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Modelling the inactivation of *Staphylococcus aureus* at moderate heating temperatures

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Abstract: The survival of bacterial contaminants at moderate processing temperatures is of interest to many food producers, especially in terms of the safety and quality of the final products. That is why the heat resistance of *Staphylococcus aureus* 2064, an isolate from artisanal Slovakian cheese, was studied in the moderate temperature range (57–61 °C) by the capillary method. The fourth decimal reduction time t_{4D}- and z-values were estimated in two steps by traditional log-linear Bigelow and non-linear Weibull models. In addition, a one-step fitting procedure using the Weibull model was also applied. All the approaches provided comparable t_{4D}-values resulting in the following z-values of 11.8 °C, 12.3 °C and 11.3 °C, respectively. Moreover, the one-step approach takes all the primary data into z-value calculation at once, thus providing a more representative output at the reasonable high coefficient of determination R^2 = 0.961.

Keywords: isothermal inactivation; *S. aureus*; capillary method; predictive microbiology

The production of foods with microbiological quality and safety requires the identification of the crucial processes when potential occurrence and reproduction of undesirable microorganisms are minimal, and the subsequent processes lead to a reduction in their numbers, if any. There are many technological processes to achieve the microbial inactivation or removal, such as thermal processes (den Besten et al. 2018; Van Impe et al. 2018). The efficacy of heat treatment to inactivate vegetative cells or spores depends on several factors including heating temperature, time and microbial heat resistance characteristics (Cebrián et al. 2017). For example, some Slovakian pasta filata cheeses are manufactured from raw milk curd, which is, except for pathogenic *Escherichia coli* and *Listeria monocytogenes*, related also to occurrence of enterotoxinogenic *Staphylococcus aureus*. In the commercial Slovakian pasta filata cheeses produced from raw milk, *S. aureus* can be present at the level up to 4.76 log CFU g^{-1} (Šipošová et al. 2020). In the traditional method of pasta filata cheese production, the steaming and stretching of the curd at 60 to 70 °C are the only heat treatments used. Therefore, acquiring the information about the capabilities of relevant vegetative bacteria to survive a moderate heat treatment applied during the stretching process is important. In this context the aim of our work was to characterize the heat resistance of *S. aureus* using three different predictive inactivation models. These allow to estimate the significant inactivation parameters and to define heat resistance of *S. aureus* at various temperatures. Concurrently, we compared and evaluated the used fitting methods to each other.

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MATERIAL AND METHODS

Preparation of bacterial culture and heat treatment method. An isolate of *Staphylococcus aureus* 2064 originated from Slovakian small-scale sheep lump cheese was used in this study. *S. aureus* 2064 was isolated by Dr. Hanzélyová (State Veterinary and Food Institute, Prešov, Slovakia) by using the cultivation method according to EN ISO 6888-1:2001. Its identity was confirmed by Gram staining, catalase test, API Staph system (bioMérieux, Marcy-l'Etoile, France), fluorescent dyeing using VIT *Staphylococcus* (Vermicon, Munich, Germany) and PCR analysis of the *nuc* gene in accordance with Akineden et al. (2008), Boynukara et al. (2008) and Pereira et al. (2009). Prior to the experiment, the bacterial culture was grown in Brain Heart Infusion Broth (BHI; Sigma-Aldrich, Steinheim, German) at 37 ± 1 °C for 18 h. Cells from the stationary phase were inoculated into the broth containing glucose, tryptone and yeast extract (GTYE broth; Biokar Diagnostics, Beauvais, France) at the initial level of approximately 8 log CFU mL^{-1}. From that medium *S. aureus* 2064 was displaced into glass capillary tubes. The total volume of inoculated GTYE broth in each capillary tube was 20 µL. The inactivation experiments were carried out in isothermal conditions (water bath) at 55 °C for 60 min, 57 °C for 120 min and 61 °C for 180 min in four parallels. The sampling was performed every 15 min during the whole heat treatment experiment. After removing the glass capillary tube from the water bath, it was immersed promptly into an ice bath. Upon cooling, the determination of *S. aureus* counts was immediately performed by using the cultivation method.

Quantification of *S. aureus*. The bacterial inoculum density was determined by a spread plate technique on GTYE agar (Biokar Diagnostics, Beauvais, France) at predefined time intervals of the experiment, subsequent incubation (at 37 ± 1 °C, 24 h, aerobic static) and calculating the initial cell concentration.

Modelling of thermal inactivation of *S. aureus* and statistical analysis. Two different fitting approaches (two- and one-step) were used to model the inactivation data set of *S. aureus* 2064. The two-step approach at first fitted the primary model to estimate the fourth decimal reduction time t_{4D}.

In this study the following primary models were used: (1) Bigelow log-linear model according to Bigelow and Esty (1920), (2) Weibull model according to Metselaar et al. (2013). Both primary models were fitted to GInaFit software (version 1.7 for Microsoft Excel; Geeraerd et al. 2005) and corresponding primary kinetic parameters were calculated. Then, the second step of the two-step procedure was used to calculate the *z*-value (the temperature increase needed to reduce the t_{4D}-value by a factor 10). When log t_{4D}-values are plotted against temperature, the reciprocal of the slope is equal to *z*-value ($z = -1/\text{slope}$). The one-step model we used to fit the Weibull model according to den Besten et al. (2017) to estimate t_{4D} along with *z*-value. The goodness of fit and variation between the experimental data set and used mathematical models were evaluated by estimating the root mean sum of square errors (RMSSE) and coefficient of determination (R^2).

RESULTS AND DISCUSSION

The lethality curves of *S. aureus* 2064 obtained in GTYE broth at temperatures 55, 57 and 61 °C in four parallels of capillary glass tubes were evaluated by two different modelling approaches: (1) two-step procedure fitting at first the primary (kinetic) model and then the effect of the environmental variables on the kinetic parameters, (2) one-step procedure directly fitting the data set to a combined model in one regression. The use of capillary tubes aimed to stimulate the inactivation kinetics of cells directly affected by heat and faster heat transfer.

As shown in Figure 1A and 1B, primary predictive models describe microbial survival as a function of time, and the major data output is t_D value, which means the time needed to reduce the microbial numbers with one log reduction. Considering the nonlinearity of obtained lethality curves, we estimated the fourth decimal reduction time t_{4D}, which has also practical relevance (Table 1). As expected, the heat resistance of isolate 2064 decreased with increasing temperature. According to the Bigelow model t_{4D}-values of 122.3 min ($n = 28$), 71.9 min ($n = 28$) and 37.0 min ($n = 25$) were calculated at 55, 57 and 61 °C, respectively. Markedly lower *D*-values were reported in the study of Kennedy et al. (2005) at similar heating temperatures. Using a linear regression analysis, they estimated D_{55}-values in the interval from 13 to 21.7 min and D_{60}-values from 4.8 to 6.6 min for the suspension of five *S. aureus* strains in Tryptic Soy Broth (TSB). On the contrary, Amado et al. (2014) defined that in cattle feed and at a similar heat load, 66.3 min, 35.7 min and 26.9 min were required to achieve a reduction of one log CFU g^{-1} in *S. aureus* counts at 55 °C, 57.5 °C and 60 °C, respectively. Observed differences may be attributed to the influence

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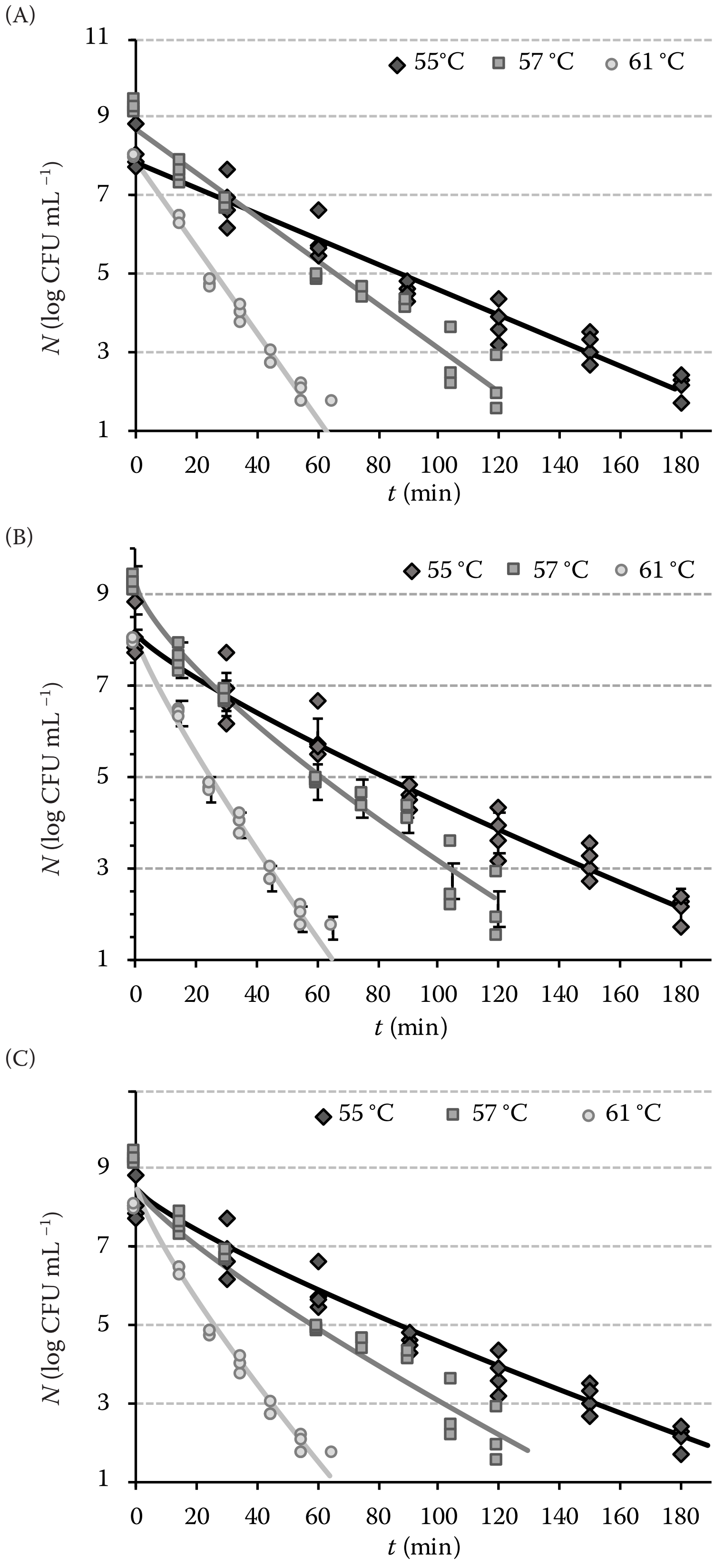

Figure 1. The lethality curves of *S. aureus* 2064 in GTYE broth as modelled by primary Bigelow (A), two-step Weibull (B) and one-step Weibull (C) models

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Table 1. The model parameters describing heat resistance of *S. aureus* 2064 in GTYE broth and corresponding statistical indices

Model	T (°C)	N_0 ± SE (log CFU mL^{-1})	δ ± SE (min)	p ± SE	k_{max} ± SE (min^{-1})	n	RMSSE	R^2	t_{4D} (min)	z (°C)	SE	R^2
Bigelow (two-step)	55	7.86 (± 0.16)	–	–	0.075 (± 0.003)	28	0.461	0.951	122.3	11.83	0.046	0.984
	57	8.66 (± 0.16)	–	–	0.128 (± 0.005)	28	0.480	0.960	71.9			
	61	7.18 (± 0.12)	–	–	0.249 (± 0.007)	25	0.307	0.980	37.0			
Weibull (two-step)	55	8.15 (± 0.21)	20.49 (± 4.59)	0.825 (± 0.079)	–	28	0.436	0.958	111.6	12.31	0.087	0.942
	57	9.17 (± 0.19)	8.86 (± 1.96)	0.739 (± 0.059)	–	28	0.400	0.973	58.8			
	61	8.04 (± 0.14)	6.85 (± 0.89)	0.865 (± 0.049)	–	25	0.274	0.985	34.5			

Model	T (°C)	N_0 ± SE (log CFU mL^{-1})	p	t_{4D} (min)	z (°C)	n	RMSSE	R^2
Weibull (one-step)	55, 57, 61	8.47 (± 0.64)	0.816	68.8	11.28	81	0.445	0.961

N_0 – initial microbial numbers; δ – the time needed to obtain the first decimal reduction of the first microbial subpopulation at temperature T; p – shape parameter; k – inactivation rate constant; t_{4D} – the time needed to obtain the fourth decimal reduction of microbial population at T; z – the temperature increase required to reduce t_{4D} by 90%; n – the number of data sets; RMSSE – root mean sum of square errors; R^2 – coefficient of determination

of environmental conditions, such as nutritional composition of the media or pH, a_w, temperature, and the physiological variability between *S. aureus* strains, as in the case of *Listeria monocytogenes* was proposed by Garre et al. (2020). Considering the environmental and nutritional composition, Shebuski et al. (2000) and Zhang et al. (2018) confirmed the increase in heat resistance of *S. aureus* by addition of an osmotic active compound into the medium. In these cases, microbial behaviour prediction by the log-linear Bigelow model could be less reliable as many microbial survival curves do not follow first-order kinetics. One of the causes of the concave/convex shape of lethality curves is the presence of the subpopulations that differ in heat resistance from each other in given conditions (den Besten et al. 2018). It was confirmed in our case as well as by Mafart et al. (2002), Coroller et al. (2006) and Daryaei et al. (2018).

Assuming the nonlinearity of survival curves, Weibull model, as the primary inactivation model, was used. By its application the lower $t_{4D,55°C}$ = 111.6 min (n = 28), $t_{4D,57°C}$ = 58.8 min (n = 28) and $t_{4D,61°C}$ = 34.5 min (n = 25) were estimated compared with t_{4D}-values obtained by the previous log-linear model. The higher coefficients of determination R^2 ranged from 0.958 to 0.985 and lower RMSSE values were calculated. If we take these statistical values into account, we can demonstrate that parameters of Weibull model give better fitted values to the experimental data set. The reason is the variation in parameter p (listed in Table 1) which allows the Weibull model to be more flexible than the log-linear model.

In order to determine the secondary inactivation parameter, the obtained log t_{4D} values were plotted versus temperature and linear regression was applied to obtain the z-value. As shown in Table 1, we estimated z = 11.8 °C (R^2 = 0.984) and 12.3 °C (R^2 = 1) based upon t_{4D}-values calculated by Bigelow and Weibull model, respectively. Consequently, to reduce t_{4D}-values by one log order we need to increase the temperature of the heating medium by approximately 12 °C for *S. aureus* 2064 isolate. Contrary to our z-values, lower z-values were reported by Dewanti-Hariyadi et al. (2011). Using the same modelling method, they estimated z-values from 3.4 to 6.1 °C for *S. aureus* AS2, NU3 and ATCC 25923 in TSB medium. In addition, Kennedy et al. (2005) reported, in the same heating medium, slightly higher z-values in the range of 7.7–8.0 °C. Considering similar nutrient medium and temperature conditions (50–60 °C) used in both our experiments and comparable studies, it can be argued that the differences

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between observed z-values were caused by different *S. aureus* origin.

The third method of inactivation modelling was a one-step fitting procedure used to fit the Weibull model to the data sets of *S. aureus* 2064 isolate (n = 81). While the two-step process is a standard approach, it has its drawbacks. A significant disadvantage of two-step approach could be the accumulation and propagation of errors in each step (primary/secondary modelling) of data analysis during the model development (Huang 2017). The alternative one-step method fitted the obtained lethality curves at the three applied temperatures in one regression (Figure 1c), and this resulted in one parameter per crop (den Besten et al. 2017). In this way, we estimated one value of log N_0 and p per total temperature range in accordance with Valdramidis et al. (2008). The calculated t_{4D}-values of isolate 2064 at 55 °C, 57 °C and 61 °C were 103.5 min, 68.8 min and 30.4 min, respectively. The z-value of isolate 2064 in GTYE broth was 11.3 °C. No significant differences ($P < 0.05$) were observed, and the statistical analyses showed a relative high correlation (R^2 = 0.961; RMSSE = 0.445) comparable with two-step approach. Nevertheless, many studies have been published to discuss and confirm whether one-step approach gives better fits to data and to reduce standard errors or not (Valdramidis et al. 2008, den Besten et al. 2017; Huang 2017).

By applying the calculated t_{4D}-values in prediction of *S. aureus* 2064 survival during the steaming and stretching process mentioned above, we could predict that the time needed to reduce *S. aureus* 2064 by 1 log CFU g^{-1} is 4.3 min at a temperature of approximately 65 °C (used during steaming and stretching of pasta filata cheeses). Then, to reduce *S. aureus* 2064 by 2 log CFU g^{-1}, a minimum of 8 to 9 min of heat treatment is required. Based on the real steaming processing conditions, when steaming usually takes 5 to 10 minutes, reduction of *S. aureus* counts higher than two logs will not be expected.

Importantly, the cheese is a complex matrix. The chemical composition, nutritional and physicochemical properties of cheese may also significantly influence the thermal resistance of microorganisms. The heat resistance of *S. aureus* and other pathogenic and technologically undesirable microorganisms can be primarily affected and controlled by addition of sodium chloride or reduction of pH value during the cheese curd preparation. The presence of sodium chloride causes the reduction in a_w of the media and the microbial cell loss of intracellular water (Cebrián et al. 2017). Based on previous inactivation experiments, addition of only 1% NaCl resulted in the enhancement of heat resistance of *S. aureus* 2064 in the model environment (Lehotová et al. 2018). In addition, the fermentation of the curd before the steaming and stretching process is also accompanied by a decrease in the pH value from 5.2 to 4.9. Contrary to the effect of a_w, changes of pH value out of the optimal values decrease the heat resistance of *S. aureus*. Montanari et al. (2015) observed increasing susceptibility of three *S. aureus* strains to heat treatment (80 °C for 20 min), when these strains were grown at pH of 6.5, 5.5 and 4.5, respectively. Importantly, the exposure of a microorganism to several sublethal stresses, such as acid, heat and salt can modify its response to other subsequent stresses, with a mechanism known as cross-protection or cross-resistance response (Van Impe et al. 2018).

Tolerance of staphylococci to heat treatment within the laboratory preparation of Slovakian pasta filata cheeses from raw cow's milk was confirmed also by Medveďová et al. (2020). The reduction of coagulase-positive staphylococci was calculated at the level 0.75 ± 1.00 log CFU g^{-1} at 63–65 °C for 5–10 min. In Kashkaval, Greek pasta filata cheese, Samelis et al. (2019) reported approximately 3 log CFU g^{-1} reduction of total staphylococci after the stretching of the curd at a significantly higher temperature of ~80 °C.

CONCLUSION

The present study demonstrates the impact of moderate heat-treatment on survival parameters of *Staphylococcus aureus* isolated from artisanal cheese curd. Depending on the inactivation data set found at moderate inactivation temperatures, we may recommend the application of one-step Weibull model proposed by den Besten et al. (2017) that may give the most precise predictions of *S. aureus* inactivation. It combines the features of primary and secondary modelling with t_{4D} and z-value as the parameters, and not only in the modelling of bacteria inactivation at high temperatures, but also in a moderate temperature range that can be taken into account during non-thermal technologies in food industry. The obtained results could help professionals to focus not only on the efficacy of key thermal processes during artisanal pasta filata cheese practice, but also other alternative processes that are accompanied by a moderate temperature increase in backgrounds, like ultrasonication or those that reduce the multiplication of *S. aureus* during cheese curd manufacture, like addition of a sufficient amount of metabolically active lactic acid bacte-

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ria. In addition, together with further planned studies dealing with the analysis of the effect of other factors affecting the survival of *S. aureus*, e.g. water activity, pH and their mutual combinations, during the production of pasta filata cheeses it would be possible to decrease the risk of staphylococcal intoxications.

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