log CFU/ml)	RMSE (log CFU/ml)	ME (log CFU/ml)	RMSE (log CFU/ml)
0.5	0.95	1.45	0.01	0.70	−0.31	0.75
1	1.56	2.02	−0.57	0.74	−0.63	0.80
2	1.09	1.59	−0.58	0.85	−0.42	0.79
5	0.88	1.29	−0.34	0.45	−0.12	0.24
10	1.16	1.59	−0.90	0.97	−0.81	0.90

parameter estimates as the Mafart model ($\delta_{60} = 2.04 \pm 0.42 min; p = 0.45 \pm 0.03; z = 3.55 \pm 0.15°C$). As illustrated in Fig. 1, this model can predict the response of *S.* Senftenberg under dynamic conditions based on isothermal data. This is further confirmed in Table 1, with ME for the predictions of this model closer to 0 log CFU/ml.

Doyle & Mazzotta (2000) gathered the *D*-values of *Salmonella* spp. reported in several publications. They observed that the *D*-value of *S.* Senftenberg at 60 °C in laboratory media ranged between 0.62 and 6.3 min. Similar findings were reported for *S.* Enteritidis in a study by Clemente et al. (2021) where D_{60} was 0.08 ± 0.02 min. The *D*-values estimated in our study fall within the range of the literature and are comparable (*S.* Senftenberg $D_{60} = 2.04 \pm 0.42$ min; *S.* Enteritidis $D_{60} = 0.08 \pm 0.03$ min).

3.2. Inactivation of Salmonella Enteritidis under isothermal and dynamic conditions

The response of *S.* Enteritidis was very different to that of *S.* Senftenberg under both isothermal and dynamic conditions, emphasising the relevance of strain variability for microbial inactivation. As expected, this bacterial strain had lower thermal resistance than *S.* Senftenberg CECT4565. Furthermore, the survivor curves were also qualitatively different. Unlike for *S.* Senftenberg, the survivor curves of *S.* Enteritidis CECT4300 did not clearly deviate from linearity. Indeed, as illustrated in Fig. 3, there is no difference between the model fits of Bigelow ($D_{57.5} = 0.52 \pm 0.02 min; z = 2.92 \pm 0.06°C$) and Mafart ($\delta_{57.5} = 0.47 \pm 0.05 min; p = 0.88 \pm 0.09; z = 2.86 \pm 0.08°C$).

Therefore, the Bigelow model was used to predict the inactivation of *S.* Enteritidis under dynamic conditions. As illustrated in Figs. 4 and 5, these model predictions based on isothermal data are clearly biassed with respect to the observations under dynamic conditions, predicting lower microbial counts than observed. Although the observed dynamic response could potentially be described using a Weibullian model fitted directly to the dynamic data, this model would not be able to describe the isothermal inactivation because these are linear (Fig. 3). On the other hand, the acclimation model by Garre et al. (2018) can be a good candidate to conciliate the microbial response observed for this strain under both isothermal and dynamic conditions. This model assumes that the earlier part of the dynamic treatment induces a physiological response that increases the stress resistance of the cell. This hypothesis is supported by the data, as the observations are higher than the predictions, indicating a higher stress resistance of the microbial cells. Furthermore, Table 2 shows that the *ME* of the model is much smaller for the profile with a heating rate of 10 °C/min than for slower heating, in line with the hypothesis of stress acclimation.

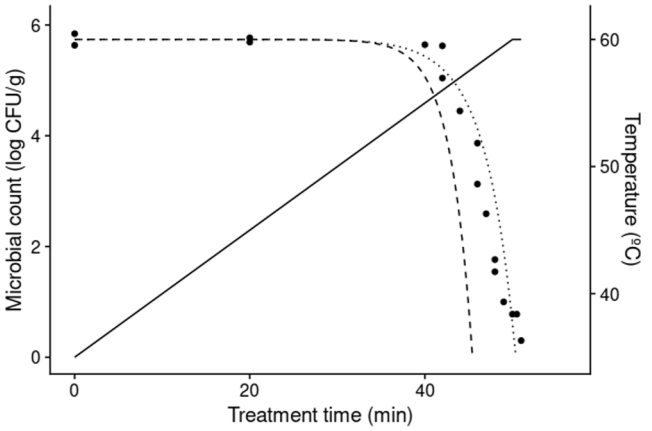

Fig. 4. Fitting of the acclimation model to the data obtained for *S.* Enteritidis for dynamic thermal profile with a heating rate of 0.5 °C/min. The dots represent the experimental data. (-) thermal profile (secondary y-axis); (··) fitting of the acclimation model; (–) prediction of the Bigelow model based on isothermal data.

Hence, the acclimation model was fitted to the experimental results obtained for a dynamic profile with a 0.5 °C/min heating rate. This profile was chosen because it had the longest heating time, allowing for a better observation of stress acclimation. Fig. 4 illustrates the fit of the model ($c = 8.97 \pm 0.26; E = 78.88 \pm 2.65°C; a = 10.36 \pm 1.63$), showing that the model could be fitted to the experimental data. Then, the model was validated by comparing the predictions of the acclimation model against independent experiments obtained for three different heating rates. As depicted in Fig. 5, for every temperature profile tested, the model was able to predict the overall response of the microbial population. This is further confirmed in Table 2, where the *ME* and

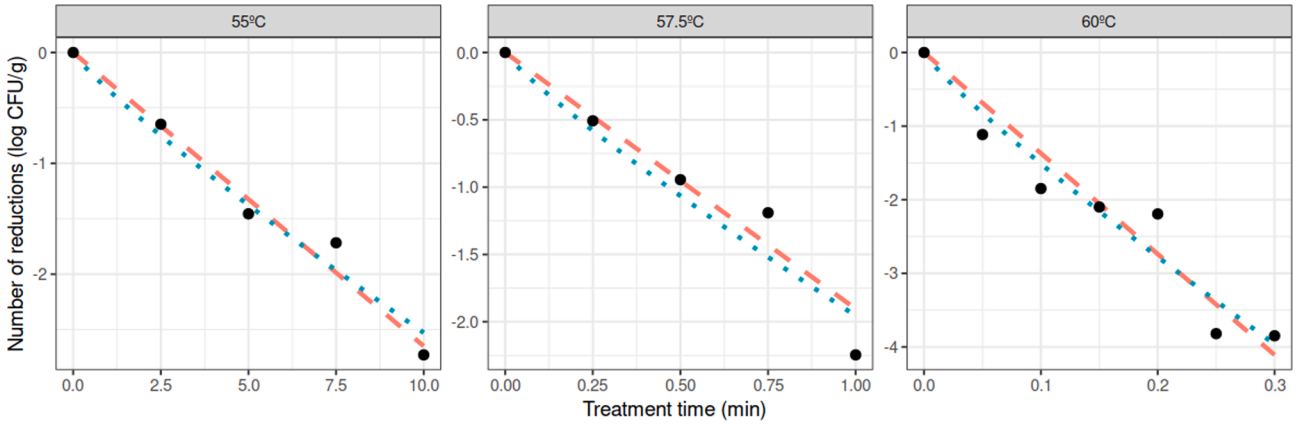

Fig. 3. Inactivation of S. Enteritidis under isothermal conditions at 55, 57.5 & 60 °C. The black dots represent the experimental data; (–) fitting of the Bigelow model; (··) fitting of the Mafart model.

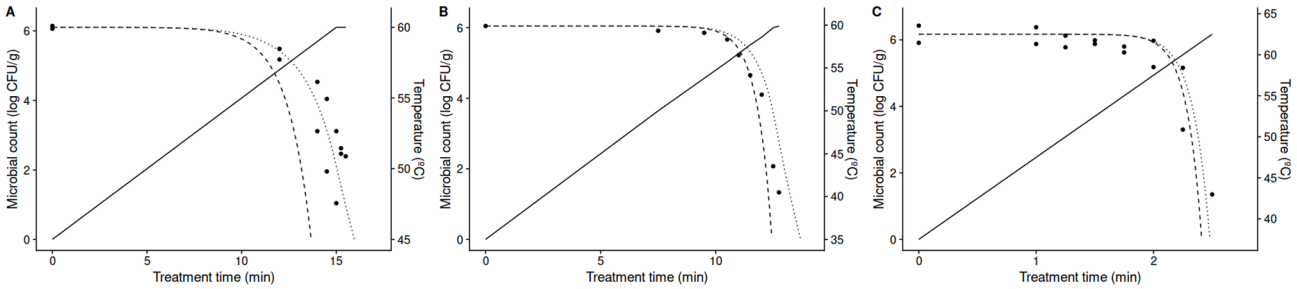

Fig. 5. Comparison between experimental data and model simulations for the profiles of *S.* Enteritidis heat treated in peptone water at different heating rates (A for 1 °C/min, B for 2 °C/min, C for 10 °C/min). The dots represent the experimental data, the dotted line is the prediction calculated by the Bigelow model whereas the dashed line is the one of the proposed model. The solid line represents the temperature profile (secondary y-axis).

Table 2
Statistical indexes evaluating the precision of the model predictions for *S. Enteritidis* for dynamic conditions based on the models fitted to data gathered under isothermal conditions.

	Bigelow model		Acclimation model	
Heating rate (°C/min)	ME (log CFU/ml)	RMSE (log CFU/ml)	ME (log CFU/ml)	RMSE (log CFU/ml)
0.5[1]	−9.08	13.07	0.19[1]	0.71[1]
1	−9.66	11.28	−0.58	1.01
2	−6.28	10.08	0.26	0.59
10	−0.36	2.17	−0.31	0.84

[1] Used to fit the acclimation model.

RMSE of the model predictions are reported.

4. Discussion

4.1. On the relevance of the hypothesis of inactivation models under isothermal and dynamic conditions

The application of predictive models to describe industrial processing treatments currently faces an important dilemma. Every treatment is dynamic (ingredients must be heated up and cooled down), but the majority of scientific data obtained in recent decades was obtained under isothermal conditions. This empirical approach is reasonable considering the type of equipment available in most microbiology laboratories, but it also raises the question about the applicability of these models for the description of actual industrial processes. This is especially the case, considering the scientific evidence pointing out that models based on isothermal data may fail at predicting the microbial response under dynamic conditions (Clemente-Carazo et al., 2020; Corradini & Peleg, 2009; Hassani et al., 2006; Stasiewicz et al., 2008; Valdramidis et al., 2007). Although some studies have been able to predict the microbial response under dynamic conditions (Milkievicz et al., 2021), it is questionable whether they are a rule or an exception. Consequently, the development of models able to describe the microbial response under both isothermal and dynamic conditions is today an active field of research.

If we wish to enhance microbial inactivation models, we must first realise that models are a collection of hypotheses, and how those hypotheses vary between isothermal and dynamic situations. Microbial inactivation by heat is extremely complex, so population-level models used in predictive microbiology apply extreme simplifications. The simplest hypothesis that can be made is that the resistance of the cells within the population is homogeneous. Therefore, differences in the time that individual cells survive a treatment would not be due to genetic or physiological differences but just pure chance (Garre et al., 2021). This hypothesis results in the first-order kinetics model that, under isothermal conditions, predicts a log-linear relationship between the microbial concentration and the treatment time.

Plenty of scientific evidence has illustrated that, in most cases, the microbial response under isothermal conditions deviates from the log-linearity predicted by this simple model (van Boekel, 2002). A variety of models have used more complex hypotheses to describe this deviation (Aspridou & Koutsoumanis, 2020). One of the most common ones is the "vitalistic" approach, which considers that stress resistance is heterogeneous within the population. The most common vitalistic approach is based on the Weibull distribution (Peleg & Cole, 1998). Under isothermal conditions, this model hypothesis predicts survivor curves with an upwards or downwards curvature (Equation (5)).

An alternative hypothesis to describe this curvature would be that stress resistance is homogeneous within the population but that it varies during exposure (e.g. due to a physiological response) according to a power law. Then, the *D*-value at any time point would be calculated as $D(t) = (t/\delta)^p$. Under isothermal conditions, this hypothesis results in predictions that are equivalent to those of the Peleg model. This does not imply that the primary models are the same, only that they are equivalent under that particular condition (in the same way that the Mafart model with $p = 1$ is equivalent to the Bigelow model). Therefore, under isothermal conditions, any differences between these models are explained by the secondary models that describe how the inactivation rates are affected by the changes in the (constant) treatment temperature.

The differences between both model approaches become evident for dynamic conditions. In order to account for a heterogeneous population, the inactivation model must include a correction term, done in the Peleg model through the term $t^\star = (-log_{10}S/b)^n$ (Peleg & Cole, 1998). This introduces an additional difference with respect to the Mafart model (van Zuijlen et al., 2010) that is only relevant under dynamic conditions. This can lead to situations where one modelling approach is more suitable for isothermal conditions (due to the secondary model), but the other one is more adequate for dynamic conditions (due to t^*). This is the case in our research, where the Mafart model fitted better the isothermal response of *S. Senftenberg*, but it failed at describing its dynamic response.

The availability of more advanced equipment during the last years has enabled the definition of novel models whose hypotheses are directly based on observations under dynamic conditions. An example of this novel approach is the acclimation model by Garre et al. (2018), which uses a similar hypothesis to the Mafart model under dynamic conditions, assuming that the stress resistance within the population is homogeneous and dynamic. However, it adds a more mechanistic hypothesis, assuming that the sublethal parts of the dynamic treatment induce a physiological response of the microbial cells, increasing their resistance (stress acclimation). Accordingly, microbial inactivation under dynamic conditions would be a "race" between microbial inactivation and the development of stress acclimation, as illustrated in Fig. 6. The application of a sublethal temperature would increase the resistance of the microbial cells (illustrated as % adaptation with a solid line in Fig. 6). If the heating is fast, the microbial cells are inactivated before they adapt. Consequently, there is barely any difference with respect to

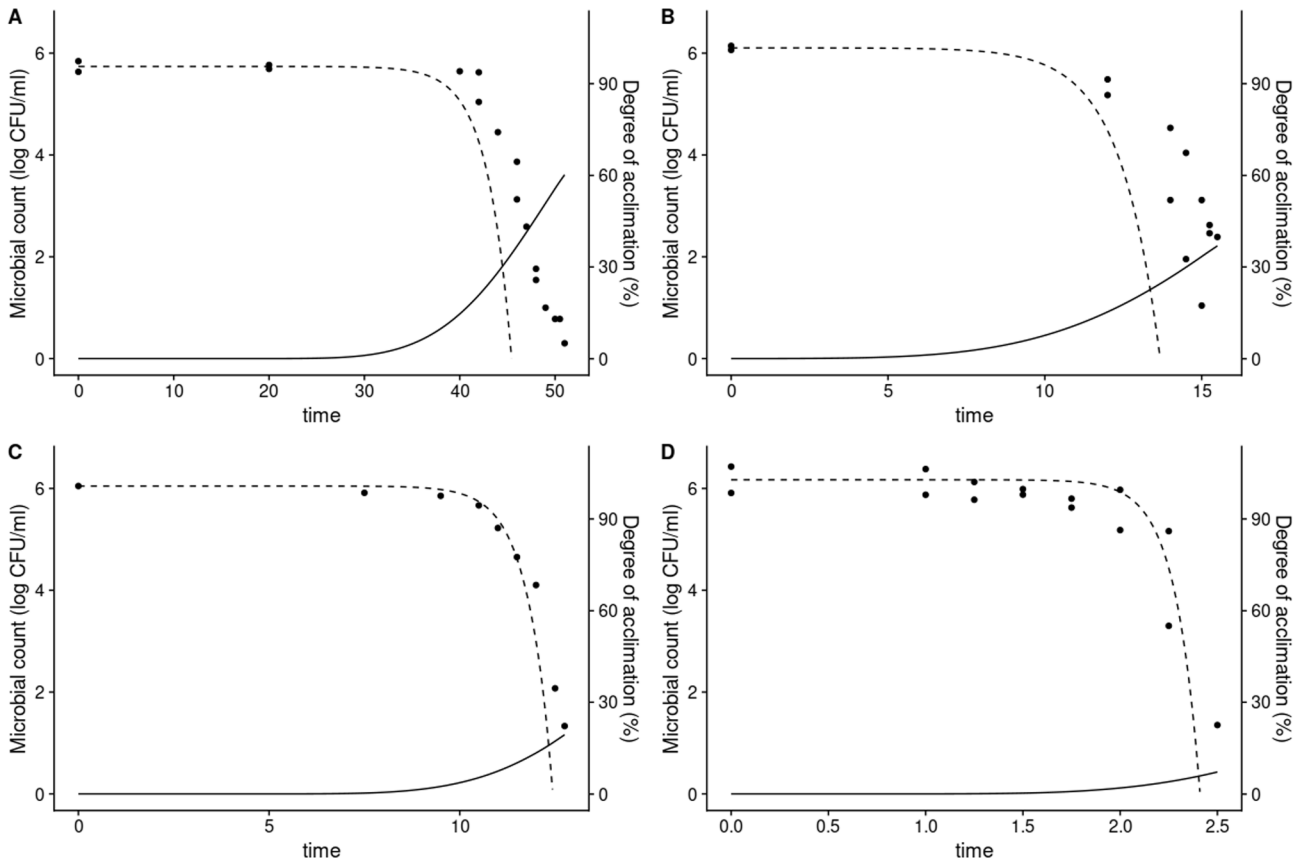

Fig. 6. Percentage of acclimation (-, calculated as $p(t)$*100) for *S.* Enteritidis heat treated in peptone water under dynamic conditions at different heating rates (A for 0.5 °C/min, B for 1 °C/min, C for 2 °C/min and D for 10 °C/min). The observed microbial concentrations are illustrated as black dots. (–) predictions of the Bigelow model based on isothermal data.

the predictions based on isothermal data (Fig. 6D). However, if the heating is slow, a significant level of acclimation takes place while the microbial population is still large, resulting in biassed predictions based on isothermal information (Fig. 6A).

It is of high importance to understand these different hypotheses. Uncovering the mechanisms by which individual cells process information and respond to changes is a major task in biological research. Differences in cell behaviour between individuals are always present to some degree in every population of cells, and the overall behaviours of a population may not be representative of the individual behaviours (Altschuler & Wu, 2010). It has been demonstrated in a variety of cell types, ranging from bacteria to mammalian cells, that heterogeneity in cellular response can exist despite isogenicity (Abdallah et al., 2013).

4.2. *Variability in microbial inactivation models is not just quantitative, but also qualitative*

Several scientific studies have focused during the last decade in the study of variability of the microbial response, trying to quantify its impact on inactivation kinetics (Abe et al., 2020; Aryani et al., 2015; den Besten et al., 2018; Garre et al., 2020; Guillén, Marcén, Álvarez, et al., 2020; Harrand et al., 2021; Luu-Thi et al., 2014; van Asselt & Zwietering, 2006). However, all these studies have applied the hypothesis that the effect of variability is only "quantitative". In other words, they have assumed that a unique model equation can describe the microbial kinetics and that strain variability can be described using different types of pooling (van Boekel, 2021). As demonstrated in this article, strain variability can also have a "qualitative" impact on microbial kinetics. In the case of *S. Senftenberg* CECT4565, the inactivation under isothermal conditions was nonlinear and the model fitted to this data was able to describe its response under dynamic conditions. *S. Enteritidis* CECT4300 had a totally different response: log-linear survivor curves under isothermal conditions and a significant deviation under dynamic conditions that could be attributed to stress acclimation. Therefore, our study shows that accounting for variability in microbial kinetics may require the use of different modelling approaches per strain, not just different model parameters.

The relevance of this fact may be small for isothermal conditions, as linear survivor curves are a particular case of the nonlinear models most commonly used (Weibullian models; Geeraerd model) (Aspridou & Koutsoumanis, 2020). However, it can be of high relevance for inactivation under dynamic conditions, due to the larger differences between the different modelling approaches. This was already indicated by Clemente-Carazo et al. (2020), who concluded that the relevance of variability under dynamic conditions may be different than under isothermal conditions. Consequently, future studies focused on the study of variability should consider the possibility that variability may also have a qualitative effect on the models, not just quantitative.

This is also of high relevance for the interpretation of experimental data obtained using cocktails of strains. According to the results of this study, it is feasible that a cocktail of strains will include strains with qualitative differences in their responses (e.g. strains able to develop acclimation) that can affect which strain is more resistant under isothermal or dynamic conditions (Garre, Egea, et al., 2018). The use of a cocktail of strains would mask this qualitative variability, allowing only the observation of the response of the strain that is the most resistant in a particular situation. Therefore, this empirical approach can mask relevant information that can be helpful in the understanding of microbial inactivation.

On the other hand, food matrices very rarely have a unique bacterial

strain. It is well known that strain variability impacts the thermal resistance of microbial cells requiring different values of the model parameters (den Besten et al., 2018). Our study has shown that, besides that "quantitative variability", variability can also cause qualitative differences between bacterial strains. This raises a fundamental question for predictive microbiology. Most models were historically developed (and, in most cases, still are) with the goal of predicting the survivor curve of a single strain. Even when models were based on cocktails of strains, they would predict a single survivor curve that would correspond to an ideal, worst-case-scenario strain. Considering that we cannot predict the particular strain that will fall in a food product, how can we validate that any model will predict the response of that strain? This is still an open question in the field that will likely be a topic of scientific discussion in the future. Nonetheless, the first step towards resolving this question is the identification of the relevant sources of variability under different scenarios. In this sense, our study provides additional insight, identifying an aspect of strain variability.

Apart from that, our results have shown that *S.* Senftenberg CECT4565, in spite of being an extremely resistant strain under static conditions, is not able to develop stress acclimation unlike *S.* Enteritidis CECT4300. This result is in-line with a recent study from our group, where we observed that the application of a heat shock would not induce an increased thermal resistance in bacterial cells of this strain (Clemente-Carazo et al., 2021). This points out the possibility of an upper limit for the stress resistance of microbial cells that cannot be surpassed by the induction of a physiological response. This hypothesis can be of high relevance for microbial risk assessment because bacterial cells within the food chain are subject to a variety of sub-lethal stresses (desiccation, acidification, competition, etc.) that can affect their resistance to stress. Hence, a better understanding of how sublethal stress can affect stress resistance of bacteria and how it is affected by variability is a potential avenue for the improvement of microbial risk assessment models.

5. Conclusions

This article has illustrated that the effect of strain variability in microbial inactivation is not just quantitative but also qualitative. For *Salmonella* Enteritidis CECT4300, we observed log-linear survivor curves under isothermal curves and stress acclimation under dynamic conditions. This behaviour was largely different from that of *Salmonella* Senftenberg CECT4565. This especially resistant strain had non-linear survivor curves under isothermal conditions and did not show stress acclimation under dynamic conditions. This different response required the application of two different modelling approaches for each strain (Weibullian models for *S.* Senftenberg, acclimation model for *S.* Enteritidis). This qualitative difference has not been described before in the context of dynamic microbial inactivation and emphasises the need to carefully evaluate different model hypotheses when describing variability in microbial inactivation.

CRediT authorship contribution statement

Leonidas Georgalis: Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. **Anna Psaroulaki:** Project administration, Funding acquisition, Supervision. **Arantxa Aznar:** Supervision, Investigation, Data curation. **Pablo S. Fernández:** Conceptualization, Supervision, Project administration, Funding acquisition. **Alberto Garre:** Conceptualization, Formal analysis, Visualization, Software.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111477.

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