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Predicting heat inactivation of *Staphylococcus aureus* under nonisothermal treatments at different pH

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The aim was to assess whether heat resistance data obtained from isothermal treatments allow the estimation of survivors of *Staphylococcus aureus* under nonisothermal conditions and to find a model that accurately predicts its heat inactivation at constantly rising heating rates (0.5–9°C/min) in media of different pH (4.0–7.4). *S. aureus* showed a higher heat resistance under isothermal treatments at pH 4.0 than at pH 5.5–7.4. However, under nonisothermal treatments *S. aureus* increased its heat resistance at pH 5.5–7.4 and became more thermotolerant than at pH 4.0. Estimations of survival curves under nonisothermal treatments obtained from heat resistance parameters of isothermal treatments did not adequately fit experimental values. Whereas the number of survivors was much higher than estimated at pH 5.5–7.4, that obtained at the slower heating rates at pH 4.0 was lower. An equation based on the Weibullian-like distribution ($\log_{10} S(t) = (t/\delta)^p$) accurately described survival curves obtained under nonisothermal conditions. A nonlinear relationship was observed among the scale parameter (δ) and the heating rate which allowed the development of two equations capable of predicting the inactivation rate of *S. aureus* under nonisothermal treatments. This study might contribute to prevent public health risks in foods requiring long heating lag phases during their processing.

Keywords: Heat inactivation / Modelization / Nonisothermal / *Staphylococcus aureus* / Weibull

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1 Introduction

The application of vacuum packaging followed by an in-pack processing, known as the sous vide cook-chill technology, is offering a new variety of cook-chilled products highly demanded by consumers. In comparison with conventional processing, it offers shelf life extension and eating quality. However, the use of vacuum, which would inhibit the usual competitive aerobic spoilage microflora, together with the longer shelf life, might allow the proliferation of those facultative anaerobes surviving the cooking/pasteurization stage [1]. The purpose of the heat treatment stage is two-fold. While ensuring microbiological food safety, it should also preserve the product quality. However, the great concern of the food industry to offer the best sensory quality has led to the design of minimal heat treatments which should be questioned in terms of pasteurization. In fact, in the last 30 years pasteurized foods have been responsible for numerous foodborne outbreaks [2].

During the cooking/pasteurization of packaged solid foods, microorganisms might be exposed to nonisothermal phases as a consequence of the slow heating penetration. The exposure of microorganisms to sublethal temperatures for short periods of time might act as heat shocks, increasing microbial heat resistance to a subsequent heat treatment [3–5]. In fact, anisothermal heating up lag phases during the pasteurization process have been implicated as possible causes of several food poisonings [6, 7]. So, the microbial inactivation rate under the current pasteurization processes involving anisothermal heating lag phases should be revised.

Different authors have tried to estimate the profile of survival curves under nonisothermal treatments from heat resistant parameters obtained under isothermal conditions; however, their conclusions are not in agreement. Whereas Mattick *et al.* [8] and Peleg *et al.* [9] concluded that heat resistance data of *Salmonella typhimurium* and *Listeria monocytogenes*, respectively, allowed the estimation of survivors under nonisothermal treatments, Stephens *et al.* [10] and Mañas *et al.* [11] observed that, depending on the treatment

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Abbreviations: CFU, colony forming units; **RMSE,** root mean square error

conditions, the number of survivors was higher than those estimated when *L. monocytogenes* and *Salmonella senftenberg* were heat treated under nonisothermal conditions followed by an isothermic treatment.

The design of nonisothermal treatments that permit the achievement of a required level of inactivation needs appropriate mathematical models that define microbial inactivation kinetics under nonisothermal conditions. In this sense, recently, our research group has described the inactivation of *L. monocytogenes* under nonisothermal treatments at different heating up lag phases (from 0.5 to 9°C/min) [12]. This study allowed us to conclude that survival curves under nonisothermal conditions might not be accurately estimated from heat resistance parameters obtained under isothermal treatments. Experimental values were much higher than estimated. Therefore, an equation based on the Weibullian-like distribution was proposed to describe the profile of the survival curves under nonisothermal heating. This model was capable of predicting survivors within five log cycles of inactivation of *L. monocytogenes* under nonisothermal treatments.

Staphylococcus aureus is an opportunistic pathogenic bacterium causing a wide range of diseases and intoxications. In fact, staphylococcal food poisoning remains a significant cause of foodborne illnesses in many parts of the world [13]. Although the risks associated to *S. aureus* are mainly related to the synthesis of exotoxins previously to the heat treatment, there are scarce studies on the influence of environmental factors affecting the heat resistance of this microorganism that allow to assess its heat inactivation under current heat treatments applied by the food industry. In fact, to the best of our knowledge, the capacity of increasing its thermotolerance under nonisothermal heating has not been previously investigated. This might be of the most concern when evaluating public health risks in foods requiring long heating up lag phases during the cooking/pasteurization stage.

The aim of this study was to assess whether heat resistance data obtained from isothermal treatments allow the estimation of survivors of *S. aureus* under nonisothermal conditions in media of different pH and to find a model that accurately predicts the heat inactivation of this bacterial species under nonisothermal treatments.

2 Materials and methods

2.1 Bacterial culture and media

The strain of *S. aureus* (ATCC 4459) was supplied by the Spanish Type Culture Collection. During these experiments the culture was maintained on slants of tryptic soy agar (Biolife, Milan, Italy) with 0.6% of yeast extract added (Biolife) (TSAYE). A broth subculture was prepared by inoculating with one single colony from a plate of TSAYE a test tube containing 5 mL of tryptic soy broth (Biolife) with 0.6% of yeast extract added (TSBYE) followed by incubation at 37°C for 18 h.

With this subculture, 250 mL Erlenmeyer flasks containing 50 mL of sterile TSBYE were inoculated to a final concentration of 10^4 cells/mL and incubated at 37°C under agitation (130 rpm) (Selecta, mod. Rotabit, Spain). Flasks were removed from incubation after 24 h, since at this time the cultures had already attained the stationary growth phase and its maximum thermotolerance (data not shown).

2.2 Microbial inactivation experiments

Before treatments, microorganisms were centrifuged at $6000 \times g$ for 5 min at 4°C and resuspended in TSBYE to a final concentration of 2×10^{10} cells/mL approximately.

Heat treatments under isothermal and nonisothermal conditions were carried out in a thermoresistometer TR-SC as previously described [14]. The thermoresistometer was operated with a compatible control thermostat which allowed the performance of heating ramps at different rates [15].

TSBYE adjusted to pH 4.0, 5.5 and 7.4 with HCl or NaOH (Panreac, Barcelona, Spain) was used as treatment medium.

Either under isothermal or nonisothermal heat treatments the inactivation of approximately four log cycles of cells was investigated. All survival curves were carried out in duplicate.

2.3 Isothermal heat treatments

Once the temperature (from 58 to 66°C) of the treatment medium (350 mL) had attained stability ($T \pm 0.05$°C), it was inoculated with 0.2 mL of the suspension. At preset intervals, 1 mL samples were collected and appropriate serial dilutions were prepared in sterile 0.1% peptone water (Biolife) and plated into TSAYE.

2.4 Nonisothermal heat treatments

The thermoresistometer was programmed to perform a linear temperature profile from 40 to 70°C at an established rate (0.5, 1, 2, 5 or 9°C/min). Once the temperature (40°C) of the treatment medium (350 mL) had attained stability, it was inoculated with 0.2 mL of the suspension and the heating ramp selected was run up. At preset intervals, 1 mL samples were collected and appropriate serial dilutions

were prepared in sterile 0.1% peptone water and plated into TSAYE. No growth was detected over the long period of exposures to moderate temperatures at any heating rate investigated.

2.5 Incubation of heated samples and survival counting

Plates were incubated at 37°C for 24 h. Previous experiments showed that longer incubation times did not influence survivor counts (data not shown). After incubation, colony forming units (CFU) were counted with a modified Image Analyser Automatic Counter (Protos, Analytical Measuring Systems, Cambridge, UK) as described elsewhere [16].

2.6 Thermotolerance parameters

The traditional model based on the first-order kinetics was used to describe survival curves. Decimal reduction times (D_T value: minutes of heating at, 'T' temperature for the number of survivors to drop one log cycle) were calculated from the slope of the survival curves. z values (°C increase in the temperature of treatment for D_T value to decrease one log cycle) were calculated from the slope of the decimal reduction time curves obtained by plotting the log D_T *versus* their corresponding heating temperature.

Correlation coefficients (r^2) and 95% confidence intervals were calculated with the appropriate statistical package (Excel 5.0, Microsoft, Seattle, Washington, DC, US).

2.7 Prediction of survivors under nonisothermal heating from heat resistance parameters obtained under isothermal conditions

Survival curves under isothermal conditions were fitted by the Eq. (1) proposed by Peleg and Cole [17]

$$\log\frac{N_t}{N_0} = -bt^n \qquad (1)$$

where t is the treatment time (min), N_t and N_0 are the population densities (CFU/mL) at time t and time zero, respectively, b is a rate parameter dependent on temperature, and n is a shape parameter dependent on the profile of the survival curve: $n < 1$ for concave upwards survival curves, $n = 1$ for linear survival curves and $n > 1$ for concave downwards survival curves. The estimated values of b and n were computed with GraphPad PRISM. The determination coefficients (R^2) and the root mean square errors (RMSE) were calculated.

The theoretical survival curves under nonisothermal conditions were estimated using a differential equation based on Eq. (1) as described by Peleg and Penchina [18]

$$\mathrm{d}\,[\log[S(t)]]/\mathrm{dt} = -b[T(t)] \times n[T(t)] \times$$

$$(-\log\,[S(t)]/b[T(t)])^{\Lambda}(n\,[T(t)] - 1)/n[T(t)] \qquad (2)$$

where $\log S(t)$ is the survival curve whose slope at any time t is the slope of an isothermal survival curve at the momentary temperature, $T(t)$, *i.e.* $b(T)n(T)t^{*n(T)-1}$ at a time t^* which corresponds to the momentary survival ratio $\log_{10} S(t)$, *i.e.* $t^* = \{-\log[S(t)]/b\,[T(t)]\}^{1/n[T(t)]}$. This momentary slope, or logarithmic inactivation rate, depends on the momentary values of the model coefficients, $b[T(t)]$ and $n[T(t)]$, which in turn depend on the thermal history $T(t)$. The thermal history, $T(t)$, is expressed algebraically by fitting the actual heating to the equation of a straight line.

2.8 Modelling of nonisothermal survival curves

Survival curves under nonisothermal conditions were fitted by the following equation [19]:

$$\log\frac{N_t}{N_0} = \left[\frac{t}{\delta}\right]^p \qquad (3)$$

where t is the treatment time (min); N_t and N_0 are the population densities (CFU/mL) at time t and time zero, respectively, and δ and p are two characteristic parameters of the equation. The δ value is called the time to the first decimal reduction (time necessary to inactivate the first log cycle of the microbial population). The p value is equivalent to the n value of Eq. (1) described above. The estimated values of δ and p were computed with GraphPad PRISM (Graph Software, San Diego, CA).

2.9 Model validation

For the model validation study, bias (Bf) and accuracy (Af) factors were used as a quantitative way to measure the performance of the model investigated [20]. The bias factor indicates by how much, on average, a model overpredicts (Bf > 1) or underpredicts (Bf < 1) the observed data. The accuracy factor indicates by how much the predictions differ from the observed data.

3 Results

Figure 1 shows typical survival curves of *S. aureus* heat treated at pH 4.0, 5.5 and 7.4 at 64°C. This figure has been included to illustrate the shape of the survival curves within

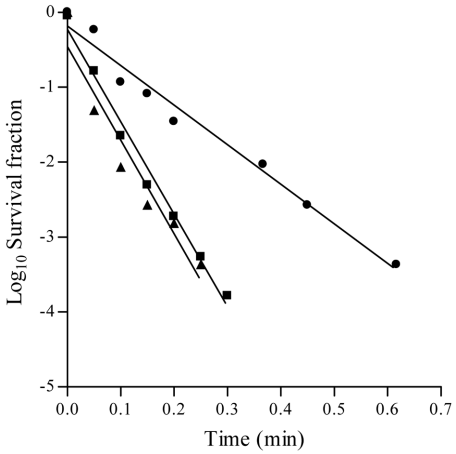

Figure 1. Survival curves of *S. aureus* heat treated at 64°C in TSB at pH 4.0 (●), 5.5 (■) and 7.4 (▲).

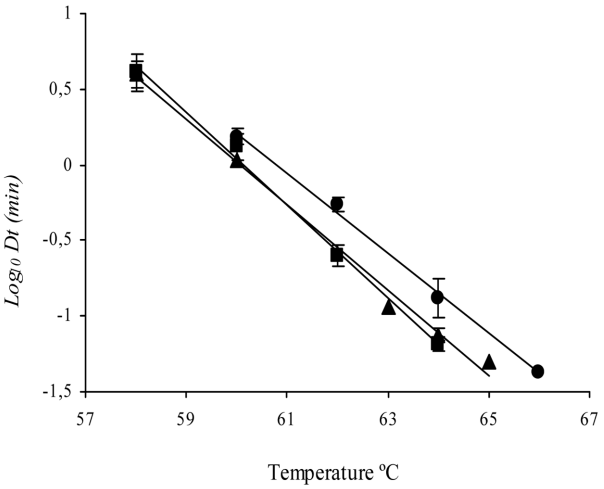

Figure 2. Influence of the treatment medium pH on the heat resistance of *S. aureus*: relationship between the log of *Dt* and the temperature at pH 4.0 (●), 5.5 (■) and 7.4 (▲).

the four log cycles investigated. As it can be observed, survival curves were nearly linear at any pH investigated. The figure includes the fitting of the first-order kinetic model to the survival curves.

Figure 2 shows the relationship between the log of D_T values calculated from the regression lines that described survival curves at every temperature and pH investigated. As seen in the figure, D_T values decreased with increased temperature and pH of treatment medium. A linear relationship was also observed between the log of the D_T value and the treatment temperature. No statistically significant differences ($p > 0.05$) were detected between z values of cells heat treated at the three pH values investigated ($z = 3.6 \pm 0.2$°C). So, in the range of temperatures investigated, the influence of the pH of treatment medium on the reduction of the resistance of *S. aureus* was constant. At any temperature investigated a reduction of the pH of treatment medium from 7.4 – 5.5 to 4.0 increased the D_T value by two times.

3.1 Prediction of survivors under nonisothermal heating from heat resistance parameters obtained under isothermal conditions

Equation (1) based on the Weibullian-like distribution accurately described isothermal survival curves corresponding to the heat inactivation of *S. aureus* in media of different pH. The estimated parameters with their 95% confidence limits, the determination coefficients (R^2) and the RMSE are listed in Table 1. As the n values were very similar and randomly changed with temperature, the curves were refitted considering a constant n value (0.96) estimated from the best fit to the experimental values and the b values were recalculated.

A linear relationship was observed between the log of b values obtained after the second fit and the treatment temperature at the three treatment medium pH values investigated as indicated by the following equations:

$$\text{pH 4.0 } \log b = 0.22\,(T) - 13.69;\ R^2 = 0.99 \quad (4)$$

$$\text{pH 5.5 } \log b = 0.28\,(T) - 17.06;\ R^2 = 0.99 \quad (5)$$

$$\text{pH 7.4 } \log b = 0.27\,(T) - 16.11;\ R^2 = 0.96 \quad (6)$$

where T is the treatment temperature (°C). These equations are valid at the lethal temperature range investigated.

Figure 3 shows nonisothermal survival curves at a heating up rate of 1, 5 and 9°C/min of *S. aureus* heat treated in TSB at pH 5.5 (Fig. 3A) and 7.4 (Fig. 3B). As shown by the fig-

Table 1. b and n values estimated from the fitting of Eq. (1) to experimental data of *S. aureus* heat treated under isothermal conditions at pH 4.0, 5.5 and 7.4

pH	T (°C)	b (min) (CL 95%)[a]	n (CL 95°C)	R^2[b]	RMSE[c]
4	60	0.50 (0.45 – 0.55)	1.23 (1.16 – 1.31)	0.99	0.10
	62	2.01 (1.56 – 0.55)	0.83 (0.45 – 1.18)	0.97	0.09
	64	5.84 (5.18 – 6.04)	1.17 (0.96 – 1.37)	0.98	0.44
	66	20.0 (0 – 25.6)	0.97 (0.46 – 1.48)	0.99	0.30
5.5	58	0.14 (0.06 – 0.21)	1.25 (1.00 – 1.50)	0.99	0.29
	60	0.77 (0.62 – 0.92)	0.95 (0.73 – 1.17)	0.98	0.23
	62	3.24 (2.81 – 3.67)	0.83 (0.60 – 1.06)	0.96	0.22
	64	9.86 (8.47 – 11.2)	0.76 (0.70 – 0.88)	0.99	0.24
7.4	58	0.28 (0.26 – 0.30)	0.94 (0.91 – 0.98)	0.99	0.14
	60	1.10 (0.98 – 1.22)	0.71 (0.53 – 0.89)	0.99	0.21
	63	7.05 (6.06 – 8.05)	0.82 (0.74 – 0.91)	0.99	0.10
	65	20.4 (18.4 – 22.4)	1.01 (0.96 – 1.05)	0.99	0.12

a) CL 95%, confidence limit.
b) R^2, determination coefficient.
c) RMSE, root mean square error.

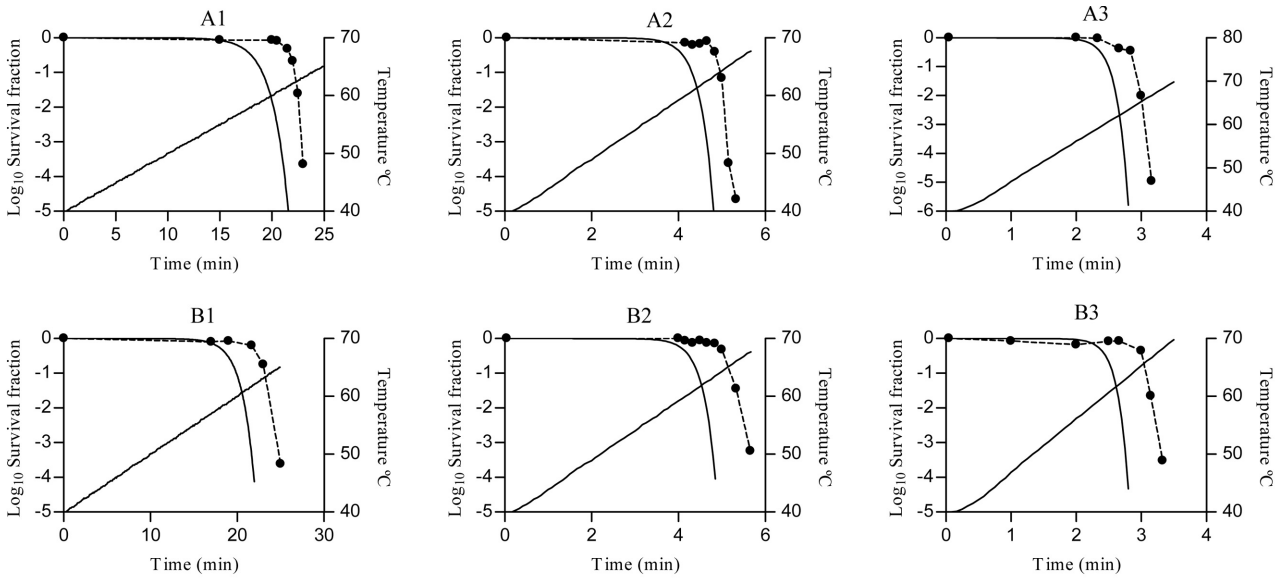

Figure 3. Influence of the heating rate on the heat resistance of *S. aureus* under nonisothermal conditions: survival curves at a heating rate of 1 (1), 5 (2) and 9°C/min (3) of *S. aureus* heat treated in TSB at pH 5.5 (A) and 7.4 (B). (- - -) Experimental data; (–) theoretical inactivation rate.

ures, experimental values were much higher than estimations at both pH. The differences between experimental and estimated values were very similar independently of the heating rate investigated. After a heat treatment at pH 5.5 or 7.4 for 22 min at a heating rate of 1°C/min the number of survivors was more than five log cycles higher than estimated.

Figure 4 shows nonisothermal survival curves at a heating up rate of 0.5, 1, 2 and 5°C/min of *S. aureus* heat treated at pH 4.0. As shown in the figure, estimations adequately fitted experimental values at the higher heating rates investigated (>2°C/min). However, at the slower heating rates, estimations were higher than experimental values. After a heat treatment for 35 min at a heating rate of 0.5°C/min the number of survivors was more than three log cycles lower than estimated.

Since inactivation under nonisothermal conditions was not accurately estimated from heat resistance parameters of isothermal treatments at any pH investigated, nonisothermal survival curves obtained at these pH were fitted by Eq. (3).

3.2 Modelling of nonisothermal survival curves

Equation 3 accurately described the complete survival curve profile obtained under nonisothermal conditions independently of the heating rate and the treatment medium pH investigated. Results demonstrated that δ values decreased with increased heating rate and p values varied as a function of the treatment medium pH. No significant differences ($p < 0.05$) were detected among p values of survival curves obtained at pH 5.5 and 7.4, but they were higher ($p > 0.05$) than those obtained at pH 4.0. Therefore, in order to reduce the number of parameters of the equation, survival curves were refitted considering a constant p value estimated from the best fit to the experimental values obtained at pH 5.5 and 7.4, and a different constant p value estimated from the best fit to the experimental values obtained at pH 4.0. The estimated parameters with their 95% confidence limits are listed in Table 2. R^2 and RMSE indicated the goodness of the fit.

Table 2. δ Values estimated from the fitting of Eq. (3) to experimental data of *S. aureus* heat treated under nonisothermal conditions at pH 7.4, 5.5 and 4.0 considering a constant p value estimated from the best fit to experimental data

pH	Hr (°C/min)	δ (min) (CL 95%)[a)]	p	R^{2}[b)]	RMSE[c)]
7.4	0.5	42.8 (42.4 – 43.1)	19.7	0.94	0.35
	1	21.7 (21.5 – 22.0)		0.89	0.38
	2	11.5 (11.4 – 11.6)		0.95	0.34
	5	4.91 (4.87 – 4.95)		0.94	0.38
	9	2.91 (2.90 – 2.92)		0.99	0.12
5.5	0.5	42.6 (42.1 – 43.0)	19.7	0.87	0.46
	1	23.4 (23.4 – 23.5)		0.99	0.05
	2	12.3 (12.3 – 12.3)		0.99	0.08
	5	5.32 (5.29 – 5.34)		0.97	0.14
	9	3.11 (3.10 – 3.14)		0.97	0.18
4	0.5	33.6 (33.5 – 33.7)	17.7	0.98	0.10
	1	19.3 (19.1 – 19.5)		0.89	0.41
	2	10.5 (10.5 – 10.6)		0.96	0.20
	5	4.77 (4.73 – 4.81)		0.95	0.15

a) CL 95%, confidence limit.
b) R^2, determination coefficient.
c) RMSE, root mean square error.

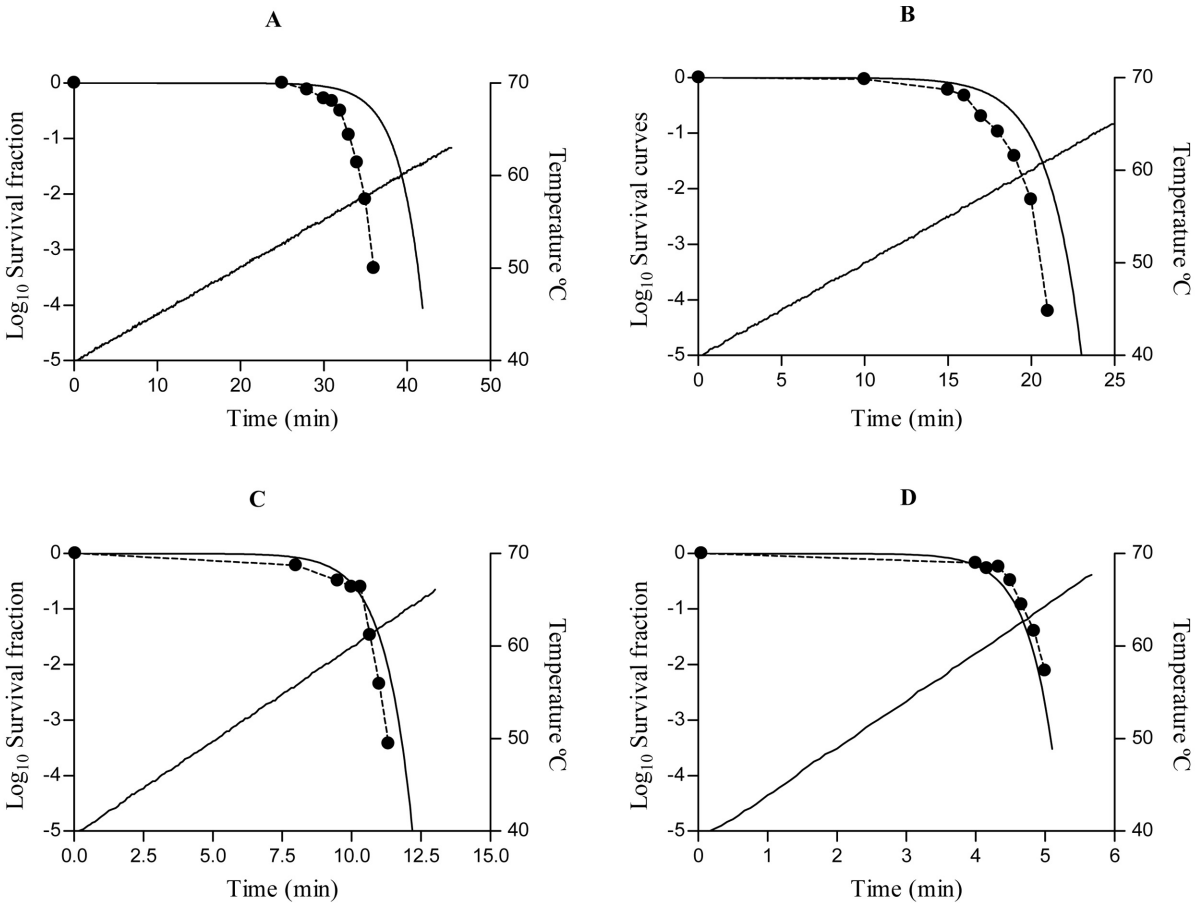

Figure 4. Influence of the heating rate on the heat resistance of *S. aureus* under nonisothermal conditions: Survival curves at a heating rate of 0.5 (A), 1 (B), 2 (C) and 5°C/min (D) of *S. aureus* heat treated in TSB at pH 4.0. (- - -) Experimental data; (–) theoretical inactivation rate.

Figure 5 illustrates the relationship between the log of the δ values and the heating rate at pH 7.4 and 5.5 (Fig. 5A) and pH 4.0 (Fig. 5B). Since there were no significant differences ($p < 0.05$) among δ values obtained at pH 5.5 and 7.4, a secondary model (Eq. (7)) was obtained to estimate the influence of the heating rate on the heat inactivation of *S. aureus* at pH 5.5–7.4. The relationship between δ values of the survival curves obtained at pH 4.0 and the heating rate is illustrated by Eq. (8)

$$\mathrm{Log}\delta = -0.40\,\mathrm{Ln(Hr)} + 1.35;\ R^2 = 0.999 \quad (7)$$

$$\mathrm{Log}\delta = -0.39\,\mathrm{Ln(Hr)} + 1.27;\ R^2 = 0.999 \quad (8)$$

where Hr is the heating rate (°C/min).

The secondary models were introduced in the primary one and two equations capable of predicting heat inactivation under nonisothermal conditions at pH 5.5–7.4 (Eq. (9)) and at pH 4.0 (Eq. (10)) were obtained

$$\log_{10}\frac{N_t}{N_0} = \left(\frac{t}{10^{-0.40\,\mathrm{Ln(Hr)}+1.35}}\right)^{19.7} \quad (9)$$

$$\log_{10}\frac{N_t}{N_0} = \left(\frac{t}{10^{-0.39\,\mathrm{Ln(Hr)}+1.27}}\right)^{17.7} \quad (10)$$

where N_t and N_0 are the population densities (CFU/mL) at time t and time zero, respectively, Hr is the heating rate (°C/min), and t is the treatment time (min) when initial treatment temperature is 40°C. When initial treatment temperature is lower or higher than 40°C, t can be substituted by the following: t–(T–40)/Hr], where T is the initial treatment temperature (°C) and Hr is the heating rate (°C/min).

Figure 6 shows the comparison between experimental values obtained at pH 7.4 or 5.5 (Fig. 6A) and at pH 4.0 (Fig. 6B) and estimated values obtained from the models developed. The bias factor was 1.64 and 1.01, and the accuracy factor 1.20 and 1.50, respectively, indicating that Eq. (3) predictions were a good fit to the measured data.

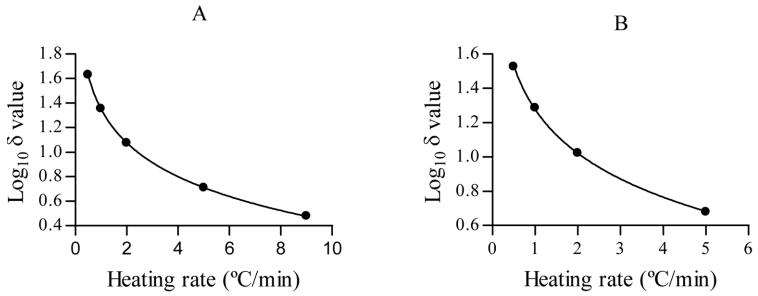

Figure 5. Influence of the heating rate on the heat resistance of *S. aureus* under nonisothermal conditions: Relationship between the δ value and the heating rate at pH 5.5 and 7.4 (A), and at pH 4.0 (B).

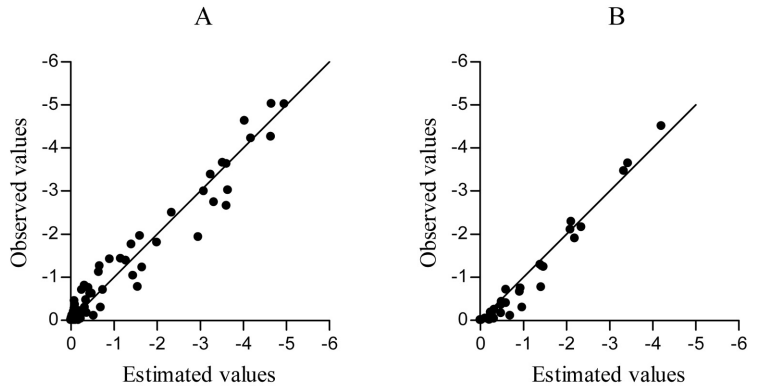

Figure 6. Comparison between observed and estimated data obtained with the tertiary model (Eqs. (8) and (9), respectively) for *S. aureus* treated under nonisothermal conditions in TSB at pH 7.4 and 5.5 (A) and at pH 4.0 (B).

4 Discussion

S. aureus is an ubiquitous organism on humans and animals and an opportunistic pathogenic bacterium causing a wide range of diseases and intoxications [13]. However, very scarce studies have been conducted in order to determine its heat resistance and how it is influenced by the environmental factors such as the pH of the treatment medium or the heat shocks previous to the heat treatment. Experimental heat resistance data obtained in this study have demonstrated that this strain of *S. aureus* might be as heat resistant as other pathogenic Gram-positive bacteria such as *L. monocytogenes* [12, 21] and its heat resistance might dramatically increase under nonisothermal treatments. To the best of our knowledge, the capacity of response of *S. aureus* under nonisothermal heating has not been previously investigated. Under isothermal treatment conditions, the kinetic of inactivation of *S. aureus* has not shown deviations from the first-order inactivation kinetics so any of the models proposed were capable of describing survival curves independently of the treatment medium pH. On the other hand, it is notable that *S. aureus*, in comparison to most bacterial species [13], has shown a higher heat resistance at pH 4.0 than at neutral pHs. These results are not in agreement with those published 40 years ago by Stiles *et al.* [22]. Also, this strain has shown a very low z value ($z = 3.6 \pm 0.2^{\circ}C$) in comparison with other Gram-positive bacterial strains [2].

4.1 Prediction of survivors under nonisothermal heating from heat resistance parameters obtained under isothermal conditions

As it happened with *L. monocytogenes* cells [12], survival curves of *S. aureus* obtained under nonisothermal treatments were not accurately estimated from heat resistance parameters obtained under isothermal treatments. Nevertheless, *S. aureus* showed a markedly different behaviour under nonisothermal conditions.

As previously described by Hassani *et al.* [12], survival curves under nonisothermal conditions were estimated, by the solution of a differential equation (Eq. (2)), from heat resistance parameters obtained under isothermal treatments following the procedure originally proposed by Peleg and Penchina [18]. The procedure assumes that no growth, damage repair or heat shock-increased thermotolerance occur, so the momentary inactivation rate only depends on the momentary temperature. This procedure was chosen since it allows estimating the survival curve under the specified nonisothermal conditions when microbial inactivation under isothermal conditions follows or not a first-order kinetic. The comparison of the theoretical and the experimental survival curves obtained under nonisothermal heating allows the estimation of the magnitude of the thermotolerance increase.

Neither survival curves of *S. aureus* obtained under nonisothermal treatments (from 0.5 to 9°C/min) at pH 5.5 and 7.4 (Fig. 3) nor those obtained at the slower heating up rates investigated (≤2°C/min) at pH 4.0 (Fig. 4) were accurately estimated from heat resistance parameters obtained under isothermal treatments. In the first case, the number of survivors was much higher than estimated which means that the thermotolerance of *S. aureus* was higher than expected. Predictions underestimated experimental values. Other authors [3, 10–12, 23–25] have previously demonstrated that anisothermal heating up lag phases may act as heat shocks inducing a thermotolerance enhancement in other bacterial species. On the contrary, in the second case, at the slower heating up rates at pH 4.0, predictions overestimated experimental values. This fact has not been previously reported and it might be related to the sensitivity of *S. aureus* to a long exposure at sublethal temperatures under acid conditions. Nevertheless, it is noticeable that, under the same experimental conditions, *L. monocytogenes* cells, which had shown a lower heat resistance at pH 4.0, did not show any susceptibility to the same slow heating up rates investigated at the same pH [12]. These results are in agreement with those reported by Stephens *et al.* [10] with *L. monocytogenes* cells and Mañas *et al.* [11] with *S. senftenberg* 775W cells. These authors were also not able to estimate survivors under nonisothermal conditions followed by an isothermic treatment from heat resistance parameters obtained after describing isothermal survival curves by a model based on a logistic algorithm, or on the traditional first-order kinetics, respectively.

Only the inactivation of *S. aureus* under nonisothermal conditions at pH 4.0 at the higher heating up rates investigated (>2°C/min) could be reasonably predicted from heat resistance parameters of isothermal treatments (Fig. 4) following the procedure established by Peleg and Penchina [18].

Our results also confirm that thermotolerance increases can also occur as a consequence of a heat shock in media at pH 5. 5. As demonstrated by Fig. 3, *S. aureus* heat treated at pH 5.5 increases its heat resistance as much as when treated at pH 7. 4. These results are in agreement with th previous studies of our research group with *L. monocytogenes* [12] and differ from those obtained by Hansen and Knochel [23]. These authors did not observe any thermotolerance increase when *L. monocytogenes* was heat shocked in ‚sous vide' cooked beef at pH 5.8 or 5.4. Perhaps, the different heating media composition might explain this disagreement.

Although *S. aureus* had shown a greater heat resistance at pH 4.0 under isothermal treatments, it was more heat resistant at pH 5.5 or 7.4 under nonisothermal heating. The lack of any microbial response at pH 4.0, as observed with *L. monocytogenes* cells under the same experimental conditions [12], indicate that the risk of survival in acid foods under nonisothermal heating would not be underestimated when predicting survivors from heat resistance parameters obtained under isothermal conditions.

Regarding the influence of the heating rate on the magnitude of the thermotolerance enhance, results obtained with *S. aureus* cells differ from those obtained with *L. monocytogenes* by Stephens *et al.* [10] and Hassani *et al.* [12] and with *S. typhimurium* by Mackey and Derrick [3] which showed a greater thermotolerance increase at the slower heating rates investigated. Heat shock-induced thermotolerance of *S. aureus* at pH 5.5 and 7.4 was just about the same independently of the heating rate (Fig. 4). Therefore, it can be deduced that the shorter exposure to sublethal temperatures at the higher heating rates would induce in *S. aureus* cells a heat shock response as intense as that caused by the slower heating rates investigated, allowing to achieve the same thermotolerance increase.

Since heat resistance data obtained from isothermal treatments overestimate or underestimate survivors of *S. aureus* under nonisothermal conditions, a new tentative based on the modelling of those survival curves obtained under nonisothermal conditions was evaluated in order to predict the inactivation of *S. aureus* under nonisothermal heating.

4.2 Modelling of nonisothermal survival curves

Survival curves under nonisothermal treatments do not follow the first-order kinetics since inactivation rate varies as temperature does. Moreover, thermal adaptation of cells, as occurred during the nonisothermal treatment, has also been shown to be responsible for the deviations of the logarithmic order of death [5, 11]. So, modelling of nonisothermal survival curves required a mathematical model capable of describing nonlinear survival curves.

Following the same procedure previously described by our research group [12], survival curves under nonisothermal conditions were described using an equation based on the Weibullian-like distribution (Eq. (3)). This equation, as others based on the Weibullian-like distribution [17, 26], is very flexible and allows describing survival curves showing shoulders or tails.

Our results have shown that this equation accurately described the complete survival curve profile of *S. aureus* obtained under nonisothermal conditions independently of the magnitude of the thermotolerance increase (Table 2).

Mathematical models should be as simple as possible describing the experimental data using the smallest possible number of parameters. Thus, the primary model was simplified by considering a constant p value estimated from the best fit to the experimental values obtained at the pH inves-

tigated. The constant p value obtained for those survival curves obtained at pH 5.5 and 7.4 differed ($p > 0.05$) from that obtained for the survival curves obtained at pH 4.0. In our opinion, the thermal adaptation suffered by those cells heat treated at pH 5.5 or 7.4 in comparison to the sensibilization suffered by those heat treated at pH 4.0 might be responsible for the different profile of the survival curves. On the other hand, p values obtained at any pH were higher than those shown by *L. monocytogenes* heat treated under the same experimental conditions [12]. Regardless of the different behaviour shown by both species in relation to the thermotolerance increase as a consequence of the heating rate, the differences in the p value might be related to the differences detected among the z values. The smaller z value observed in *S. aureus* cells (3.6 *vs.* 5.2 for *L. monocytogenes* cells [21]) means a faster increase in the inactivation rate at rising temperatures, which would cause a modification in the profile of the survival curve under nonisothermal conditions.

The other parameter of the equation (δ value) was related to the heating rate obtaining two secondary models: Eq. (7) when treated at pH 7.4–5.5 and Eq. (8) when treated at pH 4.0. As it happened with *L. monocytogenes* cells [12], there were no significant differences ($p < 0.05$) among δ values obtained at pH 7.4 and 5.5 at any heating rate investigated which allowed us to develop an equation (Eq. (9)) able to accurately predict survival counts for different times in media of pH 5.5 and 7.4 (Fig. 5A). Predicting survival curves under nonisothermal conditions at pH 4.0 required a second equation (Eq. (10)). In both cases, the model predictions were a good fit to the measured data.

In conclusion, the heat resistance of *S. aureus* varied under nonisothermal heating and survival curves could not be estimated from heat resistance parameters obtained under isothermal treatments at any pH investigated. The risk of survival of *S. aureus* in foods (pH 5.5–7.4), such as those processed by the sous vide cook-chill technology, requiring long heating up lag phases during the cooking/pasteurization stage, should be evaluated by modelling experimental data obtained during the pasteurization process. Our results have shown that the equation $\log S(t) = (t/\delta)^p$ allows describing survival curves of *S. aureus* obtained at pH 4–7.4 within the range of heating up rates investigated (0.5–9°C/min). The equations developed fitted experimental data independently of the changes in the heat resistance under nonisothermal heating. This equation might contribute to prevent public health risks in foods requiring long heating up lag phases during their processing.

Further research is needed to describe the behaviour of other pathogenic and spoiling bacteria under nonisothermal heating and to find a model that could accurately predict the number of survivors from a characteristic heat resistance parameter of each bacterial species.

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5 References

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