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The risk of food contamination with staphylococcal enterotoxins – case studies

support of practical exercises

structured tutorials for students

1. Introduction

The contamination with ***Staphylococcus aureus*** has a significant impact on the safety and quality of milk, dairy products as well as, for example, foods with a high degree of manual labour during their preparation. This bacterium is the most common cause of food-borne intoxication (staphylococcal enterotoxicosis) due to its capability to produce staphylococcal enterotoxins (SEs) directly in the foods (Hennekinne et al., 2012).

S. aureus is a commensal of warm-blooded animals, which has also been isolated from the natural environment. It is the main cause of **mastitis** in cows (Keefe, 2012; Vanderhaeghen et al., 2014) and is introduced into milk also by secondary contamination as a result of droplet infection or from the environment, udder surface, or the milker's hands. The possible presence of pathogenic microorganisms in raw milk and risks associated with its consumption has been highlighted, e.g., by Oliver et al. (2009) or Merz et al. (2016).

Delicatessen and fine bakery products are 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. S. aureus naturally colonizes the skin and nasopharyngeal region in approx. 30–50 % of the population (Soriano et al., 2002). 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 catering establishments, the deli and fine bakery products can be contaminated by employee hands and the premises themselves. *S. 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). 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 capability of about 50–75 % of *S. aureus* strains to produce, under suitable conditions, heat-stable extracellular enterotoxins presents a major risk factor in food-borne infection. **Staphylococcal enterotoxins** are members of a wide family of staphylococcal and streptococcal pyrogenic exotoxins with the potential to cause food-borne intoxications and some allergies (Balaban and Rasooly, 2000; Omoe et al., 2003). 24 types of SEs designated as A to Y are currently known (Hennekinne et al., 2012). The classical SEs: SEA, SEB, SEC1, SEC2, SEC3, SED, and SEE, are the most common causes of staphylococcal enterotoxicosis. The production of SEs is unlikely at temperatures below 10 °C (Bhunia, 2008). Although pasteurization kills *S. aureus* cells, heat-stable SEs generally retain their biological activity (Asao et al., 2003). SEs are resistant to freezing, drying, heat treatment and low pH, even to proteolytic enzymes of the gastrointestinal tract (Li et al., 2011). As stated by Hu et al. (2018), SEs represent a unique, very well adapted factor of virulence, although the evolutional function of these toxins remains unclear. *S. aureus* is among the most important causative agents of food-borne intoxications in the world (Normanno et al., 2005).

Many cases of **staphylococcal enterotoxicosis** remain unreported owing to the rapid course and similarity to other food-borne intoxications (Jablonski and Bohach, 2001). Staphylococcal enterotoxicosis has a very rapid onset and course. The first symptoms of intoxication such as vomiting, headache, abdominal pain, and diarrhoea develop as early as one to six hours after the consumption of food contaminated with SEs. The symptoms resolve spontaneously within 24–48 hours (Loir et al., 2003).

Bhunia (2008) report that at counts ranging between log 5–8 (10^5 and 10^8) CFU.g^{-1}, *S. aureus* is able to produce enterotoxin in amounts that can pose a health risk to consumers. To ensure food safety, to protect consumers' health, and to prevent the risk of staphylococcal enterotoxicosis, **Commission Regulation (EC) No 2073/2005** of 15 November 2005 on microbiological criteria for foodstuffs lay down the necessity of enumerating coagulase-positive staphylococci in selected categories of foodstuffs and of performing the screening of SEs when the count of coagulase-positive staphylococci exceeds **10^5 CFU/g** (Figure 1 and 2).

Food category	Micro-organisms/their toxins, metabolites	Sampling-plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies
		n	c	m	M		
1.21. Cheeses, milk powder and whey powder, as referred to in the coagulase-positive staphylococci criteria in Chapter 2.2 of this Annex	Staphylococcal entero-toxins	5	0	Not detected in 25g		European screening method of the CRL for Milk (13)	Products placed on the market during their shelf-life

Figure 1: Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs – criteria for the staphylococcal enterotoxins in selected categories of foodstuffs.

Food category	Micro-organisms	Sampling plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.2.7. Milk powder and whey powder (4)	Enterobacteriaceae	5	0	10 cfu/g		ISO 21528- 1	End of the manufacturing process	Check on the efficiency of heat treatment and prevention of recontamination
	Coagulase-positive staphylococci	5	2	10 cfu/g	100 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	Improvements in production hygiene. If values > 10^5 cfu/g are detected, the batch has to be tested for staphylococcal enterotoxins.
2.2.3. Cheeses made from raw milk	Coagulase-positive staphylococci	5	2	10^4 cfu/g	10^5 cfu/g	EN/ISO 6888-2	At the time during the manufacturing process when the number of staphylococci is expected to be highest	Improvements in production hygiene and selection of raw materials. If values >10^5 cfu/g are detected, the cheese batch has to be tested for staphylococcal enterotoxins.
2.2.4. Cheeses made from milk that has undergone a lower heat treatment than pasteurisation (7) and ripened cheeses made from milk or whey that has undergone pasteurisation or a stronger heat treatment (7)	Coagulase-positive staphylococci	5	2	100 cfu/g	1 000 cfu/g	EN/ISO 6888-1 or 2		
2.2.5. Unripened soft cheeses (fresh cheeses) made from milk or whey that has undergone pasteurisation or a stronger heat treatment (7)	Coagulase-positive staphylococci	5	2	10 cfu/g	100 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	Improvements in production hygiene. If values > 10^5 cfu/g are detected, the cheese batch has to be tested for staphylococcal enterotoxins.

Food category	Micro-organisms	Sampling plan (1)		Limits		Analytical reference method (2)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.4.1. Shelled and shucked products of cooked crustaceans and molluscan shellfish	*E.coli*	5	2	1 cfu/g	10 cfu/g	ISO TS 16649-3	End of the manufacturing process	Improvements in production hygiene
	Coagulase-positive staphylococci	5	2	100 cfu/g	1 000 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	Improvements in production hygiene

(1) n = number of units comprising the sample; c = number of sample units giving values between m and M.
(2) The most recent edition of the standard shall be used.

Figure 2: Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs – criteria for the count of coagulase-positive staphylococci in selected categories of foodstuffs.

The dose of toxin needed to cause intoxication is very low. Balaban and Rasooly (2000) and Omoe et al. (2003) reported the minimum infectious dose of SEA to be 100 ng. However, the individual susceptibility and body weight should also be taken into account in this regard (Roberts et al., 1996). Under favorable conditions (optimal temperature, pH, aw, salt concentration) for *S. aureus*, it takes not less than 20 h to produce enough enterotoxin for causing food poisoning (Sharma et al., 2000). The toxins are produced at a temperature range from **10 °C to 48 °C**, with the optimum between 37 °C and 40 °C. The **minimum pH suitable for their production is about 4.8**, with the optimum ranging between 6 and 7. **Minimum aw is 0.80–0.86**, but the optimum production is achieved at aw = 0.99 and higher. A higher production of toxins is observed under aerobic than under anaerobic conditions (Roberts et al., 1996).

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 (Figure 3). Throughout the experiment, the **Staphylo La Seiken test** (Denka Seiken Co., Ltd., Tokyo, Japan) was used for the identification of *S. aureus* (Figure 4).

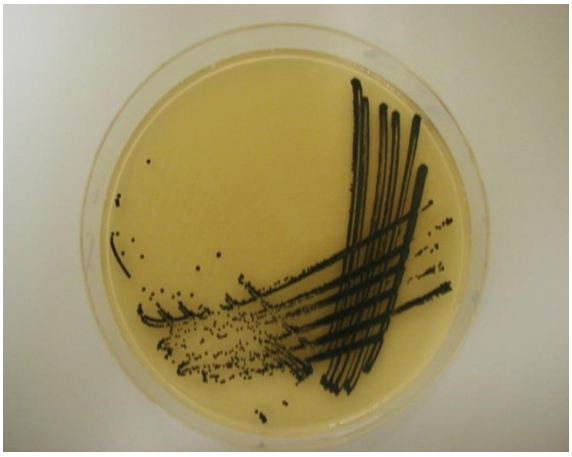


Figure 3: An example of typical *S. aureus* colonies on Baird-Parker agar. (Photo: author)

Figure 4: Staphylo La Seiken test – an inexpensive and rapid (2 minutes) test for *S. aureus* detection using latex agglutination cards. Latex particles in this set are sensitized with fibrinogen and rabbit plasma enabling distinguishing between *S. aureus* and other staphylococci. The positive reaction manifests through agglutination. (Photo: author)

Detection of staphylococcal enterotoxins

The staphylococcal enterotoxins detection using the ELFA test

The SE content can be analyzed by enzyme-linked immunofluorescence assay (ELFA) using the miniVIDAS® automated system (Vitek Immuno Diagnostic Assay System, BioMérieux, Marcy l'Étoile, France) (Figure 5). 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 (Figure 6), with test values (TVs) ≥ 0.13 indicating a positive result (Figure 7).

Figure 5: The automated **VIDAS® instrument**. All of the assay steps are performed automatically by the instrument after inserting the **SPR®s** and **strips**. (Photos: bioMérieux, France; author)

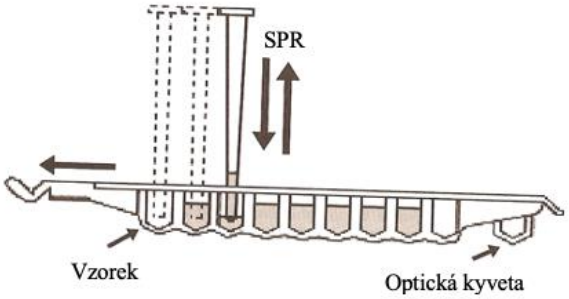
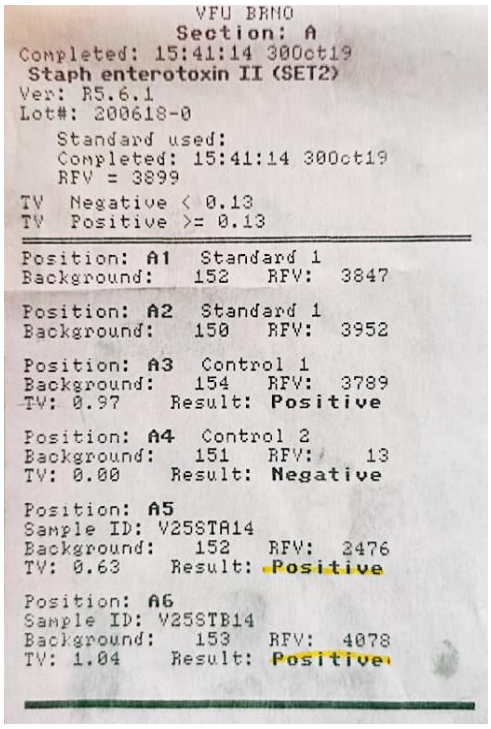


Figure 6: The **Solid Phase Receptacle (SPR®)** is a pipette coated with anti-staphylococcal enterotoxin antibodies. Reagents for the assay are ready-to-use and pre-dispensed in the sealed **reagent strips**. The proces uses a fully automated machine VIDAS®. (Photo: Baylis, 2003; bioMérieux, France)



VFU BRNO
Section: A
Completed: 15:41:14 30Oct19
Staph enterotoxin II (SET2)
Ver: R5.6.1
Lot#: 200618-0
Standard used:
Completed: 15:41:14 30Oct19
RFV = 3899
TV Negative < 0.13
TV Positive >= 0.13

Position: A1 Standard 1
Background: 152 RFV: 3847

Position: A2 Standard 1
Background: 150 RFV: 3952

Position: A3 Control 1
Background: 154 RFV: 3789
TV: 0.97 Result: Positive

Position: A4 Control 2
Background: 151 RFV: 13
TV: 0.00 Result: Negative

Position: A5
Sample ID: V25STA14
Background: 152 RFV: 2476
TV: 0.63 Result: Positive

Position: A6
Sample ID: V25STB14
Background: 153 RFV: 4078
TV: 1.04 Result: Positive

Figure 7: At the end of the assay, the results are automatically analyzed by the instrument which calculates a test alu efor each sample. **Relative Fluorescence Value (RFV)** is calculated and each result is interpreted (**positive, negative**). (Photo: author)

Detection of staphylococcal enterotoxins using the RPLA test

The production of enterotoxins can be tested using the **reverse passive latex agglutination method (RPLA)** (Denka Seiken Co., Ltd., Japan). Polystyrene latex particles are sensitised with purified antiserum from rabbits immunised individually with purified staphylococcal enterotoxins A, B, C and D. These latex particles will agglutinate in the presence of the corresponding enterotoxin. A control reagent (latex particles sensitised with non-immune rabbit globulins) is provided. The test is performed in **V-well microtitre plates**. The food extract or culture filtrate are diluted into five rows of wells, a volume of the appropriate latex suspension is added to each well and the contents are mixed. If **staphylococcal enterotoxins A, B, C or D are present, agglutination occurs**, which results in the formation of a **lattice structure**. Upon settling, this forms a diffuse layer on the base of the well. If staphylococcal enterotoxins are absent or at a concentration below the assay detection level, no such lattice structure can be formed and **a tight button** will be observed (Figure 8).

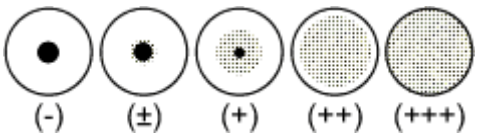


Figure 8: **Interpretation of test results**. The agglutination pattern should be judged by comparison with the following illustration. Wells marked (+), (++), and (+++) are considered positive.

Detection of staphylococcal enterotoxins using ELISA test

In the **Enzyme-linked Immunosorbent Assay (ELISA),** the enzyme catalyses the conversion of a colourless substrate to a coloured product, allowing evaluation of the test result by naked eye. The antibodies are adsorbed on a surface (e.g. wells of microtitre plate) (Figure 9). Sandwich ELISA, consisting of two antibodies which trap or sandwich the target antigen, is the simplest format most commonly used in commercially available kits (Figure 10).

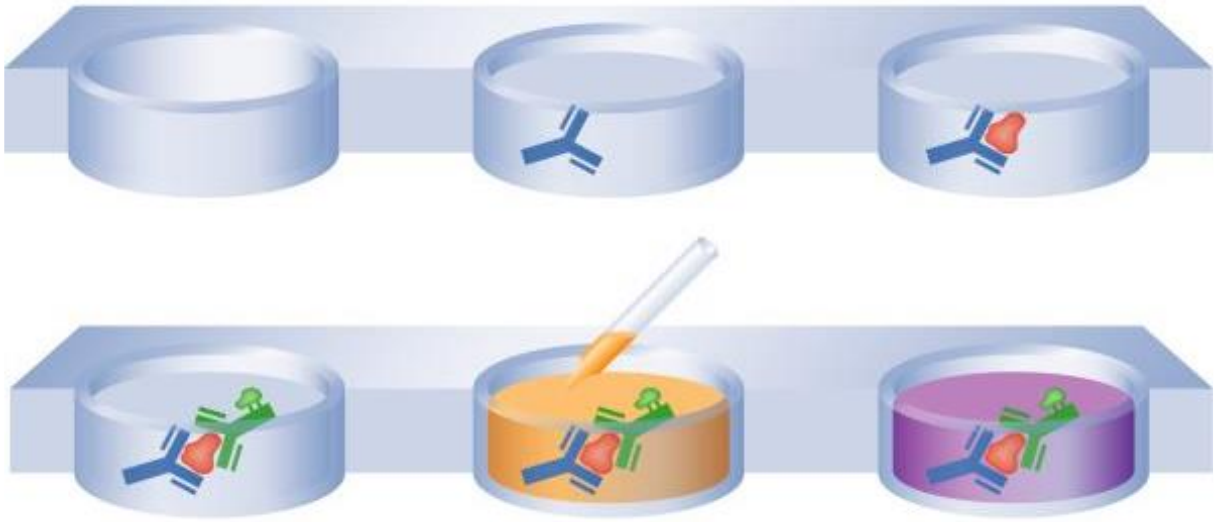


Figure 9: ELISA test – the **scheme of ELISA**: test well (upper left) is coated with specific antibodies (upper middle); antibodies capture target antigen (upper right) if present in thefood sample; labelled detection antibodies bind to the antigen forming a „sandwich" labelled with an enzyme capable of converting the chromogenic substrate into a coloured product are added (bottom left) ; chromogenic substrate is added (bottom middle); enzyme converts the colourless substrate to a coloured product (bottom right).

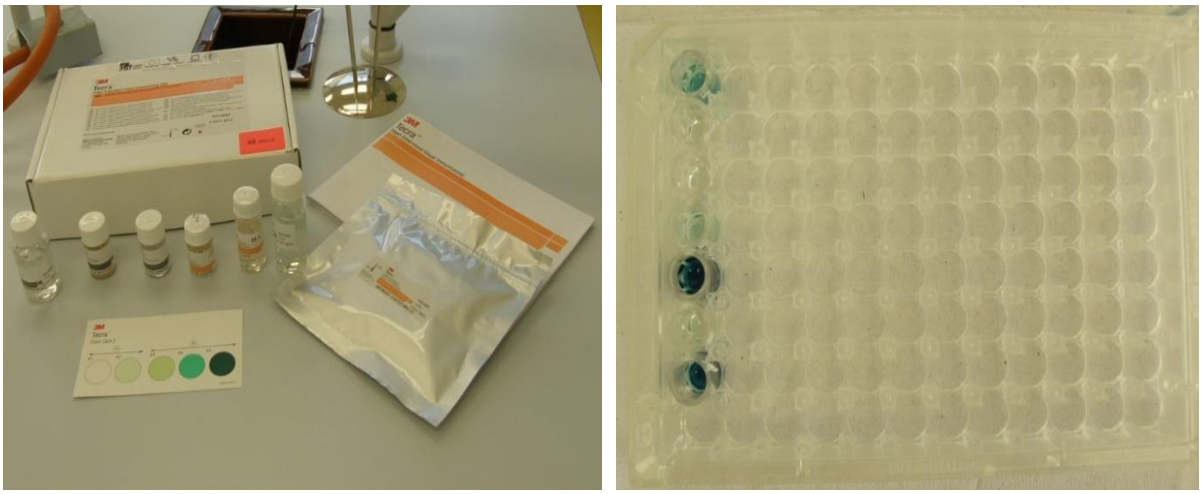


Figure 10: Commercially available ELISA test; wells with green solution indicate SE-positive samples. (Photo: author)

Questions:

A. Why are milk and milk products considered risky from the perspective of *S. aureus* occurrence?

B. Why does *S. aureus* occur more often in foods prepared with high degree of manual labour during their preparation?

C. How do we call a disease caused by consumption of foods containing staphylococcal enterotoxins (SEs)?

D. How many SE types are known, how are they designated, and which of them are the most common agents in food-borne intoxications?

E. Are SEs formed in the human gastrointestinal tract, or directly in the food? What conditions are necessary for their production?

F. Is every *S. aureus* strain capable of forming SEs?

G. What is the amount of *S. aureus* in the food representing the risk of SEs to start forming?

H. What regulation details the criteria for the count of coagulase-positive staphylococci in selected categories of foodstuffs and which categories are covered by this regulation?

I. What method is used for enumeration of coagulase-positive staphylococci (*S. aureus* and other species)?

J. What methods can be used for detection of SEs?

Answers:

A. S. aureus is one of the main agents in development of mastitis.

B. S. aureus is a natural comensal of the human skin and mucosa, thus also present in employees of food industry.

C. It is staphylococcal enterotoxicosis.

D. There are 24 types that are designated with letters A–Y; staphylococcal enterotoxicosis is caused by classical SEs: SEA–SEE.

E. SEs are extracellular enterotoxins excreted by bacteria directly into the food at a temperature range of 10–48 °C, the minimum pH is about 4.8, and minimum aw approx. 0.80–0.86.

F. No, the capability to form SEs is present only in about 50–75 % of S. aureus strains.

G. I tis more than 10^5 CFU/g.

H. Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs; the cattegories are: cheeses, milk powder and whey powder, cooked crustaceans and molluscan.

I. The Baird-Parker agar method.

J. ELFA, RPLA, ELISA.

2. Case studies

In all studies detailed below, the following methods were applied: the enumeration of *S. aureus* was performed using the Baird-Parker plate count method in accordance with ČSN EN ISO 6888-1 and the plates were cultured at 37 ± 1 °C for 24 ± 2 hours and 48 ± 2 hours. The Dry Spot Staphytect Plus test (Oxoid, UK) was used for the confirmation of suspected colonies. A fully automated miniVIDAS® instrument using the ELFA (Enzyme Linked Fluorescent Assay) technology was used to detect the production of SEs.

Study 1 – Staphyllococcal enterotoxins in milk

Ms Schwarz is used to buy raw cow milk from a milk dispenser machine at a farm near her house. At home, she always performs pasteurization of the milk at 85 °C and then, she stores the milk in the fridge. She made her last purchase on the way from her dermatologist, whom she visits because of suppurating skin lesions on her hands. She pasteurized only half of the purchased milk this time as she did not have a pot of sufficient size. While removing dirt from the milk with spoon, the spoon fell into the milk and she pulled it out with her hands. The same happened when stirring the milk during pasteurization – she, however, pulled out the spoon with her hand only after the milk cooled down. Moreover, she forgot both batches of milk on the kitchen table until morning, when she drank from both of them. The first symptoms of staphylococcal enterotoxicosis (strong vomiting and diarrhea) appeared after lunch. It is likely that both batches of milk were contaminated with toxigenic S. aureus strains.

A. Which type of milk (raw or pasteurized) was more likely to contain staphylococcal enterotoxins?

B. How should Ms Schwarz have prevented this food poisoning?

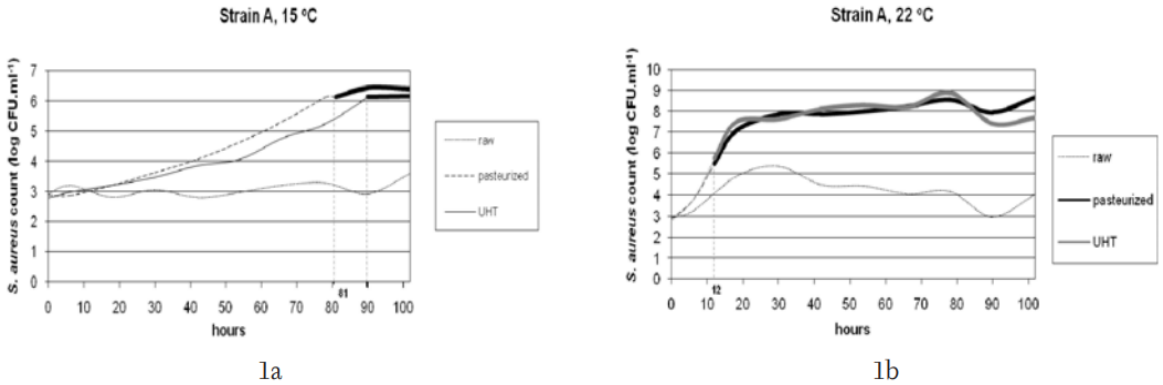
Answer these questions based on the results of the following study.

This study (Janštová et al., 2012) revealed a variation in *S. aureus* counts during the culture period and the time of SEs production, depending on the *S. aureus* strain, storage conditions, and type of milk.

S. aureus strains producing SEA, SEB, and SEC (strains A, B, and C) were used. Different types of milk that had tested negative for S. aureus were inoculated with 2.0×10^{1}–1.4×10^{3} CFU.ml^{-1} of the above strains. The tested milk samples included raw milk from

a milk vending machine (3.9–4.1 % of fat) and retail pasteurized and UHT semi-skimmed milk. Inoculated model milk samples were incubated at 15 °C and 22 °C (room temperature) to simulate inappropriate transport and storage conditions. Experiments were always conducted in parallel. Two groups of plates in each experiment were inoculated 12 hours apart to cover 24 hours. A three-hour sampling interval was used for the detection of SEs. The enumeration of *S. aureus* was performed at a 12-hour interval and the average values were calculated from the results of the parallel and repeated experiments. During the incubation, pH of the model samples was measured periodically.

When raw milk was inoculated with strain A and stored at 15 °C (Fig. 11a), the count of *S. aureus* increased from log 2.92 CFU.ml^{-l} to log 3.61 CFU. ml^{-l}, and the production of SEA was not detected during the entire storage time (102 hours). After 102 hours of incubation, *S. aureus* counts reached log 6.41 CFU.ml^{-1} in pasteurized milk and log 6.18 CFU.ml^{-1} in UHT milk. The presence of enterotoxin A was detected in pasteurized milk after 81 hours of incubation and in UHT milk after 90 hours of incubation, as shown in Figure 11a and Table 1. A more marked increase in the *S. aureus* count and earlier enterotoxin productions were both observed when inoculated samples of pasteurized and UHT milk were cultured at 22 °C (Figure 11b). At this temperature, the production of SEA was detected as early as 12 hours after inoculation. In raw milk incubated at 22 °C, the production of SEA was not detected during the entire period of incubation, despite the fact that counts of *S. aureus* reached a limit of 10^5 CFU.ml^{-1} for a short time (Figure 11b).

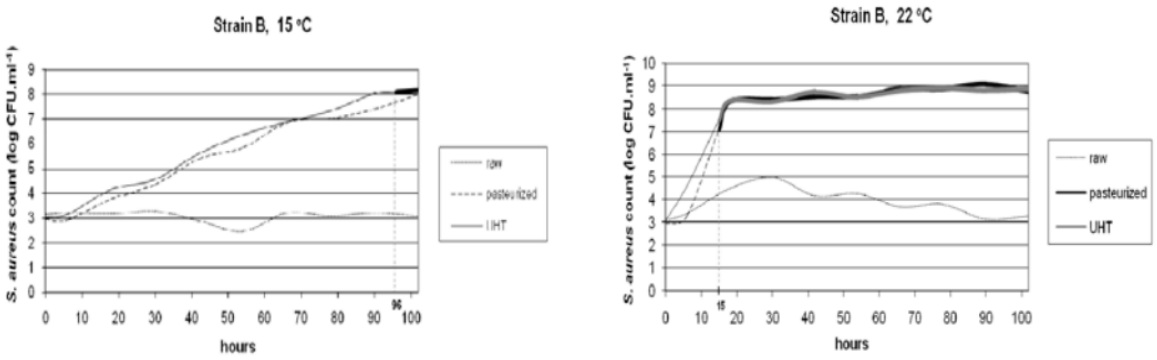


1a 1b

Figure 11: *S. aureus* growth rate and time to the first detection of SEA, SEB, SEC in raw, pasteurized, and UHT milk stored at 15 °C (1a) and 22 °C (1b).

In model milk samples inoculated with strain B and cultured at 15 °C, a critical *S. aureus* count of 10^5 CFU. ml^{-1} was only exceeded for pasteurized and UHT milk after 30 hours of culture (Figure 12a). Enterotoxin production was only detected in UHT milk after 96 hours of culture (Table 1). In pasteurized milk, no SEB production was observed even

after 102 hours of culture, although the *S. aureus* count reached log 8.00 CFU.ml^{-1}. This implies that a storage temperature of 15 °C is not optimal for SEB production in strain B. As reported by Roberts et al. (1996), under certain conditions of temperature, pH, and aw, it is possible for *S. aureus* to grow without producing enterotoxin. When cultured at 22 °C, *S. aureus* exceeded the count of 10^5 CFU.ml^{-1} early, i.e., within the first 24 hours of incubation, and SEB production was detected after 15 hours of incubation (Figure 12b). In the raw milk, strain B showed similar outcomes as strain A. SEB production was not detected during the entire incubation time despite the fact that at 22 °C, the *S. aureus* count reached the risk limit of 10^5 CFU.ml^{-1} for a short time.



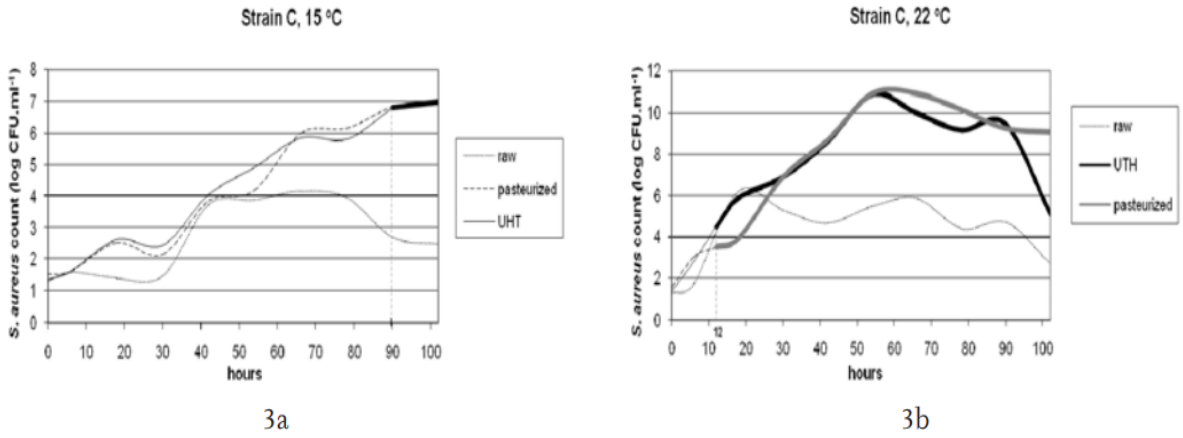
2a 2b

Figure 12: *S. aureus* growth rate and time to the first detection of SEA, SEB, SEC in raw, pasteurized, and UHT milk stored at 15 °C (2a) and 22 °C (2b).

When pasteurized and UHT milk was inoculated with strain C and cultured at 15 °C (Figure 13a), the *S. aureus* counts after 102 hours of incubation reached log 7.00 CFU.ml^{-1} and log 6.99 CFU.ml^{-1}, respectively. SEC production was only detected in UHT milk after 90 hours of culture. When cultured at 22 °C (Figure 13b), *S. aureus* showed high growth rates, and SEC production was first detected after 12 hours of incubation. In raw milk, *S. aureus* exhibited lower growth rates at both 15 °C and 22 °C, and no SEC production was detected despite the fact that at 22 °C, the risk limit of 10^5 CFU.ml^{-1} was achieved.

The fact that *S. aureus* exhibited considerably lower growth rates in raw milk in comparison with pasteurized and UHT milk that were not associated with SEs production can be explained, in accordance with Charlier et al. (2009), by the presence of natural microflora, in particular the lactic acid bacteria lowering the pH in raw milk that may prevent *S. aureus* growth and enterotoxin production. This inhibitory effect was also observed by Alomar et al. (2008). *S. aureus* is reportedly able to grow when

pH values range from 4.6 to 10 with optimal growth when the pH value is close to neutral (Charlier et al., 2008), confirmed by Necidová et al. (2009) who found the minimum pH compatible with SEs production to be 4.8. The pH of model raw milk samples measured after 102 hours of incubation at 15 °C and 22 °C ranged between 4.17 and 4.47. Respective pH values for pasteurized and UHT milk were much higher, ranging from 6.11 to 6.89, thus being in an optimum range for SEs production. *S. aureus* is able to grow in a wide range of temperatures from 7 °C to 48.5 °C, with the optimum growth at 30 °C to 37 °C. Enterotoxins are produced between 10 and 46 °C (Schmitt et al., 1990). In this study, the highest *S. aureus* counts were recorded for the strain producing enterotoxins A, B, and C when cultured in pasteurized and UHT milk at 22 °C. Our experiment, therefore, confirmed the assumption that the lower the incubation temperature, the lower the *S. aureus* growth rate and the longer the time to SEs production.



3a 3b

Figure 13: *S. aureus* growth rate and time to the first detection of SEA, SEB, SEC in raw, pasteurized, and UHT milk stored at 15 °C (3a) and 22 °C (3b).

Table 1: Time (in hours) to the first detection of SEs in raw, pasteurized, and UHT milk inoculated with enterotoxin-producing *S. aureus* strains and incubated at 15 °C and 22 °C.

	Enterotoxin production (hours)					
	SEA		SEB		SEC	
Type of milk	15 °C	22 °C	15 °C	22 °C	15 °C	22 °C
Raw milk	-	-	-	-	-	-
Pasteurized milk	81	12	-	15	-	12
UHT milk	90	12	96	15	90	12

The results of this study show that the lowest risk of SEs production is seen in raw milk, despite the critical *S. aureus* count of 10^5 CFU/g that was briefly reached during an incubation period at 22 °C. The highest risk of SEs production is associated with secondary contamination of pasteurized and UHT milk when stored at room temperature (Table 1).

Answers:

A. SEs were more likely to develop in pasteurized milk. S. aureus exhibited considerably lower growth rates in raw milk in comparison with pasteurized and UHT milk. These lower growth rates were not associated with SEs production thanks to the presence of natural microflora, in particular the lactic acid bacteria lowering the pH in raw milk that may prevent S. aureus growth and enterotoxin production. These results, however, do not support risky consumption of raw cow milk as other pathogens can be present.

B. Ms Schwarz should have cooled down both raw and pasteurized milk as soon as possible and store them at temperatures below 8 °C. She definitely should not have immersed her hands into the milk, especially as there were suppurating wounds on them.

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Study 2 - Staphylococcal enterotoxins in fresh cheese

Mr Muller produces fresh cheese at his farm. The milk come from five cows he keeps. Several customers buying fresh cheese from him informed him last week that they suffered from severe diarrhoea and vomiting, which lasted for 1 day and then recovered. The consumers did not go to a doctor and did not find out the cause. Mr Muller uses a pasteurisation process of 72 °C for 15 seconds to produce fresh cheese.

A. Could this disease have been staphylococcal enterotoxicosis caused by the consumption of Mr Muller's fresh cheese?

B. Would increasing the pasteurization temperature to 85 °C improve the safety of the product?

C. If the milk was contaminated with *S. aureus* only after the milk pasteurization and the products were stored according to the legislative requirements at temperatures below 8 °C, could *S. aureus* have grown in the cheese and produced staphylococcal enterotoxins?

Answer these question on the basis of the results of the following study.

The objective of this study (Necidová et al., 2009) was to monitor the growth characteristics of five *S. aureus* strains and their potential to produce enterotoxins at various stages of soft cheese production.

In the model experiments, raw cow's milk was inoculated separately with five *S. aureus* strains. All five strains were producers of enterotoxins of types A, B, or C (SA1185 SEA, SA1200 SEB, SA1057 SEB, SA1089 SEB, and SA843 SEC). The ability of the strains to produce enterotoxins was tested by the reverse passive latex agglutination method (Denka Seiken Co., Ltd., Japan). Each milk sample was inoculated with two different doses of *S. aureus*, low (with $< 5 \times 10^1$ to 4.8×10^3 CFU/ml) and high (with 5.3×10^4 – 2.9×10^5 CFU/ml). The inoculation was done in two ways: either 12–16 h prior to pasteurisation (experiment 1) or after pasteurisation (experiment 2). The treated milk was used to make soft cheese following the standard procedure: milk is pasteurised at different temperatures, 72 °C and 85 °C, for 15 s (as specified in the Commission Regulation (EC) No. 853/2004), $CaCl_2$ and sour cream culture (Milcom a.s., Laktoflora, Czech Republic) are added, followed by rennet (Milcom a.s., Laktoflora, Czech Republic), the mixture is renneted at 30 °C for 1 h, the curd is processed, pressed into moulds and left to drain at room temperature (24 °C) overnight, salted and seasoned. The cheese is packaged and stored at 4 °C and 8 °C for 5 days. The samples for bacterial

analysis were collected at various stages of the production and storage (Tables 1 and 2). The characteristics of the soft cheese were as follows: pH = 4.6–4.8, aw = 0.98–0.99, NaCl = 2% (w/v).

Table 2 presents the *S. aureus* counts determined in milk and at various stages of the cheese making process. In the milk inoculated with low counts of *S. aureus*, no proliferation of the agent was observed during the subsequent technological operations. Pasteurisation of 72 °C and 85 °C for 15 s completely eliminated staphylococci in the milk. When the milk was inoculated with high *S. aureus* counts, only the higher pasteurisation temperature proved to be safe. The lower pasteurisation temperature reduced the staphylococcal counts by about three orders of magnitude; nevertheless, the critical count of 10^5 CFU/ml was not exceeded in any sample at any stage of the technological process (Table 2). None of the model samples was positive in the detection of SEs. Both pasteurisation procedures used were safe enough to reduce the *S. aureus* counts to the levels unable to produce staphylococcal enterotoxins in as high amounts as needed to cause food-borne intoxications.

Table 2: *S. aureus* counts (CFU/g) at various stages of soft cheese making (milk inoculated with SA1057 strain prior to pasteurisation).

Production and storage stage	Innoculation with low counts of *S. aureus*		Innoculation with high counts of *S. aureus*	
	pasteurisation temperature per 15 s			
	72°C	85°C	72°C	85°C
Prior to pasteurisation (12 h after inoculation)	2.3×10^1	1.9×10^3	2.5×10^5	2.4×10^5
After pasteurisation	$< 5 \times 10^1$	$< 5 \times 10^1$	5.0×10^2	$< 5 \times 10^1$
After renneting	$< 5 \times 10^1$	$< 5 \times 10^1$	1.5×10^3	$< 5 \times 10^1$
During the pressing	$< 5 \times 10^1$	$< 5 \times 10^1$	4.1×10^3	$< 5 \times 10^1$
Prior to salting	$< 5 \times 10^1$	$< 5 \times 10^1$	1.7×10^4	$< 5 \times 10^1$
Storage day 1, at 4°C	$< 5 \times 10^1$	$< 5 \times 10^1$	2.6×10^3	$< 5 \times 10^1$
Storage day 1, at 8°C	$< 5 \times 10^1$	$< 5 \times 10^1$	4.1×10^3	$< 5 \times 10^1$
Storage day 2, at 4°C	$< 5 \times 10^1$	$< 5 \times 10^1$	6.3×10^3	$< 5 \times 10^1$
Storage day 2, at 8°C	$< 5 \times 10^1$	$< 5 \times 10^1$	6.9×10^3	$< 5 \times 10^1$
Storage day 5, at 4°C	$< 5 \times 10^1$	$< 5 \times 10^1$	3.3×10^3	$< 5 \times 10^1$
Storage day 5, at 8°C	$< 5 \times 10^1$	$< 5 \times 10^1$	5.9×10^3	$< 5 \times 10^1$

The model experiment described in Table 3 simulates the possible secondary contamination of soft cheese by *S. aureus* during the production and storage or during cheese making from unpasteurised milk. The milk was inoculated with toxigenic strains after pasteurisation. SEs were only detected when toxigenic strain SA1185 (SEA

production) had been used. With this strain, the highest staphylococcal counts of all experiments were obtained (up to 3.2 × 10^6 CFU/g). The first production stage at which SEs were detected was the pressing operation, 7 h after the inoculation of the pasteurised milk, with the *S. aureus* counts reaching 4.3 × 10^5 CFU/g. In this time interval, the produced soft cheese was exposed to a temperature of 30 °C for one hour while renneted. Then, the product was pressed at room temperature of 24 °C for approx. 12 hours.

Table 3: *S. aureus* counts (CFU/g) at various stages of soft cheese making (milk inoculated after pasteurisation).

Table 2. *S. aureus* counts (CFU/g) at various stages of soft cheese making (milk inoculated after pasteurisation)

Production and storage stage	Innoculation with low counts of *S. aureus*					Innoculation with high counts of *S. aureus*				
	1057	1089	843	1200	1185	1057	1089	843	1200	1185
After pasteurisation	3.3×10^3	4.6×10^3	4.8×10^3	$< 5 \times 10^1$	3.2×10^3	5.3×10^4	1.8×10^5	2.5×10^5	1.3×10^5	2.9×10^5
After renneting	3.4×10^3	4.8×10^3	4.9×10^3	$< 5 \times 10^1$	3.7×10^3	6.5×10^4	2.4×10^5	2.4×10^5	3.6×10^5	1.4×10^5
During the pressing	2.1×10^3	5.5×10^3	5.3×10^3	$< 5 \times 10^1$	3.6×10^4	6.6×10^5	2.0×10^5	1.5×10^5	2.4×10^5	$*4.3 \times 10^5$
Prior to salting	1.5×10^4	1.8×10^4	3.0×10^3	$< 5 \times 10^1$	3.5×10^4	1.0×10^6	2.5×10^5	6.9×10^4	4.2×10^5	$*1.6 \times 10^6$
Storage day 1, at 4°C	$< 5 \times 10^2$	5.5×10^2	9.3×10^2	$< 5 \times 10^1$	2.2×10^4	3.4×10^5	$< 5 \times 10^3$	2.2×10^4	6.3×10^4	$*1.6 \times 10^6$
Storage day 1, at 8°C	3.5×10^3	4.2×10^3	9.3×10^2	$< 5 \times 10^1$	3.9×10^4	4.5×10^5	2.9×10^5	1.7×10^4	2.3×10^5	$*1.2 \times 10^6$
Storage day 2, at 4°C	2.9×10^3	$< 5 \times 10^1$	8.5×10^2	$< 5 \times 10^1$	4.9×10^4	2.0×10^5	1.5×10^4	2.6×10^4	8.5×10^4	$*1.6 \times 10^6$
Storage day 2, at 8°C	3.1×10^3	1.3×10^3	7.3×10^2	$< 5 \times 10^1$	6.9×10^4	7.3×10^4	5.3×10^4	2.7×10^4	1.8×10^5	$*3.2 \times 10^6$
Storage day 5, at 4°C	7.5×10^2	$< 5 \times 10^1$	$< 5 \times 10^1$	$< 5 \times 10^1$	4.7×10^4	$< 5 \times 10^3$	$< 5 \times 10^2$	$< 5 \times 10^2$	$< 5 \times 10^3$	$*< 5 \times 10^4$
Storage day 5, at 8°C	1.6×10^3	$< 5 \times 10^1$	$< 5 \times 10^1$	$< 5 \times 10^1$	6.0×10^4	$< 5 \times 10^4$	3.9×10^4	$< 5 \times 10^2$	$< 5 \times 10^3$	$*< 5 \times 10^4$

*samples positive for staphylococcal enterotoxins

Most soft cheeses from the model experiments did not comply with the Commission Regulation (EC) No. 2073/2005 that specifies the limit for coagulase-positive staphylococci to be 10^1–10^2 CFU/g. Six out of 28 samples showed *S. aureus* counts >10^5

CFU/g and, pursuant to the regulation, should be further screened for the presence of staphylococcal enterotoxins.

The results of the study confirmed that the milk with the *S. aureus* counts higher than 10^5 CFU/g is unsuitable for the production of soft cheese. As the Commission Regulation (EC No. 853/2004) specifies the limit of the total plate count (TPC) for the supplied raw milk to be 10^5 CFU/ml, it is not expected that the safe limit for the *S. aureus* counts would be exceeded in raw milk. Under the standard production conditions, the presence of staphylococcal enterotoxins in soft cheese would indicate secondary contamination with *S. aureus*. Major prerequisites for a safe production are primarily the prevention of secondary contamination and cool chain maintenance during the storage, transportation, and distribution of soft cheeses.

Answers:

A. Yes, it is possible. Cheese is a high-risk food from the perspective of S. aureus presence, as implied by the Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs.

2.2.3. Cheeses made from raw milk	Coagulase-positive staphylococci	5	2	10^4 cfu/g	10^5 cfu/g	EN/ISO 6888-2	At the time during the manufacturing process when the number of staphylococci is expected to be highest	Improvements in production hygiene and selection of raw materials. If values > 10^5 cfu/g are detected, the cheese batch has to be tested for staphylococcal enterotoxins.
2.2.4. Cheeses made from milk that has undergone a lower heat treatment than pasteurisation (*) and ripened cheeses made from milk or whey that has undergone pasteurisation or a stronger heat treatment (*)	Coagulase-positive staphylococci	5	2	100 cfu/g	1 000 cfu/g	EN/ISO 6888-1 or 2		
2.2.5. Unripened soft cheeses (fresh cheeses) made from milk or whey that has undergone pasteurisation or a stronger heat treatment (*)	Coagulase-positive staphylococci	5	2	10 cfu/g	100 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	Improvements in production hygiene. If values > 10^3 cfu/g are detected, the cheese batch has to be tested for staphylococcal enterotoxins.

B. B. Yes, as Table 2 shows, the pasteurization temperature of 85 °C inactivated S. aureus fully even if higher counts of bacteria were present before pasteurization. As Table 3 implies, although the higher pasteurization temperature would better eradicate the bacteria from the raw milk, even lower temperature provided sufficient protection preventing the increase in bacterial count over the risky level of 10^5 CFU/ml. Therefore, it is likely that contamination occurred only after pasteurization in this case and the increased pasteurization temperature would likely not have any effect.

C. C. If milk was contaminated by higher amounts of S. aureus after pasteurization SEs could be present in the fresh cheese even if it was stored at temperatures below 8 °C, because SEs would have been formed as soon as at the beginning of the production process, i.e., during renetting and draining at higher temperatures.

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Study 3 – Staphylococcal enterotoxins in powdered milk

An intensive care unit of the paediatric ward of a regional hospital in Brno (Czech Republic) treats a case of a 4-month-old baby admitted due to persistent diarrhoea and vomiting. The only food consumed by the child is dried infant formula. The child developed symptoms of alimentary intoxication immediately after consuming the milk, which the mother had prepared at 8 o'clock in the morning, left on the kitchen table and fed it to the child only at 6 p.m., after returning from a day trip.

A. Is it possible that the child suffers from staphylococcal enterotoxicosis? Could the dried infant formula have contained *S. aureus*?

B. At what temperature and time could *S. aureus* in the reconstituted milk have reached the risk level of log 5 (>10^5) CFU/g at which SEs formation begins?

C. At what storage temperatures (Table 2) were SEs detected in the reconstituted milk? How could the mother have prevented the disease?

Answer the questions based on the results of the following study.

The aims of the study by Bogdanivičová et al. (2017) were to examine *S. aureus* growth dynamics and production of staphylococcal enterotoxins A, B, and C in reconstituted milk powder and to evaluate the potential for the production of SEA, SEB, and SEC in reconstituted milk powder that will or will not meet the parameters required by the European legislation (100 cfu.g^{-1}) and will (4 °C) or will not (15 °C, 25 °C) be stored properly.

Powdered milk samples (Hami infant formula, Nutricia Inc.) were contaminated with 1 ml suspension of *S. aureus* formed using the McFarland standard (the first equivalence corresponds to the number of 3x10^8 bacteria/ml) and mixed thoroughly. The inoculated milk powder was reconstituted with boiled water cooled to 40 °C at the ratio recommended by the manufacturer (13.5 g of infant formula + 90 ml of water). This situation created a reconstituted milk model relating to the consumer perspective. Lower and higher *S. aureus* counts were pre-designed using a mathematical calculation. The real and exact number of *S. aureus* in the reconstituted milk was determined by the method specified in each sample (9 for low count and 9 for the higher). These milk samples were also inoculated with two dilutions – low counts (5.0×10^0 – 2.7×10^1 cfu.g^{-1}) or high counts (1.3×10^4 – 2.0×10^4 cfu.g^{-1}) of one of nine *S. aureus* toxigenic strains with SEA, SEB or SEC production.

Table 4: Mean and maximal values of *S. aureus* count (log cfu.ml^{-1}) in reconstituted milk (N = 9).

Time (hours)	**Inoculation with low counts of *S. aureus*** ($5.0\times10^0 – 2.7\times10^1$ cfu.g^{-1})			**Inoculation with high counts of *S. aureus*** ($1.3\times10^4 – 2.0\times10^4$ cfu.g^{-1})		
	Storage (incubation) temperature					
	4 °C	**15 °C**	**25 °C**	**4 °C**	**15 °C**	**25 °C**
0	1.37 (2.45)	1.37 (2.45)	1.37 (2.45)	4.22 (4.30)	4.22 (4.30)	4.22 (4.30)
2	1.30 (2.00)	1.29 (1.60)	1.42 (1.70)	4.44 (5.52)	4.29 (4.57)	4.54 (4.75)
4	1.36 (1.70)	1.31 (1.60)	2.24 (2.58)	4.43 (5.20)	4.38 (4.59)	5.15 (5.45)
5	1.36 (1.70)	1.36 (1.78)	2.43 (2.72)	4.24 (4.51)	4.41 (4.75)	5.38 (5.82)
6	1.23 (1.85)	1.20 (1.70)	2.60 (2.93)	4.25 (4.52)	4.26 (4.59)	5.86 (6.30)
7	1.38 (1.85)	1.38 (1.78)	3.01 (3.30)	4.29 (4.45)	4.41 (4.60)	6.25 (6.75)
8	1.39 (1.60)	1.51 (1.90)	3.54 (4.90)	4.25 (4.46)	4.47 (4.71)	6.58 (7.04)
9	1.38 (1.78)	1.50 (2.08)	3.91 (4.40)	4.31 (4.45)	4.60 (4.90)	6.88 (7.43)
10	1.29 (1.60)	1.47 (1.95)	4.23 (4.60)	4.27 (4.38)	4.77 (4.87)	7.26 (7.73)
11	1.40 (1.85)	1.69 (2.18)	4.48 (5.18)	4.32 (4.51)	4.80 (4.93)	7.35 (7.70)
12	1.27 (1.60)	1.83 (2.11)	4.84 (5.38)	4.28 (4.52)	4.91 (5.23)	7.61 (7.87)
24	1.51 (1.70)	2.60 (3.18)	7.44 (7.76)	4.28 (4.54)	5.89 (6.23)	8.21 (8.58)
48	1.20 (1.70)	4.56 (4.95)	8.09 (9.00)	4.27 (4.41)	7.23 (7.52)	8.49 (8.64)

Table 5: The time of the first detection (hours) of SEs in powdered milk inoculated with *S. aureus* after reconstitution. The total incubation time was 48 hours.

Strains (SEs)	**Inoculation with low counts of *S. aureus*** ($5.0\times10^0 – 2.7\times10^1$ cfu.g^{-1})			**Inoculation with high counts of *S. aureus*** ($1.3\times10^4 – 2.0\times10^4$ cfu.g^{-1})		
	Storage (incubation) temperature					
	4 °C	**15 °C**	**25 °C**	**4 °C**	**15 °C**	**25 °C**
SA 393 (SEA)	-	-	24	-	48	8
SA 562 (SEA)	-	-	24	-	48	8
SA 650 (SEA)	-	-	48	-	-	24
SA 536 (SEB)	-	-	48	-	-	48
SA 652 (SEB)	-	-	24	-	48	7
SA 879 (SEB)	-	-	-	-	-	-
SA 289 (SEC)	-	-	-	-	-	-
SA 315 (SEC)	-	-	48	-	-	24
SA 360 (SEC)	-	-	-	-	-	24

All 18 inoculated samples with accurately calculated *S. aureus* counts were divided into 3 sub-samples, which were then stored at 4 °C, 15 °C, and 25 °C to simulate both suitable and improper storage conditions. Samples from each temperature were individually analysed. Experiments were run without replications, resulting in a total of 54 samples being analysed (9 strains of S. aureus / 3 different temperatures / two dilutions). *S. aureus* was enumerated in all samples at regular intervals. Sampling took place at hourly intervals during the first 12 hours of storage and then again after 24 and 48 hours. The samples were examined for *S. aureus* count and screened for the presence of SEA, SEB, and SEC. In parallel, control samples of reconstituted milk not inoculated with *S. aureus* were analyzed (Table 4 and 5).

Answers:

A. Yes, it is possible. In accordance with the Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs – criteria for the count of coagulase-positive staphylococci in milk powder are 10-100 cfu/g.

Food category	Micro-organisms	Sampling plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.2.7. Milk powder and whey powder (4)	Enterobacteriaceae	5	0	10 cfu/g		ISO 21528- 1	End of the manufacturing process	Check on the efficiency of heat treatment and prevention of recontamination
	Coagulase-positive staphylococci	5	2	10 cfu/g	100 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	Improvements in production hygiene. If values > 10^5 cfu/g are detected, the batch has to be tested for staphylococcal enterotoxins.

B. As implied by Table 4, the risk level would be reached in 11 hours (low contamination) or even as few as 4 hours (high contamination) at a temperature of 25 °C; at 15 °C, dangerous levels would be reached after 12 hours if the milk was highly contaminated.

C. SEs were detected at the temperatures of 15 and 25 °C. The mother should have put the reconstituted milk into the fridge.

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Study 4 – Staphylococcal enterotoxins in deli and fine bakery products

On his way to work on Thursday morning, Mr Harry bought sandwiches and buttercream puffs for his colleagues to celebrate his birthday. However, due to unexpected work commitments on the side of most of his colleagues, the party was postponed until Friday. It was July, the temperature outside was almost 30 °C. Mr Harry decided to leave the snacks in his office (25 °C) until the next day. The party was held on Friday after lunch. Eight out of his 13 colleagues began to suffer from severe nausea, vomiting and diarrhoea during Friday afternoon.

A. Could these symptoms have been caused by staphylococcal enterotoxicosis?

B. Could the staphylococcal enterotoxins have formed over 24 hours in the sandwiches and confectionery products?

C. Are these foods risky from the perspective of possible *S. aureus* contamination and why?

Answer these questions based on the results of the following study.

The presented study (Necidová et al., 2022) 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 staphylococcal enterotoxins and, therefore, on the risk posed to potential consumers of such foods.

Food samples (open sandwiches and buttercream puffs) were inoculated with three strains of *S. aureus* producing staphylococcal enterotoxins. Open sandwiches containing French loaf, butter, Eidam cheese and ham (initial pH = 5.55–5.71; aw = 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, selected as the model food from the category of fine bakery products (initial pH = 5.96–6.15; aw = 0.952–0.965) were purchased in 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), and fondant (sugar, glucose syrup, water). The samples were

tested for the presence of *S. aureus*; bacteria were not detected in any of the 25 g samples.

Three strains of *S. aureus* producing staphylococcal enterotoxins, 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 a sterile saline solution was prepared for each strain, with a density of approx. 8 log cfu.ml^{-1}; these partial suspensions were subsequently mixed in 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 1.7–3.58 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.

Besides staphylococci, mesophilic lactic acid bacteria 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).

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 (Figure 14). *S. 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 6). 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 (Figure 14, Table 6).

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 exceed the risk limit of 5 log cfu.g^{-}

[1], although pH and aw values as well as storage temperature supported *S. aureus* growth for the greater part of the study period (Table 6).

Results of this 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, which was expected at 25 °C a 30 °C, was not observed in most of the samples. SEs were detected only in buttercream puffs after 24 and more hours 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 lactic acid bacteria. Lactic acid bacteria are highly metabolically active at 30 °C and their metabolic products can be responsible for the lower *S. aureus* growth at the temperature higher than 25 °C.

Table 6: Mean ± standard deviation of *S. aureus* count (log cfu.g^{-1}) in sandwiches and desserts inoculated with 3 log cfu.g^{-1} and stored for 72 hours at 15 °C, 25 °C and 30 °C.

Time (hours)	Count of *S. aureus* (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

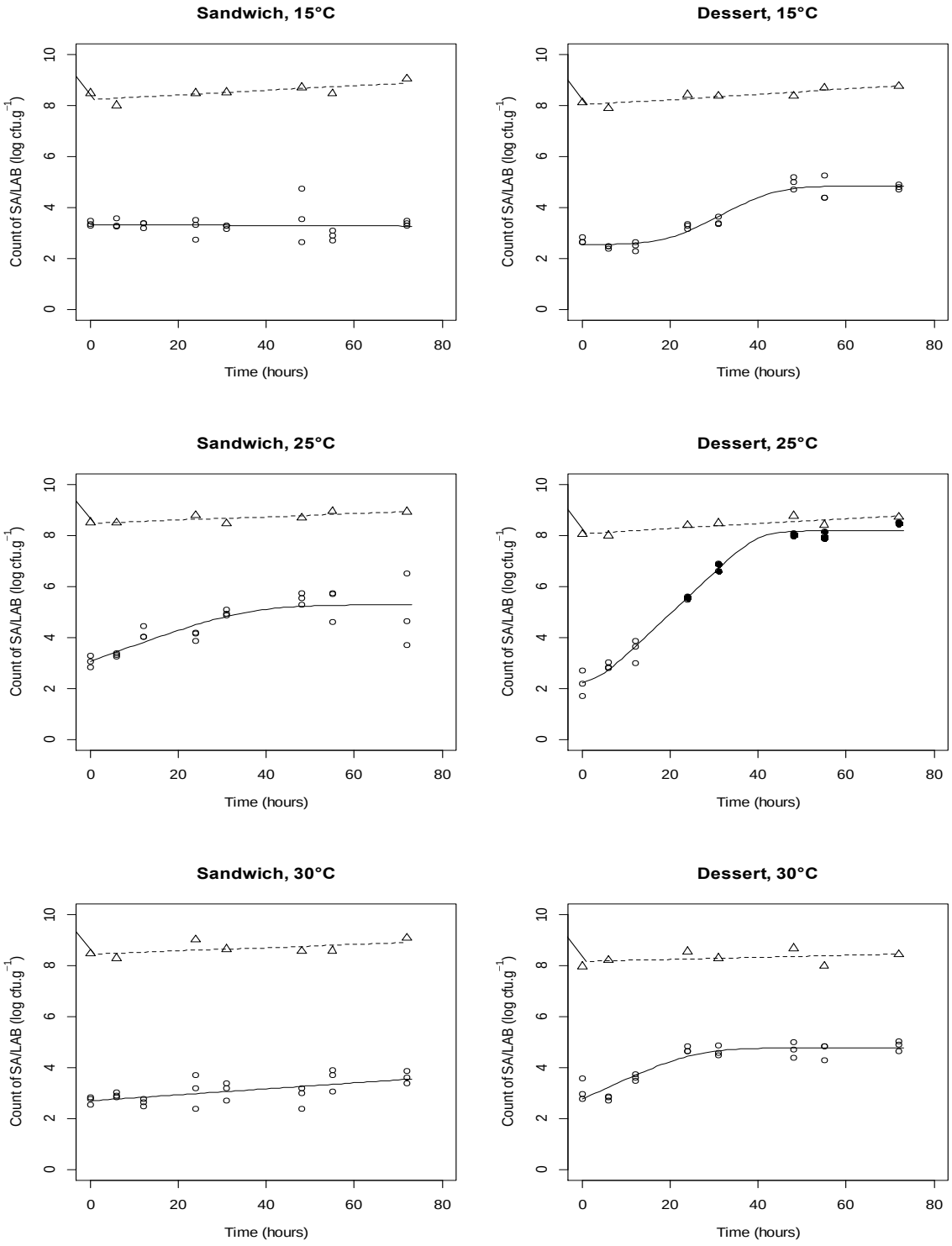

Figure 14: Growth curves of *S. aureus* (SA) and lactic acid bacteria (LAB) in open sandwich and buttercream puffs dessert stored for 72 hours at 15 °C, 25 °C and 30 °C. Observed *S. aureus* data (○ symbols) and predicted Baranyi models (— curves) or linear models (— lines) and the time of detection of toxins (● full symbols). Observed lactic acid bacteria data (△ symbols) and predicted linear models (– – – lines).

Answers:

A. Yes, these could be symptoms of staphylococcal enterotoxicosis, as evidenced by the rapid onset of symptoms with high intensity in a large proportion of persons shortly after consumption of contaminated food.

B. Yes, SEs may have been formed in the foods mentioned, especially in dessert (buttercreams puffs) as shown by the study results in Table 6.

C. Both sandwiches and buttercream puffs are high-risk foods in terms of possible contamination with S. aureus because of the high degree of manual labour during their preparation. The hands and mucous membranes of workers, especially purulent wounds and talking or sneezing during the preparation of these foods, can be a source of S. aureus.

D. Chlebíčky i věnečky jsou rizikovými potravinami z hlediska možné kontaminace bakteriemi S. aureus z důvodu high degree of manual labour during their preparation. Zdrojem S. aureus mohou být ruce a sliznice pracocníků, zvláště pak hnisavá poranění a mluvení nebo kýchání během přípravy těchto potravin.

3. References

ALHASHIMI, H. M. M., Nasal carriage of enterotoxigenic *Staphylococcus aureus* among food handlers in Kerbala city. *Karbala Internationat Journal of Modern Science*, 2017, vol. 3, p. 69–74.

ALOMAR, J., LOUBIERE, P., DELBES, C., NOUAILLE, S., MONTEL, M. C. Effect of *Lactococcus garvieae, Lactobacillus lactis* and *Enterococcus faecalis* on the behaviour of *Staphylococcus aureus* in microfiltered milk. *Food Microbioogy*, 2008, vol. 25, no. 3, p. 502–508.

ASAO, T., KUMEDA, Y., KAWAI, T., SHIBATA, T., ODA, H., HARUKI, K., NAKAZAWA, H., KOZAKI, S. An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: Estimation of enterotoxin A in the incriminated milk and powdered skim milk. *Epidemiology and Infection*, 2003, vol. 130, no. 1, p. 33–40.

BALABAN, N., RASOOLY, A. Staphylococcal enterotoxins. *International Journal of Food Microbiology*, 2000, vol. 61, no. 1, p. 1–10.

BOGDANOVIČOVÁ, K., KAMENÍK, J., DOROTÍKOVÁ, K., STREJČEK, J., KŘEPELOVÁ, S., DUŠKOVÁ, M., HARUŠTIAKOVÁ, D. 2019. Occurence of Foodborne Agents at Food Service Facilities in the Czech Republic. *Journal of Food Protection*, 2019, vol. 82, no. 7, p. 1096–1103.

BOGDANOVIČOVÁ, K., NECIDOVÁ, L., HARUŠTIAKOVÁ, D., JANŠTOVÁ, B. Milk Powder Risk Assessment with Staphylococcus aureus Toxinogenic Strains. *Food Control*, 2017, vol. 73, p. 2–7.

BHUNIA, A. K. Staphylococcus aureus. In: BHUNIA, A. K. (Ed.): Foodborne microbial pathogens. Mechanisms and pathogenesis. 1st ed. New York, USA: Springer Science+Business Media, LLC. 2008. p. 125–134. ISBN: 978-0-387-74536-7.

COMMISSION REGULATION (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Official Journal of the European Union, 2007, L 332, pp. 12–29.

HENNEKINNE, J. A., DE BUYSER, M. L., DRAGACCI, S. *Staphylococcus aureus* and its food poisoning toxins: Characterization and outbreak investigation. *FEMS Microbiology Reviews*, 2012, vol. 36, no. 4, p. 815–836.

HU, D.-L., WANG, L., FANG, R., OKAMURA, M., ONO, H. K. *Staphylococcus aureus* enterotoxins. In: FETSCH, A. (Ed). Staphylococcus aureus. London, United Kingdom: Academic Press, Elsevier, Inc. 2018. p. 39–55. ISBN: 978-0-12-809671-0.

CHARLIER, C., CRETENET, M., EVEN, S., LE LOIR, Y. Interactions between *Staphylococcus aureus* and lactic acid bacteria: An old story with new perspectives. *International Journal of Food Microbiology*, 2009, vol. 131, no. 1, p. 30–39.

CHARLIER C., EVEN, S., GAUTIER M., LE LOIR, Y. Acidification is not involved in the early inhibition of *Staphylococcus aureus* growth by *Lactobacillus lactis* in milk. *International Dairy Journal*, 2008, vol. 18, no. 2, p. 197–203.

CHAVES, R. D., PRADELLA, F., TURATTI, M. A., AMARO, E. C., DA SILVA, A. R. FARIAS, A. S., PEREIRA, J. L., KHANEGHAH, A. M. Evaluation of *Staphylococcus* spp. in food and kitchen premises of Campinas, Brazil. *Food Control*, 2018, vol. 84, p. 463–470.

ISO 6888-1. (1999). Mikrobiologie potravin a krmiv – Horizontální metoda stanovení počtu koagulázopozitivních stafylokoků (*Staphylococcus aureus* a další druhy) - Část 1: Technika s použitím agarové půdy podle Baird-Parkera. Praha: Český normalizační institut. 16 p.

ISO 15214. (2000). Mikrobiologie potravin a krmiv – Horizontální metoda stanovení počtu mezofilních bakterií mléčného kvašení – technika počítání kolonií vykultivovaných při 30 °C. Praha: Český normalizační institut. 15 p.

JABLONSKI, L. M., BOHACH, G. *Staphylococcus aureus*. In: DOYLE, M. P., BEUCHAT, L. R., MONTVILLE, T. J. (Eds): Food microbiology: Fundamentals and Frontiers, Second edition. Washington, D. C., USA: ASM Press. 2001. p. 411–434. ISBN: 978-1-55581-208-9.

JANŠTOVÁ, B. jr., NECIDOVÁ, L., JANŠTOVÁ, B., VORLOVÁ, L. *Staphylococcus aureus* growth and enterotoxin production in different types of milk. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, 2012, vol LX., no 5., p. 103–108.

JANSTOVA, B. jr., NECIDOVA, L., SKOCKOVA, A., JANSTOVA, B. Staphylococcal enterotoxin production in model samples of milk and fresh cheese. *Journal of Food and Nutrition Research*, 2014, vol. 53, no. 4, p. 389–392.

KEEFE, G. Update on control of *Staphylococcus aureus* and *Streptococcus agalactiae* for management of mastitits. *Veterinary Clinics of North America: Food Animal Practice*, 2012, vol. 28, no. 2, p. 203–216.

LI, S. J., HU, D.-L., MAINA, E. K., SHINAGAWA, K., OMOE, K., NAKANE, A. Superantigenic activity of toxic shock syndrome toxin-1 is resistant to heating and digestive enzymes. *Journal of Applied Microbiology*, 2011, vol. 110, no. 3, p. 729–736.

LOIR, Y., BARON, F., GAUTIER, M. Staphylococcus aureus and food poisoning. Genetics and Molecular Research, 2003, vol. 2, no. 1, p. 63–76.

MERZ, A., STEPHAN, R., JOHLER, S. *Staphylococcus aureus* isolated from goat and sheep milk seem to be closely related and differ from isolates detected from bovine milk. *Frontiers in Microbiology*, 2016, vol. 7, p. 319.

NECIDOVÁ, L., BURSOVÁ, Š., HARUŠTIAKOVÁ, D., BOGDANOVIČOVÁ, K. Evaluation of *Staphylococcus aureus* growth and staphylococcal enterotoxin production in delicatessen and fine bakery products. *Acta Veterinaria Brno*, 2022, vol. 91, no. 4, p. 417–425.

NECIDOVÁ, L., ŠŤÁSTKOVÁ, Z., POSPÍŠILOVÁ, M., JANŠTOVÁ, B., STREJČEK, J., DUŠKOVÁ, M., KARPÍŠKOVÁ, R. Influence of Soft Cheese Technology on the Growth and Enterotoxin Production of *Staphylococcus aureus*. *Czech Journal of Food Science*, 2009, vol. 27, no. 2, p. 127–133.

NORMANNO, G., LA SALANDRA, G., DAMBROSIO, A., QUAGLIA, N. C., CORRENTE, M., PARISI, A., SANTAGADA, G., FRINU, A., CRISETTI, E., CELANO, G. V. Occurrence, characterization and antimicrobial resistence of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products. *International Jornal of Food Microbiology*, 2007, vol. 115, no. 3, p. 290–296.

OLIVER, S. P., BOOR, K. J., MURPHY, S. C., MURINDA, S. E. Food safety hazards associated with consumption of raw milk. *Foodborne Pathogens and Disease*, 2009, vol. 6, no. 7, p. 793–806.

OMOE, K., HU, D.-L., TAKAHASHI-OMOE, H., NAKANE, A., SHINAGAWA, K. Identification and characterization of a new staphylococcal enterotoxin-related putative toxin encoded by two kinds of plasmids. *Infection and Immunity*, 2003, vol. 71, no. 10, p. 6088–6094.

REGULATION (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. *Official Journal of the European Union*, 2004, L 139, pp. 55–205.

ROBERTS, T. A., BAIRD-PARKER A. C., TOMPKIN R. B. Staphylococcus aureus. In: INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF): Microorganisms in Food 5: Characteristics of Microbial Pathogens. London: Blackie Academic and Professional, 1996. pp. 299–333. ISBN: 9780412473500.

SHARMA, N. K., REES, C. E. D., DODD, C. E. R. Development of a single-reaction multiplex PCR toxin typing assay for *Staphylococcus aureus* strains. *Applied and environmental mikrobiology*, 2000, vol 66, no. 4, p. 1347–1353.

SCHMITT, M., SCHULER-SCHMID, U., SCHMIDT-LORENZ, W. Temperature limits of growth, TNase and enterotoxin production of *Staphylococcus aureus* strains isolated from foods. *International Journal of Food Microbiology*, 1990, vol. 11, no. 1, p. 1–19.

SOARES, K., MOURA, A. T., GARCÍA-DÍEZ, J., OLIVEIRA, I., ESTEVES, A., SARAIVA, C. Evaluation of hygienic quality of food served in universities canteens of northem Portugal. *Indian Journal of Microbiology*, 2019, p. 1–8.

SOLANO, R., LAFUENTE, S., SABATE, S., TORTAJADA, C., DE OLALLA, P. G., HERNANDO, A. V., CAYLA, J. Enterotoxin production by *Staphylococcus aureus*: An outbreak at a Barcelona sports club in July 2011. *Food Control*, 2013, vol. 33, p. 114–118.

SORIANO, J. M., FONT, G., MOLTO, C., MANES, J. Enterotoxigenic staphylococci and their toxins in restaurant foods. *Trends in Food Science & Technology*, 2002, vol. 13, p. 60–67.

SUNDARARAJ, N., KALAGATUR, N. K., MUDILI, V., KRISHNA, K., ANTONYSAMY, M. Isolation and identification of enterotoxigenic *Staphylococcus aureus* isilates from Indian food samples: evaluation of in-house developer aptamer linked sandwich ELISA (ALISA) method. *Journal of Food Science and Technology*, 2019, vol. 56, no. 2, p. 1016–1026.

VANDERHAEGHEN, W., PIEPERS, S., LEROY, F., VAN COILLIE, E., HAESEBROUCK, F., DE VLIEGHER, S. Invited review: effect, persistence, and virulence of coagulase-negative *Staphylococcus* species associated with ruminant udder health. *Journal of Dairy Science*, 2014, vol. 97, no. 9, p. 5275–5293.

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