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Mathematical modelling of the stress resistance induced in *Listeria monocytogenes* during dynamic, mild heat treatments

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ABSTRACT

Modelling of stress acclimation induced by thermal inactivation of *Listeria monocytogenes* under dynamic conditions is analyzed in this work. A mathematical model that separates the effect of the instantaneous temperature from the one of stress acclimation, was used. The model was trained using isothermal inactivation experiments, and one biphasic dynamic treatment with a heating rate of 1 °C/min and a holding phase of 60 °C. These experiments were performed in laboratory media (Tryptic Soy Broth; TSB). The model parameters estimated through these experiments ($D_{55} = 12.87 \pm 0.82 min$, $z = 4.58 \pm 0.04°C$, $a = 0.11 \pm 0.01 min^{-1}$, $E = 0.50 \pm 0.01°C$ and c= 1.23 ± 0.03) were successfully used to predict the microbial inactivation for another seven inactivation profiles, with and without a holding phase. Moreover, similar experiments were performed using milk as heating media, obtaining a good agreement between the model predictions and the empirical observations.

The results of this study are compatible with the hypothesis that *L. monocytogenes* is able to develop a physiological response during dynamic treatments that increases its thermal resistance. Also, that the model used can be used to predict microbial inactivation of this microorganism taking into consideration stress acclimation.

1. Introduction

Governmental agencies, industries and academic institutions related to food safety are fully aware of the risks associated to foodborne diseases. Foodborne illnesses have several adverse effects in human and animal health, which may involve large economic costs (e.g. treatment of patients or litigations). On top of that, a food outbreak may cause severe damage in the corporate image of the producing company. Microbial Risk Assessment (MRA) is a science based methodology that enables an evaluation of the risk associated to the consumption of a selected food product (Coleman and Marks, 1999). Although in its origin MRA was mostly qualitative, the development of more accurate predictive models have enabled a shift towards Quantitative Microbial Risk Assessment (QMRA), which is currently the reference approach to ensure food safety (Brul et al., 2012; Dong et al., 2015; Havelaar et al., 2008; Lindqvist and Lindblad, 2008; Nauta et al., 2009; Pielaat et al., 2015; Zwietering, 2009). QMRA is based on a quantitative description of the microbial response during each step of the farm-to-fork chain of the product analyzed. For this, mathematical models able to predict the bacterial response (growth or inactivation) for the environmental conditions encountered by the product are basic tools.

Listeria monocytogenes is the foodborne pathogen responsible for listeriosis and it is being closely monitored by health authorities worldwide. This disease is especially relevant for food safety due to its severity on the consumers. It was ranked first among the foodborne diseases in the UK according to the number of hospitalizations and deaths attributed to it (European Food Safety Authority and European Centre for Disease Prevention and Control, 2017). It has a high mortality rate, (20–30%), with respect to other foodborne diseases. Since 2008, there have been 760 notifications in the Rapid Alert System for Food and Feed (RASFF) of *L. monocytogenes* in foods. More than a third of them were notified in “fish and fish products” (288). The category “milk and milk products” ranked second (186), accounting for almost the 25% of all the notifications (European Food Safety Authority and European Centre for Disease Prevention and Control, 2017). In spite of several preventive measures taken at a European level, an increasing trend in the number of cases of listeriosis has been observed in the EU over the 2009–2013 period (European Food Safety Authority and

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European Centre for Disease Prevention and Control, 2017). This could be attributed to the uncertainty associated to the response of *L. monocytogenes* in different environments (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2018). Therefore, further research is needed to fill-in the gaps of previous risk assessments for *L. monocytogenes*. Mathematical models able to accurately describe the response of the bacterial cells to the different steps of the farm-to-fork cycle can help in this task.

One of the steps of the farm-to-fork chain of a food product is processing. During this step, a stressful agent is applied to the product, in order to destroy or damage bacterial cells so that they will not harm the consumer (Kilcast and Subramaniam, 2011). Although several technologies are available for this step, such as high hydrostatic pressure, pulsed electric fields, microwaves or the application of ultraviolet light (Cebrián et al., 2016), thermal treatment is still the most popular method in food industry (Peng et al., 2017). The first studies dealing with the mathematical modelling of the microbial inactivation during thermal inactivation were published in the early 1920s (Bigelow, 1921; Bigelow and Esty, 1920). Although thermal treatments applied in industry are usually dynamic, the equipment available at the time only allowed the performance of isothermal studies. Consequently, the microbial response during the industrial, non-isothermal treatments had to be predicted based on data observed from isothermal experiments.

Several scientific studies have been published during the last decades, shedding light on the microbial response during heat processing. First, the microbial response during isothermal treatments was studied over extended periods, concluding that the relationship between the microbial density and the elapsed time can deviate from log-linearity under some conditions (Peleg and Cole, 1998). Next, several studies reported that the inactivation models trained using isothermal data may be biased for predicting the microbial response during non-isothermal profiles (Dolan et al., 2013; Garre et al., 2018d; Hassani et al., 2006a; Valdramidis et al., 2006). The shape of the temperature profile appears to have an effect on the microbial inactivation, and may increase or reduce it (Dolan et al., 2013; Huertas et al., 2016).

Hassani et al. (2005) observed that, during mild, non-isothermal treatments, the inactivation rate of *L. monocytogenes* was lower than expected from isothermal data. Similar responses were observed for *E. coli* (Hassani et al., 2007; Mackey et al., 1993; Valdramidis et al., 2006), *Enterococus faecium* (Hassani et al., 2007), *Staphylococcus aureus* (Hassani et al., 2006b, 2006a), *Salmonella* (Hansen and Knøchel, 1996; Mackey and Derrick, 1987; Quintavalla and Campanini, 1991) and for vegetative cells of *Bacillus stearothermophilus* (Mackey et al., 1993). All these results suggest that bacterial cells may develop a resistance/adaptation mechanism when they are exposed to a mild stress. This phenomenon has been named stress acclimation, stress adaptation or induced stress resistance by different authors.

Stress acclimation can result in an increase of the number of cells able to survive an inactivation treatment. Consequently, it is especially relevant for food safety. Dolan et al. (2013) reviewed several articles where mathematical models were proposed to describe this process. More recently, Garre et al. (2018d) developed a new model to describe non-isothermal microbial inactivation taking into consideration the induced stress resistance. For the development of the model, inactivation data of two *E. coli* strains in laboratory media were used. However, this model had not yet been validated for other microorganisms or in a food product. The objective of this work was to test whether the model proposed by Garre et al. (2018d) can describe the inactivation of *L. monocytogenes* (a reference microorganism in heat inactivation studies) during non-isothermal experiments, taking into account the induced stress resistance. Moreover, the ability of the model to describe microbial inactivation in a food product is also evaluated, using non-isothermal inactivation data obtained in milk as heating medium.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Experiments were performed using *L. monocytogenes* CECT 4032, supplied by the Spanish Type Culture Collection. The origin of the strain was a case of meningitis after eating soft cheese in the United Kingdom. We decided to work with this strain because it has been used extensively for heat resistance and other inactivation studies and it provides a good indication of the resistance of *L. monocytogenes* (Garre et al., 2018c; González-Tejedor et al., 2017, 2018; Maté et al., 2017). We did not use a cocktail because we were trying to characterize the impact of acclimation on heat inactivation very precisely. And the use of a cocktail would introduce the variability among strains in the overall effect. As this effect has not been characterized before, it was important to be able to describe it reducing all factors that could hamper its description.

A freeze-dried sample was transferred to 10 mL of Tryptic Soy Broth (TSB) (Scharlab Chemie S.A., Barcelona Spain) for rehydration during 30 min. Then, 5 mL of culture were inoculated in 500 mL of TSB and incubated for 21 h at 37 °C with constant stirring at 200 rpm. At that time, cells reached stationary growth phase. Subsamples of the culture were frozen with glycerol (1:1) at −20 °C and stored until use. To perform experiments, a frozen sample was inoculated in a Tryptic Soy Agar (TSA) plate and incubated at 37 °C for 24 h. Then, a colony was selected, inoculated in TSB and grown with constant stirring overnight until cells reached the stationary growth phase.

2.2. Thermal treatments and enumeration of survivors

Thermal treatments were carried out using a Mastia thermoresistometer (Conesa et al., 2009). This device allows to perform thermal treatments in liquid media with a temperature profile that can be programmed within the maximum heating and cooling rates of the equipment (40 °C/min). The vessel of the thermoresistometer is constantly stirred during the treatment, ensuring a homogeneous temperature distribution.

Before starting the treatment, the vessel was filled with 400 mL of the heating medium. Sterile TSB and skimmed milk were used as heating media.

Isothermal experiments were performed at 55, 57.5, 60, 62.5 and 65 °C. For each treatment, the thermoresistometer was programmed at constant temperature. Once the temperature in the vessel was stable, a 0.2 mL volume of the microorganism suspension was inoculated. For non-isothermal conditions, eight different temperature profiles were tested, that can be grouped in two different categories: monophasic profiles with constant heating rate, and biphasic profiles with a holding phase after the initial heating rate. Different combinations of heating rate and holding temperatures were tested (see Tables 1–3; or Fig. 3).

Viable counts were determined following the same procedure for both isothermal and dynamic profiles. Samples with a volume of 3 mL were taken at preset times and collected in sterile test tubes, which were immediately cooled in ice. They were based on duplicate counts from appropriate dilutions, of 1 mL aliquots, that were plated and mass homogenized in TSA. Plates were incubated at 37 °C for 24 h and then counted. Triplicate experiments were performed.

2.3. Mathematical modelling

Microbial inactivation was described using the mathematical model proposed by Garre et al. (2018d), which has been built as an extension of the log-linear inactivation model (Bigelow, 1921) with added terms to account for the stress acclimation. In this model, the first derivative of the microbial density (N) with respect to time (t) is proportional to the microbial density, as shown in Equation (1).

Table 1
Statistical indexes describing the goodness of the model predictions for biphasic temperature profiles in TSB.

	Heating rate (°C/min)	Holding temperature (°C)	ME[a] (logCFU/ml)	RMSE[b] (logCFU/ml)
Bigelow model	0.5	57.5	−1.73	4.92
	0.5	60	−0.40	2.64
	1	57.5	−1.01	2.80
	1	60	−1.47	5.03
	2	60	−1.13	2.89
Stress acclimation model	0.5	57.5	0.32	0.42
	0.5	60	0.41	0.44
	1	57.5	0.53	0.44
	1	60	−0.04	0.07
	2	60	0.11	0.05

[a] Mean Error.
[b] Root Mean Squared Error.

Table 2
Statistical indexes describing the goodness of the model predictions for monophasic temperature profiles in TSB.

	Heating rate (°C/min)	ME[a] (log CFU/ml)	RMSE[b] (log CFU/ml)
Bigelow model	0.5	−1.14	3.75
	1	−1.42	4.90
	3	−0.22	1.39
Stress acclimation model	0.5	−0.34	0.82
	1	−0.81	2.48
	3	0.13	0.31

[a] Mean Error.
[b] Root Mean Squared Error.

$$\frac{dN}{dt} = -k(<env>, <phys>)N \quad (1)$$

The model parameter $k(<env>, <phys>)$ is the inactivation rate, which depends on the environmental conditions and the physiological state of the cell. In predictive microbiology, it is very common the use the D-value ($D = \frac{\ln 10}{k}$.), rather than the inactivation rate. This parameter represents the time required to cause a ten-fold reduction of the microbial density under constant environmental conditions.

For thermal inactivation, the relationship between the D-value and the treatment temperature (T) is usually expressed using the relationship proposed by Bigelow (1921). This model, shown in Equation **(2)**, assumes that the D-value has an exponential relationship with the treatment temperature. The sensitivity of the D-value to temperature changes is quantified through the z-value (z). This parameter is equal to the temperature increase that would cause a ten-fold reduction of the D-value. This model uses a reference temperature (T_{ref}), without any biological meaning, but that impacts the parameter identifiability of the model (Schwaab and Pinto, 2007). The model parameter D_{ref} represent the D-value estimated at the reference temperature.

$$D(T) = D_{ref} \cdot 10^{\frac{T - T\_ref}{z}} \quad (2)$$

The model proposed by Garre et al. (2018d) considers that the inactivation rate can be divided in two independent, multiplicative terms, as shown in Equation (3). The first term, $k_1(<env>)$, models the effect that the environmental conditions have on the inactivation rate, whereas the second, $k_2(<phys>)$, describes the influence of the physiological state of the cell.

$$k(<env>, <phys>) = k_1(<env>) \cdot k_2(<phys>) \quad (3)$$

This model considers that the first term follows the model proposed by Bigelow (Equation (2)) and the second factor describes the induced stress resistance, taking the algebraic form shown in Equation (4). The degree of acclimation of the bacterial cells is described through the variable $p(t)$, which takes values between zero (no acclimation) and one (maximum stress acclimation). Hence, if the bacterial cells have not had time to develop a stress resistance ($p = 0$), this model predicts the same inactivation rate as the Bigelow model. If, however, a mild stress has been applied inducing a bacterial stress resistance, the D-value predicted by the Bigelow model is multiplied by a factor of $1 + c \cdot p(t)$. The maximum value that variable $p(t)$ can take is one. Hence, the model parameter c describes how much the D-value is increased when the bacterial cell is fully adapted, with respect to the one observed for isothermal conditions. For instance, if $c = 0.3$, the bacterial cell increases its D-value a 30% when it is fully adapted to the thermal stress.

$$k_2(<phys>) = \frac{1}{1 + c \cdot p(t)} \quad (4)$$

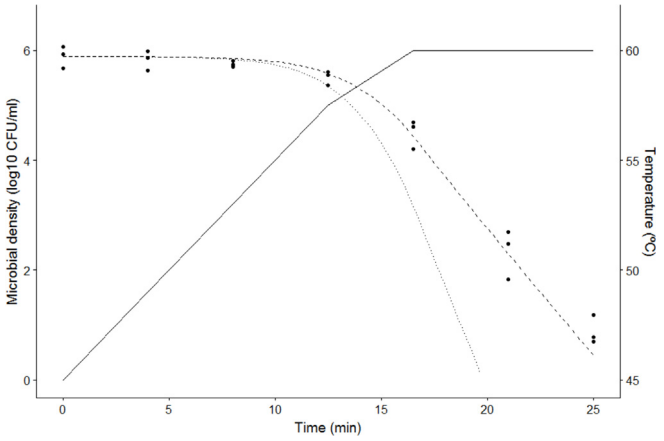

Fig. 1. Data used for the estimation of the model parameters (dots) of the stress acclimation model. The dashed line represents the curve fitted and the solid line the temperature profile (secondary y-axis). The dotted line represents the prediction of the Bigelow model based on isothermal experiments. The experiments were performed in TSB.

Table 3
Statistical indexes describing the goodness of the model prediction sfor thermal inactivation in milk.

	Type of profile	ME[a] (log CFU/ml)	RMSE[b] (log CFU/ml)
Bigelow model	Monophasic, 0.5 °C/min	−1.10	3.64
	Monophasic, 3 °C/min	−0.16	1.31
	Biphasic, 0.5 °C/min, 57.5 °C	−1.65	4.54
	Biphasic, 0.5 °C/min, 60 °C	−0.85	3.43
Stress acclimation model	Monophasic, 0.5 °C/min	−0.26	0.55
	Monophasic, 3 °C/min	0.09	0.23
	Biphasic, 0.5 °C/min, 57.5 °C	0.42	0.54
	Biphasic, 0.5 °C/min, 60 °C	0.13	0.18

[a] Mean Error.
[b] Root Mean Squared Error.

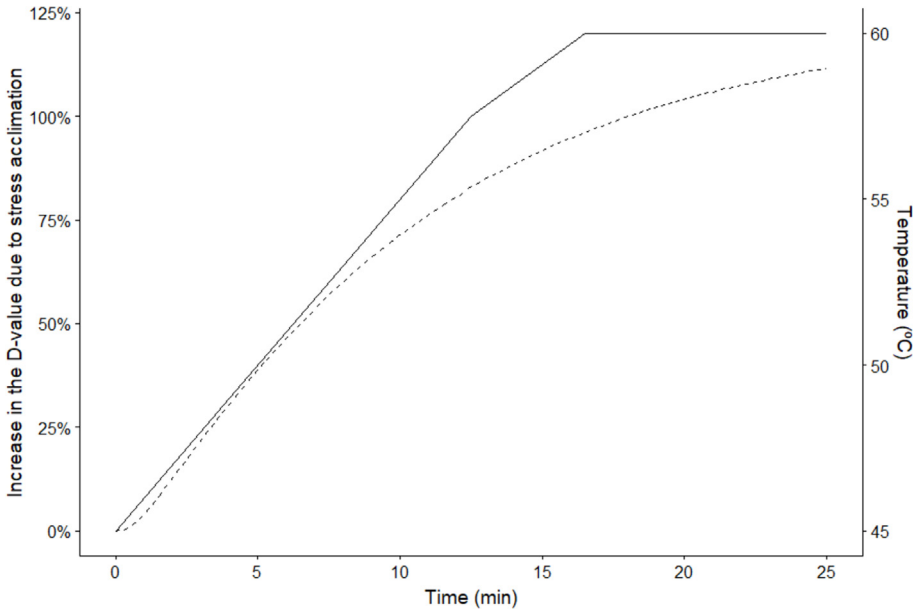

Fig. 2. Evolution of the factor describing the induced stress resistance ($1 + c{\cdot}p(T)$, dashed line) during the thermal treatment in Fig. 1, according to the model parameters fitted. The thermal profile is represented by the solid line (secondary y-axis).

This inactivation models considers that the variable $p(t)$ grows only when the cell is under stress, i.e. when the temperature is above the stress inducing temperature, T_{si}. If this is the case, $p(t)$ increases its value according to Equation (5), where a and E are unknown model parameters. The logistic growth term (Verhulst, 1838) is introduced in this equation, so that the value of $p(t)$ is bounded between zero and one.

$$\frac{dp}{dt} = \begin{cases} 0, & T \leq T_{si} \\ a{\cdot}e^{-E/(T-T_{si})}(1-p), & T > T_{si} \end{cases} \quad (5)$$

The combination of Equations (1)–(5) gives rise to the microbial inactivation model proposed by Garre et al. (2018d), shown in Equation (6).

$$\frac{dN}{dt} = -\left(\frac{\ln 10}{D(T_{ref})10^{-\frac{T-T_{ref}}{z}}}\right)\left(\frac{1}{1+c{\cdot}p}\right)N \quad \frac{dp}{dt}$$
$$= \begin{cases} 0, & T \leq T_{si} \\ a{\cdot}e^{-E/(T-T_{si})}(1-p), & T > T_{si} \end{cases} \quad (6)$$

2.4. Numerical methods and model fitting

Using the experimental data of inactivation for *L. monocytogenes* described above, the model in Equation (6) was fitted following the procedure suggested by Garre et al. (2018d, 2018b). On a first step, the D and z-values were estimated using isothermal experiments. On a second step, the three model parameters describing the stress acclimation (a, E and c) were estimated using one non-isothermal profile. This procedure avoids potentially parameter correlation issues between the components of k_1 and k_2. The stress inducing temperature, T_{si}, was fixed to 45 °C, as a maximum temperature at which *L. monocytogenes* is able to grow (Augustin and Carlier, 2000).

The *Bioinactivation* software (Garre et al., 2018a, 2017) was used for the model fitting of isothermal experiments and to calculate the predictions of the Bigelow model during non-isothermal experiments. The predictions of the model by Garre et al. (2018d) were calculated by solving the system of differential Equations **(6)**, using the *deSolve* package for R (Soetaert et al., 2010). The estimation of the model parameters from non-isothermal experiments was performed using the Adaptive Monte Carlo algorithm proposed by Haario et al. (2006), using the implementation included in the *FME* package for R (Soetaert and Petzoldt, 2010).

The convergence of the fitting algorithm was assessed following the recommendations by Brooks (2011). A trace and running mean plot was used to assay the quality of the mixing and the stability of the solution. Through an autocorrelation plot, the independence of the iterations was inspected. Finally, the test by Heidelberger and Welch (1983) was used to ensure the stationarity of the solution. The R package *coda* (Plummer et al., 2006) was used for these operations.

The Adaptive Monte Carlo algorithm used for the model fitting provides an estimate of the posterior probability distribution for the model parameters. Due to its skewness, the mode of the posterior distribution was selected as an estimate of the most likely parameter values. The procedure followed is described in Venter (1967), and was carried out using the R package *modeest* (Poncet, 2012). The standard error of the Monte Carlo Markov Chain iterations was used as an estimate of the uncertainty of the model parameter (Brooks, 2011).

The goodness of the model predictions was evaluated using the Mean Error (*ME*) and Root Mean Squared Error (*RMSE*). Both indexes are defined in Equations **(7)** and **(8)**, where N_i and $\hat{N}_i$ are the observations and model predictions for the sampling point i, and n represents the total number of observations. The mean error diagnoses if the model predictions are biased with respect to the experimental observations, by analyzing the mean of the residuals. Values of the *ME* lower than zero imply that the model underpredicts the microbial counts, whereas values greater than zero mean the opposite. This index can, thus, be used to compare the bias of several models; the one with the lowest *ME* is the one whose predictions are less biased with respect to the observations. The *RMSE* measures the overall accuracy of the model predictions. An *RMSE* of zero implies a perfect fit, whereas higher values imply a higher spread of the experimental observations with respect to the model predictions.

$$ME = \sum_i \frac{1}{n}(\log_{10}\hat{N}_i - \log_{10}N_i) \quad (7)$$

$$RMSE = \sum_i \sqrt{\frac{1}{n}(\log_{10}\hat{N}_i - \log_{10}N_i)^2} \quad (8)$$

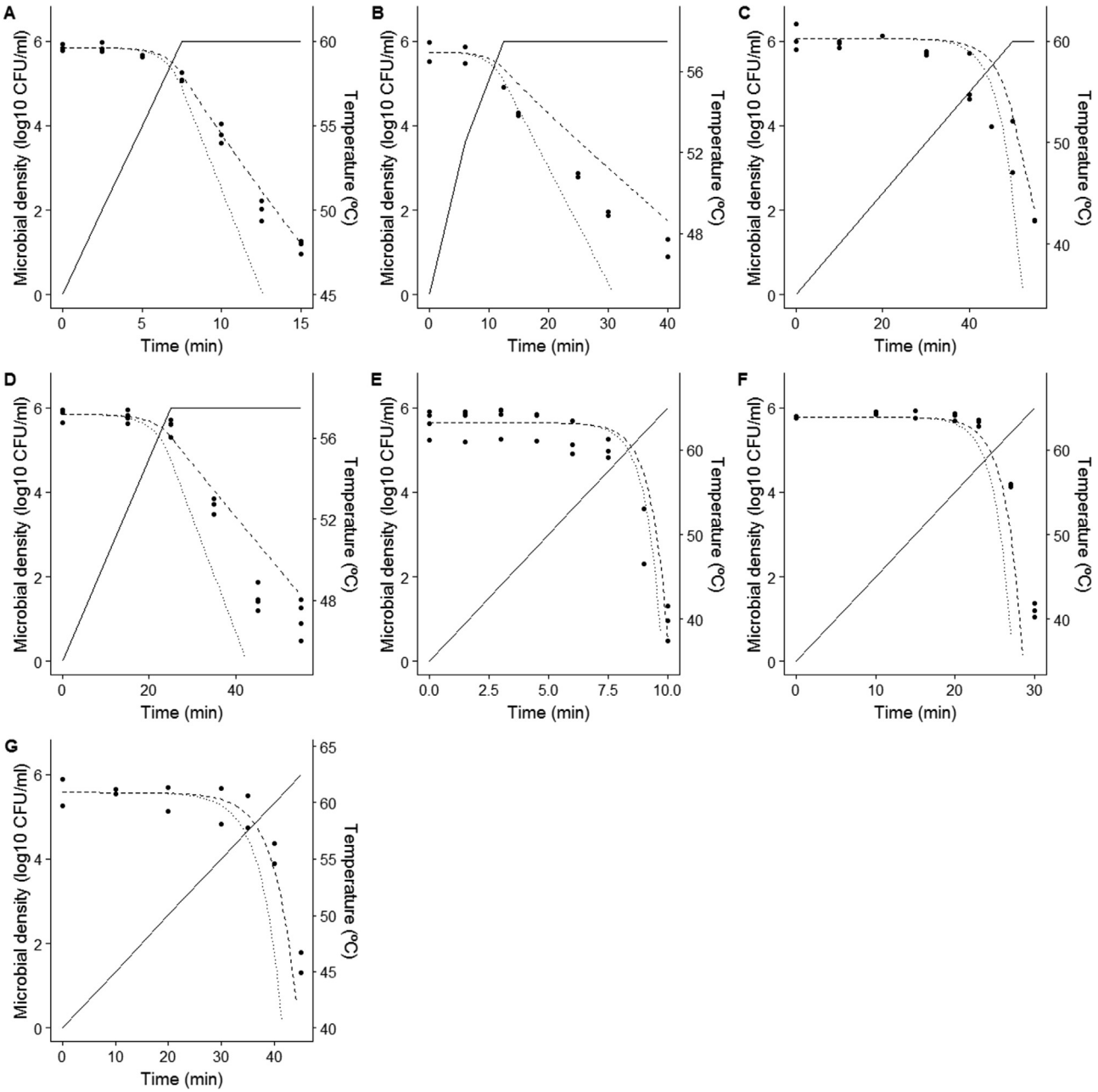

Fig. 3. Comparison between the empirical observations (dots) and the model predictions of the acclimation model (dashed line) fitted to the data in Fig. 1. Different temperature profiles were assayed (solid line; secondary y-axis) using TSB as heating media. The dotted line represents the predictions of the Bigelow model based on isothermal experiments.

3. Results and discussion

According to Datta (1993), several values of the reference temperature were tested before fitting the model, with little impact in the estimated parameter values (data not shown). Hence, a reference temperature of 55 °C was set, estimating a D-value at 55 °C of 12.87 min (S.D. = 0.37) and a z-value of 4.58 °C (S.D. = 0.04). *L. monocytogenes* is known to present large differences in thermal resistance among different strains (Aryani et al., 2015; Rosnes et al., 2012; van Asselt and Zwietering, 2006). Golden et al. (1988) calculated D-values between 16.8 and 31.6 min for different strains of *L. monocytogenes* at 54 °C, and between 5.7 and 16 min at 56 °C using tryptose phosphate broth as heating medium. The D-value estimated in this study falls within that range. Sörqvist (2003) gathered the D-values of *Listeria monocytogenes* reported in several publications obtaining a z-value of 6.1 °C (1.2). A two-sided Student's t-test at the 0.95 confidence level shows that there are no significant differences between the z-values estimated in our study and the one reported by Sörqvist (2003).

On a second step, model parameters describing the induced stress resistance (i.e, *a, E* and *c* in Equation **(6)**) were estimated using a single non-isothermal inactivation profile. A biphasic profile with a heating rate of approx. 1 °C/min followed by holding at 60 °C was used. The following values were estimated for the model parameters describing the stress acclimation: $a = 0.11(0.01) min^{-1}$, $E = 0.50(0.01)°C$, c= 1.23(0.03). Fig. 1 shows the experimental observations, as well as the curve fitted (dashed line) using the model by Garre et al. (2018d), whereas the solid line represents the temperature profile. In order to visualize the effect of the stress acclimation, the predictions of the Bigelow model based on the D and z-values estimated using isothermal experiments have been added to the plot (dotted line). This curve

represents the microbial response predicted under the assumption that the inactivation rate depends only on temperature, without any acclimation induced by the shape of the thermal profile. The Bigelow model predicted a microbial inactivation higher than the one actually observed. Therefore, the bacterial cells are more resistant than expected based solely on the isothermal experiments, indicating that stress acclimation is relevant in this thermal profile. The model by Garre et al. (2018d), on the other hand, showed a good agreement with the experimental observations (Fig. 1), being able to accurately predict the microbial inactivation taking into account the increased resistance.

The model parameter c of the model by Garre et al. (2018d) can be interpreted as the percentage increment in the D-value of the bacterial cells when it has reached its maximum level of acclimation. Therefore, the value of $c = 1.23$ estimated for *L. monocytogenes* CECT 4032 indicates that, when fully adapted, its D-value was increased by a factor of 2.23. This value has the same order of magnitude as the one obtained by Garre et al. (2018d) for *Escherichia coli* ($c = 1.98$).

In the inactivation model used in this work, the stress acclimation is reflected in the term $(1 + c{\cdot}p(t))$, which increases the D-value. In order to illustrate the kinetics of the stress acclimation, Fig. 2 plots the evolution of this factor during the experiment used to fit the model. At the beginning of the treatment, the bacterial cells are not exposed to any stress. Consequently, no stress acclimation is developed and the model prediction is equivalent to the one predicted by the Bigelow model. When the treatment temperature is risen above T_{si} (which, in our case, happens during all the experiment), the stress acclimation begins, increasing the D-value with respect to the one predicted by the Bigelow model. After approximately 10 min of treatment, the difference between both model predictions can be observed (see Fig. 1), with the induced stress resistance resulting in a relative increase of the D-value of approx. a 75% (i.e., $(1 + c{\cdot}p(t)) \approx 1.75$). When the thermal treatment enters the holding phase (at around 18 min, see Fig. 1), the induced stress resistance is close to its maximum. Consequently, the Verhulst logistic term is relevant and the rate of variation of $p(t)$ falls.

In order to validate the model parameters estimated using the isothermal experiments and the non-isothermal profile in Fig. 1, seven non-isothermal profiles with different shapes were used. These profiles were divided in two categories: monophasic profiles with constant heating rate and biphasic profiles with a holding phase after a constant heating rate. Different heating rates (between 0.5 °C/min and 3 °C/min) and holding temperatures (57.5 °C and 60 °C) have been used for both categories. Recall that these profiles were not taken into consideration for the parameter estimation problem, but just for model validation. Fig. 3 compares the predictions of the acclimation model (dashed lines) against experimental data for the different profiles described above. The predictions of the Bigelow model fitted to isothermal data have been added to the plot (dotted lines), allowing the visualization of the induced stress resistance. Moreover, Tables 1 and 2 report the *ME* and *RMSE* calculated, respectively, for the biphasic and monophasic profiles. In every tested profile, the *ME* of the Bigelow model is lower than zero. Therefore, the predictions based on isothermal experiments overpredict microbial inactivation, indicating the development of a stress acclimation. Nevertheless, the absolute *ME* value of the Bigelow model was the largest for thermal profiles with slow heating rates. When the heating rate was low, the heating phase has a longer duration and the bacterial cells developed a bigger stress acclimation, increasing the relevance of this effect. Consequently, the bias of the Bigelow model, which only takes into account the instantaneous temperature, is higher in these cases, and a model taking into account stress acclimation is more suitable. This is confirmed by our results where the higher differences between Bigelow and Garre models are precisely observed in those profiles with lower heating rates, being the model by Garre et al. (2018d) the one that best fits the experimental data, with values of the *ME* and *RMSE* closer to zero for every profile tested. For profiles with a high heating rate (e.g., the monophasic profile with heating rate of 3 °C/min, Fig. 3E) the difference between both models is insignificant, showing when no stress acclimation occurs, the acclimation model provides similar results to Bigelow, since the term $c{\cdot}p(t)$ approaches 0. Indeed, the *ME* is similar for both models for this profile (−0.22 log CFU/ml for Bigelow, 0.13 log CFU/ml for Garre), whereas differences are higher than two log-units for the biphasic treatments with a heating rate of 0.5 °C/min (2.05 log CFU/ml with a holding temperature of 57.5 °C, Fig. 3D; 2.81 log CFU/ml with a holding temperature of 60 °C, Fig. 3B).

The model predictions for the biphasic profiles showed an excellent agreement with the data, with *RMSE* and *ME* lower than 0.6 log CFU/ml. The acclimation model predictions for the monophasic profiles, despite improving those from the Bigelow model, are not as accurate as those for the biphasic profiles. The acclimation model considers a lower stress acclimation at the end of the experiment and, thus, predicts a higher inactivation than the one that actually takes place. The dynamic temperature profile used to train the model has a maximum temperature of 60 °C, several Celsius lower than the maximum temperature reached during the monophasic treatment. Therefore, the reason for the model bias may be an extrapolation error, because lower temperatures were used for estimation of the D and z-values. Nevertheless, the inactivation rate at temperatures higher than 60 °C is very high (D-value of 5s at 65 °C), making it very hard to design a thermal profile with slow heating and high holding temperature. Optimal experimental approaches (Garre et al., 2018c; van Derlinden et al., 2010) could help reach a more accurate characterization of the model.

Several authors have reported that model predictions trained using laboratory media may be biased when describing the microbial response in a food product (González-Tejedor et al., 2018; Periago et al., 2004). For this reason, it is essential to validate the models using data generated in a food product (Koutsoumanis et al., 2016). Therefore, experiments similar to those shown in Fig. 3 have been performed using skimmed milk as heating medium, instead of TSB. Two monophasic profiles with heating rates of 0.5 °C/min and 3 °C/min have been carried out, as well two biphasic treatments with a heating rate of 0.5 °C/min and holding temperatures of 57.5 and 60 °C. Fig. 4 compares the predictions of the model by Garre et al. (2018d) based on the model parameters estimated using TSB (dashed lines) against the experimental observations. Again, the predictions of the Bigelow model (dotted lines) based on isothermal inactivation data in TSB are included, allowing the visualization of the induced stress resistance and possible differences due to the medium.

The microbial response during the treatments in skimmed milk is analogous to the one observed in TSB. Therefore, this laboratory medium is suitable for testing thermal inactivation in skimmed milk under similar conditions than the ones of this study, even when stress acclimation effects are relevant. The *ME* and *RMSE* of the Bigelow and Garre model in skimmed milk (Table 3) are similar to those obtained for the same profiles using TSB as heating media (Table 1), further confirming the adequacy of TSB to predict thermal inactivation (with possible acclimation) in skimmed milk.

The stress acclimation model was able to describe microbial inactivation in milk for every dynamic profile tested. As shown in Table 3, the model predictions had a low bias, with mean residual lower than 0.5 log CFU/ml. Furthermore, they have a low spread, with a *RMSE* lower than 0.6 log CFU/ml. This was expected, due to the similarity between the results obtained in both heating media. Therefore, the model by Garre et al. (2018d) can be used to describe the microbial inactivation of *L. monocytogenes* during non-thermal treatments in a food substrate (skimmed milk), taking into account the stress resistance induced during mild phases of the treatment.

4. Conclusions

The stress resistance induced in *L. monocytogenes* during non-isothermal treatments has been described using the model proposed by Garre et al. (2018d). Experiments were performed using both TSB and

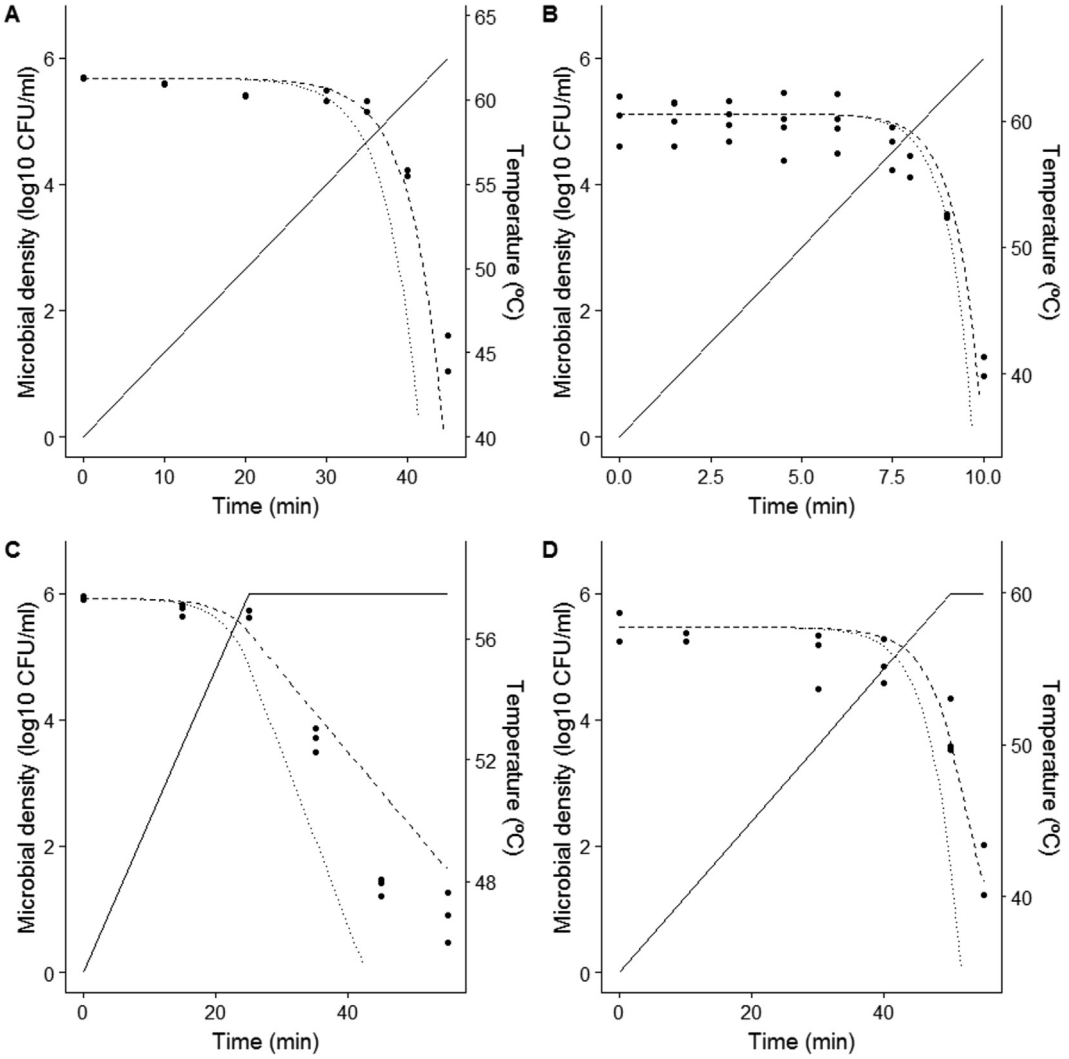

Fig. 4. Validation of the model predictions using skimmed milk as heating media. The dashed line represents the model predictions of the temperature profile illustrated by the solid line (secondary y-axis). The dotted line represents the predictions of the Bigelow mode3l based on isothermal experiments.

skimmed milk as heating media. The results obtained have been similar, showing that TSB is suitable for studying inactivation of *L. monocytogenes* in milk, even when stress acclimation is relevant.

In every profile tested, the predictions of the Bigelow model using D and z-values estimated from isothermal experiments overpredicted microbial inactivation, evidencing the relevance of stress acclimation in these thermal profiles. The inactivation model by Garre et al. (2018d), on the other hand, has been able to predict experimental data obtained, with a mean error between plus and minus one log CFU/ml for every profile tested. Nevertheless, the model was less accurate when temperatures in non-isothermal experiments was higher than the one in isothermal experiments used to estimate D and z.

In conclusion, the mathematical model proposed by Garre et al. (2018d), can be used to describe the inactivation of *L. monocytogenes* in both laboratory media and a liquid food product, such as milk, quantifying acclimation to heat processes. Nevertheless, further research would be required for the application of this model in more heterogeneous food products.

Declarations of interest

None.

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