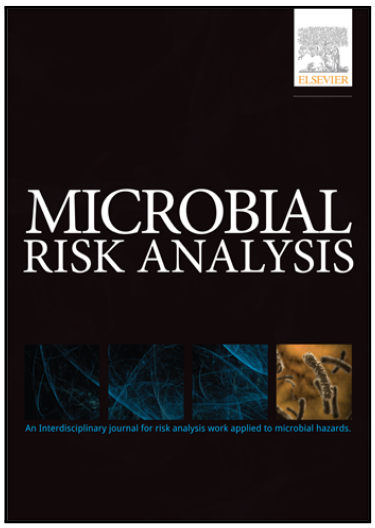

Modelling the kinetics of Staphylococcus aureus in goat's raw milk under different sub-pasteurisation temperatures

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Title:

Modelling the kinetics of *Staphylococcus aureus* in goat's raw milk under different sub-pasteurisation temperatures

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Highlights

- *S. aureus* inactivation parameters were estimated using the Weibull model.
- Temperature has an impact on the inactivation kinetics of *S. aureus*.
- The omnibus approach showed improved parameter estimation, compared to the two-step approach.
- A quadratic relationship between the Weibull parameters and temperature was observed.
- Thermisation can be used to reduce the burden of *S. aureus* in goats' raw milk.

Abstract: In this study, the heat resistance of *S. aureus* in goats' raw milk subjected to thermisation temperatures was characterised through tests at various temperatures and modelling the survival curves using the Weibull model, through a two-step and an omnibus approach, which can

model a full dataset covering all experimental conditions in one step. The fitting capacity of the secondary models obtained from the two-step approach was reasonable (adj. $R^2 > 0.639$) and both demonstrated the negative linear effects of temperature on $\sqrt{\chi}$ ($p = 0.0004$) and $\sqrt{\beta}$ ($p = 0.017$). The fitting capacity of the omnibus model was more satisfactory (adj. $R^2 = 0.996$) and also hinted at the negative linear effect of temperature on $\sqrt{\chi}$ ($p < 0.0001$), with the added advantage that, in this model, random effects can be used to account for the variability in the parameters. Our study estimated the significant inactivation parameters and established a model capable of predicting *S. aureus* behaviour at various temperatures. This information is useful to create time-temperature tables to reach target log reductions of *S. aureus* in goats' raw milk to be used by artisanal cheesemakers; hence providing an opportunity to increase the microbiological safety of cheeses made from unpasteurised milk.

Keywords: Weibull, thermisation, heat treatment, artisanal cheese, inactivation

1. Introduction

Artisanal cheeses are highly appreciated by consumers for their unique organoleptic properties, particularly their richness of taste, aroma and texture. They are frequently produced from goat and/or sheep raw milk due to tradition and the enhanced organoleptic properties attributed, at farm level, by small local dairies or by cheese industries working at regional level (Gonzales-Barron et al., 2017).

The use of raw milk in small-scale production plants, where the control of processing variables and of environmental parameters may be challenging, implies a potential risk of microbial contamination and growth (Pasquali et al., 2022). Accordingly, *S. aureus* is among the main bacterial pathogens of interest concerning the safety of cheeses, particularly those made from raw milk (Engstrom et al., 2021; Gonzales-Barron et al., 2017; Possas et al., 2021). The average incidence rates of *S. aureus* for goats' raw milk and goats' raw milk cheeses were estimated to be as high as 30-40% (Donnelly, 2018; Gonzales-Barron et al., 2017). Moreover, several outbreaks attributed

to milk and dairy products (including raw milk cheeses) caused by *S. aureus* have been reported in the past years, and raw milk cheeses have been involved in most of the outbreaks reported in relation with staphylococcal enterotoxins (European Food and Safety Authority, 2021; Johler, Giannini, et al., 2015; Johler, Weder, et al., 2015). For these reasons, the presence of *S. aureus* in these products appears to remain a public health hazard (Gonzales-Barron et al., 2017).

To this, milk thermisation has been proposed as a strategy to improve the safety of cheeses made from unpasteurised milk (Engstrom et al., 2021; Lindsay et al., 2021). Thermisation is the generic description for a range of sub-pasteurisation (< 72 °C) heat treatments of milk prior to pasteurisation and/or cheese manufacture, generally from 57 to 68 °C, with a holding time of 5 seconds up to 30 minutes, which may promote a bacterial reduction of 3 to 4 log (Codex Committee on Food Hygiene, 2013; Dash et al., 2022; Eugster & Jakob, 2019; Lindsay et al., 2021; Panthi et al., 2017; Rukke et al., 2016). This milk treatment markedly reduces the number of spoilage bacteria, and, in the case of *S. aureus*, the log reduction is such that toxin formation in the cheese, which requires a microorganism count greater than 5 log CFU/g, is highly unlikely (Eugster & Jakob, 2019). Simultaneously, thermisation causes minimum collateral heat damage to milk constituents and milk renneting properties, mild effect on the raw milk flora and the functionality of milk caseins and salts, and reduced impact on the sensory profile of the final cheeses (Eugster & Jakob, 2019; Giaccone et al., 2016; Panthi et al., 2017; Rukke et al., 2016; Samelis et al., 2009). For example, since the heat load is lower compared to that used in pasteurisation, enzymes involved in cheese flavour development, such as lipoprotein lipase, are less inactivated, thus avoiding changes in ripening and in aroma and flavour improvement of the cheese (Eugster & Jakob, 2019). Pasteurisation of milk, on the other hand, modifies the biochemistry and the microbiology of ripening to a greater extent, as well as the flavour and texture of the cheese. This does not allow for the characteristic and desirable special features of raw milk cheeses to emerge; thus, making this heat treatment inappropriate for such a product, unlike thermisation (Grappin & Beuvier, 1997).

To our knowledge, there is no literature available describing the effects of thermisation temperatures against *S. aureus* in goats' raw milk. Only one study has reported on *S. aureus* populations

after thermisation, but in a composite raw milk consisting of 90% ewes' and 10% goats' milk (Samelis et al., 2009). As a result, the temperature and time combinations needed to enhance the safety of goats' raw milk and reduce *S. aureus* counts are not characterised.

For this, a range of mathematical models can be used to estimate kinetic parameters from constant-temperature inactivation experiments, where microbial counts are modelled as a function of time (primary models). If properly formulated and validated, in conjunction with secondary models (which describe the effects of environmental factors, such as temperature, on the primary model parameters), these models facilitate prediction of the effects of a treatment regime and can be used for the design of thermal inactivation processes (Condron et al., 2015).

Frequently, primary and secondary models are fitted sequentially (two-step modelling) (Pennone et al., 2021). However, using a mixed-effects nonlinear regression approach (also known as omnibus or global modelling), a full dataset covering all experimental conditions can be modelled at once, fitting the primary and secondary models simultaneously (Juneja et al., 2015; Juneja et al., 2016; Pennone et al., 2021; Saraiva et al., 2016). The omnibus method has advantages compared to the two-step modelling as there is no loss of information associated with the uncertainty of the primary model kinetic parameters, and random effects can be used to account for the variability in parameters that environmental conditions may not explain (Pennone et al., 2021).

In this context, the aim of our research was to characterise the heat resistance of *S. aureus* in goats' raw milk at sub-pasteurisation temperatures and to compare the standard two-step modelling approach with the omnibus modelling.

Through these models, it was possible to estimate the significant inactivation parameters and to determine the heat resistance of *S. aureus* at various temperatures, information that is valuable and can be employed to derive time/temperature tables to reach target *S. aureus* log reductions, which can be used by artisanal cheesemakers and improve the microbiological safety of cheeses made from unpasteurised milk.

2. Materials and Methods

2.1. Inoculum preparation

Staphylococcus aureus ATCC 6538, obtained from the Polytechnic Institute of Bragança stock collection, was used. A loop of culture kept on Nutrient Agar slant was inoculated in 10 mL of Mueller Hinton broth (Ref. 4017412, Biolife, Italia). Broth tubes were incubated at 37 °C for 24 h, to achieve a concentration of approximately 8 log CFU/mL, verified by measurement of the absorbance at 600 nm using a spectrophotometer (Peak Instruments Inc., Version 1701).

2.2. Sample inoculation and heat treatment

The heat treatment trials were performed as described by Engstrom et al. (2021) with some modifications. Fifty mL of raw goat's milk was pipetted into a sterile centrifuge tube and inoculated at 1% (*v/v*) with *S. aureus* to yield approximately 7 log CFU/mL. After vortexing, five mL aliquots of inoculated milk was pipetted into sterile sample bags, which were flattened to a uniform thickness.

Sample bags were then attached to a sampling rack to ensure their even distribution within the water bath and to allow for simultaneous and efficient immersion. A stirred 30 L water bath (Clifton Range, United Kingdom) was used, to reduce heat transfer delays. The sampling rack was submerged in the water bath heated to 55 °C, 56.5 °C, 58 °C, 61 °C, 62.5 °C and 64 °C, and samples were removed at six appropriate pre-defined time intervals, according to preliminary trials conducted to ensure that a minimum of 20 colonies could be counted when performing *S. aureus* quantification. At each sampling point, sample bags were removed and promptly immersed into an ice bath to reach approximately 15 °C. Chilled sample bags were removed from the ice bath, dried, and sanitised (on the outside) with ethanol 70% (*v/v*) before opening.

Determination of *S. aureus* counts was then performed. For every treatment, two runs (two sets of sample bags) were conducted.

2.3. Quantification of S. aureus

For every test unit, appropriate serial dilutions were prepared by homogenising the heat-treated milk in 45 mL of buffered peptone water (Ref. 414944.1210, PanReac AppliChem, Spain) for 30 seconds in a stomacher (BagMixer 400, Interscience, France). To determine *S. aureus* concen-

tration, 0.1-mL aliquot of the dilutions was plated on Baird-Parker agar (Ref. 4011162, Biolife, Italy), supplemented with Egg Yolk Tellurite (Ref. FD046-100MLX5VL, HiMedia, India), following ISO 6888-1:2001 (International Organization of Standardization, 2021). Typical colonies were counted after 48 h after incubation at 37 °C. Microbiological determinations were done in duplicate.

2.4. Statistical analysis

The statistical analyses described below were performed in R software (version 4.1.0, R Foundation for Statistical Computing, Vienna, Austria) using the 'nlme' and 'stats' packages.

2.4.1. Two-step modelling approach

Primary model. For the survival curves obtained at 55 °C, 58 °C, 61 °C and 64 °C, *S. aureus* behaviour was modelled using the three-parameter Weibull equation as the primary model (i.e., a model describing microbial concentration as a function of time), defined as:

$$Y(t) = Y_0 - \left(\frac{t}{\chi}\right)^{\beta} \quad (1)$$

where Y_0 and $Y(t)$ represent the logarithms of microbial concentrations (log CFU/mL) at an initial time point (t = 0) and actual time t minutes, respectively; and χ and β are the scale and shape parameters of the underlying Weibull distribution, respectively. The scale parameter χ indicates the time for first decimal reduction (minutes), whereas the shape parameter accounts for upward concavity of a survival curve (β < 1), a linear survival curve (β = 1), or a downward concavity (β > 1) (Van Boekel, 2002). After separately fitting the Weibull primary model to each of the survival curves, the parameters Y_0, χ and β were extracted. To ensure that the estimated χ and β were positive, natural logarithmic transformations of those parameters were used for the fitting.

Secondary model. Since the survival experiments were conducted under different temperatures, secondary models (i.e., models describing one or more parameters of a primary model as a

function of an intrinsic or extrinsic variable) were developed to assess the effects of temperature on χ and β.

Initially, a log-linear Bigelow model was tested, but the logarithmic transformation produced estimates with high errors associated. For that reason, the four estimates of the scale and shape parameters from the three-parameter Weibull models underwent a square root transformation ($\sqrt{\chi}$ and $\sqrt{\beta}$), which proved to reduce heteroscedasticity and produced estimates with smaller errors associated, thus proving to be more adequate. Then, the transformed estimates were plotted against the corresponding temperature, and the following equations were adjusted to describe $\sqrt{\chi}$ and $\sqrt{\beta}$ as a function of temperature:

$$\sqrt{\chi} = a_1 + a_2 \times Temperature \quad (2)$$

$$\sqrt{\beta} = b_1 + b_2 \times Temperature \quad (3)$$

Model validation: The model was validated by parametric bootstrapping (1000 iterations) (Cox et al., 1994), comparing the set of experimental data collected at 56.5 °C with the predicted survival curve obtained by fitting the primary and secondary models at that temperature. For the bootstrapping, it was assumed that the residuals of the model follow a normal distribution with mean zero and standard deviation calculated from the square root of the residual sum of squares. The confidence intervals were calculated at a significance level of α=0.05. For the evaluation of the performance of the model, two statistical internal validation indices were calculated from the observed and predicted values: the bias factor (Bf) and the accuracy factor (Af) (Ross, 1996).

2.4.2. Global modelling approach: omnibus model

An omnibus model is one that fits the primary and secondary models simultaneously, using all the data from the experimental curves and jointly estimating the parameters of both models (Juneja et al., 2015, 2016).

Various equations were tested to be added as secondary models to the omnibus model, including those described by Equations (2) and (3), considering that the parameters of the Weibull model

could be expressed as a function of the temperature, as shown by the previous step-wise-regressions tested. In this sense, several mixed-effects models were assessed, and among all, that of Equation 4 fitted significantly better than the others and was the most parsimonious, revealing the best goodness-of-fit measures (Akaike Information Criterion and Bayesian Information Criterion) and behaviour of the residuals. Therefore, it is the only model presented here. In this omnibus approach, the effect of temperature on $\sqrt{\chi}$ was described by a linear model, as it was in the two-step approach, whereas $\sqrt{\beta}$ was maintained fixed.

The log CFU/mL concentration measured at time *i* when subjected to condition *j* was estimated as:

$$Y_{ij} = Y_{0_j} - \left(\frac{t}{\chi_i}\right)^{\beta_j} + \varepsilon_{ij} \quad (4)$$

$$Y_0 = Y_{0\ mean}$$

$$\sqrt{\chi_j} = a_1 + a_2 * Temperature + v_j$$

$$\sqrt{\beta_j} = b + u_j$$

Two random-effects terms, *u* and *v*, were added to the expression predicting $\sqrt{\beta}$ and to the mean of the intercept a_1 of the expression predicting $\sqrt{\chi}$, respectively. This was done because a fraction of the variability in the shape and scale parameters could not be explained solely by their fixed-effect predictors. Hence, the random effects *u* and *v* were assumed to take in random shifts subject to a given condition *j* defined by the inactivation temperature. The two random effects were assumed to follow normal distributions with means zero and covariance matrix $[s_u^2, 0, 0, s_v^2]$. The residual error ε_{ij} followed a normal distribution with mean zero and variance s^2.

Model validation. The model was validated with two independent sets of data (external validation; Schvartzman et al., 2014): one collected at 56.5 °C and another at 62.5 °C. Briefly, the

procedure consisted of fitting the omnibus model to each set of data, separately, and calculating the mean predicted bacterial concentrations and confidence intervals (at a significance level of α=0.05) along the time. The predicted inactivation curves were then compared with the experimental values. Like in the two-step approach, the performance of the omnibus model was evaluated by calculating the bias and accuracy factors.

3. Results and Discussion

3.1. Two-step modelling approach

Primary modelling. In the present study, *S. aureus* survival curves (Figure 1) presented various shapes, which may be due to the presence of subpopulations that differ in heat resistance, bacterial clumps (Den Besten et al., 2018; Abee et al., 2016), and/or vital cellular components that are being destroyed before inactivation starts (Geeraerd et al., 2000), for example. Since the primary model selected had to be flexible to portray the various shapes observed in this study and considering that the Weibull model can be used to describe nonlinear survival curves and may be helpful to pinpoint relevant physiological effects caused by heating (Van Boekel, 2002), the three-parameter Weibull equation was considered adequate and representative of all the survival curves. Ninety-five percent confidence intervals were calculated and are displayed in Figure 1 to account for the uncertainty in the estimates, which may be a result of potential laboratory errors, biological variation and stochasticity.

Table 1 compiles the means and standard errors of the parameters of the Weibull equation fitted separately to each of the thermisation temperatures tested.

S. aureus initial concentrations (Y_0) were significant ($p < 0.05$), and a fast decline in their numbers with increasing thermisation temperature was observed, as suggested by the decreasing χ values. These indicate smaller times for the first decimal reduction as temperature rises: for example, at 55 °C, the time needed for one log reduction is around 38 minutes, whereas at 64 °C, 19.32 seconds achieve the same decrease.

The general decrease of the shape parameter (β) values from 55 °C to 64 °C suggests that, as temperature increases, more damage and stress is progressively caused to *S. aureus*, although, between them, the values of the shape parameter at 61 °C (0.630) and 64 °C (0.936) are not aligned with this descending trend.

Even though the Weibull model is empirical, the value of β can be somewhat associated with the physiological effects of the heat treatment on the bacterial cells (Van Boekel, 2002). According to Van Boekel et al. (Van Boekel, 2002), $\beta < 1$ suggests cell adaptation and $\beta > 1$ alludes to accumulated cell damage. In this sense, our results suggest that, at any point in the inactivation curve, the surviving bacteria become increasingly heat-susceptible in all of the temperature-specific experimental curves, although at 61 °C this behaviour was less evident.

Secondary modelling. The results from Table 1 show that temperature has an impact on the inactivation kinetics of *S. aureus*. For this reason, the relationships between the transformed parameters $\sqrt{\chi}$ and $\sqrt{\beta}$ of the primary model and the thermisation temperatures were explored by scatter plots and, subsequently, by separate stepwise regression analyses (i.e., secondary models). Parameters of the resulting secondary models predicting $\sqrt{\chi}$ and $\sqrt{\beta}$ as a function of temperature are presented in Table 2.

From Table 2, the positive intercept estimates (40.59, $p = 0.0003$ for $\sqrt{\chi}$; 4.776, $p = 0.006$ for $\sqrt{\beta}$) and the negative linear effects of temperature (-0.638, $p = 0.0004$ for $\sqrt{\chi}$; -0.062, $p = 0.017$ for $\sqrt{\beta}$) were anticipated considering the results of χ and β from Table 1, and since higher temperatures should lead to shorter inactivation times.

The fitting capacity of the secondary models was reasonable, as shown by the adjusted R^2 values, 0.895 and 0.639, thus supporting the robustness of the models. Nonetheless, these R^2 values indicate that the models could not account for all the variability in the results, and that some of it remains unexplained. For both models, to further assess the quality of the fitting, the relationship between residuals and predicted values was assessed through scatter plots, which showed that the spread of the residuals over the fitted values was randomly distributed around the

zero of the horizontal axis (plots not shown). Such results additionally corroborated the fitting quality of the models. Nevertheless, it is worth mentioning that the model should not be extrapolated for temperatures below 55 ºC or beyond 64 ºC, since the behaviour of $\sqrt{\chi}$ at those temperature ranges is not accounted for in our model.

The plots in Figure 2 illustrate the change in $\sqrt{\chi}$ and $\sqrt{\beta}$ taking into account the impact of temperature, as described by the linear models built. Visual inspection of the plots further supports the agreement between the observed data and the predicted values suggested by the adjusted R^2, as most experimental values lay well within the 95% confidence bands.

Model validation. The inactivation curve displayed in Figure 3 was obtained by iteratively (N=10000) calculating the values of χ and β for the temperature of 56.5 °C using the secondary models, considering that χ and β parameters follow a normal distribution with zero mean and constant variance, and placing such estimates of χ and β on the Weibull equation to obtain predicted *S. aureus* counts. From this iteration process, confidence intervals and predictions intervals could also be calculated and are presented in Figure 3.

From this bootstrapping approach, using both primary and secondary models, it was possible to adequately describe the inactivation curve for the temperature of 56.5 °C, considering that it provided a good coverage of the experimental data points (all the observations are well within the 95% prediction bands), as shown in Figure 3.

The agreement between the predicted survival curve and the observed data was also verified by calculating the accuracy factor, Af = 1.06, and bias factor, Bf = 0.96. The Af is a measure of average deviation that indicates the spread of the results about the predictions (Ross, 1996) and, in this case, the Af value suggests that, on average, predictions are 1.06 factors of difference with respect to observations. The Bf, in turn, is a measure of the agreement between the predictions made by the model and the actual observations (Ross, 1996). In this case, the Bf value suggests that the model may tend to underestimate the microbial concentrations by approximately 10%, and, for that reason, may be deemed as "fail-dangerous".

3.2. Global modelling approach: omnibus model

Omnibus model. The final omnibus model presented a total of seven parameters (Equation 4), from which four were fixed effects or predictors of $\sqrt{\chi}$, $\sqrt{\beta}$ and $Y_{0\ mean}$, and two were variances of the random effects and the residual error. This global approach allowed a good description of all the inactivation curves. Table 3 compiles the parameter estimates for the omnibus model.

The positive intercept a_1 and the negative linear effect of temperature on $\sqrt{\chi}$ (reflected by a_2) observed in the two-step modelling approach were also observed in the omnibus model. With regards to the model's random effects, the two variances s_u^2 and s_v^2 were significant ($p < 0.05$). Analysing the standard errors of the predictors of $\sqrt{\chi}$, it can be stated that the omnibus model reduced the error associated with those parameters, when comparing with the standard errors obtained by the two-step modelling approach (Table 2). These results indicate that, by simultaneously fitting both primary and secondary models, this global approach minimises the error propagation that occurs when using the two-step methodology and, thus, improves parameter estimation. This is further supported by the fitting capacity of the model, given by the adjusted R^2 of 0.996, which is higher than those of the secondary models of the two-step approach.

Model validation. The omnibus model was successfully validated with the separate data sets obtained. Predictions for two temperatures (56.5 and 62.5 °C) are shown in Figure 4, and, in both cases, the plots reveal a good agreement between the predicted survival curve for each temperature and its observed data (i.e., all the observations lay well within the 95% confidence bands).

Such agreement was further supported by the bias factors (1.05 and 0.84) and accuracy factors (1.23 and 1.25) of each model fitted (56.5 and 62.5 °C, respectively). While the accuracy factors suggest that, on average, predictions are 1.23 to 1.25 times the value of observations, i.e., there is similar discrepancy between observed and predicted values at different temperatures, the values of the bias factors suggest that the ability of the model to accurately estimate the microbial concentrations is dependent on the temperature for which the predictions are made. In this case, it

seems that when the model was adjusted for a temperature closer to the limits of the range of temperatures tested (62.5 °C, in this case), it became less capable of accurately making predictions, and instead, underestimated the microbial concentrations. Nevertheless, it is important to note that the omnibus model is capable of making accurate predictions for temperatures that were not used to build the model (Figure 4), while the two-step approach revealed some difficulty in adequately fitting two of the temperatures included in the data set used to produce the model: 61 and 64 °C (Figure 1). This fact strongly supports the superiority and advantage of the global modelling approach.

4. Discussion

Comparing the estimates of Tables 2 and 3, there was general agreement between the outcomes of the secondary and the omnibus models. Nonetheless, our work shows that the omnibus approach is better at avoiding loss of information and error propagation, as occurs with the two-step method, which is reflected in the lower standard errors associated with the model estimates. Moreover, the global approach allows to identify potential systematic errors in a dataset from one environmental condition and to explore them through an appropriate choice of fixed and random effects incorporated in the model (Pennone et al., 2021).

To our knowledge, this is the first work using modelling to obtain *S. aureus* kinetics in goats' raw milk at different sub-pasteurisation temperatures, and it contributes to the body of work using predictive microbiology to describe pathogen heat-inactivation in milk, which is scarce, particularly if pasteurisation studies are disregarded. Lehotová et al. (2021) studied the heat resistance of *S. aureus* in the 57–61 °C temperature range using the capillary method and broth containing glucose, tryptone and yeast extract. Then, the authors modelled the bacterial survival and estimated the fourth decimal reduction time t_{4D-} and *z*-values through log-linear Bigelow and non-linear Weibull models. Although these models are useful, their accuracy to predict the real behaviour of bacteria in foods may be questioned, as using experimental data from homogeneously well-mixed broth media implies disregarding the food microstructure and composition

(Verheyen et al., 2019). For this reason, in our work, raw milk was chosen over broth media, aiming to produce meaningful estimates that may be used in real life applications.

Other authors have reported on the effects of heat treatments for pathogen inactivation using raw milk instead of broth media, but usually the results are conveyed as a comparison of microbial populations before and after the treatment, or as decimal reduction values (D-values). Samelis et al. (2009), for example, observed an effective reduction of coagulase-positive staphylococci, from 3.3 log CFU/mL to < 2 log CFU/mL, when applying thermisation treatments of 60 °C and 67 °C for 30 seconds to a mixture of ewe's and goat's milk (90:10). Zottola et al. (1969) applied sub-pasteurisation treatments of 147 to 150 °F (63.8 to 65.6 °C) for 16 to 21 seconds to raw milk, and reduced *S. aureus* concentration to such an extent that the pathogen was undetected. In turn, Engstrom et al. (2021) determined and validated D-values for *L. monocytogenes* and STEC in raw milk at thermisation temperatures of 65.6, 62.8 and 60.0 °C (also at 57.2°C for *L. monocytogenes* only). The results from such studies are also valuable and validate the usefulness of thermal treatments for pathogen control and improved food safety. However, they do not enable predictions nor interpolations for other temperatures, which is an advantage of using predictive modelling.

5. Conclusions

The present study estimated the inactivation parameters of *S. aureus* in goats' raw milk at several thermisation temperatures using the Weibull model in two distinct approaches: two-step modelling vs. omnibus modelling.

The results showed that the temperature influenced the time needed for the first decimal reduction, as expected, and produced distinct physiological effects on the pathogenic cells, as suggested by the different values of the shape parameter β. A quadratic relationship was found between each of the parameters of the Weibull model and the temperature, meaning that the effect of temperature is not constant over the range tested.

Validation of the models produced at temperatures within the models' domain was performed successfully, demonstrating their aptitude to predict inactivation kinetics of *S. aureus* in

goats' raw milk. Nonetheless, the omnibus approach showed improved parameter estimation, considering the reduced standard errors associated, and revealed its value as a complementary approach to the traditional two-step modelling by enabling further exploration and insight of the experimental inactivation data.

The models described in this work can be used to design lethality treatments to achieve specific reductions of *S. aureus* in goats' raw milk, thus contributing to the enhancement of the microbiological quality and safety of raw milk cheeses.

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Table 1. Kinetic parameters of the Weibull decay model describing *S. aureus* behaviour in goats' raw milk heated at different thermisation temperatures (°C)

Thermisation temperature (°C)	Y_0 [1]	χ [2]	β [3]
55	6.819 ± 0.054 *	37.92 ± 1.036 *	2.241 ± 0.221 *
58	6.821 ± 0.006 *	9.703 ± 0.301 *	1.145 ± 0.058 [ns]
61	6.907 ± 0.169 *	0.573 ± 0.076 *	0.630 ± 0.041 [ns]
64	6.734 ± 0.008 *	0.322 ± 0.017 *	0.936 ± 0.053 [ns]

[1] Y_0: initial counts (log CFU/mL); [2] χ: scale parameter (minute); [3] β: shape parameter (dimensionless) (these parameters were expressed as means and standard error). Asterisks (*) represent the significance of the estimated parameter at $p < 0.05$; ns: non-significant ($p > 0.05$).

Table 2. Parameter estimates of the secondary models predicting the square root transformed parameters χ and β in goats' raw milk as a function of temperature (°C)

Parameters	**Mean**	**Standard error**	**Pr > |t|**	**AIC/BIC**
Predictors of $\sqrt{\chi}$ ($min^{0.5}$)				
a_1 (Intercept)	40.59	5.322	0.0003	23.7/24.0
a_2 (Temperature)	-0.638	0.089	0.0004	
Variance				
s^2 (residual)	0.615		Adj. R^2	0.895
Predictors of $\sqrt{\beta}$				
b_1 (Intercept)	4.776	1.137	0.006	-0.95/-0.71
b_2 (Temperature)	-0.062	0.019	0.017	
Variance				
s^2 (residual)	0.028		Adj. R^2	0.639

Table 3. Parameter estimates of the mixed-effects omnibus model predicting the non-log-linear decay of *S. aureus* in goats' raw milk as a function of temperature (°C)

Parameters	Mean	Standard error	Pr > |t|	AIC/BIC
Predictors of $\sqrt{\chi}$ ($min^{0.5}$)				
a_1 (Intercept)	39.62	4.618	0	
a_2 (Temperature)	-0.622	0.077	0	71.8/84.0
b	1.053	0.088	0	
$Y_{0\ mean}$	6.840	0.058	0	
Variances				
s_u^2 ($\sqrt{\beta}$)	0.236			
s_v^2 (a_1)	0.716			
s^2 (residual)	0.181		Adj. R^2	0.996

Figure 1. *S. aureus* experimental observations (markers), mean predicted values (full line) and 95% confidence intervals (dashed lines), as obtained by the two-step modelling approach, in goats' raw milk heated at 55, 58, 61 and 64 °C over time. For each temperature, same markers represent observations from the same experiment (n=2).

Figure 2. Mean (full line) and 95% confidence intervals (dashed lines) of the effect of temperature (°C) on the square root transformed scale parameter χ (left) and shape parameter β (right). For each temperature, different markers represent χ values obtained from different experiments (n=2).

Figure 3. Mean (full line), 95% confidence intervals (dark grey) and 95% prediction intervals (light grey) of the concentration of *S. aureus* in goats' raw milk treated at 56.5 °C against time, as predicted by the two-step modelling approach. For each time point, a marker represents the mean of two replicates (n=2).

Figure 4. Mean and 95% confidence intervals of the concentration of *S. aureus* in goats' raw milk against time, as predicted by the omnibus model. Model external validation for temperatures 56.5 and 62.5 °C is shown (left to right). For each temperature, same markers represent observations from the same experiment (n=2).

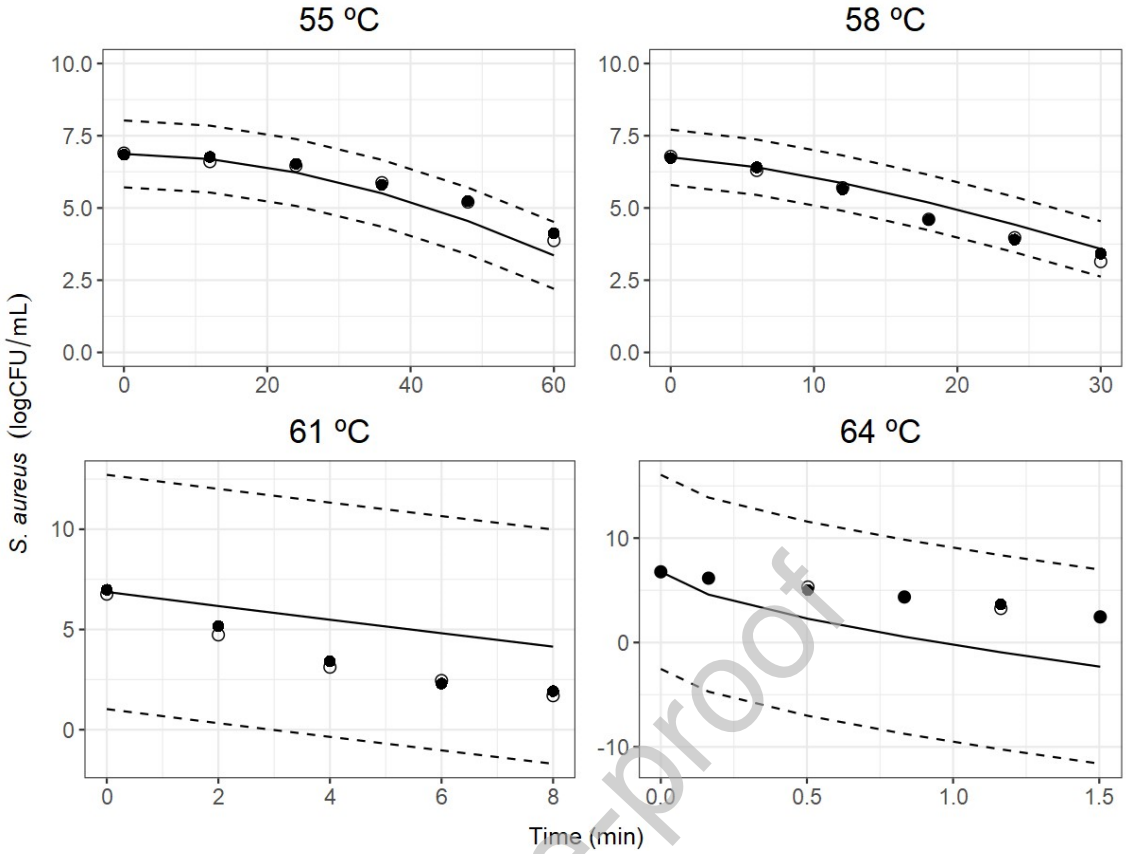

Figure 1

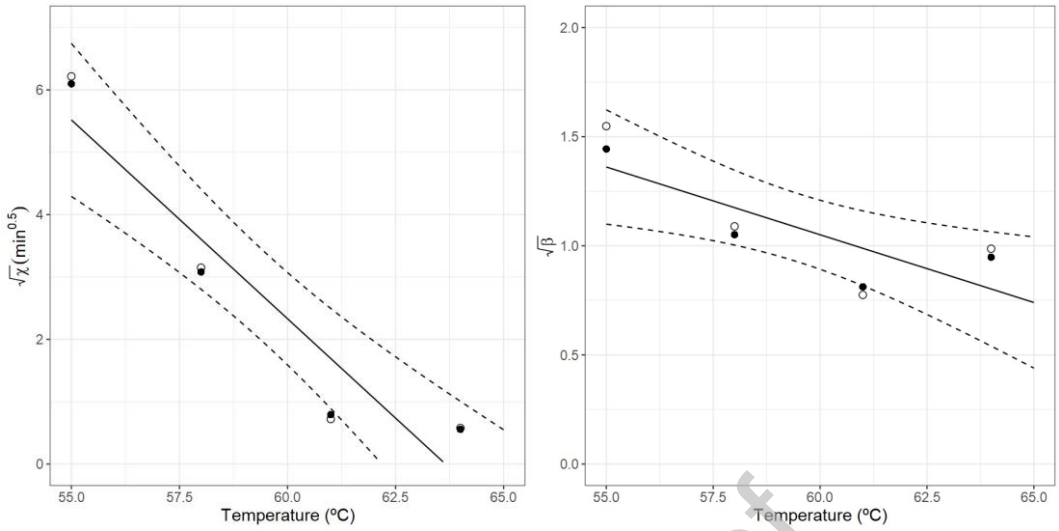

Figure 2

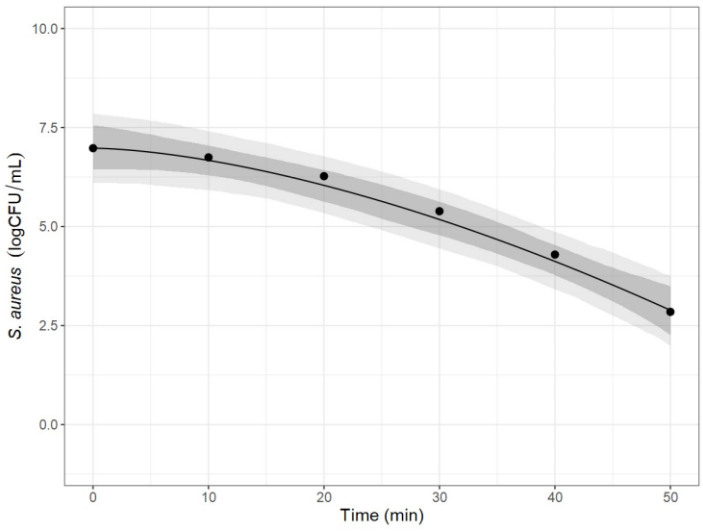

Figure 3

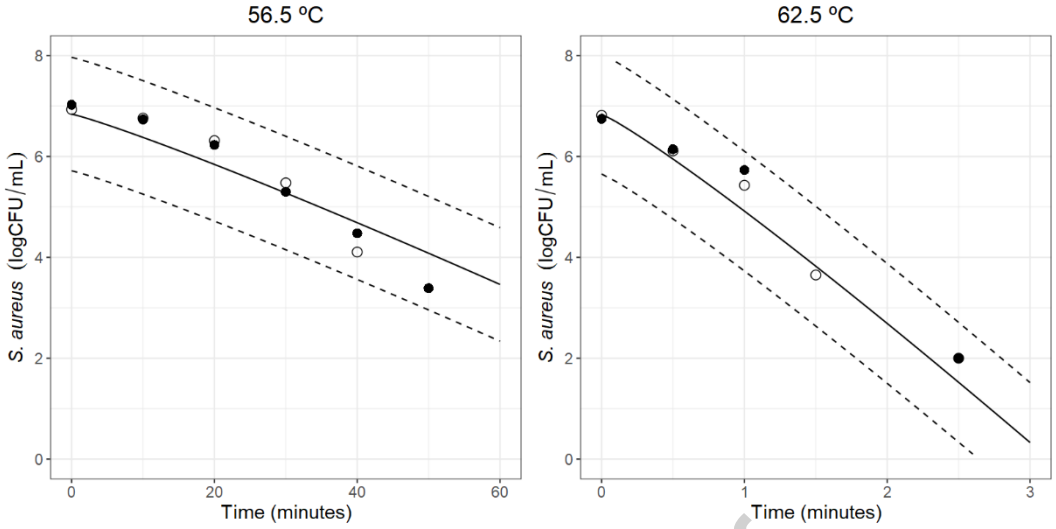

Figure 4

Author Contributions: Conceptualization, U.G.-B. and V.C.; methodology, S.C.-F., V.C. and U.G.-B.; software, B.N.S., U.G.-B. and V.C.; validation, B.N.S., U.G.-B. and V.C.; formal analysis, B.N.S., U.G.-B. and V.C.; investigation, B.N.S. and S.C.-F.; resources, U.G.-B. and V.C.; data curation, B.N.S. and U.G.-B.; writing—original draft, B.N.S. and U.G.-B.; writing—review and editing, B.N.S. and U.G.-B.; visualization, B.N.S., J.A.T., U.G.-B. and V.C.; supervision, U.G.-B. and V.C.; project administration, U.G.-B. and V.C.; funding acquisition, U.G.-B. and V.C. All authors have read and agreed to the published version of the manuscript.

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