Multiplication of Staphylococcus (S.) aureus and production of enterotoxins during the experimental manufacturing of Camembert cheese

G. Hahn, M. Müller

Institute for Hygiene, Federal Dairy Research Centre, Kiel

1 Introduction

The enterotoxins (SE) A-E produced by Staphylococcus (S.) aureus world-wide belong to the most frequent causes of food intoxications. One of the origins for raw milk and raw milk products may be cows suffering from subclinical mastitis. About 10^6 cfu/ml/g are sufficient to produce an amount of enterotoxins which may be dangerous for humans (1). Because of similar conditions for the production thermonuclease (TNase) may be used as a screening for the potential presence of enterotoxins in the product (3). It is important to know that especially in soft cheeses because of the high water content and pH value during the ripening process there are a lot favourable conditions for the multiplication of S.aureus (4). In the EU Directive 92/46 (6), therefore, limits are given for S.aureus in raw milk used for manufacturing of raw milk products (m = 500, M = 2 000) and for cheese from raw milk and thermized milk (m = 1000, M = 10 000). If M is exceeded the presence of toxin has to be examined in the product. Aim of the presented work was to show the kinetics of multiplication and of enterotoxin production of S.aureus during the experimental manufacturing of raw milk soft cheese under different conditions.

2 Material and methods

2.1 Material

- Reference strains of S.aureus producing SEA (staphylococcus enterotoxin A) and SEE (Staphylococcus enterotoxin E)
- inhibitor free raw bulk milk for cheese production from cows of the experimental station of the institute
- starter cultures (Probat 505-Wiesby);
- P.candidum (SC, DIP D1-Wiesby)
- rennet extract standard (Chr. Hansen)

2.2 Methods

The manufacturing of raw milk soft cheese was done according to Prokopek and Voss (7).

- thermization of the raw milk to 32° C and additional inoculation of S.aureus culture of reference strains with different counts
  starter culture (concentration 0.75 - 1.6 %, preincubation: 1 or 3 days at 22° C in UHT milk) and P.candidum culture (0.025 %) at manufacturing step 0.5 h
- after filling the cheese curd in forms three turnings after every hour
- ripening (2. to 11. day): 18° C, 80 % relative air humidity, wrapping of the cheeses
- storage (12.-32. day): 15° C, 20 % relative air humidity

The steps for taking samples during manufacturing are given in Table 1.

Table 1: Sampling during the manufacturing of raw milk soft cheese

manufacturing step		time of sampling
bulk milk before thermization		0 h
after addition of S.aureus, starter and mould culture		0,5 h
after addition of rennet		1,5 h
after cutting of cheese curd	whey	2,5 h
	curd	2,5 h
after filling in form	whey	3,5 h
	curd	3,5 h
after third turning		6,5 h
after brine bath		8,5 h**
2., 4., 6., 8., 11., 18., 25., 32. day		indication of manufacturing (d)

All samples were examined according to official standard methods (8) for:

Count of coagulase positive staphylococci

- preparation of the cheese samples (L 03.001-1)
- determination of the count of coagulase positive staphylococci (L 01.00-24)
- differentiation of colony types on BAIRD PARKER-medium
- determination of TNase (L 01.00-33)
- determination of coagulase (L 01.00-24) for TNase positive colonies

Isolation of TNase directly from the food (L 01.00-33)

- the enterotoxins were determined directly from the food for SEA and SEE by methods (sandwich ELISA) which were developed in the institute (9, 10)
- measuring of pH value and temperature

All these measurements were done at the steps given in Table 1.

3 Results

The different lots of cheese varied regarding the count of enterotoxigenic S.aureus strains (2×10^3 - 4.5×10^4 cfu/ml) and the mentioned starter activities and concentrations.

3.1 Course of pH values

Starting in raw milk with pH 6.8 the value decreased till 6.5 h resp. 8.5 h to pH < 5.0. These values were constant up to the 6. resp. 8. day, then increased during the following steps and reached 6.0 - 7.5 between 25. - 32. day.

3.2 Course of S.aureus counts (see Figure 1)

Regarding different conditions the geometric mean values of the different lots are demonstrated starting at 0.5h after inoculation of the test organisms. Lots with identical test conditions are summarized in Figure 1. A general tendency of multiplication during the first days of manufacturing is a clear increase up to 8.5h, followed by a plateau and then within the last weeks of storage again by a further multiplication.

The counts in the whey are not shown in the figure. After 3.5h they were generally two potencies lower than in the cheese curd, which may be caused by a physical enrichment.

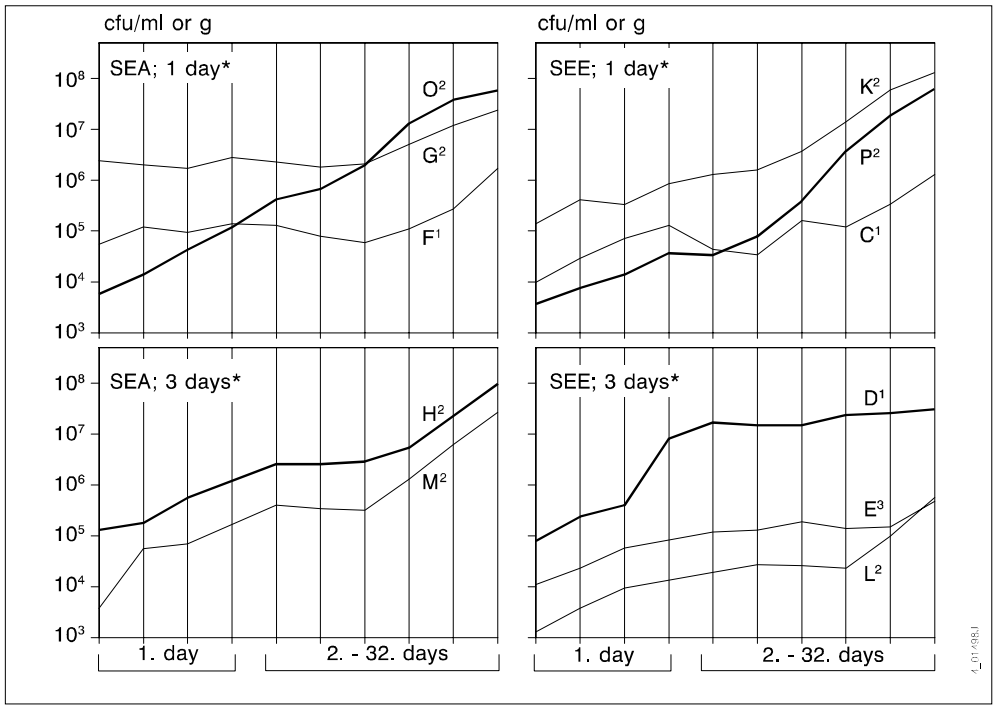

Fig. 1: Manufacturing of raw milk soft cheese. Course of S.aureus counts (cfu/ml/g) after inoculation as geometric means.
Starter concentration (%): [1] 1.0; [2] 0.75; [3] 1.6
* Starter pre-incubation: lots F, G, O and C, K, P (1 day), lots H, M and D, E, L (3 days)

The influence of the initial counts of S.aureus (0.5h), the concentration (0.75%, 1.0%, 1.6%) and activity (pre-incubation one or three days) regarding the growth course and count of S.aureus at the end of ripening may be interpreted as follows.

The initial count seems to have less importance if the starter concentration is equal (e.g. lots O^2, G^2; H^2, M^2; K^2, P^2). It shows that the lowest starter concentration allows the highest final count of staphylococci. Comparing the activities of starter (1 or 3 days) the fresh one day starters, obviously, for lot F an G prevent a multiplication of S.aureus in spite of higher initial counts. The staphylococci of lot O, however, show a continuous multiplication in spite of a low beginning, maybe caused by the lower starter concentration. Summing up the starter concentration seems to have more influence than the starter activities.

3.3 TNase and enterotoxin production (see Figures 2 and 3)

The most important question of these examinations was to see the possible production of enterotoxins during a usual manufacturing process of raw milk soft cheese and the prove of TNase as a screening for potential enterotoxin presence also under the identical combinations as described above.

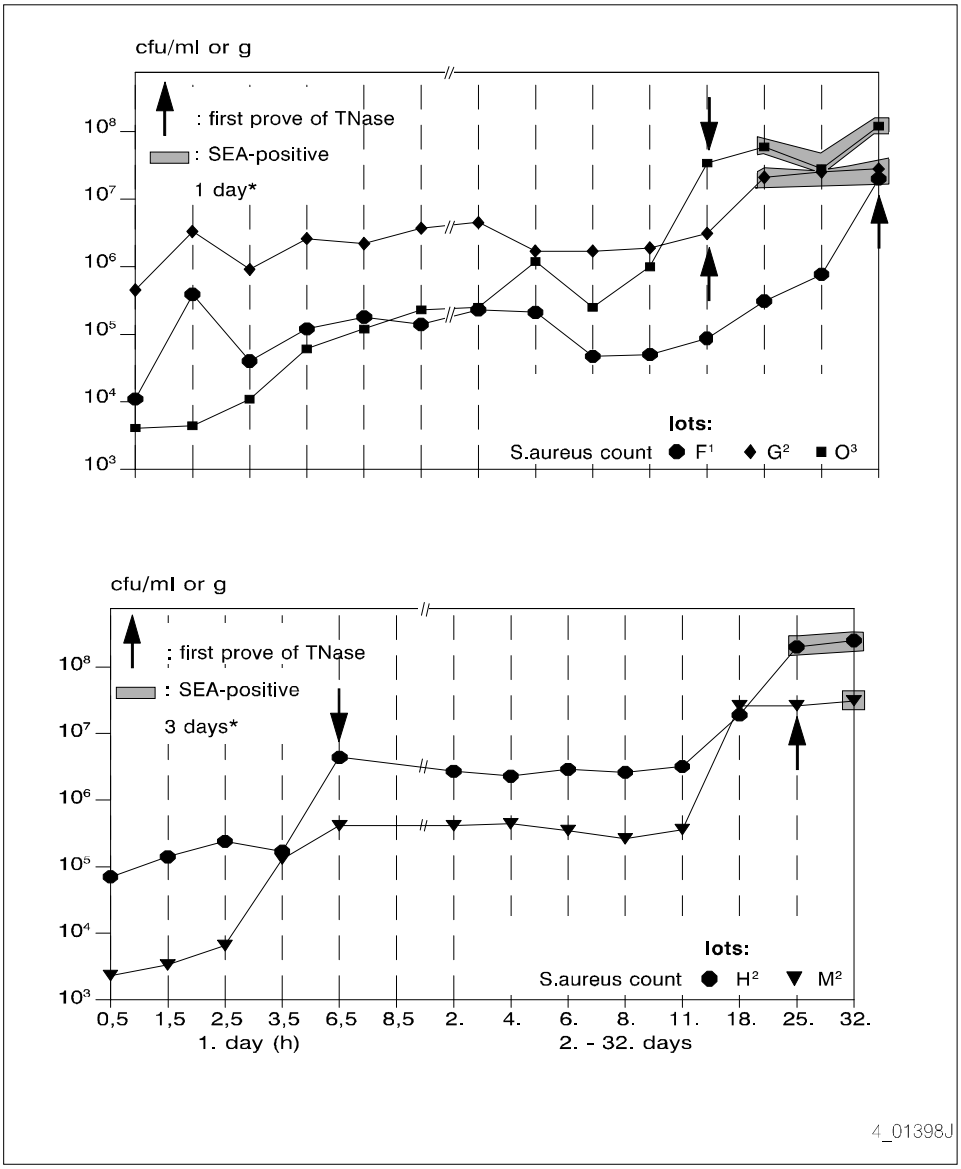

Fig. 2: Manufacturing of raw milk soft cheese with inoculation of an SEA-producing S.aureus
First ocurrence of TNase-and Enterotoxin in relation to the growth of staphylococcus starter concentration (%): [1] 1.0; [2] 0.75; [3] 1.6
* starter pre-incubation: lots F,G,O (1 day), lots H,M (3 days)

In ten of eleven lots during the manufacturing and ripening enterotoxin could be found. All SEA samples showed also a positive TNase except one lot (F) earlier to the presence of enterotoxin. This, however, was not true for the SEE samples, where TNase was detected at the same time or later as enterotoxin. As it is known from the literature TNase and/or enterotoxin only can be detected if more than at least 10^6cfu/g or ml are present. However, detectable amounts only are present if the bacteria had the chance to multiply several times in the food. That means that high initial counts also need a distinct time of incubation for production of these substances. A direct correlation to initial count of staphylococci, starter concentration and activity is not to be seen. These factors only are relevant for the multiplication of the staphylococci and these consequently are responsible for TNase and toxin production.

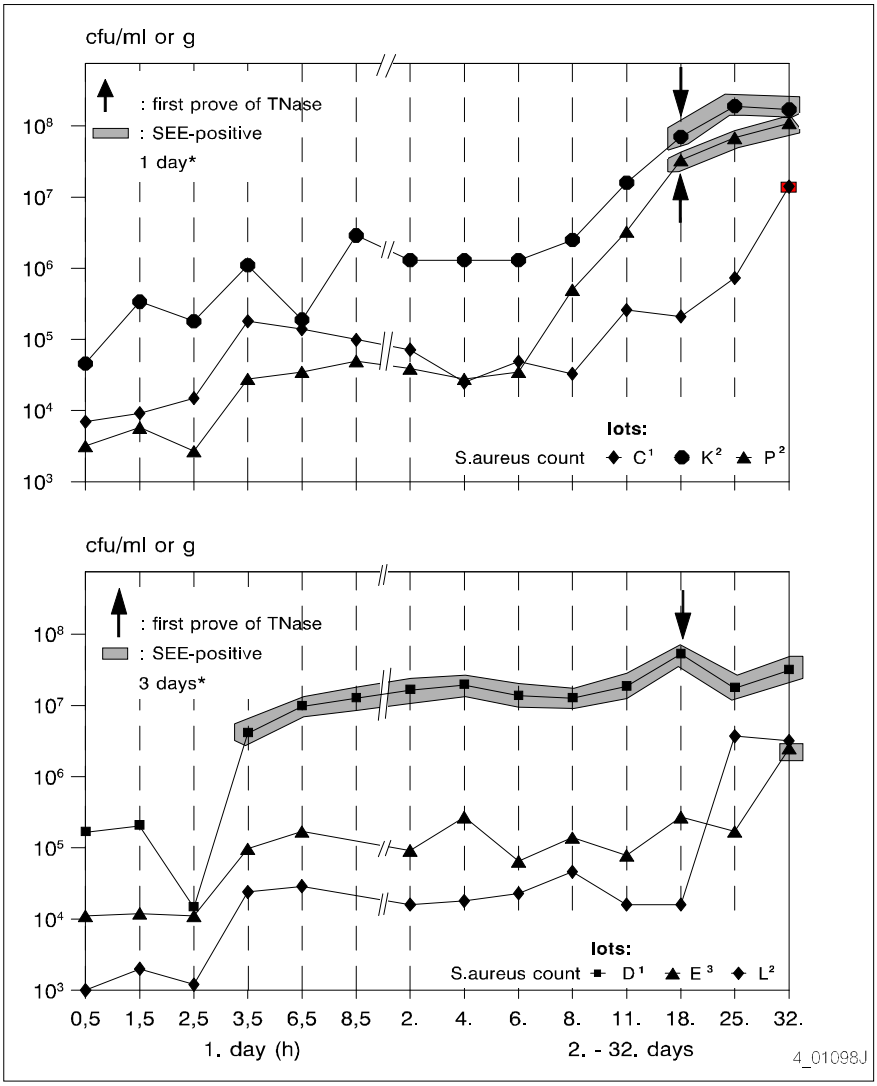

Fig. 3: Manufacturing of raw milk soft cheese with inoculation of an SEE-producing S.aureus First ocurrence of TNase-and Enterotoxin in relation to the growth of staphylococcus starter concentration (%): [1] 1.0; [2] 0.75; [3] 1.6
* starter pre-incubation: lots C, K, P (1 day), lots D, E, L (3 days)

4 Discussion

On the background of the EU Directve 92/46 resp. the German MilchVO '95 (5) it should be shown whether the demanded maximum values for S.aureus in raw milk and raw milk soft cheese are realistic under practical conditions. This concerns especially the multiplication of S.aureus and the production of enterotoxins and TNase. For this purpose enterotoxin producing reference strains in different initial counts were experimentally inoculated in raw ex-farm bulk milk for manufacturing raw milk camembert cheese. Besides the varying S.aureus counts different starter concentrations and activities were used.

The increase of S.aureus counts during the first day of manufacturing was also described by others (12, 13, 14) and may be explained besides the multiplication by a physical enrichment by the cheese curd (15, 16). Generally, this phenomenon depends on the kind of cheese and its stage during ripening and storage. In hard cheese a decrease is usual (16, 17) while at a higher water activity in soft cheeses counts of 10^6 - 10^7 cfu/g were observed (18, 19).

In the beginning of manufacturing the concentration and activity of starter cultures have a clear effect on the multiplication of pathogens (16, 20, 21, 22). In our experiments the concentration seems to have a more distinct influence than the activity. When low concentrations and/or low activities were used yet in the early stage of manufacturing S.aureus counts of $>10^6$ cfu/g can be reached. Especially, under non-professional conditions on farms this may happen. The counts of S.aureus in our experiments exceed by far the maximum (M) of 10,000 cfu/g as it is demanded in the EU Directive 92/46. These counts in any case are high enough to produce detectable amounts of enterotoxin (2).

The described relation of TNase- and enterotoxin production regarding the S.aureus counts of at least 10^6/g (2, 16) could also be proved by our own experiments as well as the fact that TNase mostly occurs earlier than the enterotoxin (13). In ten from eleven lots enterotoxin could be detected in a more or less early stage of manufacturing.

Regarding the standards for raw milk cheese on the level of manufacturing our results show that a maximum (M) of 10,000 cfu/g acc. to the EU Directive easily may be exceeded latest at the end of the ripening . Similar results were obtained from examinations for Brie and red smear cheese produced from naturally contaminated raw milk in dairy plants (23). For the postulated examination for toxins if M is exceeded, therefore, the TNase test as a rapid and reliable screening can be recommended.

Summing up, from our experiments it could be demonstrated that during the manufacturing process even at "allowed" counts of S.aureus in the milk may lead to a multiplication which enables the production of detectable amounts of enterotoxins. The methods used in the presented paper are feasible and reliable also for routine laboratories to fulfil the demands of the EU Directive.

5 Summary

The EU-directive 92/46 has laid down criteria for the presence of S.aureus in raw milk used for the production of raw milk products and in raw milk cheese. The regulations were transferred to German national law by means of the Milk Ordinance ‘95. The scope of the present investigation was to monitor the kinetics of multiplication and production of enterotoxins during the experimental manufacturing of camembert-cheese from artificially inoculated milk.

For the experimental production of camembert cheese, raw milk was used containing levels of 10^3-10^5 cfu S.aureus/ml after artificial inoculation with SEA- or SEE producing reference strains. The conditions of the starter culture varied in concentration (0.75 - 1.6 %) and time of incubation (1 or 3 days). Thermonuclease and/or enterotoxins were produced in 10 out of 11 lots. They even occurred in cheese manufactured from milk with low initial counts of 7.0×10^3 cfu S.aureus/ml, using starter culture (1.0 % concentration; 1 day incubation).

Thermonuclease- and/or enterotoxin-positive samples in all cases contained counts of $> 10^6$ cfu S.aureus/g and were observed mostly in the last stages of ripening (days 18-32). If milk with high initial counts of 10^4 - 10^5 cfu S.aureus/ml was used in combination with a long incubation period of starter cultures (3 days), S.aureus reached counts sufficient for thermonuclease and/or enterotoxin already on the first day of manufacturing. On the other hand, neither thermonuclease nor enterotoxin was detected in one lot produced from milk with 1.0×10^3 cfu S.aureus/ml, although the starter cultures were less active (0.75 % concentration, 3 days incubation).

The thermonuclease-test is recommended as an additional method for the screening or the confirmation of the occurrence of enterotoxins in cheese.

The present combination of methods is suggested as a basis for the development of a routine method according to the Milk Ordinance'95.

References

1. Bergdoll, M.S.: Staphylococcus aureus. In: Doyle, M.P., Foodborne bacterial pathogens, M. Dekker, Inc., New York und Basel, 463-523 (1989)
2. Cords, B.R., Tatini, S.R.: J. Dairy Sci. **56** 1512-1519 (1973)
3. Zaadhof, K.J.: Arch. Lebensmittelhyg. **29** 138 (1978)
4. Nooitgedagt, A.J., Hartog, B.J.: Neth. Milk Dairy J. **42** 57-72 (1988)
5. Verordnung über Hygiene- und Qualitätsanforderungen an Milch und Erzeugnisse auf Milchbasis (Milchverordnung) vom 24. April 1995, BGBl. I Nr. 21 S. 544
6. Richtlinie 92/46/EWG des Rates vom 16. Juni 1992 mit Hygienevorschriften für die Herstellung und Vermarktung von Rohmilch, wärmebehandelter Milch und Erzeugnissen auf Milchbasis, Abl. EG Nr. L 268 S. 1
7. Prokopek, D., Voss, E.: Deutsche Molkerei Zeitung **35** 1437-1450 (1972)
8. Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG: Methode L 03.00-1: Vorbereitung der Proben für mikrobiologische Untersuchungen, Verfahren für Käse. Methode L 01..00-24: Bestimmung Koagulase-positiver Staphylokokken in Milch und Milchprodukten, Koloniezählverfahren. Methode L 01.00-33: Nachweis von Staphylokokken-Thermonuklease in Milch. BgVV Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (Hrsg.), Beuth Verlag GmbH, Berlin, Wien, Zürich
9. Hahn, G.: Arch. Lebensmittelhyg. **43** 6-8 (1992)
10. Müller, M.: Vet. Med. Diss., Berlin (1996)
11. Müller, M., Hahn, G., Heeschen, W.: Häufigkeit von Enterotoxin-bildenden S.aureus in Rohmilch und Rohmilch-Weichkäse vor dem Hintergrund der Milchverordnung. Deutsche Veterinärmedizinische Gesellschaft, 36. Arbeitstagung des Arbeitsgebietes Lebensmittelhygiene, Garmisch-Partenkirchen, 26.-29. September 1995 (1995)
12. Abo-Elnaga, I., Kandler, O.: Milchwissenschaft **20** 416-419 (1965)
13. Van Schouwenburg-Van Foeken, W.J., Stadhouders, J., Jans, J.A..: Neth. Milk Dairy J. **32** 217-231 (1978)
14. Erkmen, O.: J. Food Prot. **58** 1201-1205 (1995)
15. Tatini, S.R., Jezeski, J.J., Morris, H.A.: J. Dairy Sci. **54** 815-825 (1971)

16. Otero, A., Garcia, M.C., Garcia, J.L., Santos, J.A., Moreno, B.: Int. Dairy J. **3** 85-96 (1993)
17. Nunez, M., Bautista, L.., Medina, M., Gaya, P.: J. Appl. Bacteriol. **65** 29-34 (1988)
18. Santos, E.C. Dos, Genigeorgis, C.: J. Food Prot. **44** 177-184 (1981)
19. Stecchini, M.L., Sarais, I., De Bertoldi, M.: Intern. J. Food Microbiol. **14** 99-109 (1991)
20. Genigeorgis, C.A.: Intern. J. Food Microbiol. **9** 327-360 (1989)
21. Stadhouders, J., Cordes, M.M., Van Schouwenburg-Van Foeken, A.W.J.: Neth. Milk Dairy J. **32** 193-203 (1978)
22. Gomez-Lucia, E., Goyache, J., Blanco, J.L., Vadillo, S., Garayzabal, J.F.F.: Z. Lebensm. Unters. Forsch. **184** 304-307 (1987)
23. Maier, S.: Staphylococcus aureus in Weichkäse aus Rohmilch. Diplomarbeit, Institut für Lebensmitteltechnologie, Universität Hohenheim (1994)