Influence of Soft Cheese Technology on the Growth and Enterotoxin Production of *Staphylococcus aureus*

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Abstract

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The aim of this study was to monitor *S. aureus* growth and toxin production in soft cheese during the technological processing. In model experiments, raw milk was inoculated separately with five *S. aureus* strains isolated from milk and milk products. All the strains were producers of staphylococcal enterotoxins (SEs) of types A, B, or C. SEs were detected by the enzyme-linked fluorescence assay (ELFA) performed in the MiniVIDAS device. This study has shown that the amount of SEs varied with the tested strains and stages of the technological process. SEs were detected in soft cheese made from pasteurised milk inoculated with 2.9×10^5 CFU/g of *S. aureus*. The prevention of *S. aureus* contamination and multiplication during the cheese making process is a prerequisite for the production of safe soft cheese. The most important enterotoxin dose build-up factor can be overcome by strict compliance with the cooling requirements during the manufacture, distribution and storage of the product.

Keywords: dairy products; enterotoxin; bacteria; soft cheese processing; growth curve

Staphylococcus aureus is an important human and animal pathogen known to produce a range of toxic substances that can cause various diseases. From the perspective of food microbiology, the most relevant characteristic of *S. aureus* is the production of heat-stable enterotoxins implicated in food-borne intoxications. Currently, 19 staphylococcal enterotoxins (SEs) are known: 5 classical and 14 newly described ones (Thomas *et al*. 2007). The potential to cause food-borne intoxications has been reported in all classical and some new enterotoxins (Omoe *et al*. 2002).

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 & Bohach 2001).

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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 (Zhang *et al.* 1998; Atanassova *et al.* 2001; Loir *et al.* 2003). The symptoms resolve spontaneously within 24–48 h (Loir *et al.* 2003).

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. During their production, storage, and distribution, the bacteria can proliferate and subsequently produce SEs.

For instance, in France and the UK, *S. aureus* is the major causative agent of food-borne intoxications due to milk and dairy products. The research conducted in 1993–1998 has shown that 4.8% of staphylococcal intoxications reported in Europe were associated with the consumption of milk and dairy products (Lopez-Pedemonte *et al.* 2007).

S. aureus counts in the range as low as 10^3 to 10^5 CFU/g of food can produce the amount of toxin that poses a risk to consumers (Jablonski & Bohach 2001; Balaban & Rasooly 2000). From this point of view, the critical value of *S. aureus* counts has been determined at the level of 10^5 CFU/g. This is also the value determined by the current legislation as hazardeous (European Commission. Commission Regulation (EC) No. 1441/2007).

The dose of toxin needed to cause intoxication is very low. Balaban and Rasooly (2000) and Omoe *et al.* (2002) 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 favourable conditions (optimum temperature, pH, a_w, salt concentration) for *S. aureus*, it takes not less than 20 h to produce enough enterotoxin (Sharma *et al.* 2000). The toxins are produced at a temperature range from 10°C to 48°C, with the optimum between 37°C to 40°C. The minimum pH suitable for their production is about 4.8, with the optimum ranging between 6 and 7. Nevertheless, the type of enterotoxin also plays a role. At a pH between 5.3 and 6, the production of enterotoxins B and C declines while that of enterotoxin A continues unchanged. In the products with a lower water activity and under aerobic growth conditions, the production of toxins is possible at an a_w as low as 0.80–0.86, but the optimum production is achieved at a_w = 0.99 and higher. A higher production of toxins is observed under aerobic than under anaerobic conditions (Roberts *et al.* 1996).

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

MATERIAL AND METHODS

Samples. In the model experiments, raw cow's milk was inoculated separately with five *S. aureus* strains. They originated from milk and dairy products and were kept in the collection of microorganisms of the Centre of the Hygiene of Food Chains, National Institute of Public Health, at a temperature of minus 75°C in nutrient broth, with 20% glycerine added. 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) according to the manufacturer's instructions.

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. 1662/2006), $CaCl_2$ and sour cream culture (Milcom a.s., Laktoflora, Czech Republic) are added, rennet (Milcom a.s., Laktoflora, Czech Republic) is added, the mixture is renneted at 30°C for 1 h, the curd is processed, pressed into moulds and let 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, a_w = 0.98–0.99, NaCl = 2% (w/v).

***Quantitative detection of** S. aureus.* The individual samples were examined, coagulase-positive staphylococci were enumerated according

to CSN EN ISO 6888-1 by the technique using Baird-Parker agar medium (Bio-Rad, France). The identification of typical colonies was based on the growth measurements on the Sa*Select* selective chromogenic medium (Bio-Rad, France) and blood agar, and the results of the coagulase tests ITEST STAFY-coagulase (ITest, Hradec Králové, Czech Republic) and Staphylo LA Seiken (DENKA SEIKEN Co. Ltd., Japan).

Detection of staphylococcal enterotoxins. Staphylococcal enterotoxins were detected by the enzyme-linked fluorescence assay (ELFA) using a MiniVIDAS analyzer (Vitek Immuno Diagnostic Assay System, bioMérieux, France), able to detect the sum of enterotoxins SEA–SEE, with the detection limits of 0.5 ng/g or per ml of food for SEA and SEB and of 1.0 ng/g or per ml of food for SEC–SEE.

The samples were processed according to the manufacturer's instructions as follows: The cheese samples were first homogenised with sterile distilled water (at a ratio of 25 g of sample to 40 ml of distilled water), then pH was adjusted to a range from 3.5 to 4, the supernatant was obtained by centrifugation and its pH was adjusted to 7.5–8. The final sample yielded after an additional centrifugation was transferred directly into the well of the VIDAS SET2 strip. The pH of the liquid samples was adjusted without the addition of distilled water.

As the ELFA technique does not allow the quantitative detection of SEs, the results are expressed as either positive or negative. To monitor the dynamics of the production of SEs at various stages of the cheese making process, the Relative Fluorescence Values (RFV) were determined. RFV is calculated by subtracting the background reading from the final result. This calculation appears on the result sheet. The RFV obtained for each sample is interpreted by the VIDAS system as follows: test value < 0.13 – interpretation negative, test value ≥ 0.13 – interpretation positive.

RESULTS AND DISCUSSION

Model experiment 1 was focused on the study of the growth of *S. aureus* and the production of enterotoxins in naturally contaminated milk, subsequently pasteurised and processed into soft cheese. Table 1 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 at temperatures

Table 1. *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	$< 5 \times 10^1$

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

of 72°C and 85°C for 15 s completely eliminated staphylococci in milk.

Milk pasteurisation has been reported to reduce significantly *S. aureus* counts or even to eliminate completely the agent (Contreras *et al*. 2003). Nevertheless, when the *S. aureus* counts prior to pasteurisation were higher than 10^5 CFU/ml, heat-stable enterotoxins could be present in the milk. The same may be observed when the milk is not cooled properly after milking. Ikeda *et al*. (2005) reported an outbreak in Japan in 2000, with more than 10 000 cases of staphylococcal enterotoxicosis, milk powder produced from pasteurised milk having been identified as the source of the infection.

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 1). 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.

To guarantee the food safety, attention should be paid to the possible secondary contamination by *S. aureus*, particularly in foods intended for direct consumption such as soft cheeses. The requirements for the detection of coagulase-positive staphylococci and SEs in soft cheeses are also included in the Commission Regulation (EC) No. 1441/2007 on microbiological criteria for foodstuffs. The presence of staphylococci in these products is often a result of improper handling of the food while processed and failure to comply with the safety regulations (Acco *et al*. 2003).

The model experiment described in Table 2 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 re-

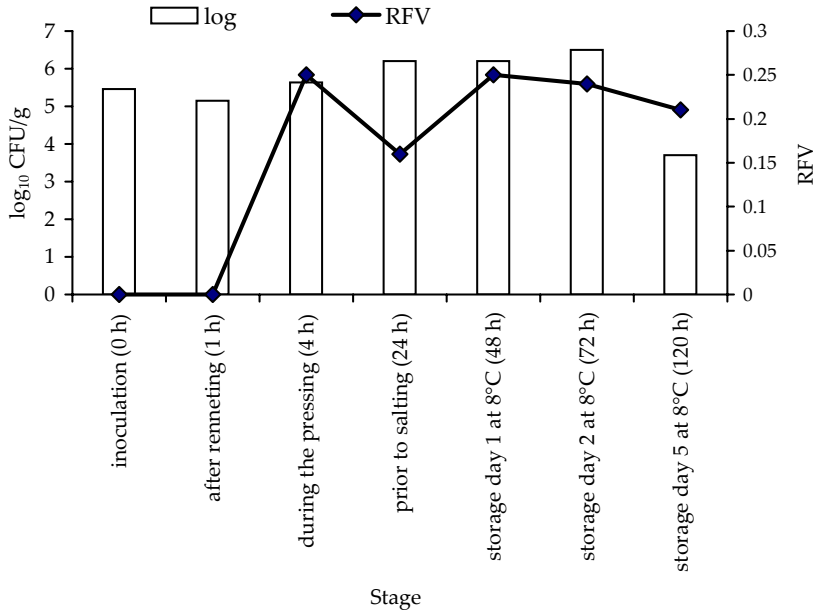

Figure 1. The relationship between *S. aureus* SA1185 count and RFV at various stages of soft cheese making and storage at 8°C

lationships between *S. aureus* counts and RFVs, i.e. the amounts of staphylococcal enterotoxins at various production stages, are given in Figure 1.

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 ca 12 hours. After the separation of the whey from the curd, i.e. prior to salting, RFV decreased considerably, i.e. much less staphylococcal enterotoxin remained in the product. This was in agreement with the fact that SEs, proteins with a short polypeptide chain, soluble in water and saline solution (Loir *et al*. 2003), are partly removed from the cheese together with the whey.

Towards the end of the cool storage (Day 5), the *S. aureus* counts either showed a plateau or slightly decreased, yet the production of enterotoxins was detected. This finding clearly shows that the enumeration of *S. aureus* in the final product is only generally indicative and provides no information on the presence of staphylococcal enterotoxins.

On the other hand, based on the results obtained, it can be confirmed that although *S. aureus* is able to grow slowly at the refrigerator temperature, the production of SEs is limited (at 7–10°C) as proved in previously published studies (Bergdoll 1979; Schmitt *et al*. 1990; Roberts *et al.* 1996) or any growth and toxin production are not recorded at all (at temperature about 4°C) (Halpin-Dohnalek & Marth 1989), not even at an inoculum of 10^6 CFU/ml (Anunçiacao *et al*. 1995).

Most soft cheeses from the model experiments did not comply with the Commission Regulation (EC) No. 1441/2007 that specifies the limit for coagulase-positive staphylococci to be 10^1–10^2 CFU/g. Six of 28 products showed *S. aureus* counts > 10^5 CFU/g and, pursuant to the regulation, should be further screened for the presence of staphylococcal enterotoxins.

CONCLUSION

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. 1662/2006) 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. Other steps to reduce microbial counts including staphylococci are heating (pasteurisation) of the milk used in the production of soft cheese and the inhibitory effect of the starter lactic acid bacteria. 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 pri-

marily the prevention of secondary contamination and cool chain maintenance during the storage, transportation, and distribution of soft cheeses.

From the results of the study it follows that the production rates and produced amounts of staphylococcal enterotoxins in soft cheese vary with *S. aureus* strains. In the present study, the most potent enterotoxin producer was a *S. aureus* strain producing enterotoxin A.

The knowledge obtained can be helpful in establishing the limits for the critical control points within HACCP (Hazard Analysis Critical Control Points) and in testing the whole system of critical control points in the technological process of soft cheese making.

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