

## STAPHYLOCOCCUS AUREUS GROWTH AND ENTEROTOXIN PRODUCTION IN DIFFERENT TYPES OF MILK

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### Abstract

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The aim of our study was to assess *Staphylococcus aureus* growth and the time of first detection of staphylococcal enterotoxins type A, B and C (SEA, SEB, SEC) in different type of milk, depending on the strain and storage conditions. Raw, pasteurized, and UHT milk were inoculated with three strains of *S. aureus*, and growth patterns were determined by the plate method in accordance with EN ISO 6888-1. Baird-Parker agar medium was used for the detection of *S. aureus* and the Enzyme Linked Fluorescent Assay (ELFA) used with a miniVIDAS analyzer tested the production of staphylococcal enterotoxins. The results of model experiments showed the dependence of the growth rate and subsequent production of staphylococcal enterotoxins on incubation (storage) temperature, *S. aureus* strain, and type of milk. A significant finding was that the growth of *S. aureus* and production of enterotoxins in raw milk was inhibited by natural microflora, and production of enterotoxins was therefore not detected in raw milk within 102 hours of storage either at 15 °C or 22 °C. The highest risk of SEs production is associated with secondary contamination of pasteurized and UHT milk when stored at room temperature, where production was first detected after 12 hours of incubation.

*Staphylococcus aureus*, staphylococcal enterotoxins, milk, food safety

The contamination with *Staphylococcus aureus* has a significant impact on the safety and quality of dairy products. This bacterium is the most common cause of food-borne intoxication due to its production of staphylococcal enterotoxins (SEs).

*S. aureus* is a commensal of warm-blooded animals and has also been isolated from the natural environment. It is the main cause of mastitis in cows (RABELLO *et al.*, 2007) 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 (SATTAR *et al.*, 2001). Because of its heat resistance, *S. aureus* can be detected even in pasteurized milk (JIČÍNSKÁ and HAVLOVÁ, 1995). The capability of about 50–75% of *S. aureus* strains to produce, under suitable conditions, heat-stable extracellular enterotoxins is a major risk factor in food-borne infection. Staphylococcal enterotoxins are members of the

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.*, 2002). 22 types of SEs designated with letters A–V are currently known (ARGUDÍN *et al.*, 2010). The causes of staphylococcal enterotoxigenicosis are classical SEs: SEA, SEB, SEC1, SEC2, SEC3, SED, and SEE. The production of SEs is unlikely at temperatures below 10 °C (SCHMITT *et al.*, 1990).

Although pasteurization kills *S. aureus* cells, heat-stable SEs generally retain their biological activity (EVENSON *et al.*, 1988; ASAO *et al.*, 2003). Thus, because of the importance of these toxins in the public health and food sectors, an efficient screening to detect the prevalence of enterotoxigenic strains in foods is required.

*S. aureus* is among the most important causative agents of food-borne intoxications in the world

(NORMANNO *et al.*, 2005). Many cases of staphylococcal enterotoxigenesis remain unreported owing to the rapid course and similarity to other food-borne intoxications (JABLONSKI and BOHACH, 2001). Staphylococcal enterotoxigenesis 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 (ZHANG *et al.*, 1998; ATANASSOVA *et al.*, 2001; LOIR *et al.*, 2003). The symptoms resolve spontaneously within 24–48 hours (LOIR *et al.*, 2003).

JABLONSKI and BOHACH (2001) report that even at counts ranging between  $10^3$  and  $10^5$  CFU.g<sup>-1</sup>, *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 enterotoxigenesis, 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 is  $> 10^5$  CFU/g.

## MATERIALS AND METHODS

*S. aureus* strains producing SEA, SEB, and SEC (strains A, B, and C) were obtained from the Czech Collection of Microorganisms (CCM 5756, 5757, and 5971). Different types of milk that had tested negative for *S. aureus* were inoculated with  $2.0 \times 10^1$ – $1.4 \times 10^3$  CFU.ml<sup>-1</sup> of the above strains. The milk samples tested were raw milk from a milk vending machine (3.9–4.1% of fat) and retail pasteurized and UHT half fat 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.

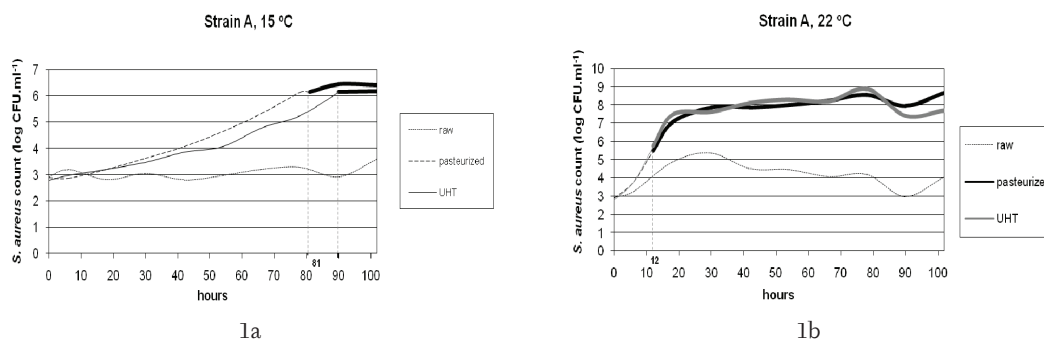
Enumeration of *S. aureus* was performed using the Baird-Parker plate count method in accordance with ČSN EN ISO 6888-1 and the plates were cultured at  $37 \pm 1$  °C for  $24 \pm 2$  hours and  $48 \pm 2$  hours. The Dry Spot Staphytect Plus test (Oxoid, UK) was used for the confirmation of suspected colonies. A fully automated miniVIDAS<sup>®</sup> instrument using the ELFA (Enzyme Linked Fluorescent Assay) technology was used to detect the production of SEs.

Statistical evaluation was carried out and statistical significance of differences was detected by comparing the results of mutual relations between two selections using the Mann-Whitney non-parametric test. Differences were compared with data sets that reflect the influence of strains of *S. aureus*, the type of milk, and storage temperature on the growth of *S. aureus* and enterotoxin production (MATOUŠKOVÁ *et al.*, 1992).

## RESULTS AND DISCUSSION

Our experiments revealed 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.

When raw milk was inoculated with strain A and stored at 15 °C (Fig. 1a), the count of *S. aureus* increased from log 2.92 CFU.ml<sup>-1</sup> to log 3.61 CFU.ml<sup>-1</sup>, 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<sup>-1</sup> in pasteurized milk and log 6.18 CFU.ml<sup>-1</sup> 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 Fig. 1a and Tab. I. 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 (Fig. 1b). 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<sup>-1</sup> for a short time (Fig. 1b).



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

With samples inoculated by strain A we evaluated the statistical insignificance of *S. aureus* count differences between pasteurized and UHT milk at both storage temperatures. Statistically insignificant differences were also in the evaluation of the influence of temperature on each type of milk. Statistically significant differences ( $P = 0.05$ ) were found by comparing the *S. aureus* count in raw milk versus pasteurized milk and UHT at the same storage temperature.

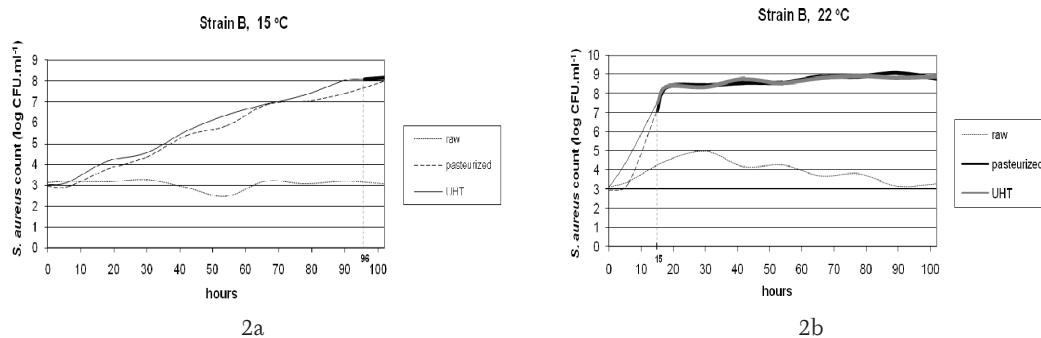
In model milk samples inoculated with strain B and cultured at 15 °C, a critical *S. aureus* count of  $10^5$  CFU.ml<sup>-1</sup> was only exceeded for pasteurized and UHT milk after 30 hours of culture (Fig. 2a). Enterotoxin production was only detected in UHT milk after 96 hours of culture (Tab. I). 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<sup>-1</sup>. 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  $a_w$ , 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<sup>-1</sup> early within the first 24 hours of incubation, and SEB production was detected after 15 hours of incubation (Fig. 2b). 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<sup>-1</sup> for a short time.

With samples inoculated by strain B we compared the different *S. aureus* counts in all three types of milk stored at different temperatures. The differences were statistically significant in pasteurized milk ( $P = 0.05$ ) and highly significant ( $P = 0.01$ ) with raw milk and UHT. In the mutual comparison of *S. aureus* count in raw milk and pasteurized significant differences were determined at both storage temperatures ( $P = 0.05$ ); when comparing raw milk and UHT, an even higher significant difference ( $P = 0.01$ ) was found. There was no significant difference in *S. aureus* count between UHT milk and pasteurized milk at both storage temperatures.

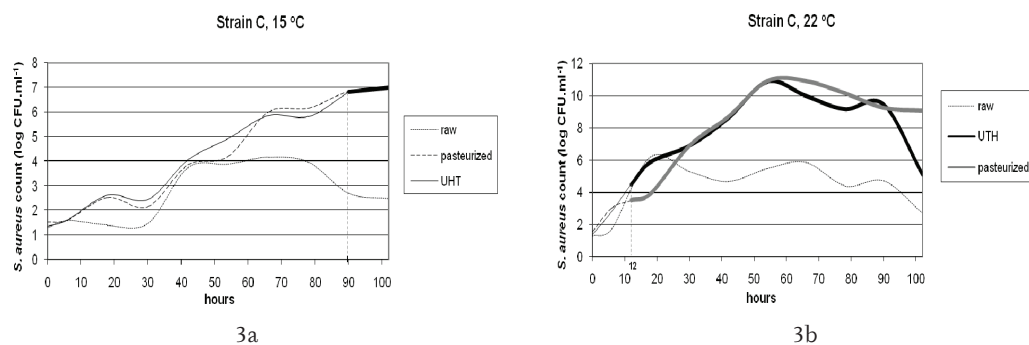
When pasteurized and UHT milk was inoculated with strain C and cultured at 15 °C (Fig. 3a), the *S. aureus* counts after 102 hours of incubation were log 7.00 CFU.ml<sup>-1</sup> and log 6.99 CFU.ml<sup>-1</sup>, respectively. SEC production was only detected in UHT milk after 90 hours of culture. When cultured at 22 °C (Fig. 3b), *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 despite the fact that at 22 °C, the risk limit of  $10^5$  CFU.ml<sup>-1</sup> was achieved.

Statistical evaluation of results of the samples inoculated by strain C were exactly the same as in the case of milks inoculated by strain B.

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



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



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

I: 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

Type of milk	Enterotoxin production (hours)					
	SEA		SEB		SEC	
	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

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) having 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, and were 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 being observed at 30 °C to 37 °C. Enterotoxins are produced between 10 and 46 °C (SCHMITT *et al.*, 1990). In our 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.

The results of our 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(ml)<sup>-1</sup> 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 (Tab. I).

## CONCLUSIONS

Given the risk of food-borne intoxications caused by SEs, to monitor the presence of *S. aureus* and its toxins is a crucial step in food safety and quality control. The results of the model experiments showed the correlation between the *S. aureus* growth rate and subsequent SEs production, incubation (storage) temperature, *S. aureus* strain, and type of milk. It should be noted that *S. aureus* growth and SEs production in raw milk, in contrast to pasteurized and UHT milk, is inhibited by natural competitive microflora and low pH. The production of SEA, SEB, and/or SEC was not detected in raw milk within 102 hours of storage at both 15 °C and 22 °C. Nevertheless, this finding should not be interpreted as our endorsement for consumption of raw milk. The limit of  $10^5$  CFU.ml<sup>-1</sup> specified by the law as associated with SEs production is credible, however we first detected SEC toxins in pasteurized and UHT milk below the legal limit. The results of our study amplify the critical importance of maintaining the cold chain below 8 °C from producer through retailer and on to the consumer to ensure the safety of milk and dairy products. Study results could be relevant not only for the food industry areas of quality control and food safety but also with dairy product endusers.

## SUMMARY

Our experiment's goal was to assess *Staphylococcus aureus* growth and identify the first detection of staphylococcal enterotoxins type A, B, and C (SEA, SEB, SEC) in different types of milk, depending on strain and storage conditions.

*S. aureus* strains producing SEA, SEB, and SEC (strains A, B, and C) were obtained from the Czech Collection of Microorganisms (CCM 5756, 5757, and 5971). Different types of milk (raw milk from a milk vending machine, retail pasteurized, and UHT half fat milk) were inoculated with  $2.0 \times 10^1$ – $1.4 \times 10^3$  CFU.ml<sup>-1</sup> of the above strains. Inoculated model milk samples were incubated at 15 °C and 22 °C. A three-hour sampling interval was used for the detection of SEs. *S. aureus* was enumerated at 12-hour intervals using the Baird-Parker plate count method in accordance with ČSN EN ISO 6888-1. The Dry Spot Staphytest Plus test was used to confirm suspected colonies. A miniVIDAS<sup>®</sup> instrument using the ELFA method (Enzyme Linked Fluorescent Assay) was used to detect the production of SEs. Production of SEA, SEB, SEC was not detected in raw milk during 102 hours of storage either at 15 °C or 22 °C. Presence of SEA was detected in pasteurized milk after 81 hours of incubation and in UHT



milk after 90 hours of incubation at 15 °C. Production of SEA in pasteurized and UHT milk at 22 °C was detected as early as 12 hours after inoculation. In samples with strain B at 15 °C, enterotoxin production was only detected in UHT milk after 96 hours of culture. 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<sup>-1</sup>. SEB production was detected at 22 °C in pasteurized and UHT milk after 15 hours of incubation. SEC production during incubation at 15 °C was only detected in UHT milk after 90 hours of culture. When *S. aureus* was cultured at 22 °C, SEC production was first detected after 12 hours of incubation.

The results of our study showed the dependence of the growth rate and subsequent production of SEs on incubation (storage) temperature, *S. aureus* strain, and type of milk. The lowest risk of SEs production is seen in raw milk, despite the critical *S. aureus* count of 10<sup>5</sup> CFU.g(ml)<sup>-1</sup> 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.

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