



COMPARING THE GROWTH OF *S. AUREUS* AND PRODUCTION OF STAPHYLOCOCCAL ENTEROTOXIN C IN SHEEP'S AND GOAT'S MILK

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ABSTRACT

In sheep's and goat's milk inoculated with a strain of *Staphylococcus aureus*, the staphylococcal growth was tested by the plate method according to EN ISO 6888-1 on Baird-Parker agar. The automated instrument miniVIDAS[®] was used for the detection of staphylococcal enterotoxin C by enzyme-linked immunofluorescent assay (ELFA). The influence of poor storage conditions (15 °C, 22 °C) and of the type of milk on the monitored parameters was evaluated. The results of the model experiments showed the dependence of the multiplication of *S. aureus* and subsequent production of staphylococcal enterotoxins on the culture/storage temperature and the type of milk. It is noteworthy that in raw milk, the *S. aureus* growth rate and production of enterotoxins can be suppressed by competitive microflora.

Keywords: *Staphylococcus aureus*, staphylococcal enterotoxins, miniVIDAS, ELFA, sheep's milk, goat's milk

INTRODUCTION

Staphylococcus aureus is considered the world's third most important cause of food-borne illnesses (Tirado and Schimdt, 2001). Milk is a good substrate for *S. aureus* growth, and dairy products are a known source of intoxication (De Buyser et al., 2001; Jorgensen et al., 2005). *S. aureus* is the major causative agent of mastitis in cows (Rabello et al. 2007). Therefore, milk and dairy products pose a risk to consumers.

The products contaminated by *S. aureus* are often the cause of food-borne intoxications due to the production of enterotoxins. 20 types of staphylococcal enterotoxins denoted A - U are currently known (Loir et al., 2003; Løvseth et al., 2004; Ono et al., 2008). Staphylococcal enterotoxins (SEs) are encoded by the respective genes (*sea* - *seu*). A strain of *S. aureus* can carry two or more genes of this range at the same time, with *seg* and *sei* being the most common pair, but the presence of a single SE gene has also been reported (Ercolini et al. 2004). Bautista et al. (1988), Foschino et al. (2002), and Scherrer et al. (2004) have found that *S. aureus* strains isolated from sheep's and cow's milk are most often producers of SEC.

Many cases of staphylococcal enterotoxigenicosis remain unreported, owing to the rapid course and similarity to other food-borne intoxications (Jablonski and Bohach 2001). Staphylococcal enterotoxigenicosis 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).

Although pasteurization kills *S. aureus* cells, thermostable 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. The currently available enzyme-linked immunosorbent and fluorescent assay kits, ELISA and ELFA, detect only SEA, SEB, SEC, SED and SEE.

Jablonski and Bohach (2001) have found the *S. aureus* counts in the range from 10^3 - 10^5 CFU.g⁻¹ to be able to produce enterotoxin in such high quantities that may pose a health risk to the consumer. To ensure the food safety, to protect the consumer's health, and to reduce as much as possible the risk of staphylococcal enterotoxigenicosis, Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for

foodstuffs and **Commission Regulation (EC) No 1441/2007** of 5 December 2007 amending Regulation (EC) No 2073/2005 lay down the requirements for the detection and enumeration of coagulase-positive staphylococci in cheeses (including sheep's milk and goat's milk cheeses) and for ruling out the presence of staphylococcal enterotoxins when the count of coagulase-positive staphylococci detected in such products reaches $>10^5$ CFU.g⁻¹. Another microbiological limit for raw goat's and sheep's milk, i.e. the limit for the moving geometric average of the total microbial count, is specified by **Commission Regulation (EC) No 853/2004** laying down specific hygiene rules for food of animal origin. The microbiological limits for the *S. aureus* counts in both raw and pasteurized sheep's and goat's milk are laid down by Slovak standards **STN 57 0510 Sheep's milk and STN 57 0520 Goat's milk**. The limit counts are 1.10^3 CFU.ml⁻¹ for raw sheep's milk and 2.10^3 CFU.ml⁻¹ for raw goat's milk. For pasteurized milk, the limit count is $< 5.10^1$ CFU.ml⁻¹ for both sheep's and goat's milk.

The most common milk available on the Czech market is cow's milk, but it would be desirable to increase the production and sales of goat's milk because of its positive effects. Goat's milk differs from cow's milk in its better digestibility (**Park, 2000**), higher alkalinity (**Aganga et al., 2002**), higher buffering capacity, and higher content of basic milk components (**Park, 2000**). Raw goat's milk also has different sensory properties. Goat's milk is clearly less allergenic in comparison with cow's milk (**Martín-Diana et al., 2003**).

Sheep's milk is most often used for the production of cheeses. It is usually processed into cheese directly on farm to be sold there or at selected points of the retail market. The dairy products of this kind need to be monitored for the safety because of the possible presence of microbial pathogens in them (**Samarzija et al., 2007**).

Although the production of sheep's and goat's milk cheeses in the Czech Republic has not a long tradition as is the case in Slovakia, these fine and delicious cheeses are very popular with Czech consumers. The safety of raw materials is a vital prerequisite for the production of safe and high-quality foodstuffs.

MATERIAL AND METHODS

The *S. aureus* strain producing enterotoxin C (CCM 5971) was obtained from the Czech Collection of Microorganisms. This strain was inoculated into the goat's and sheep's milk at 4.78 to 5.0 CFU.ml⁻¹ after ruling out the presence of *S. aureus* in these milk samples.

The inoculated model samples were stored at 15 °C (a temperature previously reported to be suitable for the production of SEs) and at 22 °C (room temperature) to simulate poor transport

and storage conditions. The enumeration of *S. aureus* and detection of enterotoxin were performed at 24-hour intervals for 7 days, using the plate method according to ČSN EN ISO 6888-1 on Baird-Parker agar. The plates were cultured at $37 \pm 1^\circ\text{C}$ for 24 ± 2 hours and 48 ± 2 hours. The coagulase test Dry Spot Staphytest Plus (Oxoid, UK) was used for the confirmation of the selected suspected *S. aureus* colonies. The automated instrument miniVIDAS[®] was used for the detection of SEs by enzyme-linked immunofluorescent assay (ELFA).

RESULTS AND DISCUSSION

In our model experiments, the *S. aureus* counts varied during culture and at the beginning of SEs production, depending on the storage conditions and the type of milk (raw vs. pasteurized).

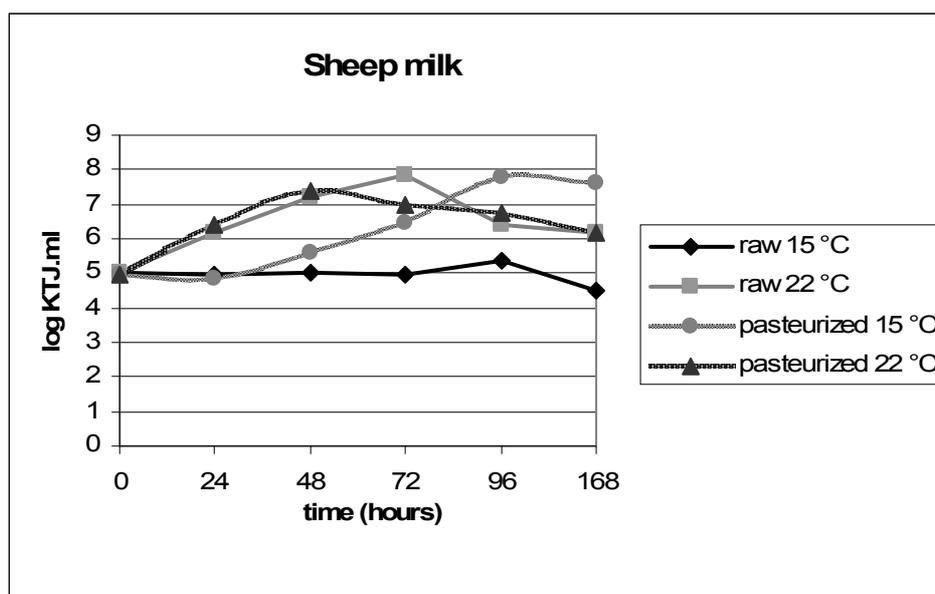


Figure 1 *S. aureus* growth in raw and pasteurized sheep's milk at 15°C and 22°C

In the raw sheep's milk stored at 15°C, the *S. aureus* count increased from the baseline $\log 5.00 \text{ CFU.ml}^{-1}$ to $\log 5.36 \text{ CFU.ml}^{-1}$. This count was recorded after 96 hours of culture, i.e. on storage day 4, but on storage day 7, the *S. aureus* count dropped to $\log 4.51 \text{ CFU.ml}^{-1}$. At the higher storage temperature, i.e. at 22°C, a more rapid increase in the *S. aureus* count was observed, from $\log 5.00 \text{ CFU.ml}^{-1}$ to $\log 6.15 \text{ CFU.ml}^{-1}$, with a peak of $\log 7.86 \text{ CFU.ml}^{-1}$ on culture day 3. Given the high *S. aureus* count at inoculation, the critical limit of 10^5 CFU.ml^{-1} was reached as early as within 24 hours of culture.

In **pasteurized sheep's milk** inoculated with *S. aureus* producing enterotoxin C and cultured at **15 °C** for 168 hours, the *S. aureus* count increased from the baseline log 4.96 CFU.ml⁻¹ to log 7.62 CFU.ml⁻¹. When the inoculated milk was cultured at **22 °C**, the limit of 10⁵ CFU.ml⁻¹ was exceeded as early as after 24 hours of culture and on day 7, the *S. aureus* count was log 6.18 CFU.ml⁻¹.

As reported by some authors, sheep's milk is often contaminated with high counts of *S. aureus*. **Necidová et al. (2010)** has detected *S. aureus* in 14 samples (67%) of raw sheep's milk in counts ranging from < log 5.0.10¹ CFU.ml⁻¹ to > log 7.5.10⁴ CFU.ml⁻¹. In two samples (9.5%), the limit for *S. aureus* laid down by standard STN 57 0510 Sheep's milk was exceeded. The presence of *S. aureus* in raw sheep's milk has also been detected by others (**Dudriková et al., 1999; Muehlherr et al., 2003**). **Přidalová et al. (2010)** have found the limit count of *S. aureus* to be exceeded in 12 of 32 samples of sheep's milk cheese for retail sale. **Bautista et al. (1988)** have reported that as many as 81.8% of *S. aureus* strains isolated from sheep's milk have the capability of producing staphylococcal enterotoxins (SEs), most often SEC.

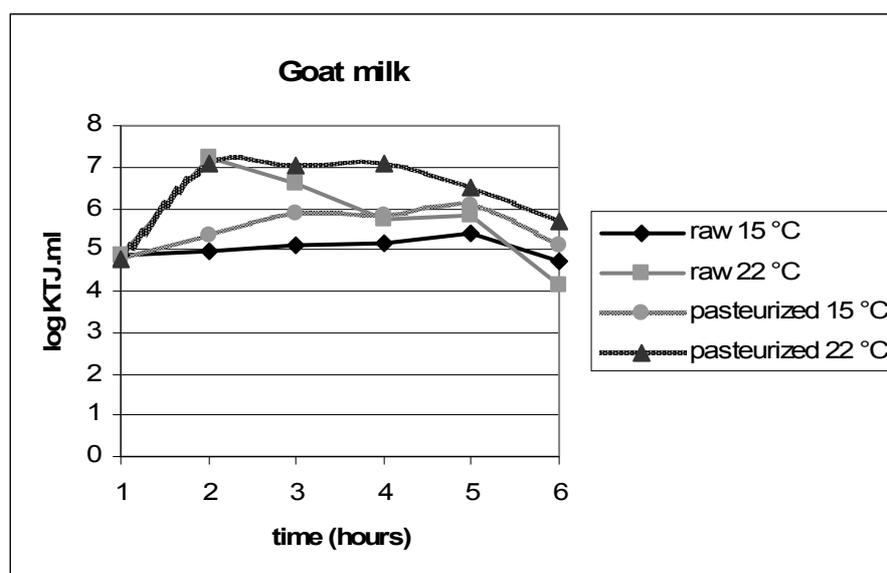


Figure 2 *S. aureus* growth in raw and pasteurized goat's milk at 15°C and 22°C

In **raw goat's milk** inoculated with *S. aureus* producing enterotoxin C at log 4.86 CFU.ml⁻¹ and cultured at **15 °C**, the count of *S. aureus* on day 7 was log 4.74 CFU.ml⁻¹. A similar *S. aureus* count was also detected on the last day of culture at **22 °C**, with an initial sharp rise to log 7.23 CFU.ml⁻¹ (after 24 hours of culture) followed by a downward trend to log 4.15 CFU.ml⁻¹ on day 7.

Pasteurized goat's milk was inoculated with *S. aureus* producing enterotoxin C at log 4.78 CFU.ml⁻¹. After 168 hours of culture at 15°C, the *S. aureus* count increased to log 5.11 CFU.ml⁻¹, but a peak count of log 6.08 CFU.ml⁻¹ was achieved on day 4. The culture at 22°C resulted in higher growth rates of *S. aureus*, peaking at log 7.08 CFU.ml⁻¹ after 24 hours of culture and then following a downward trend to log 5.70 CFU.ml⁻¹.

The presence of *Staphylococcus aureus* in raw goat's milk was studied by **Cupáková et al. (2006)**. They detected *S. aureus* in 69 % of raw goat's milk samples. Nearly all their isolates were carriers of the *sec* gene encoding staphylococcal enterotoxin C (SEC). The production of this enterotoxin was confirmed by a reverse passive latex agglutination (RPLA) assay. None of the enterotoxigenic genes was detected by PCR in one *S. aureus* isolate, while two isolates were positive for the *seb*, *seg*, and *sei* genes, and a single isolate was a carrier of the *seg* and *sei* genes. The production of enterotoxin B (SEB) was confirmed by a RPLA assay again. A single isolate of *S. aureus* from pasteurized goat's milk was a producer of SEC. The *S. aureus* counts detected in raw milk were lower than 10⁵-10⁶ CFU.ml⁻¹ and therefore, did not reach the infection dose needed to produce the amount of enterotoxins required to cause food intoxication, as reported by **Ercolini et al. (2004)**. However, under poor storage conditions, *S. aureus* can multiply in raw milk, producing highly heat-stable toxins that cannot be safely controlled by pasteurization. The detection of *Staphylococcus aureus* in pasteurized goat's milk was rare.

SEC producers were described as the most prevalent enterotoxin-producing *S. aureus* isolates from goat's milk (**Foschino et al., 2002; Scherrer et al., 2004**), raw goat's milk cheese (**Cremonesi et al., 2007**), and the goat's udder skin, teats, and milk (**Valle et al., 1990**).

Table 1 Time to the first detection of staphylococcal enterotoxin C in sheep's and goat's milk

	Raw		Pasteurized	
	15 °C	22 °C	15 °C	22 °C
Sheep's milk	-	48 h	48 h	24 h
Goat's milk	-	24 h	48 h	24 h

Tab 1 shows the times (in hours) to the first detection of staphylococcal enterotoxin C in different types of milk inoculated with *S. aureus*. From the table, it is evident that the storage temperature significantly influences the growth of *S. aureus* and production of SEs.

In **raw milk** stored at **15 °C**, no enterotoxin production was detected during the whole storage period, i.e. within 7 days, in either sheep's or goat's milk.

In raw sheep's milk samples cultured at **22 °C**, enterotoxin production was detected within 48 hours, while in raw goat's milk samples, enterotoxin production was revealed as early as within 24 hours of culture.

Pasteurized milk was a good substrate for enterotoxin production. At **15 °C**, SEC was detected within 48 hours of culture in both sheep's and goat's milk, while at **22°C**, the enterotoxin production was detected as early as within 24 hours of culture.

The environmental conditions such as temperature, pH, water activity, salt concentration, and competing microflora influence the *S. aureus* growth and SE production (**Genigeorgis, 1989**).

The fact that the *S. aureus* growth in raw milk was clearly lower in comparison with pasteurised milk and no SE production was detected can be explained, according to **Charlier et al. (2008)**, by the natural microflora of raw milk that can inhibit the *S. aureus* growth and thus also SE production. An important part of this natural microflora are lactic acid bacteria (LAB) that reduce the pH of milk. This inhibitory effect has also been reported by **Alomar et al. (2008)**. Lactococci (*Lactococcus lactis*) are the most rapid LAB to multiply in raw milk. Several strains of *L. lactis* produce the bacteriocin nisin. Nisin has been shown to be bactericidal for *S. aureus* (**Klaenhammer, 1993**).

CONCLUSION

With food-borne intoxication risk heightened by presence of caused by staphylococcal enterotoxins, the monitoring of *S. aureus* and staphylococcal enterotoxins is a crucial step in the food quality and safety control.

The results of our model experiments have shown that *S. aureus* multiplication and subsequent staphylococcal enterotoxin production depend on the culture/storage temperature and type of milk. It is noteworthy that in raw milk, *S. aureus* growth and SE production are suppressed by competitive microflora. Nevertheless, this finding cannot be used as an argument to encourage consumption of raw, unpasteurized milk. The limit count of 10⁵ CFU/g laid down by regulations as a prerequisite for the production of SEs was confirmed by our experiments. The ELFA assay using the miniVIDAS[®] instrument proved suitable for detecting of SEs.

The results of our study support the importance of maintaining the cold chain (at a safe temperature under 8°C) from production to retail sale for the safety of milk and dairy products.

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