Evaluation of *Staphylococcus aureus* growth and staphylococcal enterotoxin production in delicatessen and fine bakery products

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Abstract

Staphylococcal food poisoning is one of the most prevalent causes of foodborne intoxication worldwide. Sandwiches and desserts are susceptible to contamination by *S. aureus* due to the high proportion of manual work during their production. Our study aimed to evaluate the impact of storage conditions on staphylococcal enterotoxin production in sandwiches and buttercream puffs. Foods were inoculated with different *S. aureus* strains capable of producing type A, B, and C staphylococcal enterotoxins and incubated at 15, 25, and 30 °C. During the storage, samples were analysed for *S. aureus* counts and for staphylococcal enterotoxins. An enzyme-linked fluorescence assay was used to detect staphylococcal enterotoxins. The influence of inappropriate storage on *S. aureus* growth and staphylococcal enterotoxin production was evaluated. No staphylococcal enterotoxins were detected in sandwiches stored for 72 h at any of the tested temperatures. In buttercream puffs, enterotoxins type A, B, and C were detected within 24 h of storage at 25 °C.

Food safety, bacterial toxins, predictive microbiology, Baranyi-Roberts model, linear model

Bacterial toxins can cause a wide range of food poisonings. One of the most common food poisonings is the staphylococcal enterotoxicosis (staphylococcal food poisoning, SFP) caused by *Staphylococcus aureus* strains capable of staphylococcal enterotoxins (SEs) production directly in the foods (Hennekinne et al. 2012). The most common cause of SFP is the classic types of SEA-SEE. The characteristics in detail are known mainly for SEA, SEB, and SEC, which were also used in this study.

Delicatessen and fine bakery products count among the riskiest foods from the perspective of possible *S. aureus* occurrence. One of the reasons is a high degree of manual labour during their preparation, representing the principal source of *S. aureus* contamination in these types of foods. *Staphylococcus aureus* naturally colonizes the skin and nasopharyngeal region in approx. 30–50% of the population (Soriano et al. 2002). Soriano et al. (2002) described certain types of foods (chicken salad, sandwiches, ham, scrambled eggs, etc.), which were identified as causes of catering-associated SFP. Alhashimi et al. (2017) proved the presence of *S. aureus* in nasopharyngeal swabs in 30.1% of food handlers. Chaves et al. (2018) isolated enterotoxigenic strains of *Staphylococci* from swabs taken from six types of surfaces in catering establishments as well as in home kitchens (sink, fridge, cooker, cutting board, knives, towels) as well as from the hands and mucosal surfaces of cooks/workers on these premises. As reported by Bogdanovičová et al. (2019) in their study on the catering establishments, the deli and fine bakery products can be contaminated by employee hands and the premises themselves. *Staphylococcus aureus* were identified in 17.9% of swabs of the surfaces on the premises or employees' hands; genes encoding SE production were found in 58.5% of these (70.0% from hands swabs, 52.0% from surfaces).

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Sundararaj et al. (2019) isolated 34 *S. aureus* samples from 100 samples of ready-to-eat foods; in 14 of those, strains capable of production of staphylococcal enterotoxin B (SEB) were detected.

Forty two cases of staphylococcal food poisoning were caused by foods from a single catering establishment producing pasta, tomatoes, fish fingers and yoghurt (Solano et al. 2013). Soares et al. (2019) evaluated the microbiological quality of foods served in 20 catering establishments in northern Portugal. The highest numbers of microbiological hygiene indicators (*Escherichia coli*) and pathogens (*S. aureus*) were detected in sandwiches, salads and pastry. Reasons for such contamination may include unsuitable disinfection methods, cross-contamination, and absence of any thermal treatment.

The presented study aimed to evaluate the growth and multiplication of enterotoxigenic strains of *S. aureus* in model deli and fine bakery products. Special attention was paid to the assessment of storage conditions and their influence on the production of SEs and, therefore, on the risk posed to potential consumers of such foods.

Materials and Methods

Food samples and their inoculation

Open sandwiches (n = 36) containing French loaf, butter, Edam cheese and cooked ham (initial pH = 5.55–5.71; a_w = 0.946–0.964) were chosen as representatives of the delicatessen products. The open sandwiches were prepared in the laboratory immediately before the beginning of the experiment from purchased retail products. Buttercream puffs (n = 36), selected as the model food from the category of fine bakery products (initial pH = 5.96–6.15; a_w = 0.952–0.965) were purchased on the market at a local producer declaring the following ingredients: wheat flour, eggs, water, salt, vegetable oil, dried milk powder, butter, sugar, cream powder (corn flour, aroma, β-carotene, lemon yellow), vanillin sugar (aroma - ethylvanillin), fondant (sugar, glucose syrup, water). The samples were tested for the presence of *S. aureus* (enrichment in buffered peptone water, isolation on Baird-Parker agar with egg yolk emulsion and tellurite (Oxoid, Ltd., Basingstoke, UK); bacteria were not detected in any of the 25 g samples.

Three strains of *S. aureus* producing SEs, namely *S. aureus* No. 562 (SEA producing strain), *S. aureus* CCM 5757 (SEB) and *S. aureus* CCM 5971 (SEC) were aerobically cultured on blood agar at 37 °C for 24 h. Subsequently, a bacterial suspension in sterile saline was prepared for each strain, with a density of approx. 8 log cfu·ml^{-1}; these partial suspensions were subsequently mixed at a 1:1:1 ratio. The resulting suspension mix was homogenized by stirring, diluted as needed and used for inoculation of food samples. The resulting initial *S. aureus* concentrations in the samples were 2.54–3.48 log cfu·g^{-1} in open sandwiches and 1.70–3.58 log cfu·g^{-1} in buttercream puffs. Four replicates were prepared for each storage temperature and sample type; three replicates were always inoculated with the mixed suspension and the fourth sample served as a blank.

Inoculated samples were homogenized using a stomacher homogenizer and kept in sterile bags at temperatures simulating cold chain disruption (15, 25, and 30 °C) for 72 h. Partial food samples were aseptically taken immediately (0 h) and 6, 12, 24, 31, 48, 55 and 72 h after inoculation; 10 g were taken at each time point.

Determination of coagulase-positive staphylococci

Enumeration of coagulase-positive staphylococci (*S. aureus* and other species) in the partial samples was performed by the ISO 6888-1 (1999) horizontal method using Baird-Parker agar with egg yolk emulsion and tellurite. Throughout the experiment, the Staphylo La Seiken test (Denka Seiken Co., Ltd., Tokyo, Japan) was used for the identification of *S. aureus*.

Enumeration of mesophilic lactic acid bacteria

Besides staphylococci, mesophilic lactic acid bacteria (LAB) were also enumerated in the partial samples using the horizontal method according to ISO 15214 (2000) on De Man, Rogosa and Sharpe agar (Oxoid, Ltd., Basingstoke, UK).

Detection of staphylococcal enterotoxins

The SE content was analyzed in the partial samples by enzyme-linked immunofluorescence assay (ELFA) using the miniVIDAS® automated system (Vitek Immuno Diagnostic Assay System, BioMérieux, Marcy l'Étoile, France). This method is capable of detecting SEA–SEE enterotoxins (without specification of individual types) with a detection limit of 0.5 ng·g^{-1} or ml^{-1} of food for SEA and SEB, and 1.0 ng·g^{-1} or ml^{-1} of food for SEC–SEE. Partial samples (25 g) were homogenized with extraction buffer (25 ml), processed according to manufacturer's instructions and analysed using the VIDAS SET2 strip test, with test values (TVs) ≥ 0.13 indicating a positive result.

Determination of the pH value and water activity

A microprocessor pH meter 211 (Hanna Instruments, Woonsocket, Rhode Island, USA) was used to determine the pH value from the aqueous infusion (10 g sample + 100 ml deionized water, 15 min at room temperature). The pH value was measured at a temperature of 25 °C. Water activity was determined using a LabMaster water activity meter (Novasina AG, Lachen, Switzerland) at a temperature of 25 °C.

Statistical analysis

The obtained experimental data (cfu·g^{-1}) were $\log_{10}$ transformed, and the mean values and standard deviations were calculated. The growth of *S. aureus* in sandwiches at 15 °C and 30 °C was estimated by a linear model; at 25 °C and in desserts stored at all temperatures by the Baranyi-Roberts model (Baranyi and Roberts 1994) using the 'nlsMicrobio' library (Baty and Delignette-Muller 2014) in R software. Parameters such as the duration of the lag phase, exponential growth rate, and the final *S. aureus* count were used to interpret the model. The growth of LAB in both food products was fitted by a linear model.

Results

Initial values of both internal and external factors in foods tested in this study (open sandwiches: pH = 5.75–5.89, a_w = 0.954–0.965; dessert/buttercream puffs: pH = 6.89–6.94, a_w = 0.945–0.962) supported SE formation. After 72 h, pH decreased to 5.42–5.69 in open sandwiches and 4.08–4.34 in puffs. A decrease in pH below 4.5 (limiting for SE production) occurred, therefore, only in buttercream puffs; even that happened only on the last day of storage (i.e., between 48–72 h). Water activity in open sandwiches decreased to 0.934–0.946 after 72 h while it remained within the range of 0.942–0.966 in desserts.

The *S. aureus* growth and multiplication in open sandwiches and fine bakery products with buttercream at the temperatures of 15, 25 and 30 °C is characterised by growth curves created using Baranyi-Roberts and linear models for individual storage temperatures (Fig. 1). *Staphylococcus aureus* did not grow in open sandwiches stored at 15 °C and only negligible growth from 2.72 to 3.62 log cfu·g^{-1} was observed at 30 °C. In open sandwiches stored at 25 °C, however, the growth was more pronounced. In buttercream puffs, *S. aureus* growth was observed at all experimental temperatures. The growth was relatively slower at 15 and 30 °C; similarly to open sandwiches, the growth was the fastest at 25 °C (Table 1). The assumption that the fastest growth of *S. aureus* population would occur at 30 °C was, therefore, not confirmed and the fastest growth (as well as the highest counts of *S. aureus* at the end of the study period) was observed in both open sandwiches and buttercream puffs at 25 °C (Fig. 1, Tables 1 and 2).

Table 1. Mean ± standard deviation of *Staphylococcus aureus* count (log cfu·g^{-1}) in sandwiches and desserts inoculated with 3 log cfu·g^{-1} and stored for 72 h at 15 °C, 25 °C and 30 °C.

Time (h)	*S. aureus* count (log cfu·g^{-1})					
	Sandwich			Dessert		
	15 °C	25 °C	30 °C	15 °C	25 °C	30 °C
0	3.37 ± 0.09	3.06 ± 0.23	2.72 ± 0.16	2.71 ± 0.10	2.19 ± 0.50	3.11 ± 0.42
6	3.37 ± 0.17	3.32 ± 0.06	2.93 ± 0.10	2.45 ± 0.05	2.89 ± 0.13	2.81 ± 0.10
12	3.33 ± 0.13	4.18 ± 0.24	2.64 ± 0.15	2.49 ± 0.18	3.51 ± 0.45	3.61 ± 0.13
24	3.20 ± 0.41	4.08 ± 0.17	3.09 ± 0.65	3.27 ± 0.11	5.54 ± 0.06*	4.72 ± 0.12
31	3.25 ± 0.09	4.96 ± 0.10	3.09 ± 0.36	3.47 ± 0.16	6.79 ± 0.16*	4.65 ± 0.21
48	3.64 ± 1.05	5.52 ± 0.22	2.86 ± 0.41	4.96 ± 0.24	8.02 ± 0.07*	4.70 ± 0.30
55	2.90 ± 0.21	5.35 ± 0.65	3.56 ± 0.43	4.69 ± 0.50	7.98 ± 0.15*	4.67 ± 0.32
72	3.39 ± 0.09	4.95 ± 1.43	3.62 ± 0.23	4.81 ± 0.09	8.49 ± 0.03*	4.86 ± 0.20

*Samples with detected staphylococcal enterotoxins

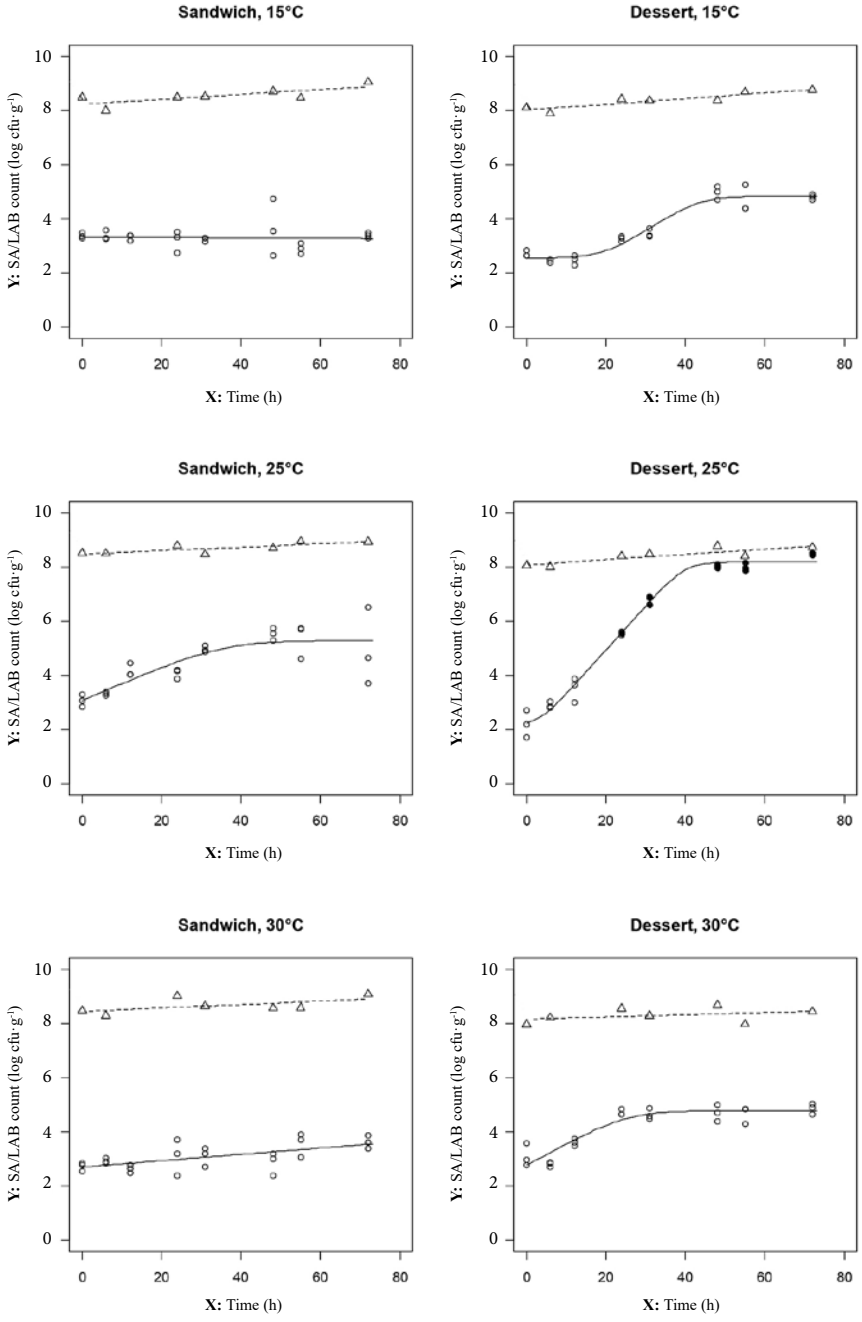

Fig. 1. Growth curves of *Staphylococcus aureus* (SA) and lactic acid bacteria (LAB) in open sandwich and buttercream puffs dessert stored at 15 °C, 25 °C, and 30 °C for 72 h. Observed *S. aureus* data (○ symbols) and predicted Baranyi models (— curves) or linear models (— lines) and the time of detection of toxins (● full symbols). Observed lactic acid bacteria data (△ symbols) and predicted linear models (– – – lines).

Table 2. Parameters of *Staphylococcus aureus* and lactic acid bacteria growth models in sandwiches and desserts stored at 15 °C, 25 °C, and 30 °C for 72 h.

	S/D	T (°C)	Model	Lag time (h)	Growth rate (log cfu·g^{-1}·h^{-1})	Initial value (log cfu·g^{-1})	Final value (log cfu·g^{-1})	R^2
SA	S	15	linear		–0.001 ± 0.004	3.332 ± 0.137		0.003
SA	S	25	B-lag		0.062 ± 0.017	3.076 ± 0.250	5.287 ± 0.219	0.696
SA	S	30	linear		0.012 ± 0.003	2.702 ± 0.122		0.389
SA	D	15	B	20.325 ± 3.281	0.103 ± 0.030	2.549 ± 0.097	4.845 ± 0.111	0.945
SA	D	25	B	3.535 ± 1.719	0.164 ± 0.010	2.233 ± 0.163	8.175 ± 0.098	0.988
SA	D	30	B-lag		0.081 ± 0.015	2.760 ± 0.152	4.780 ± 0.105	0.856
LAB	S	15	linear		0.009 ± 0.003	8.215 ± 0.144		0.582
LAB	S	25	linear		0.006 ± 0.002	8.476 ± 0.091		0.621
LAB	S	30	linear		0.006 ± 0.004	8.437 ± 0.164		0.351
LAB	D	15	linear		0.011 ± 0.002	8.011 ± 0.093		0.816
LAB	D	25	linear		0.010 ± 0.003	8.072 ± 0.106		0.746
LAB	D	30	linear		0.004 ± 0.004	8.168 ± 0.179		0.131

SA – *S. aureus*, LAB – lactic acid bacteria, S – sandwich, D – dessert, Model: B – Baranyi model, B-lag – Baranyi model without lag phase, R^2 – coefficient of determination (in the case of linear model) and square of the correlation of the observed and model-predicted values (in the case of Baranyi model)

The *S. aureus* growth curve at 25 °C in Fig. 1 shows that the last major increase in the bacterial population in buttercream puffs (by an order of magnitude, i.e., by log = 1) occurred between 31–48 h. After 48 h, the bacteria stopped to grow, which corresponded with the pH decrease below 4.5. This reduced pH could, therefore, have contributed to the growth stagnation. Similarly, we can observe the growth stagnation corresponding to the decrease in pH in the *S. aureus* growth curve in the buttercream puffs stored at 30 °C.

The initial count of LAB in our study was high (8–9 log cfu·g^{-1}), their number kept slowly increasing over the 72 h of storage at a practically identical rate in all cases (ranging between 0.004 and 0.011 log cfu·g^{-1}·h^{-1}; Fig. 1, Appendix A).

In our study, SE production was recorded only in a single scenario, namely in buttercream puffs stored at 25 °C; there, it was detected as soon as after 24 h. In all other scenarios, *S. aureus* counts did not significantly cross the risk limit of 5 log cfu·g^{-1}, although pH and a_w values as well as storage temperature supported *S. aureus* growth for the greater part of the study period (Table 1).

Discussion

The level of coagulase-positive staphylococci considered capable of SE production in amounts causing staphylococcal food poisoning is ≥ 5 log cfu·ml^{-1} or g^{-1}; Commission Regulation (EC) No. 2073/2005; Necidová et al. 2012). In foods, generally, SEs are produced at temperatures of 10–46 °C, pH 4.5–9.6 and a minimum water activity of 0.87 (Bhunia 2008).

Most foodborne outbreaks involving staphylococcal intoxication are associated with food preparation and handling (Tallent and Sheehan 2018). Sandwiches contaminated with *S. auerus*, which were stored for 8 to 10 h without refrigeration, were reported as the cause of outbreaks by Todd et al. (2007). In another study on egg products inoculated with 1, 3, and 5 log cfu·g^{-1} of *S. aureus*, no production of SEs was observed over the entire study period in samples stored at 18 °C, unlike at the temperatures of 22 and 37 °C (Yang et al. 2001).

Although SEs can be, therefore, produced at temperatures as low as 10 or 15 °C, their occurrence in foods is typically associated with higher temperatures (Hennekinne et al. 2012).

Competitive flora is a major preservative factors for foods (Leistner 2000), inhibiting the growth of many pathogens. *Staphylococcus aureus* does not grow well in the presence of competitive flora (Hennekinne 2018). In fermented foods, *S. aureus* is inhibited by lactic acid (Martin and Myers 2018). In our study, LAB were enumerated both in the open sandwiches and buttercream puffs during storage, ranging between 8–9 log cfu·g^{-1} (Fig. 1, Appendix A). We can assume that the SE formation might be influenced, besides the storage temperature, also by the presence of competitive microbiota represented by LAB.

Some *Lactobacillus lactis* strains produce a bacteriocin nisin. The inhibition effect of nisin (at 1 µg·g^{-1}) on *S. aureus* growth and on SEA production in model cheese samples was demonstrated also by Mohammad and Jodeiri (2014). The inhibition effect of nisin was higher at the storage temperature of 8 °C than at 25 °C. Bromberg et al. (2004) evaluated the antimicrobial activity of bacteriocins produced by LAB isolated from samples of meat and meat products. These strains were capable of inhibiting *S. aureus* growth; besides, the results revealed that *S. aureus* was the most sensitive of all tested microorganisms. The study of Klimešová et al. (2018) confirms that *S. aureus* is sensitive to antibacterial substances in the environment. Baran et al. (2017) evaluated the influence of temperature on SE production in cheeses maturing at 4 and 12 °C. The cheeses were inoculated with 6 log cfu·g^{-1} of SEB-producing *S. aureus*. No formation of SEBs was observed throughout the entire study period, although *S. aureus* concentration in the cheese exceeded the risk value of 5 log cfu·g^{-1} for as long as 40 days. The likely reasons include the relatively low storage temperatures along with the activity of the used starting culture. The effect of the probiotic microorganism *Lactobacillus acidophilus* on the suppression of *S. aureus* growth in a cream dessert was demonstrated e.g. also in a study by Rosa et al. (2016).

The growth of *S. aureus* and the associated risk of intoxication by SEs in sandwiches and other ready-to-eat foods was studied by many authors. Ding et al. (2010) evaluated the growth of *S. aureus* in sandwiches using three primary models, namely the Gompertz, logistic, and Baranyi models. Of these, the Gompertz model was shown to be the most suitable one for the statistical evaluation of acquired data. Min et al. (2013) quantified the SE amounts in ready-to-eat foods, namely in kimbab (pH 6.53, a_w 0.976; ingredients: rice, minced beef, fresh vegetables) and sandwiches (pH = 6.21, a_w = 0.984; ingredients: ham, eggs, tomatoes, salad, cucumber, cheese, white bread) during storage. The foods were inoculated with a mixture of three strains of SEA-, SEC- and SED-producing *S. aureus* at a concentration of 2.5–3.5 log cfu·g^{-1} and stored at temperatures of 17, 20, 23, 28, and 30 °C. The authors used ELISA for SE detection (TECRA SE Visual Immunoassay kit). The highest SE production was recorded in kimbab at 30 °C after 30.5 h of storage (1.8 ng/g); in sandwiches, the highest concentration was achieved at 30 °C after 52 h (0.15 ng/g), which confirms the strong dependence of SE production on the food matrix. Of the tested temperatures, only the lowest tested storage temperature of 17 °C was recommended by the authors as sufficiently low for the prevention of SE production.

Kang et al. (2010) compared the growth of *S. aureus* in eight ready-to-eat foods inoculated with three SE-producing *S. aureus* strains with the initial inoculation concentration of 2.9–3.4 log cfu·g^{-1}. The study shows an increase in the growth rate with the increasing storage temperature in all eight studied food matrices. In bread, the *S. aureus* growth rates at temperatures of 17, 24, and 30 °C were 0.048 log cfu·g^{-1}·h^{-1}, 0.181 log cfu·g^{-1}·h^{-1}, and 0.453 log cfu·g^{-1}·h^{-1}, respectively. Results of our study imply that in both open sandwiches and buttercream puffs, the growth rate was higher at 25 °C than at 30 °C; the lowest rates were observed at 15 °C (sandwiches) and 30 °C (puffs). As shown in

Appendix A, the growth rate in the open sandwiches at 25 °C (0.062 ± 0.017 log cfu·g^{-1}·h^{-1}) was significantly lower than that recorded in bread at a comparable temperature by Kang et al. (2010), i.e., (0.181 log cfu·g^{-1}·h^{-1}). In buttercream puffs, the growth rate at 25 °C was 0.164 ± 0.010 log cfu·g^{-1}·h^{-1} (Appendix A). A possible explanation for the slower growth of *S. aureus* in the food matrices in our study could lie in the presence of high concentrations of the competitive microflora (Fig. 1) and its higher activity at 30 °C (the optimal temperature for mesophilic LAB). The probable reduced metabolic activity of this competitive microflora at 25 °C likely promoted the growth of *S. aureus* and, in the case of buttercream puffs, even SE production.

The comparison of the duration of the lag phase as a parameter of the model for *S. aureus* growth in puffs (Appendix A) with that reported for bread in the study by Kang et al. (2010) revealed that the lag phase for puffs was significantly longer at 15 °C (20.325 ± 3.281 h) than at 25 °C (3.535 ± 1.719 h; Appendix A). In bread, the duration of the lag phase at 17 °C was 23.04 h; at 24 °C, it dropped to 6.72 h (Kang et al. 2010). The lag phase durations at 15 °C (puffs) and 17 °C (bread) were comparable. The significantly shorter lag phase in puffs at 25 °C compared to the lag phase in bread at 24 °C can be explained by the type of matrix and, therefore, by the presence of nutrients potentially supporting *S. aureus* growth.

Available studies focusing on fine bakery products/desserts in association with pathogenic *S. aureus* strains focus predominantly on the evaluation of the frequency of the occurrence of these strains in selected products (Shimamura et al. 2006; Cokal et al. 2012; Ulusoy et al. 2017; Harada et al. 2018) or describe food poisoning outbreaks caused by the consumption of fine bakery products or desserts containing toxigenic *S. aureus* strains. SFP of 24 restaurant guests in Italy, caused by Chantilla cream dessert, was described by Ercoli et al. (2017). The investigation revealed high amounts of coagulase-positive staphylococci (8.53 log cfu·g^{-1}) and the presence of SE (2.12 ng SEA·g^{-1}) in the cream dessert. In addition, *S. aureus* was detected also in swabs from the kitchen worktop and in the nasopharyngeal swabs from five kitchen workers. The investigation also revealed that the dessert was stored in unsuitable conditions, i.e., at room temperature, for approx. 5 h (Ercoli et al. 2017). Various types of fine bakery products with subsequently proven occurrence of toxicogenic strains of *S. aureus* originating from a bakery in Illinois, USA, caused food poisoning in more than 100 consumers (Hait et al. 2012).

Results of our study indicate that if open sandwiches or buttercream puffs are contaminated with toxigenic strains of *S. aureus* at a concentration of approx. 3 log cfu·g^{-1}, these toxigenic bacteria are capable of growth in buttercream puffs at all tested temperatures (15, 25, and 30 °C) while in open sandwiches, no growth was observed at 15 °C. Nevertheless, the production of staphylococcal enterotoxins (SEA, SEB, and SEC), which was expected at 25 °C and 30 °C, was not observed in most of the samples. These enterotoxins were detected only in buttercream puffs after 24 h and more of storage at 25 °C. The results of our study indicate that the formation of enterotoxins depends, besides the food matrix, also on the presence of competitive microflora such as LAB. Lactic acid bacteria are highly metabolically active at 30 °C and their metabolic products can be responsible for the lower *S. aureus* growth at that temperature than at 25 °C.

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