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Research Note

Effect of Scalding Temperature on Growth of *Staphylococcus aureus* and Formation of Staphylococcal Enterotoxin during the Production of Alpine Cheese in a Laboratory Cheesemaking Model

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ABSTRACT

To reduce the number of cheese with potential *Staphylococcus aureus* contamination reaching consumers, European legislation has stipulated that all cheese must be tested for coagulase-positive staphylococci (CPS) at the point in production when numbers are expected to be highest. When CPS counts exceed 10^5 CFU/mL, staphylococcal enterotoxin (SE) tests must be conducted. When SE tests are positive, the cheese must be destroyed. Manufacturers of Swiss Alpine cheese are exempt from this legislation because SE formation in hard cheese is expected to be very unlikely because of the high scalding temperatures used for cheeses during production, which inactive CPS in the curd. However, this assumption has not been scientifically tested. A laboratory-scale cheese production experiment was performed in which the conditions corresponded to certain limitations in practical cheesemaking conditions such as temperature and time exposure as for production of Gruyere or Tete de Moine Swiss type cheeses. Raw milk aliquots (200 mL) were inoculated with five strains of CPS, and scalding temperatures of 46 to 56°C were used during cheese production. The temperatures applied after the curd was pressed were meant to reproduce the temperature curve in the peripheral zone of a real cheese wheel. Contrary to expectations, SE formation occurred and differed according to the scalding temperature (52 to 56°C). The differences in SE formation were more associated with strain type rather than temperature. These results indicate that the mechanisms of SE formation in cheese require further study.

HIGHLIGHTS

- Higher scalding temperatures (56°C) do not protect cheese from SE contamination.
- *S. aureus* genotype B, common in the Swiss Alps, poses a special risk for SE formation.
- *S. aureus* in Swiss Alpine hard and extra-hard raw milk cheese may cause food poisoning.

Key words: Cheese production; Gruyere; Scalding temperature; Staphylococcal enterotoxin; *Staphylococcus aureus*; Tete de Moine

Coagulase-positive staphylococci (CPS) are foodborne pathogens that can produce staphylococcal enterotoxins (SEs) *(21)*. According to the European Food Safety Authority *(10),* SEs are a major cause of foodborne illness in humans, and many studies have reported the role of SEs in foodborne outbreaks *(20, 25, 26)*. Although staphylococci are killed during food processing, SEs remain viable because of their high thermal stability *(22)*. In contrast to tests aimed at determining the viability of CPS, tests for the viability of SEs are difficult and relatively expensive, and therefore not usually performed. Currently available enzyme-linked immunosorbent assay kits for SEs cannot be used to detect all known SEs *(2),* and detection of enterotoxin genes in staphylococcal strains with a PCR assay does not always provide accurate information on enterotoxin gene expression *(14, 15, 30)*.

The foods associated with foodborne outbreaks are usually ready-to-eat products, often meat and dairy products *(14)*. Among dairy products, raw milk cheese is very susceptible to contamination with SEs *(5)*. The enterotoxins in cheese often originate from raw milk from cows with *Staphylococcus aureus* mastitis *(25)*. However, poor food safety practices and poor personal hygiene also can result in contamination *(14)*.

With the ribosomal spacer PCR method, >100 genotypes and variants of *S. aureus* from bovine intramammary infections have been identified, 17 subtypes of which belonged to genotype B (GTB) and genotype C (GTC) representing 81% of the isolates *(11)*. The remaining genotypes were rare and accounted for only 1.0 to 4.0% of all isolates *(11)*.

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TABLE 1. *Description of the* S. aureus *strains used in this study (15)*[a]

Strain	Genotype	SE1 PCR	SE2 PCR	Vidas SE2	SE RPLA	*spa* type	Comment
a	C	c	g, i	+	C+	t529	
b	B	a, d, r	j	+	A+ D+	t2953	Involved in SFPO[b]
c	B	a		+	A+	t5271	
d	B	d, r	j	+	D+	t2953	
e	O	d	g, i	+	D+	t7013	Rare genotype

[a] SE, staphylococcal enterotoxin; RPLA, reversed passive latex agglutination; *spa* type, specific gene sequence of the *S. aureus* staphylococcal protein A.
[b] SFPO, staphylococcal food poisoning outbreaks.

Staphylococcal genotypes are highly associated with virulence gene patterns *(4, 11)*. Various enterotoxin genes, including *sea, sed, sej,* and *ser,* have been identified in *S. aureus* GTB *(4, 6)*. This genotype also can persist along the food chain and its enterotoxin genes have been linked to foodborne outbreaks involving raw milk cheeses *(15, 21)*. In contrast, *S. aureus* GTC strains were typically positive for *sec, seg, sei,* and *tst (11, 12)* and were not linked to outbreaks traced to raw milk cheeses *(15)*. For the remaining genotypes, the virulence gene pattern was heterogeneous *(11),* and a few genotypes were associated with staphylococcal food poisoning *(15)*.

The clinical characteristics of *S. aureus* infections differ depending on the genotype *(12)*. For *S. aureus* GTB, up to 87% of cows in an infected herd had intramammary infections, indicating that *S. aureus* GTB is a contagious mastitis pathogen that spreads easily *(11, 12, 28)*. In contrast, *S. aureus* GTC and most of the other genotypes were detected in only a few individual cows with mastitis *(6, 11, 12)*. In previous research, *S. aureus* GTC was isolated from milk of cows with intramammary infections throughout Europe, whereas *S. aureus* GTB was detected in milk from cows with intramammary infections only in countries bordering Switzerland *(4)*. Other genotypes, except GTR, were rarely detected, although some of the genotypes were increasing in specific countries *(4)*.

Growth of *S. aureus* can occur before cheese processing because the raw milk for production may be stored at 8 to 18°C for up to 24 h before use. During cheese production, temperature, pH, competitive pressure from starter flora, and lactose starvation influence the growth of *S. aureus* and SE formation. Salt stress may also play a role in *S. aureus* growth and SE formation, although in hard and semihard Swiss cheeses, CPS counts start to decline during pressing or a few days after brining when the salt concentration in the cheese is still low *(1)*. Interactions between these parameters may enhance SE production *(8, 19, 29)*.

According to Commission Regulation 2073/2005 on microbiological criteria for foodstuffs *(9),* staphylococcal tests of cheese must be conducted when levels are assumed to be the highest. For hard and extrahard cheese, this is usually before the scalding process step. When the CPS level is $>10^5$ CFU/g, SE tests of the entire lot must be conducted.

Based on the practical experience of the advisory service for cheesemakers, a scalding temperature of 52°C has been considered sufficient to inactivate all CPS present, and at this and higher temperatures no SE production takes place. All hard and extrahard Swiss cheese production includes a process step in which the scalding temperature exceeds 52°C. Thus, provisional best practice guidelines for the manufacture of hard and extrahard Swiss Alpine cheese *(19)* do not include a requirement for staphylococcal and SE tests of these cheeses. The objective of this study was to provide data on the microbiological safety of hard and extrahard cheeses and to evaluate the effect of the current process and scalding protocols on SE formation. The Swiss Federal Office for Food Safety and Veterinary Affairs, as the responsible authority, has questioned the current practices and commissioned the National Reference Laboratory for Coagulase Positive Staphylococci (Agroscope) to research this issue.

MATERIALS AND METHODS

Strains and inoculum preparation. Five *S. aureus* strains (a through e), all of which had been previously isolated from cheese and analyzed by Hummerjohann et al. *(15),* were used for the inoculation of raw milk (Table 1). Some of the strains, such as strain b, were previously reported to be involved in staphylococcal food poisoning outbreaks *(12)*. All strains used were stored at –20°C and revived on Colombia agar plates with 5% sheep blood (bioMérieux, Geneva, Switzerland). For the preparation of the inocula, one plate colony of each strain (a through e) was transferred into 5 mL of Oxoid *Staphylococcus* medium (no. 110, Thermo Fisher Scientific, Pratteln, Switzerland) and incubated for 18 h at 37°C. These cultures were then diluted (0.9% NaCl) to obtain inocula containing about 10^5 CFU/mL and stored at –20°C until used (maximum storage of 3 months). Before use, the bacterial count of the inocula was determined by plating on Columbia agar with 5% sheep blood. This method was used to obtain an exact bacterial level in the inocula. SEs were detected with a kit (mini Vidas SET2, bioMérieux) according to the manufacturer's instructions.

Cheese production and experimental setup. A laboratory model cheese system (Supplemental Fig. S1) was set up that complied with the parameters and conditions (temperature profile, pH, culture, and rennet) encountered during the first 24 h of the cheesemaking process. In the model system, cheese was produced in a 250-mL Schott bottle containing 200 mL of raw milk. The raw milk was obtained from a local cheese dairy and was also tested for the presence of CPS.

In each batch, five cheeses inoculated with *S. aureus* (strains a through e) and one negative control were produced at the same time, with one scalding step. The six Schott bottles (with stir bars) were placed in a water bath in which the temperature was controlled by a thermostat (Ecoline Staredition RE 310, LAUDA-Brinkmann, Delran, NJ). The water bath was stirred with a multiposition magnetic stirrer (Variomag, Daytona Beach, FL) at an agitator speed of 220 rpm. Five of the bottles (containing strains a through e) were kept closed. The sixth bottle was used to record the temperature during curdling and scalding. The water bath was covered with floating balls to prevent evaporation.

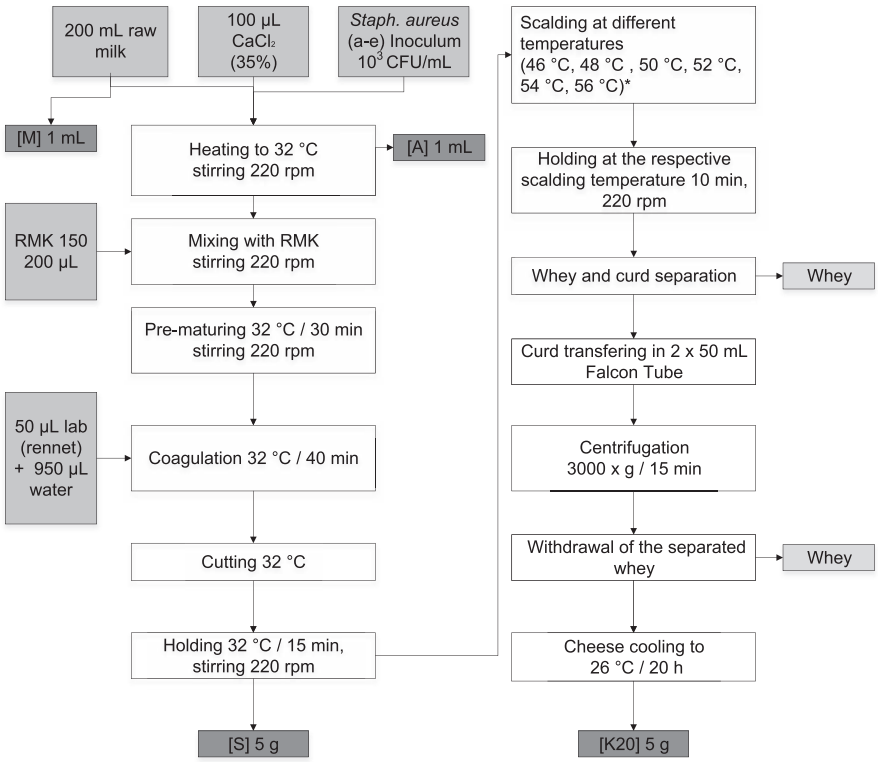

FIGURE 1. *Flow chart for the production of cheese in the laboratory cheesemaking model. The open boxes indicate the processes applied, the light shaded boxes provide information on material added or removed during the cheesemaking process, and the dark shaded boxes refer to sample collection at different times in the cheesemaking process. Heating and holding times and temperatures are described in Table 2.*

Figure 1 provides an outline of the experiments. An appropriate volume of thawed inoculum (calculated based on the level in the frozen inoculum, reaching ~10^4 CFU/mL in the milk) and 100 µL of calcium chloride (35%) were added to each bottle containing 200 mL of raw milk. The mixture was then heated to 30 to 32°C (as determined with a thermostat) with stirring. Then 200 µL of thermophilic starter RMK 150 (Agroscope, Bern, Switzerland) was added, and the milk was left for 30 min for prematuration. Subsequently, 50 µL of calf rennet (Winkler GR orange, Winkler AG, Konolfingen, Switzerland) was added to induce mild coagulation. The coagulum was cut 40 min later with a kitchen knife to obtain a curd size of 5 mm by cutting the coagulum horizontally and then rotating the bottle to make vertical cuts. The resulting curd (size and homogeneity) was not the same as that in commercial facilities but this difference was not expected to impact the outcome of the experiment. The curd was then heated to a scalding temperature while stirring gently. The temperature-time profiles for this stage were comparable to those used in commercial cheese production (Table 2).

TABLE 2. *Thermostat settings used for the different scalding procedures with a heating rate of 0.5°C/min*[a]

Scalding temp (°C)	Heating to scalding		Hold at scalding	
	Temp (°C)	Time (min)	Temp (°C)	Time (min)
46	47	32	46.5	10
48	49	35	48.5	10
50	51	39	50.5	10
52	53	40	52.5	10
54	55	45	54.5	10
56	57	48	56.5	10

[a] The final temperature was 1°C higher than the target to compensate for the temperature differences between the water bath and the milk.

After scalding, the whey and curd were separated with kitchen sieves. The remaining curd was then placed in two 50-mL Falcon tubes and centrifuged for 15 min at 3,000 rpm (Heraeus Megafuge 16 R, Thermo Scientific). The remaining whey was removed, the cheese was weighed (approximately 20 g), and the tubes were immediately returned to the water bath, which was still at a scalding temperature. The experimental cheeses were then cooled to 26°C for 20 h. The temperatures applied were the same as those applied in artisan cheese factories. Therefore, these temperature-time profiles reflected the situation in the peripheral zones of the cheese samples.

In each batch, six experimental cheeses (five contained one of the five *S. aureus* strains and one control) were produced with different scalding temperatures (48, 50, 52, 54, or 56°C). Each batch was replicated five times, resulting in 25 batches, 125 inoculated cheeses, and 25 control cheeses. The temperature and pH were measured in the control cheeses with specific measuring equipment (Almeno 2590, Ahlborn, Holzkirchen, Germany).

Microbial analysis and SE detection. The raw milk, raw milk with inoculum, curd, and cheese samples for microbiological examinations were obtained as illustrated in Figure 1. *S. aureus* cell viability in the samples was analyzed with the Tempo STA method (bioMérieux). This method was previously validated in house against the ISO 6888-3:2003 method *(17)*. SEs were detected in only the cheese samples using the ISO 19020:2017 method *(18)* and an immunoenzymatic assay (Vidas SET2, bioMérieux). Instead of the 25 g of sample material required by ISO 19020:2017, only 5 g of cheese was analyzed because of the miniaturized system. All reagents used were proportionally adapted, without affecting the limit of detection.

Statistical evaluation. For statistical analysis, Student's *t* test and an analysis of variance were used with Excel 2016 (Microsoft, Redmond, WA). This model also accounted for repeated measurements. The *P* value for significant differences was 0.05.

Data were analyzed for significant differences in strain levels (a through e, taking into account the five measurements) in cheeses processed with different scalding temperatures (46 to 56°C) and the changes in CPS levels between production steps (raw milk, curd, and cheese).

RESULTS AND DISCUSSION

Model system for cheese production. Because no pilot plant exists in Switzerland for large-scale experiments with class 2 microorganisms, the present study was conducted with a laboratory cheesemaking model. During the development of the cheesemaking model, care was taken to ensure that the cheesemaking parameters (temperature and pH) corresponded to those used in Alpine cheeseries (Figs. S2 and S3). A microprocessor-controlled water bath and continuously recorded process parameters (pH and temperature) were monitored during each experiment. Because the parameters and results were reproducible on different experimental days, the system was considered stable and reliable. The temperature and pH of the laboratory cheeses were comparable to those of hard and semihard Swiss cheeses found in Alpine cheeseries (Tete de Moine and Gruyere were used as reference cheeses based on consult experience and published reports *(13)*). An average pH of 5 ± 0.04 was recorded in all the cheese batches after 20 h, which corresponds to the pH of 5 found in Alpine cheeseries for the production of Tete de Moine and Gruyere. The pH was measured in the sample without inoculum, but because the *S. aureus* level (10^2 to 10^7 CFU/mL depending on the scalding temperature) was much lower than that of the starter culture (ca. 10^9 CFU/mL), the inoculum did not affect the pH. All raw milk used was negative for CPS, and *S. aureus* from natural contamination was therefore excluded from consideration.

Effect of scalding temperatures on *S. aureus* levels and SE production. The time course of increases in *S. aureus* levels during cheese production was independent of the strain used; no significant differences were found in strain levels at various time points in either raw milk or curd ($P > 0.05$). Up to a 2-log increase was found in each batch showed (Fig. 2). This increase was attributed to the physical entrapment of the bacteria in the curd, as reported previously *(27)*.

The initial *S. aureus* level was the same in the inoculated raw milk (ca. 10^4 CFU/mL, $P > 0.05$) and in the curds (ca. 10^6 CFU/mL, $P > 0.05$), However, after 20 h, significant differences in *S. aureus* levels were found in the cheese, both between strains ($P = 0.02$) and between scalding temperatures ($P = 0.0006$) (Fig. 2). For example, the level of *S. aureus* strain d increased to 6 to 7 log CFU/g (Fig. 2) even after applying a scalding temperature of 50 or 52°C. Based on these cell counts, the viability of strains a, c, and e clearly declined after 20 h after exposure to scalding temperatures of ≥50°C and fell to <100 CFU/g after scalding at 56°C (Fig. 2).

Cheese industry stakeholders have assumed that CPS are inactivated or damaged to such an extent that toxin production in cheese is no longer possible after scalding at 52°C. This inactivation occurred for strain d (Table 3), which produced SEs up to a scalding temperature of 50°C. At 52°C, toxin concentrations were near the limit of detection and not detectable at 54 and 56°C. However, for strains b and c, SEs were produced up to a scalding temperature of 56°C, whereas strains a and e did not produce SEs even at 46°C (Table 3). Strain-specific differences in SE production have been described previously *(9)*.

The *S. aureus* strains used in the present study differed genotypically and phenotypically. Strains b, c, and d are GTB *(6, 12),* whereas strains a and e are GTC and GTO, respectively. GTB is associated with contagious mastitis and causes herd problems, GTC or GTO cause illness in single animals *(6, 11, 12, 24, 28)*. GTB is able to persist in the food chain *(21),* and the persistence and adaption of this genotype to the dairy environment, including elevated temperatures, explains its survival and enterotoxin production in the present study. Adaptation to a temperature similar to that used in this study (58°C) has been described *(3)*.

In terms of SE production, not all five strains produce the same enterotoxins. Strain a produces SEC, strain b produces SEA and SED, strain c produces SEA, and strains d and e produce SED. According to the literature, not all strains begin to produce enterotoxins at the same CPS level *(16)*. As per the Commission Regulation *(9),* SE tests must be conducted only when the *S. aureus* level reaches 10^5 CFU/g. Previous research revealed only SEA production at this level, and SEC was produced only at 10^8 CFU/g *(16),* which explains why SEs were not detected from strain a. However, in the present study, SE production occurred at *S. aureus* levels $<10^5$ CFU/g. The presence of SE in the inoculum can be ruled out, because tests were conducted at the time of inoculum preparation. The following two explanations are proposed.

Strains b and c are generalized SE producers, able to produce toxin outside the optimal temperature range of 10 and 48°C *(27)* and possibly even at low levels ($<10^5$ CFU/g). In the present study, this level was reached in curd after a short time (due to the physical enrichment) but immediate scalding decreased the CPS level, and no enterotoxin production occurred in this short period. Thus, SEs could have been produced in the heating phase and/or after scalding during the 20 h of cooling, as was found for strain b (56°C scalding temperature) where the *S. aureus* level was 10^3 CFU/g after 20 h. Johler et al. *(20)* found SE in ripened cheese at an *S. aureus* level of 10^3 CFU/g.

SE production at lower *S. aureus* levels in the present study may also be explained by the fact that strains b and c can reach the log phase very quickly and thus achieve $>10^5$ CFU/g during heating to the scalding temperature and can produce enterotoxins in this phase. However, no published studies have been conducted to test this hypothesis. In the present study, a thawed inoculum instead of an overnight culture was used, which may have had an impact on the duration of the bacterial lag phase. Based on published reports *(23),* the *S. aureus* levels reached with the thawed inoculum should be comparable to those with an overnight

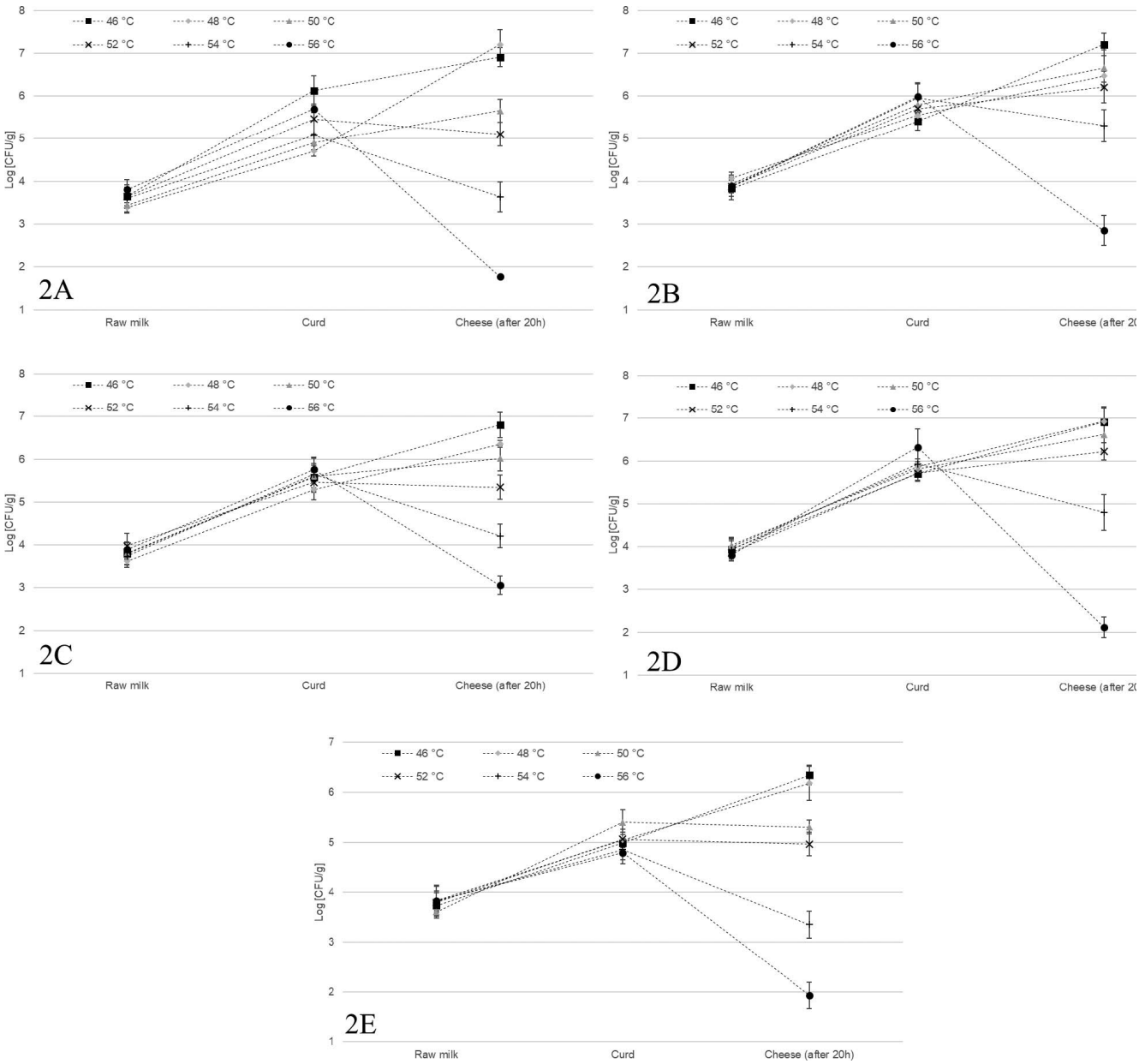

FIGURE 2. S. aureus *strain levels (means and standard deviations) during the cheesemaking process (raw milk samples with inoculum, curd, and cheese after 20 h) conducted with different scalding temperatures (46 to 56°C) (see also Fig. 1). (A through E) Strains a through e, respectively.*

TABLE 3. *SE production (SEA through SEE) by five* S. aureus *strains in cheese 20 h after production*

		SE production after scalding at[a]:					
Strain	Genotype	46°C	48°C	50°C	52°C	54°C	56°C
a	C	0	0	0	0	0	0
b	B	5	5	5	5	3	2
c	B	5	5	5	5	4	1
d	B	5	5	5	3	0	0
e	O	0	0	0	0	0	0

[a] Number of positive results from five cheeses produced at five scalding temperatures (46 to 56°C). The five cheeses were produced in different batches at different times. SE production was evaluated with the Vidas SET2 method.

culture. A longer lag phase, due to the additional stress on the bacteria from thawing, would mean that the present study represents a best-case scenario. However, *S. aureus* in the milk may not have the same growth potential as an overnight culture. Duquenne et al. *(8)* reported that temperature and time were key parameters for controlling SE production during cheese production. However, the temperatures (32 to 38°C) and times (15 to 45 min) used in their study were markedly different than those used in the present study, which may explain the SE production in their study. The parameters used in the present experiment should result in no risk of increased SE production. We conclude that prematuration of milk for <30 min at <35°C is sufficient to minimize the risk of SE production.

Strains from starter cultures added to milk, such as the commonly used *Lactococcus lactis,* may affect *S. aureus* growth and viability *(7)*. However, when the scalding temperature is >45°C, mesophilic starters are inappropriate. Therefore, we used a thermophilic starter (RMK 150) containing *Lactobacillus delbrueckii* subsp. *lactis* and *Streptococcus thermophilus*.

The laboratory model cheesemaking system used had a specific starting *S. aureus* level of 10^4 CFU/mL and reproduced conditions for the temperature curve in the peripheral zone of a commercial cheese (parameters specific for Gruyere and Tete de Moine) was used; however, the initial hypothesis that *S. aureus* in Swiss Alpine hard and extrahard raw milk cheese production poses no risk of staphylococcal food poisoning outbreaks was not supported. Some of the specific strains used produced SE even when scalding temperatures were >52°C. *S. aureus* GTB, which is common in the Swiss Alps, seem to have this ability.

SE production in this study was evaluated under only very specific conditions of *S. aureus* level, curd size, model cheese size, and processing parameters. To obtain more generalizable results, further study of the behavior of *S. aureus* and its mechanisms of enterotoxin production in the cheese matrix is needed. Futures studies should use cheeses the same size as commercial cheeses and various cheese production parameters and additional starting *S. aureus* levels (especially lower ones such as 10^2 to 10^3 CFU/mL). Further study is also needed to determine why *S. aureus* strains, especially GTB strains, differ in their ability to produce SEs.

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SUPPLEMENTAL MATERIAL

Supplemental material associated with this article can be found online at: https://doi.org/10.4315/JFP-19-600.s1

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