Turkish Journal of Veterinary and Animal Sciences
http://journals.tubitak.gov.tr/veterinary/
Research Article

Turk J Vet Anim Sci
(2014) 38: 318-324

doi:10.3906/vet-1307-2

Staphylococcus aureus growth and enterotoxin production in Italian caciotta cheese

Francesca PEDONESE*, Carlo D'ASCENZI, Beatrice TORRACCA, Clizia ZINGONI, Barbara TURCHI, Filippo FRATINI, Roberta NUVOLONI
Department of Veterinary Sciences, University of Pisa, Pisa, Italy

Received: 03.07.2013 • **Accepted:** 14.01.2014 • **Published Online:** 21.04.2014 • **Printed:** 20.05.2014

Abstract: To evaluate growth and enterotoxin production of *Staphylococcus aureus* in raw milk caciotta, an Italian soft cheese, experimental cheesemaking trials were carried out with and without a commercial starter. Two different inocula of selected enterotoxin-producing *S. aureus* strains were tested: high (mean value 5.03 log CFU/mL of milk) and low (mean value 3.22 log CFU/mL of milk). Uninoculated cheeses with milk containing 2.15 log CFU/mL mean value of coagulase-positive staphylococci were also examined. With higher inocula coagulase-positive staphylococci counts reached 7.57 log CFU/g without starter and had significantly higher values after the acidification phase than those with starter (below 6.5 log CFU/g). Using an enzyme immunoassay, enterotoxins were detected in cheeses both without and with starter, but the latter showed significantly lower photometrical absorbance values than the former. In low inoculum and uninoculated cheeses without starter, coagulase-positive staphylococci maximum values were between 5 and 6 log CFU/g, without enterotoxin production. To enhance the safety of this cheese it is fundamental to strictly prevent coagulase-positive staphylococci contamination and multiplication, particularly at the primary production level, and to correctly acidify the curd by starter cultures, which can substantially limit total coagulase-positive staphylococci increase.

Key words: Caciotta cheese, enterotoxin production, experimental cheesemaking process, *Staphylococcus aureus*

1. Introduction

In Italy, dairy cattle breeding takes place both on large farms, mainly in the northern regions where industrial dairy plants are concentrated, and on small farms. On the latter, which are located primarily in the central and southern regions, raw milk is often utilised for direct on-farm production of traditional cheeses such as caciotta cheeses, which are easily saleable on the local market.

The term caciotta refers to soft or semisoft cheeses made with cow, ewe, or mixed milk; these are among the most ancient Italian cheeses. The traditional production is concentrated in Central Italy (mainly Tuscany, Latium, Marches, and Umbria).

Today, caciotta production is spreading to all Italian regions and is often made on an industrial scale with pasteurised milk, reaching about 23,000,000 kg per year according to the Italian Dairy Association (Assolatte) (http://www.bancodibrescia.it/page/c/document_library/get_file?uuid=fd918ae2-96b3-4d33-b1f1-c8a4fc5ec73b&groupId=563303).

There are fundamentally 2 variants of caciotta; the cow milk type, which has a white, sweet, and soft interior, and the ewe or mixed milk type, with a stronger, more piquant taste and a more yellowish paste consistency. The cheeses are small-to-medium-sized, usually up to 2 kg in weight, and the ripening period varies from 7–15 to about 45 days. Moisture is between 40% and 45% depending on variants, and salt content can reach a maximum 2.5% in more ripened cheeses (1).

Among the microbiological hazards to be considered in raw milk soft cheeses, staphylococcal enterotoxins, which can be produced by about one-third of the coagulase-positive food origin *Staphylococcus* strains (2), are of major concern. This is mainly due to the fact that *Staphylococcus aureus* is one of the most important pathogens associated with bovine mastitis (3) and is highly inclined to persist in a herd in the form of subclinical infections, which have the tendency to become chronic (4).

Thus, *S. aureus* is frequently related to food-borne diseases from milk and milk product consumption; and raw milk cheeses in particular have been repeatedly involved in staphylococcal outbreaks (5–7). Studies in different cheeses (8–15) have proven that both *S. aureus* growth and enterotoxin production depend on many factors, mainly cheesemaking characteristics and the use of lactic acid starter cultures.

Since farmers in Central Italy often manufacture caciotta cheeses with raw milk, the aim of this work was to

* Correspondence: fpedones@vet.unipi.it

evaluate the behaviour of *S. aureus* during the experimental production of raw milk caciotta cheeses on a laboratory scale, according to the traditional cheesemaking procedures applied by small plants in Central Italy (Tuscany). Two sets of experimental cheesemaking trials were carried out in order to verify the growth and enterotoxin production of *S. aureus* strains inoculated at high and low dosages and explore the role of lactic acid starters in limiting them.

2. Materials and methods

2.1. *Staphylococcus aureus* strains

Two strains of *Staphylococcus aureus* (C21 and FB5) belonging to the culture collection of the Laboratory of Food Microbiology, Department of Veterinary Sciences, University of Pisa, and isolated from raw milk and cheese, respectively, were used. Strain identification was confirmed both phenotypically by API STAPH (bioMérieux, Marcy-l'Etoile, France) and genotypically at species level by polymerase chain reaction using oligonucleotide primers that amplified the thermonuclease encoding gene (*nuc*) (16). The presence of genes encoding for production of A, B, C, and D staphylococcal enterotoxins (SEs) was tested in the examined strains genotypically (17). A, B, C, and D SE production was also tested phenotypically by reversed passive latex agglutination using SET-RPLA Toxin Detection Kit (Oxoid, Basingstoke, Great Britain). *S. aureus* ATCC 25923 and 6538 strains were used as controls.

2.2. Cheesemaking trials

Raw cow milk was obtained from the experimental farm of the Interdepartmental Centre for Agro-Environmental Research, Enrico Avanzi (University of Pisa). Experimental mini-cheeses of about 150 g were manufactured in the laboratory following the cheesemaking procedure used for traditional caciotta cheese produced in a Tuscan dairy factory with the usual starter cultures (*Streptococcus thermophilus*) and rennet. In a first phase (phase 1), 4 experimental groups of cheeses were defined: with *S. aureus*-inoculated milk and without a lactic acid starter (SA+ st–), with *S. aureus*-inoculated milk and with a starter (SA+ st+), without *S. aureus* inoculum and without a starter (SA– st–), and without *S. aureus* inoculum and with a starter (SA– st+). For each group, 3 independent batches were prepared for a total of 12 batches. Each batch was prepared in duplicate using 5 + 5 L of the same milk (and the same *S. aureus*-inoculated milk for SA+ groups). In a second set of trials (phase 2), 2 independent batches (not duplicated) were prepared for each of the 4 groups (2SA+ st–, 2SA+ st+, 2SA– st–, and 2SA– st+) using a lower *S. aureus* inoculum.

In the trials with *S. aureus*-adjunct cultures the inoculum was prepared as follows: strains were previously transferred twice on brain–heart infusion broth (Oxoid) and incubated at 37 °C. Then, a 1% inoculum of both strains in the same medium was made, and bacterial cells were pelleted, washed twice, and prepared by dilution in sterile, reconstituted skim milk (Oxoid) to obtain a staphylococcal count in the range 10^6–10^8 and 10^5–10^6 CFU/mL in phases 1 and 2, respectively. Inoculated milk was then added (1% v/v) to raw milk in order to obtain a final amount of inoculated *S. aureus* in the range 10^4–10^6 and 10^3–10^4 CFU/mL, respectively, for phases 1 and 2. In all trials milk was warmed to 38 ± 2 °C. For SA+ st+ and SA– st+ cheeses, a commercial thermophilic starter (*Streptococcus thermophilus*, MIX CR 02, Uniferm, Saviano, Napoli, Italy) was added. The starter contains approximately 10 log CFU/g of thermophilic streptococci. Following the supplier's instructions, a count of approximately 6 log CFU of thermophilic streptococci/mL of milk was obtained. For the coagulation 0.05 g/L of a commercial calf powder rennet (Caglificio Clerici, Cadorago, Como, Italy; chymosin 96%, pepsin 4%) were used. The curd was cut in 2 phases into hazelnut-sized grains, extracted after about 1 h from the rennet addition, and placed into plastic moulds. Acidification and draining were completed into a covered plastic box where the cheese temperature was maintained between 33 °C and 37 °C with hot water and steam (steaming phase). For the SA+ st+ and SA– st+ groups the steaming phase lasted until a pH value of at least 5.3 was reached, an index of good acidification. For the SA+ st– and SA– st– groups the steaming phase lasted 3 h. This is the usual length of the steaming phase in the traditional cheesemaking process and the average time necessary to obtain a pH value of 5.3 using starter. Cheeses were then dry salted and stored at 7 ± 1 °C for 14 days.

2.3. Milk and cheese samples

Samples of uninoculated and inoculated milk, samples of cheese before the steaming phase (BS) and after the steaming phase (AS), and at 24 h (24H) and 14 days (14D) after manufacture were immediately submitted to physicochemical and microbiological analyses. A portion of the same samples was frozen at – 20 °C for SE determination.

2.4. Physicochemical analyses

The pH was measured in each milk sample from phases 1 and 2 and in each cheese sample from phase 1. For phase 2, the pH was measured only in cheese samples after the steaming phase (AS). The pH values were determined by using a GLP 21 pH meter (Crison Instruments S.A., Barcelona, Spain) with a penetration electrode and an automatic temperature compensator.

A_w values were registered in four 24H-cheeses of phase 2, one for each group, using a Rotronic PBI AWYD device (PBI International, Milano, Italy).

2.5. Microbiological analyses

Total bacterial count was determined on raw, uninoculated milk on plate count agar (Oxoid) at 30 °C for 72 h.

Coagulase-positive staphylococci (CPS) were enumerated applying the UNI EN ISO Standard 6888-1:2000 (18) on uninoculated and inoculated milk and on cheese samples.

2.6. Enterotoxin determination

For the combined detection of SEs in cheese samples the sandwich enzyme immunoassay RIDASCREEN SET Total (R-Biopharm AG, Darmstadt, Germany) was used. The results were evaluated photometrically by measuring the absorbance at 450 nm against a reference filter of 630 ± 10 nm with a Multiskan FC v. 1.00.79 microwell plate photometer (Thermo Fisher Scientific, Waltham MA, USA). In this method the cut-off value for evaluation of results as positive or negative is calculated by adding 0.15 to the optical density (OD) value registered for the negative control. OD values higher than the cut-off are considered positive. For phase 1, all cheese samples were tested. For each batch and technological stage (BS, AS, 24H, and 14D) a pool of the 2 corresponding cheeses made in duplicate was used. For phase 2, only 24H-cheese samples of 2SA+ st+ and 2SA+ st– groups were tested. OD values of RIDASCREEN-positive samples of phase 1, beginning from AS phase, were used as relative absorbance values to make a comparison between SA+ st– and SA+ st+ results. According to the supplier's instructions, OD values higher than 2.5 are considered positive; however, there is no linearity between OD values and enterotoxin amounts. Thus, a fixed OD value of 2.5 was assigned in these cases.

In addition, the production of each tested SE was verified on SA+ st– 24H-cheese samples by SET RPLA Toxin Detection Kit.

2.7. Statistical analysis

Statistical analyses were performed using R v. 2.15 software (R Foundation for Statistical Computing, Vienna, Austria). Data from phase 1 and 2 milk and cheeses were subjected to the 1-way ANOVA test and Tukey's HSD test for post-hoc comparisons. The experimental group was used as the factor to determine the effect of starter cultures on pH values and CPS counts for phase 1 and on CPS counts for phase 2 for each stage of the cheesemaking process. Significance of differences was determined at the 0.01 probability level. Results from microbial counts were previously converted into log CFU/g. As to the SE production data, the differences in OD values were evaluated with the 2-way ANOVA test and Tukey's HSD test for post-hoc comparisons using the experimental group and the cheesemaking stage as factors; values were considered statistically different if associated with a P value lower than 0.05.

3. Results

3.1. Physicochemical measurements

The milk used for the trials in phases 1 and 2 had a pH of about 6.7, which is the normal value for cow milk.

First phase: Mean values of pH evolution in the 4 experimental groups are reported in the Figure. Significantly different values were obtained beginning from AS stage, both between SA+ st– and SA+ st+ and between SA– st– and SA– st+.

Second phase: Mean pH values measured in AS samples were 6.54 ± 0.16 for 2SA+ st–, 5.32 ± 0.02 for 2SA+ st+, 6.51 ± 0.13 for SA– st–, and 5.31 ± 0.03 for SA– st+.

The mean A_w value in 24H samples was 0.977 ± 0.010. This rather low value is probably due to the very small size of the experimental cheeses which created a high surface/volume ratio.

3.2. Microbiological analyses and enterotoxin production

On the basis of the API STAPH results, C21 and FB5 strains were identified as *Staphylococcus aureus* with an identification percentage (%ID, given by the supplier's

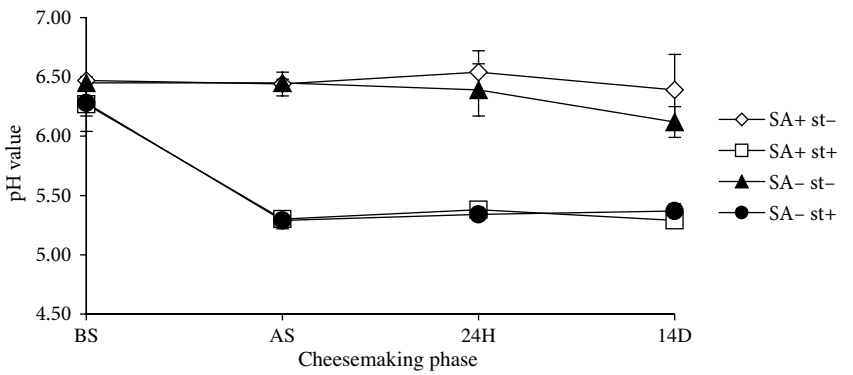

Figure. Evolution of pH values of phase 1 cheeses examined at the different cheesemaking stages. SA+: cheeses with experimental inoculum of *Staphylococcus aureus*; SA–: cheeses without experimental inoculum of *Staphylococcus aureus*; st+: cheeses with lactic acid starter; st–: cheeses without lactic acid starter. BS: cheeses before the steaming phase; AS: cheeses after the steaming phase; 24H: cheeses 24 h after manufacture; 14D: cheeses 14 days after manufacture.

software) higher than 97%, as confirmed by genotypical analyses, which revealed the presence of the *nuc* gene for both strains. PCR revealed the presence of the C toxin gene for C21 and the A and D toxin genes for the FB5 strain. SET-RPLA Toxin Detection Kit results confirmed the production of these toxins.

Raw milk used for the cheesemaking trials of phases 1 and 2 constantly showed total bacterial counts within the legal limit of 5 log CFU/mL (4.66 ± 0.32 log CFU/mL), and the CPS mean value (2.15 ± 0.17 log CFU/mL) was acceptable with respect to Italian microbiological criteria for raw milk intended for direct human consumption (500 CFU/mL) (19).

First phase: Results regarding the behaviour of CPS during the cheesemaking process in phase 1 are shown in Table 1. The statistical comparison between cheese samples with and without starter showed that CPS counts were significantly different in all cheesemaking phases beginning from AS, both for SA+ and SA– cheeses. As expected, significant differences were consistently found between the SA+ and SA– groups, beginning from the milk.

SEs were detected in all SA+ st– and SA+ st+ cheese samples with the exception of BS samples, among which only 1 SA+ st– sample deriving from the batch with the strongest inoculum of *S. aureus* was positive. SA– samples never revealed the presence of SEs. Total OD mean values of positive AS, 24H, and 14D samples were 2.212 ± 0.603 and 0.591 ± 0.305 for SA+ st– and SA+ st+, respectively. As shown in Table 2, OD mean values of positive SA+ st+ samples were significantly lower than those of SA+ st– samples. Indeed, the 2-way ANOVA test showed the statistical significance of both factors (experimental group and cheesemaking stage), and post-hoc comparisons revealed that the decrease in OD values observed in 14D samples was statistically significant.

With regard to the type of SEs found in cheeses, results demonstrated the simultaneous production of A, C, and D SEs, proving that the 2 experimental *S. aureus* strains were able to grow sufficiently without reciprocal inhibition.

Second phase: In Table 3 the results (CPS counts) of trials carried out with a low *S. aureus* inoculum are shown. The highest mean value of CPS was 5.37 log CFU/g in 2SA+ st– cheeses. SEs were never detected in the examined samples.

4. Discussion

Among the different categories of cheeses, soft cheeses such as Italian caciotta represent a very suitable environment for the growth of CPS and, consequently, for SE production due to their high A_w values and their technological cycle, which maintains temperature values close to the optimum range for mesophilic bacteria for some hours in the early stages of the cheesemaking process. In these cases, especially if raw milk is used, the acidification process is of the utmost importance.

In our study commercial thermophilic starter gave rise to rapid acidification and active proliferation of lactic acid bacteria, both in SA+ and SA– caciotta cheeses in which pH values gradually fell as far as 5.3 during cheesemaking. As expected, the most effective cheesemaking period was the steaming phase when the cheeses remained at the best temperature range for acidifying action; thus, the highest pH decrease was noted between BS and AS samples. As to the trials carried out without a lactic acid starter, pH values decreased only slightly because of the very weak acidifying activity of autochthonous lactic acid bacteria from both the milk and the environment. The low-temperature ripening did not permit a valid multiplication of lactic acid microorganisms, and pH remained substantially unchanged, approximately 5.3 when a starter was used and over 6 without a starter.

In SA+ cheeses with a starter CPS (maximum mean value: 6.43 log CFU/g) showed an increase between 0.5 and 1 log CFU/g during the steaming phase, whereas in SA+ cheeses produced without starter (maximum

Table 1. Coagulase-positive staphylococci counts (CPS) (mean ± standard deviation, log CFU/mL or g) in phase 1 cheeses of different experimental groups examined at the different cheesemaking stages.

	M	BS	AS	24H	14D
SA+ st–	4.88 ± 0.70[a]	5.59 ± 0.66[a]	7.27 ± 0.34[a]	7.57 ± 0.13[a]	7.51 ± 0.09[a]
SA+ st+	5.18 ± 0.56[a]	5.60 ± 0.44[a]	6.27 ± 0.31[b]	6.43 ± 0.45[b]	5.92 ± 0.38[b]
SA– st–	2.20 ± 0.17[b]	3.04 ± 0.17[b]	5.56 ± 0.14[c]	5.65 ± 0.12[c]	5.24 ± 0.16[c]
SA– st+	2.10 ± 0.17[b]	2.62 ± 0.03[b]	3.15 ± 0.22[d]	3.39 ± 0.26[d]	3.46 ± 0.17[d]

SA+: cheeses with experimental inoculum of *Staphylococcus aureus*; SA–: cheeses without experimental inoculum of *S. aureus*; st+: cheeses with lactic acid starter; st–: cheeses without lactic acid starter. M: CPS count in milk; BS: CPS count before the steaming phase; AS: CPS count after the steaming phase; 24H: CPS count 24 h after manufacture; 14D: CPS count 14 days after manufacture. Different letters in the same column indicate significant differences ($P < 0.01$).

Table 2. Optical density (OD) values (mean ± standard deviation) of phase 1 cheeses of the SA+ st– and SA+ st+ groups examined at the different cheesemaking stages.

	SA+ st–	SA+ st+
AS	2.500 ± 0.000[a]	0.866 ± 0.043[c]
24H	2.500 ± 0.000[a]	0.609 ± 0.326[c]
14D	1.637 ± 0.841[b]	0.298 ± 0.146[d]

SA+: cheeses with experimental inoculum of *Staphylococcus aureus*; st+: cheeses with lactic acid starter; st–: cheeses without lactic acid starter. AS: OD values after the steaming phase; 24H: OD values 24 h after manufacture; 14D: OD values 14 days after manufacture. Different letters in the same row and column indicate significant differences ($P < 0.05$).

mean CPS value: 7.57 log CFU/g) the corresponding increase was between 1.5 and 2 log CFU/g. Similarly, in SA– cheeses with starter (maximum mean value: 3.46 log CFU/g) the acidification determined by lactic acid bacteria gave rise to a strong limitation in the autochthonous CPS in the same phase. Indeed, CPS easily multiplied in SA– cheeses without starter where they reached (even without SE production) the EU CPS level considered a risk with respect to staphylococcal food poisoning (10^5 CFU/g) (20). This finding is not uncommon in Italian traditional raw milk soft cheeses produced without starter (http://www.agricoltura.regione.lombardia.it/shared/ccurl/143/792/AL_20090412_3654_quaderno_78_AGR_MS.pdf). Considering the absence of starter in both products, the CPS increase in SA– st–, which was higher than in SA+ st–, could be due to CPS counts close to the maximum limits of the bacterial growth curve in the latter, after which a slowing down begins and/or greater multiplying impetus in the autochthonous strains in comparison with the inoculated ones (see also phase 2). Nevertheless, samples of both SA– st– and SA– st+ never revealed the presence of SEs, in contrast with SA+ st– and SA+ st+, which had a strong *S. aureus* initial inoculum.

In all groups total CPS increases revealed very poor or no multiplying action in the manufacturing stages after the steaming phase, and in the case of SA+ st+ they even showed a decrease during the ripening period. This decrease, which should be further verified, could be due to the lower vitality of inoculated *S. aureus* strains (C21 and FB5) in the presence of the starter cultures; in 2SA+ st+ the wild CPS, not wiped out by a great number of inoculated *S. aureus* strains, could have limited the decrease. A similar decrease between 24H and 14D samples, corresponding to a slight pH decrease, was also noted in SA– st– group, where autochthonous lactic acid bacteria probably played a role.

In absence of the thermal treatment of milk, as in the examined cheeses, even low initial levels of *S. aureus* can lead to the production of SEs. In particular, counts greater than 10^3 CFU/mL in milk can reach unsafe levels (7) due to their multiplication potential. In the case of the *S. aureus* values tested in phase 2 milk (maximum mean value 3.34 log CFU/mL), it was indeed possible to reach counts of about 10^5 CFU/g during the cheesemaking process with increases analogous to those of phase 1, but this did not lead to SE production. In fact, based on the current knowledge (21) enterotoxin detection in cheeses is usually correlated with higher *S. aureus* counts (about 10^6–10^7 CFU/g).

Considering the SE positive samples found in phase 1 SA+ cheeses, even if the RIDASCREEN method gives only qualitative results in absence of a titration curve, it is noteworthy that the OD mean values of SA+ st– AS, 24H, and 14D samples were significantly higher than those of SA+ st+. This fact, together with evidence that the staphylococcal counts of SA+ st+ cheeses were

Table 3. Coagulase-positive staphylococci counts (CPS) (mean ± standard deviation, log CFU/mL or g) in phase 2 cheeses of different experimental groups examined at the different cheesemaking stages.

	M	BS	AS	24H	14D
2SA+ st–	3.09 ± 0.12[a]	3.88 ± 0.31[a]	5.18 ± 0.13[a]	5.37 ± 0.10[a]	5.30 ± 0.39[a]
2SA+ st+	3.34 ± 0.19[a]	3.95 ± 0.10[a]	4.75 ± 0.35[a]	5.02 ± 0.11[a]	4.91 ± 0.16[a]
2SA– st–	1.94 ± 0.34[b]	2.87 ± 0.12[a]	4.85 ± 0.14[a]	4.99 ± 0.19[a]	4.94 ± 0.13[a]
2SA– st+	2.17 ± 0.13[b]	2.53 ± 0.39[a]	3.08 ± 0.25[b]	3.30 ± 0.09[b]	3.39 ± 0.16[b]

2SA+: cheeses with experimental inoculum of *Staphylococcus aureus*; 2SA–: cheeses without experimental inoculum of *S. aureus*; st+: cheeses with lactic acid starter; st–: cheeses without lactic acid starter. M: CPS count in milk; BS: CPS count before the steaming phase; AS: CPS count after the steaming phase; 24H: CPS count 24 h after manufacture; 14D: CPS count 14 days after manufacture. Different letters in the same column indicate significant differences ($P < 0.01$).

significantly lower than those of the corresponding SA+ st– cheeses, confirms that starter cultures were effective in limiting staphylococcal growth and the subsequent SE production, even in the presence of very high initial *S. aureus* contamination. The inhibitory effect of starter cultures is well known and related not only to the pH decrease through lactic acid production, but also to competition mechanisms and the release of substances such as hydrogen peroxide and bacteriocins (22–24).

Moreover, lactic acid bacteria can also exert their action on SEs. A decreasing action of some lactic acid bacteria on SEA levels (*Lactobacillus*, *Streptococcus thermophilus*, and *Pediococcus*) was found when partially purified SEA was added to microbiological media inoculated with these microorganisms. This reduction was measured by a serological method and was not simply due to decreases in pH, since uninoculated SEA-containing media, acidified up to pH 3 with lactic acid, had no effect (25). Such an action, probably causing modifications in the antigenic profile of SEs with the consequent loss of immunological reactivity, could have had a role in determining the decrease in OD values shown by 14D samples of SA+ st+ (starter lactic acid bacteria) and SA+ st– (autochthonous lactic acid bacteria). Some studies (26,27) have demonstrated that the loss of C- and N-terminal amino acids causes loss of conformation, superantigenic, and serological activity in SEs. Lactic acid bacteria could act in such a way via their proteolytic properties. Weight loss in cheeses during the ripening could also have contributed to the decrease. Since these aspects have been very poorly explored, our findings must be adequately supported by additional data.

In spite of their merits, raw milk cheeses are still traditionally manufactured in the absence of lactic acid starter cultures on many small dairy farms; in other cases commercial starters are misused and fail to produce effective acidification. In these situations, the greatest attention should be paid to maintaining the CPS values in milk as low as possible, to avoid unnecessary prolongation of manufacturing and optimise the production process at the lowest temperatures suitable for typical cheesemaking. Since these precautions cannot be sufficient to avoid SE poisoning, there is a need for the widest diffusion of basic information with the aim of sharing with artisanal raw milk soft cheese makers the benefits of correctly using lactic acid starters. In fact, these are cheap and easy tools for enhancing the safety of cheeses that generally do not cause undesirable changes in typical traits; rather, they can improve flavour and lead to higher standardisation. When the use of a commercial starter is not possible, natural autochthonous milk starter cultures could be substituted. In both cases, the starter acidifying action has to be properly managed by applying adequate time-temperature parameters and by carefully monitoring pH evolution.

Obviously, the correct employment of starter cultures is not sufficient to ensure food safety if CPS counts are not adequately low in the milk (within 3.5 log CFU/mL, in our experience) and good hygiene practices are not fully applied during each phase of milk and cheese production. In these cases, milk pasteurisation is strongly recommended.

Acknowledgements

The authors wish to thank the Interdepartmental Centre for Agro-Environmental Research, Enrico Avanzi (University of Pisa), for kindly supplying milk and the Official Veterinary Service of Azienda Unità Sanitaria Locale 5, Pisa for inspiring this work based on the actual needs of small cheesemakers.

References

1. Salvadori del Prato O. Trattato di Tecnologia Casearia. Bologna, Italy: Edagricole–Edizioni Agricole, 1998 (in Italian).
2. Halpin-Dohnalek M, Marth E. *Staphylococcus aureus*: production of extracellular compounds and behaviour in foods: a review. J Food Protect 1989; 52: 267–282.
3. Vasudevan P, Mohan Nair MK, Annamalai T, Venkitanarayanan KS. Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Vet Microbiol 2003; 92: 179–185.
4. Gill JJ, Pacan JC, Carson ME, Leslie KE, Griffiths MW, Sabour PM. Efficacy and pharmacokinetics of bacteriophage therapy in treatment of subclinical *Staphylococcus aureus* mastitis in lactating dairy cattle. Antimicrob Agents Chemother 2006; 50: 2912–2918.
5. De Buyser ML, Dufour B, Maire M, Lafarge V. Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. Int J Food Microbiol 2001; 67: 1–17.
6. Kerouanton A, Hennekinne JA, Letertre C, Petit L, Chesneau O, Brisabois A, De Buyser ML. Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. Int J Food Microbiol 2007; 115: 369–375.
7. Cretenet M, Even S, Le Loir Y. Unveiling *Staphylococcus aureus* enterotoxin production in dairy products: a review of recent advances to face new challenges. Dairy Sci Technol 2011; 91: 127–150.
8. Tatini SR, Jezeski JJ, Morris HA, Olson JC, Casman EP. Production of staphylococcal enterotoxin A in Cheddar and Colby cheeses. J Dairy Sci 1971; 54: 815–825.

9. Gomez-Lucia E, Goyache J, Orden JA. Growth of *Staphylococcus aureus* and synthesis of enterotoxin during ripening of experimental Manchego-type cheese. J Dairy Sci 1992; 75: 19–26.

10. Bachmann HP, Spahr U. The fate of potentially pathogenic bacteria in Swiss hard and semihard cheeses made from raw milk. J Dairy Sci 1995; 78: 476–483.

11. Vernozy-Rozand C, Meyrand A, Mazuy C, Delignette-Muller ML, Jaubert G, Perrin G, Lapeyre C, Richard Y. Behaviour and enterotoxin production by *Staphylococcus aureus* during the manufacture and ripening of raw goats' milk lactic cheeses. J Dairy Res 1998; 65: 273–281.

12. Daminelli P. Dynamics of the survival of *Staphylococcus aureus* in Crescenza and Italico cheeses artificially contaminated. Selezione Veterinaria 1999; 11: 819–829 (article in Italian with an English abstract).

13. Meyrand A, Vernozy-Rozand C, Gonthier A, Mazuy C, Ray-Gueniot S, Jaubert G, Perrin G, Lapeyre C, Richard Y. Main differences in behavior and enterotoxin production of *Staphylococcus aureus* in two different raw milk cheeses. Rev. Méd Vét 1999; 150: 271–278.

14. Delbes C, Alomar J, Chougui N, Martin JF, Montel MC. *Staphylococcus aureus* growth and enterotoxin production during the manufacture of uncooked, semihard cheese from cows' raw milk. J Food Protect 2006; 69: 2161–2167.

15. Pexara A, Solomakos N, Sergelidis D, Govaris A. Fate of enterotoxigenic *Staphylococcus aureus* and staphylococcal enterotoxins in Feta and Galotyri cheeses. J Dairy Res 2012; 79: 405–413.

16. Kalorey DR, Shanmugam Y, Kurkure NV, Chousalkar KK, Barbuddhe SB. PCR-based detection of genes encoding virulence determinants in *Staphylococcus aureus* from bovine subclinical mastitis cases. J Vet Sci 2007; 8: 151–154.

17. Johnson WM, Tyler SD, Ewan EP, Ashton FE, Pollard DR, Rozee KR. Detection of genes for enterotoxins, exfoliative toxins and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. J Clin Microbiol 1991; 29: 426–430.

18. UNI EN ISO Standard 6888-1:2000. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species). Technique using Baird-Parker agar medium (in Italian).

19. Intesa tra il Governo e le Regioni in materia di vendita di latte crudo per l'alimentazione umana n. 5 del 25-01-2007. Supplemento alla Gazzetta Ufficiale della Repubblica Italiana n. 36 del 13-02-2007 (in Italian).

20. Commission Regulation EC No. 1441/2007 of 5 December 2007 amending Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs. Official Journal of the European Union L322/12, 07-12-2007.

21. Meyrand A, Vernozy-Rozand C. Croissance et entérotoxinogenèse de *Staphylococcus aureus* dans différents fromages. Rev. Méd. Vét. 1999; 150: 601–616 (article in French with an English abstract).

22. Iandolo JJ, Clark CW, Bluhm L, Ordal ZJ. Repression of *Staphylococcus aureus* in associative culture. Appl Microbiol 1965; 13: 646–649.

23. Haines WC, Harmon LG. Effect of selected lactic acid bacteria on growth of *Staphylococcus aureus* and production of enterotoxin. Appl Microbiol 1973; 436–441.

24. Stadhouders J, Cordes MMO, Schouwenburg-van Foeken AWJ. The effect of manufacturing conditions on the development of staphylococci in cheese. Their inhibition by starter bacteria. Neth Milk Dairy J 1978; 32: 193–203.

25. Chordash RA, Potter NN. Stability of staphylococcal enterotoxin A to selected conditions encountered in foods. J Food Sci 1976; 41: 906–909.

26. Harris TO, Hufnagle WO, Betley MJ. Staphylococcal enterotoxin type A internal deletion mutants: serological activity and induction of T-cell proliferation. Infect Immun 1993; 61: 2059–2068.

27. Metzroth B, Marx T, Linnig M, Fleischer B. Concomitant loss of conformation and superantigenic activity of staphylococcal enterotoxin B deletion mutant proteins. Infect Immun 1993; 61: 2445–2452.