Short communication: Pasteurization as a means of inactivating staphylococcal enterotoxins A, B, and C in milk

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ABSTRACT

Our aim was to assess the effect of pasteurization temperature on inactivation of staphylococcal enterotoxins (SE). Milk samples were inoculated with log 4.38 to 5.18 cfu/mL of 40 different *Staphylococcus aureus* strains having the ability to produce types A, B, or C SE and incubated at 37°C for 24 h to develop SE. This incubation was followed by heat treatment for 15 s at 72, 85, and 92°C. Samples were analyzed for *Staph. aureus* count by plate method and, specifically, for SE presence. An enzyme-linked immunofluorescent assay on a MiniVIDAS analyzer (bioMérieux, Marcy l'Étoile, France) was used to detect SE, which were determined semiquantitatively based on test values. The *Staph. aureus* count in milk before pasteurization did not affect the amount of SE. Before pasteurization, SEB was detected in the lowest amount compared with other SE types. Staphylococcal enterotoxins were markedly reduced with pasteurization and inactivated at pasteurization temperatures to an extent depending on the amount in the sample before pasteurization. After pasteurization at 72°C, SE were detected in 87.5% of samples (35/40), after pasteurization at 85°C in 52.5% of samples (21/40), and after pasteurization at 92°C in 45.0% of samples (18/40). We determined that SE may still persist in milk even when *Staph. aureus* bacteria are inactivated through pasteurization. Although pasteurization may partially inactivate SE in milk, a key measure in the prevention of staphylococcal enterotoxicosis linked to pasteurized milk consumption is to avoid any cold chain disruption during milk production and processing.

Key words: heat-resistant staphylococcal enterotoxin, *Staphylococcus aureus*, pasteurization, inactivation

Short Communication

Staphylococcal food poisoning is one of the most prevalent causes of food-borne intoxication worldwide. Up to 50 to 70% of *Staphylococcus aureus* strains are able to produce, under suitable conditions, extracellular heat-stable staphylococcal enterotoxins (SE). In 2014, 393 food-borne outbreaks caused by staphylococcal toxins were reported in the European Union. This represents 7.5% of all outbreaks, a small increase compared with 2013 when 386 outbreaks caused by staphylococcal toxins were reported. As in previous years, France reported the majority (89.6%) of the outbreaks. (EFSA, 2015).

Staphylococcal enterotoxin A (SEA), either alone or in combination with other classical SE (SEB, SEC, SED, and SEE), is considered to be the main cause of staphylococcal food poisoning throughout the world (Dinges et al., 2000; Rola et al., 2015). In France, staphylococcal food-poisoning outbreaks are the second cause of food-borne diseases after *Salmonella*. The *sea* gene is dominant in *Staph. aureus* strains linked to staphylococcal food-poisoning outbreaks in France (Kérouanton et al., 2007). In Austria, a staphylococcal food-poisoning outbreak affected 40 children in 2007 after consumption of pasteurized milk products. Unopened milk products leftover at the affected institutions yielded SEA and SED. The cows, not the dairy owner, were likely reservoirs of the SE producing *Staph. aureus* (Schmid et al., 2009). A study of *Staph. aureus* obtained from dairy products responsible for 16 outbreaks in Brazil revealed that the most frequently encountered enterotoxin gene was *sea* followed by *seb* (Veras et al., 2008).

Staphylococcal enterotoxicosis has a very rapid onset and course. The first symptoms of intoxication, such as vomiting, headache, abdominal pain, and diarrhea, develop as early as 1 to 6 h after the consumption of food contaminated with SE. The symptoms resolve spontaneously within 24 to 48 h (Le Loir et al., 2003; Johler et al., 2015).

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Intoxication by *Staph. aureus* is most frequently identified as the cause of human outbreaks resulting from raw milk consumption or products made thereof (Yamashita et al., 2003; Barker and Gomez-Tome, 2013; Claeys et al., 2013; Janstova et al., 2014). By contrast, with commercially processed milk, consumer health is potentially at risk from *Staph. aureus* enterotoxin production during improper delivery and storage practices. Although boiling would successfully kill pathogens, the consumer could be vulnerable to *Staph. aureus* enterotoxin produced before heat processing (Ding et al., 2016). Minimum pasteurization treatments are set out in the European legislation: 72°C for 15 s or 63°C for 30 min (European Commission, 2004). High-temperature pasteurization, a commonly used practice, is defined as heat treatment of milk at not less than 85°C (Czech Republic, 2003).

Staphylococcus aureus bacteria present in raw materials and foods can be inactivated with proper heat treatment. Staphylococcal enterotoxins produced, under suitable conditions, by *Staph. aureus* strains proliferating in a food product are generally considered as heat resistant and may persist in foods even after heat treatment (Balaban and Rasooly, 2000; Larkin et al., 2009). Staphylococcal enterotoxins are active even after boiling for 30 min and may remain stable at 121°C for 28 min (Bhumia, 2008; Fernandes, 2009). Initial studies of thermal inactivation of SE used biological experiments and confirmed inactivation of enterotoxins by temperatures above 100°C (Read and Bradshaw, 1966; Humber et al., 1975).

We aimed to analyze the thermal stability of SE and possibility for inactivation of SEA, SEB, and SEC by temperatures commonly used in milk pasteurization (i.e., those under 100°C). We hypothesized (1) that SE would be reduced by pasteurization, (2) SE did not depend on *Staph. aureus* count, and (3) inactivation by pasteurization varies with the amount of SE produced before pasteurization.

Forty toxigenic strains of *Staph. aureus* were recovered from 228 raw cow milk samples collected from 40 farms in the Czech Republic between 2012 and 2014. Colonies from the plates were examined with a plasma coagulase test (Staphylo LA Seiken, Denka Seiken Co. Ltd., Tokyo, Japan), and suspected *Staph. aureus* strains were confirmed by PCR based on detection of a species-specific genomic fragment, SA442 (Martineau et al., 1998). Three of the study strains were from the Czech Collection of Microorganisms (Brno, Czech Republic; CCM 5765, CCM 5757, and CCM 5971). For the purposes of the study, 13 strains carrying the *sea* gene, 13 strains carrying the *seb* gene, and 14 strains carrying the *sec* gene were selected. All strains were

screened by PCR to detect the presence of the genes encoding enterotoxins *sea*, *seb*, or *sec*. Multiplex PCR according to Løvseth et al. (2004) was used to detect the genes for enterotoxins SEA to SEE and SEH. The strains were tested for the ability to produce enterotoxins by enzyme-linked fluorescence assay (ELFA) after enrichment in both pasteurized milk and brain heart infusion medium (Oxoid, Basingstoke, UK) at 37°C.

Retail pasteurized cow milk (fat 1.5%) from the Czech Republic was used as a test medium. This milk was tested for *Staph. aureus* presence. Milk samples were inoculated with log 4.38 to 5.18 cfu/mL of 1 of 40 toxigenic strains of *Staph. aureus* and incubated at 37°C for 24 h to allow the proliferation of *Staph. aureus* and production of SE. *Staphylococcus aureus* counts after incubation reached log 8.28 to 9.41 cfu/mL. Subsequently, 10-mL aliquots of the samples were divided into test tubes and pasteurized in water bath at 3 temperatures commonly used in the milk industry (72, 85, and 92°C) for 15 s. After heating, the samples were cooled and screened for *Staph. aureus* count and presence of SEA, SEB, and SEC. Samples were examined, and coagulase-positive staphylococci were enumerated according to CSN EN ISO 6888-1 (ISO, 2000) by a technique using Baird-Parker agar medium supplemented with egg yolk-tellurite emulsion (Bio-Rad, Hercules, CA).

Staphylococcal enterotoxins were detected by an ELFA using the SET2 tests and MiniVIDAS analyzer (bioMérieux, Marcy l'Étoile, France) capable of detecting the sum of enterotoxins SEA to SEE, with detection limits of 0.5 ng/g or mL of food for SEA and SEB and 1.0 ng/g or mL of food for SEC to SEE. As the ELFA used does not allow for quantitative SE detection, results are expressed as either positive or negative. To monitor SE amount, relative fluorescence values and test values (TV) were assessed for semiquantitative detection. A sample TV less than 0.13 was considered negative, whereas a sample TV greater or equal to 0.13 was labeled as positive.

Differences in the *Staph. aureus* count and amount of SE (interpreted from the TV) among SEA, SEB, and SEC producers were analyzed using the Kruskal-Wallis test followed by multiple comparison whenever the test yielded a significant result. The Wilcoxon matched-pair test was used to assess the decline in the amount of SE after pasteurization. The proportions of SE-positive and SE-negative samples for 3 types of SE (SEA, SEB, and SEC) before and after pasteurization were analyzed by the Fisher's exact test. The relationship between *Staph. aureus* count and the amount of SE before pasteurization was calculated by means of nonparametric Spearman rank correlation. Finally, differences

Table 1. *Staphylococcus aureus* counts (log cfu/mL) in milk before and after pasteurization (mean $\pm$ SD)¹

Item	All samples (n = 40)	SEA (n = 13)	SEB (n = 13)	SEC (n = 14)	Kruskal-Wallis test, <i>P</i> -value	Multiple comparison
Before pasteurization	8.73 $\pm$ 0.26	8.70 $\pm$ 0.30	8.66 $\pm$ 0.23	8.83 $\pm$ 0.23	0.096	
After pasteurization at 72°C	1.81 $\pm$ 1.71	2.18 $\pm$ 1.73	2.47 $\pm$ 1.73	0.85 $\pm$ 1.30	0.024	SEB – SEC
After pasteurization at 85°C	0.19 $\pm$ 0.69	0.22 $\pm$ 0.56	0.05 $\pm$ 0.19	0.28 $\pm$ 1.04	0.739	
After pasteurization at 92°C	0.03 $\pm$ 0.21	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.09 $\pm$ 0.35	0.395	

¹SEA, SEB, and SEC = strains producing staphylococcal enterotoxins A, B, and C, respectively.

in the amounts of SE and *Staph. aureus* counts before pasteurization between SE-positive and SE-negative samples after pasteurization were assessed with the Mann-Whitney U test. Significance was established as $P < 0.05$. Statistical analyses were performed with IBM SPSS Statistics, version 22 (IBM Corp., Armonk, NY) and Statistica Software, version 12 (StatSoft, Tulsa, OK).

The concentration of *Staph. aureus* in milk before pasteurization was 8.73 ± 0.26 log cfu/mL and no significant difference was found between producers of different SE types (Kruskal-Wallis test, $P = 0.096$). As expected, the bacteria count decreased after pasteurization. *Staphylococcus aureus* presence after pasteurization at 72°C varied significantly between producers of different SE types, the lowest being associated with SEC production. Pasteurization at 85 and 92°C reduced bacterial amount to nonthreatening levels, with no difference found between producers of different SE types (Table 1).

Staphylococcal enterotoxin quantities defined by respective TV ranged from 0.25 to 3.79 and differed significantly between SE types before pasteurization (SEA = 2.36, SEB = 1.12, SEC = 2.06; Kruskal-Wallis test, $P = 0.025$; significant difference recorded between SEB and SEA). The SE detected in the lowest amount was SEB, and this trend persisted after pasteurization at any temperature (Table 2). The amount of SE significantly decreased after pasteurization (Wilcoxon test: $P < 0.001$ by comparing TV at baseline and after pasteurization at 72°C). Moreover, higher pasteurization temperatures continued to reduce SE ($P < 0.001$ when comparing TV at 72 and 85°C; $P = 0.016$ when comparing TV at 85 and 92°C).

Considering TV of 0.13 as the cutoff level to classify samples as SE-negative or -positive, all samples (100%; 40/40) were SE-positive before pasteurization. Altogether, 87.5% (35/40) of samples were positive after pasteurization at 72°C, 52.5% (21/40) after pasteurization at 85°C, and 45.0% (18/40) after pasteurization at 92°C (Figure 1). Positive sample proportions differed significantly between SEA, SEB, and SEC producers after pasteurization at higher temperature; that is, at 85°C (positivity rates of 76.9% for SEA, 15.4% for SEB, and 64.3% for SEC; Fisher's exact test: $P = 0.004$) and 92°C (76.9% for SEA, 15.4% for SEB, and 42.9% for SEC; Fisher's exact test: $P = 0.006$).

Staphylococcus aureus counts in milk before pasteurization did not affect the amount of SE (Spearman correlation = 0.078, $P = 0.631$) or SE positivity. Indeed, comparing SE-negative and SE-positive samples after pasteurization, no relationship with the *Staph. aureus* count before pasteurization was noted (Mann-Whitney U test: $P = 1.000$, $P = 0.903$, and $P = 0.514$ for the *Staph. aureus* count in SE-positive and SE-negative samples after pasteurization at 72, 85, and 92°C, respectively). We observed SE inactivation by pasteurization to be dependent on the amount of SE before pasteurization. Comparing baseline amounts in SE-positive and SE-negative samples, the difference was highly significant with all 3 pasteurization regimens. The SE-positive samples after pasteurization at 72°C had significantly higher TV before pasteurization than SE-negative samples (respective TV of 2.043 and 0.502, Mann-Whitney U test: $P = 0.004$). Similarly, TV before pasteurization was higher in samples that were SE-positive after pasteurization at 85 and 92°C compared with negative samples (85°C: 2.740 and 0.866,

Table 2. Detection of staphylococcal enterotoxin by MiniVIDAS analyzer (bioMérieux, Marcy l'Étoile, France) in milk before and after pasteurization (mean $\pm$ SD of test value)¹

Item	All samples (n = 40)	SEA (n = 13)	SEB (n = 13)	SEC (n = 14)	Kruskal-Wallis test, <i>P</i> -value	Multiple comparison
Before pasteurization	1.85 $\pm$ 1.11	2.36 $\pm$ 0.97	1.12 $\pm$ 0.88	2.06 $\pm$ 1.13	0.025	SEA – SEB
After pasteurization at 72°C	1.32 $\pm$ 1.21	2.00 $\pm$ 1.17	0.53 $\pm$ 0.79	1.41 $\pm$ 1.23	0.007	SEA – SEB
After pasteurization at 85°C	0.74 $\pm$ 0.97	1.46 $\pm$ 1.05	0.28 $\pm$ 0.64	0.50 $\pm$ 0.81	0.006	SEA – SEB
After pasteurization at 92°C	0.71 $\pm$ 1.00	1.52 $\pm$ 1.06	0.25 $\pm$ 0.64	0.40 $\pm$ 0.76	0.026	SEA – SEB

¹SEA, SEB, and SEC = strains producing staphylococcal enterotoxins A, B, and C, respectively.

Figure 1. Proportions of staphylococcal enterotoxin (SE)-positive samples after pasteurization at 72, 85, and 92°C. SEA, SEB, and SEC = strains producing staphylococcal enterotoxin A, B, and C, respectively. All samples were SE-positive before pasteurization.

respectively, Mann-Whitney U test: $P < 0.001$; 92°C: 2.894 and 0.996, respectively, Mann-Whitney U test: $P < 0.001$).

The limit of 10^5 cfu/g or mL is considered a high-risk count of *Staph. aureus* in food, which enables the production of enterotoxins (European Commission, 2005; Necidova et al., 2012). From the results of our study, it is evident that diverse strains of the same species may produce various amounts of enterotoxins. Many environmental factors affect the expression of enterotoxins and their presence in foods. Environmental conditions (i.e., pH, temperature, or oxygenation) affect the development of *Staph. aureus* and SE gene expression and production in culture media or in foodstuffs (Le Loir et al., 2003). Enterotoxin expression is coordinated by a complex network of regulatory elements. Among them, the *agr* regulatory system plays a crucial role. The *agr* system positively regulates the expression of many virulence genes (including some genes for enterotoxins: *seb*, *sed*, and, presumably, *sec*), and this expression increases simultaneously with increasing cell density (Novick and Geisinger, 2008). Our results recorded that SEB is produced in the lowest amount of all SE, which agrees with the conclusions of Duquenne et al. (2016), who compared the relative expression levels of *sea*, *seb*, *sec*, and *sed* in *Staph. aureus* strains during the manufacturing of uncooked, semihard cheeses and found the lowest expression found for the *seb* gene. The most likely reason for quick degradation and lowest detection

of SEB in our study is connection with low frequency of SEB or *seb* genes in *Staph. aureus* strains in milk and milk products. Through the structure, AA sequence, and length of single-chain polypeptides, there are no significant differences between SEA, SEB, and SEC (Argudin et al., 2010). The molecular weight 28,336 Da is higher at SEB than at SEA and SEC (Hennekinne et al., 2012).

Staphylococcal enterotoxins are proteins with a short polypeptide chain, soluble in water and saline solution (Le Loir et al., 2003). One of the basic characteristics of SE is that they are heat-resistant and, unlike *Staph. aureus* bacteria, they can persist in foods even after heat treatment (Pepe et al., 2006). Staphylococcal enterotoxins are more heat-resistant in foodstuffs than in laboratory culture media (Bergdoll, 1983). This characteristic is illustrated by an extensive outbreak caused by SEA present in powdered skim milk manufactured in Japan (Asao et al., 2003). The SEA exposed at least twice to pasteurization at 130°C for 4 or 2 s retained both immunological and biological activities, although it had been partially inactivated. In this powdered milk outbreak, thermal processes destroyed staphylococci in milk, but SEA had retained enough activity to cause intoxication.

Protein denaturation denotes a reversible or irreversible change of native conformation (tertiary structure) without cleavage of covalent bonds (except for disulfide bridges); this can be accomplished by changing the

temperature. Denaturation of biologically active proteins is usually associated with loss of activity (Belitz et al., 2009); it is possible that the short length of the polypeptide chain itself is cause of their thermostability. Our results confirmed that pasteurization temperatures can partially inactivate SE. For instance, Claeys et al. (2013) noticed that already formed and heat-resistant enterotoxins of *Staph. aureus* were not destroyed by pasteurization (e.g., 71–74°C for 15–40 s). Sterilization (110–120°C for 10–20 min), UHT (indirect UHT at 135–140°C for 6–10 s and direct UHT at 140–150°C for 2–4 s), or innovative steam injection (150–200°C for <0.1 s) destroyed toxins of *Staph. aureus* in milk.

At temperatures above 100°C, Denny et al. (1966) noted that SEA at a concentration of 7 µg/mL gave heat inactivation end points of 8 to 11 min at 121.1°C, as compared with 22 min at 121.1°C at an enterotoxin concentration of 21 µg/mL. Intravenous injection of enterotoxin B heated at 115.6°C for 32.5 min and longer did not induce an emetic response in cats (Read and Bradshaw, 1966). Humber et al. (1975) reported a significant effect of pH on heat inactivation of SEA; SEA given to monkeys through a stomach tube, after heat treatment (at 100°C and pH 5.3 for 1 min), did not induce an emetic response in test animals. Heat treatment at the same temperature (100°C), but at a higher pH of 7.8, achieved the same result (negative monkey emetic responses) after 50 min. Milk pH varies from 6.4 to 6.7; heat treatment time used in previous studies was much longer than that for which our pasteurization temperatures were applied.

Our study determined that even pasteurized milk with zero or minimal counts of *Staph. aureus* bacteria detected can cause staphylococcal enterotoxigenosis due to the presence of heat-stable SE. Although SE can be inactivated with specific pasteurization temperatures, a crucial factor is the amount of SE in milk. *Staphylococcus aureus* count before pasteurization has no effect on the amount of SE in pasteurized milk. The SE detected in the lowest amount among our samples was SEB. Higher pasteurization temperatures reduced the amounts of SEA, SEB, and SEC in milk samples. Although milk pasteurization is a crucial step toward ensuring milk safety, a key measure to prevent staphylococcal enterotoxigenosis among pasteurized milk consumers is to prevent any cold chain disruption, as mandated by the European legislation in force (European Commission, 2004).

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