Tracing and inhibiting growth of *Staphylococcus aureus* in barbecue cheese production after product recall

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ABSTRACT

Staphylococcal food poisoning is one of the most prevalent causes of foodborne intoxication worldwide. It is caused by ingestion of enterotoxins formed by *Staphylococcus aureus* during growth in the food matrix. Following a recall of barbecue cheese due to the detection of staphylococcal enterotoxins in Switzerland in July 2015, we analyzed the production process of the respective dairy. Although most cheese-making processes involve acidification to inhibit the growth of pathogenic bacteria, barbecue cheese has to maintain a pH >6.0 to prevent undesired melting of the cheese. In addition, the dairy decided to retain the traditional manual production process of the barbecue cheese. In this study, therefore, we aimed to (1) trace *Staph. aureus* along the barbecue cheese production process, and (2) develop a sustainable strategy to inhibit growth of *Staph. aureus* and decrease the risk of staphylococcal food poisoning without changing the traditional production process. To this end, we traced *Staph. aureus* in a step-wise blinded process analysis on 4 different production days using *spa* (*Staphylococcus* protein A gene) typing, DNA microarray profiling, and pulsed-field gel electrophoresis analysis. We subsequently selected a new starter culture and used a model cheese production including a challenge test assay to assess its antagonistic effect on *Staph. aureus* growth, as well as its sensory and technological implications. We detected *Staph. aureus* in 30% (37/124) of the collected samples taken from the barbecue cheese production at the dairy. This included detection of *Staph. aureus* in the final product on all 4 production days, either after enrichment or using quantitative detection. We traced 2 enterotoxigenic *Staph. aureus* strains (t073/CC45 and t282/CC45) colonizing the nasal cavity and the forearms of the cheesemakers to the final product. In the challenge test assay, we were able to show that the new starter culture inhibited growth of *Staph. aureus* while

meeting the sensory and technological requirements of barbecue cheese production.

Key words: *Staphylococcus aureus*, contamination routes, process analysis, barbecue cheese, starter culture

INTRODUCTION

In July 2015, the Swiss Federal Food Safety and Veterinary Office (Bern) issued a warning concerning the consumption of barbecue cheese, in which *Staphylococcus aureus* and staphylococcal enterotoxins (SE) had been detected. The dairy producing the barbecue cheese issued a recall, followed by a comprehensive external process analysis to trace *Staph. aureus* in the cheese-making process.

Staphylococcus aureus can cause staphylococcal food poisoning (SFP), the most prevalent foodborne intoxication worldwide. Ingestion of major or newly described SE (Hennekinne et al., 2010; Johler et al., 2015) formed during growth of the organism in food leads to symptoms of acute gastroenteritis and violent emesis (Hu and Nakane, 2014). Although symptoms usually subside within 24 h, SFP can, in rare cases, be fatal for children and the elderly. The Centers for Disease Control and Prevention (Atlanta, GA) estimates 240,000 cases per year in the United States, resulting in 1,000 hospitalizations and 6 deaths (Scallan et al., 2011).

As SE are heat-stable and will not be inactivated during the cooking process, preventive measures focus on inhibiting growth of *Staph. aureus* in the food matrix (Le Loir et al., 2003). To this end, starter cultures are used in the production of a wide range of foods including cheese. A suitable starter culture will outcompete the organism, thus effectively preventing *Staph. aureus* growth and SE formation. However, starter cultures need to meet several criteria to be suitable for the production of barbecue cheese. Although the antagonistic effect of many starter cultures is due to acidification of the food matrix, the pH of barbecue cheese cannot be lowered to values <6.0, as this would result in melting of the cheese when it is exposed to high temperatures during preparation by the customer. In addition, suitable starter cultures must have no negative sensory implications.

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In this study, we aimed to (1) trace *Staph. aureus* in the barbecue cheese production process, and (2) develop a sustainable strategy to inhibit growth of *Staph. aureus* and decrease the risk of SFP without changing the traditional production process.

MATERIALS AND METHODS

Sampling Along the Production Process and Isolation of Coagulase-Positive Staphylococci

To identify potential sources for contamination of the barbecue cheese at the dairy, 4 production cycles (see Figure 1) of the barbecue cheese were screened for coagulase-positive staphylococci in a blinded setup. To avoid bias, the dairy and the cheesemakers were only informed of the results upon completion of the study. Samples were taken from cheesemakers and at each step of the cheese-making process on August 24 (**T**₁), August 27 (**T**₂), September 1 (**T**₃), and September 3 (**T**₄), 2015 (see Table 1). Before the start of cheese production, swabs from the anterior nares and forearms of the cheesemakers were taken, as well as a swab from the inner and outer side of the end of the milk hose after pasteurization. All swabs were moistened using 0.85% NaCl. During cheese production, 10-mL samples of milk, whey, and starter cultures, and 30-g samples of curd and cheese were taken. Samples were screened for coagulase-positive staphylococci (**CPS**) using enrichment in Mueller-Hinton broth with 6.5% NaCl and plating on rabbit plasma fibrinogen (RPF) agar (Oxoid, Pratteln, Switzerland) and quantitatively, following the EN ISO 6888-2 protocol (ISO, 1999). Species identification of *Staph. aureus* was achieved through subsequent *spa* typing and the detection of species-specific markers by Staphytype DNA microarray profiling (Alere, Jena, Germany).

Cell Lysis and DNA Extraction

For cell lysis and DNA extraction, reagents of the Staphytype genotyping kit 2.0 and the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) were used according to the manufacturers' instructions. The concentration of nucleic acids was measured using a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE).

spa Typing

The polymorphic X region of *spa* was amplified as previously described (Wattlinger et al., 2012). Each PCR product was subsequently purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich, Buchs, Swit-

zerland) and sequencing was outsourced (Microsynth, Balgach, Switzerland). Subsequently, *spa* types were determined using the *spa* server (<http://spa.ridom.de/>; Harmsen et al., 2003).

DNA Microarray-Based Genotyping

The Staphytype genotyping kit 2.0 was used to detect the presence or absence of over 300 virulence and resistance genes and their allelic variants in *Staph. aureus* strains that were traced from the cheesemakers to the final product. Detection included genes encoding the major SE (*sea*, *seb*, *sec*, *sed*, *see*), as well as genes coding for newly described SE and enterotoxin-like superantigens (*seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *selm*, *seln*, *selo*, *seq*, *ser*, *selu*). Microarray profiles also allowed for assignment of the strains to *agr* types, as well as clonal complexes (**CC**; Monecke et al., 2008).

Pulsed-Field Gel Electrophoresis Analysis

Preparation of chromosomal DNA and pulsed-field gel electrophoresis (**PFGE**) analysis of *Sma*I-digested fragments was performed as previously described (Bannerman et al., 1995). Electrophoresis was carried out in a Bio-Rad CHEF-DR III electrophoresis cell (Bio-Rad, Hercules, CA). *Salmonella enterica* serovar Braenderup strain H9812 digested with 50 U of *Xba*I (12 h, 37°C) was used as a molecular size standard. Gels were analyzed with Gel Compar II software (Applied Maths, Sint-Martens-Latem, Belgium) using the dice coefficient and were represented by unweighted pair grouping by mathematical averaging (UPGMA) with an optimization of 0.5% and position tolerance of 1%.

Model Cheese Production Including Challenge Test Assay Using New Starter Culture

A new starter culture, consisting of CNS, was chosen (START Crudo 500, Christl Gewürze GmbH, Moosdorf, Austria). Species identification of CNS was performed by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS). To evaluate the suitability of the new starter culture for the barbecue cheese production, the cheesemakers produced 4 batches of model cheese (batches A to D) in a laboratory setting.

To assess growth of the starter culture during the cheese-making process, we determined CNS counts in 2 barbecue cheese production batches (batches A and B) at 3 time points. The first sample was taken from the curd directly after coagulation, the second sample was obtained from the cheese after pressing (same day), and the third sample was taken in the morning of the

Figure 1. Flowchart providing an overview of the barbecue cheese production process until the ripening stage. The average batch size equaled 500 kg of milk, resulting in 55 kg of cheese.

following day. At this time, we also measured the pH of the cheese.

The inhibitory effect of the new starter culture on *Staph. aureus* growth was assessed in a challenge assay using 2 batches of barbecue cheese (batches C and D) produced with milk contaminated with *Staph. aureus*. To this end, a *Staph. aureus* strain isolated during the step-wise analysis of the barbecue cheese production process (strain 1, isolated at processing step P30, t282) was grown in brain-heart infusion (BHI) broth (Oxoid, Pratteln, Switzerland) at 37°C (225 rpm shaking) overnight to 2.5×10^9 cfu/mL. From the overnight broth, as well as from its 10-fold dilution in 0.85% NaCl, 1.0 mL was added to 3 L of milk, resulting in contamination levels of 10^6 cfu/mL milk in batch C and 10^5 cfu/

mL milk in batch D. The cheeses were sampled after 24 h and CPS counts were determined following the EN ISO 6888-2 protocol (ISO, 1999).

RESULTS AND DISCUSSION

We detected *Staph. aureus* in 30% (37/124) of the samples collected along the production process of the 4 barbecue cheese production cycles at the dairy (see Table 1). This included detection of *Staph. aureus* in the final product on all 4 production days, either after enrichment (T₁) or by quantitative detection methods (T₂ to T₄). The highest CPS count detected in a sample equaled 6.3×10^5 cfu/g in the final product, with cell density levels of 10^5 to 10^6 cfu/mL generally

Table 1. Tracing *Staphylococcus aureus* in a step-wise blinded process analysis on 4 different days (T₁ to T₄)¹

Sample ID	Source	Sampling details	Result ²			
			T ₁	T ₂	T ₃	T ₄
Before processing (swabs)						
BP1	Cheesemaker 1	Nose	t282 (B)	—	t282 (B)	t282 (B)
BP2	Cheesemaker 2	Nose	t073 (A)	t073 (A)	t073 (A)	t073 (A)
BP3	Cheesemaker 1	Forearms	t282	t362 _e	—	—
BP4	Cheesemaker 2	Forearms	—	t073 (A)	—	t5264
BP5	Milk hose after pasteurization	Inside and outside	—	—	—	—
Processing						
P1	Raw milk		t529	t3992	—	—
P2	Milk after pasteurization	Production start	—	—	—	—
P3	Milk after pasteurization	Mid-production	—	—	—	—
P4	Starter culture		—	—	—	—
P5	Curd before cutting	Sample 1	—	—	—	—
P6	Curd before cutting	Sample 2	—	—	—	—
P7	Curd before cutting	Sample 3	—	—	t073 _e (A)	—
P8	Curd after 1st stirring	Sample 1	—	t073 _q (A)	—	t073 _e (A)
P9	Curd after 1st stirring	Sample 2	—	—	—	—
P10	Curd after 1st stirring	Sample 3	—	t073 _e	—	—
P11	Whey	1st draining	—	—	—	—
P12	Curd after 2nd stirring	Sample 1	—	—	—	t282 _e (B)
P13	Curd after 2nd stirring	Sample 2	—	—	—	t073 _e (A)
P14	Curd after 2nd stirring	Sample 3	—	—	—	—
P15	Whey	2nd draining	—	—	—	—
P16	Curd after mixing	Sample 1	—	—	—	—
P17	Curd after mixing	Sample 2	—	—	—	—
P18	Curd after mixing	Sample 3	—	—	—	—
P19	Whey	3rd draining	—	—	—	t282 _e (B)
P20	Cheese after pressing I	Sample 1	—	—	—	—
P21	Cheese after pressing I	Sample 2	—	—	—	t282 _e
P22	Cheese after pressing I	Sample 3	—	—	—	t282 _e
P23	Cheese after pressing II	Sample 1	—	—	—	t5264 _q
P24	Cheese after pressing II	Sample 2	—	—	—	t282 _e
P25	Cheese after pressing II	Sample 3	—	—	—	t073 _e
P26	Cheese after pressing III	Sample 1	—	—	—	t282 _q
P27	Cheese after pressing III	Sample 2	—	—	—	t5264 _q
P28	Cheese after pressing III	Sample 3	—	—	—	t282 _q
P29	Cheese after pressing IV	Sample 1	—	t073 _q (A)	—	t5264 _q
P30	Cheese after pressing IV	Sample 2	—	t073 _q	t282 _q (B)	t282 _q
P31	Cheese after pressing IV	Sample 3	t282 _e	t073 _q	t073 _q (A)	t282 _q

¹The table indicates at which production steps *Staph. aureus* isolates were detected and provides an overview of the respective *spa* types (t073, t282, t5264, t529, t3992) and pulsed-field gel electrophoresis (PFGE) patterns (A, B) determined for selected isolates. Three strains could be traced from the cheesemakers to the final product: t072 (A), t282 (B), and t5264.

²Letters (A, B) in parentheses indicate assignment of isolates to PFGE patterns. Subscript q (_q) indicates that quantitative detection was possible for coagulase-positive staphylococci counts >10² cfu/g; subscript e (_e) indicates that lower levels were detectable only after enrichment.

Table 2. Molecular characteristics of the 3 *Staphylococcus aureus* strains traced from the cheesemakers to the final product

Characteristic	Strain 1	Strain 2	Strain 3
Typing			
<i>spa</i> typing	t282	t073	t5264
PFGE ¹	Pattern B	Pattern A	Not determined
Clonal complex	CC45	CC45	CC88
<i>agr</i> typing	<i>agrI</i>	<i>agrI</i>	<i>agrIII</i>
Enterotoxin genes			
Major enterotoxins ²	—	<i>sec</i>	—
Others ³	<i>seg, sei, selm, seln, selo, selu</i>	<i>seg, sei, sel, selm, seln, selo, selu</i>	—

¹Pulsed-field gel electrophoresis.²Screening included all major enterotoxin genes (*sea, seb, sec, sed, and see*).³Screening included *seg, seh, sei, sej, sek, sel, selm, seln, selo, seq, ser, and selu*.

being regarded as sufficient for enterotoxin production. The step-wise analysis showed that, in most cases, low-level contamination occurred early in the production process, with *Staph. aureus* being detected only after enrichment.

To determine the source of the contamination, we traced the isolates along the production process using *spa* typing, DNA microarray analysis, and PFGE analysis. The 37 isolates represented 6 different *spa* types: t282 (n = 15), t073 (n = 15), t5264 (n = 4), t362 (n = 1), t529 (n = 1), and t3992 (n = 1). The 3 *spa* types that were detected only once were found in *Staph. aureus* strains obtained from raw milk and the forearm of a cheesemaker. As these strains were not present in the final product, we excluded them from further characterization experiments. The DNA microarray analysis and *spa* typing showed that the remaining 34 isolates could be assigned to 3 distinct *Staph. aureus* strains. Genotypic characteristics, including typing results, as well as enterotoxin gene profiles of these strains are provided in Table 2. Strains 1 (t282/CC45) and 2 (t073/CC45) harbored enterotoxin genes and were repeatedly detected in samples from cheesemakers and in the final product. Strain identity of selected isolates was confirmed by PFGE analysis, resulting in only 2 distinct patterns. A third *Staph. aureus* strain (t5264/CC88) was only found in one production cycle (T₄) and was shown not to be enterotoxigenic.

Staphylococcus aureus colonizes the skin and mucosa of humans, with nasal carriage rates between 30 and 50% in the adult population (Diederer et al., 2006; Munckhof et al., 2008; Halablab et al., 2010; Wattinger et al., 2012). The 3 *Staph. aureus* strains detected in the nasal cavity and on the forearms of the cheesemakers, as well as in the final product, were assigned to t282/CC45, t073/CC45, and t5264/CC88. *Staphylococcus aureus* isolates of these *spa* types and clonal complexes have been previously reported in association with asymptomatic colonization and cases of infections in humans (Bloemendaal et al., 2009; Luedicke et al.,

2010; Wattinger et al., 2012; Gómez-Sanz et al., 2013). Nasal carriage status of *Staph. aureus* is not necessarily permanent but has been shown to change frequently over time (Sakwinska et al., 2009). Although decolonization strategies such as intranasal application of mupirocin and chlorhexidine washing have high success rates immediately after treatment, many carriers will become recolonized during a longer follow-up period (van Rijen et al., 2008; Ammerlaan et al., 2009). Thus, decolonization of cheesemakers does not represent a sustainable approach to increase food safety and promotes the risk of nasal colonization with mupirocin-resistant *Staph. aureus* (Ammerlaan et al., 2009).

As the dairy decided to retain the traditional manual production of the barbecue cheese, the most promising strategy to increase food safety and reduce the risk of SFP was the inhibition of *Staph. aureus* growth by use of a new starter culture. The candidate starter culture was identified as *Staphylococcus vitulinus* by MALDI-TOF MS. As shown in Table 3, the new starter culture was able to grow under the conditions of the cheese-making process. It had no negative sensory implications and resulted in a favorable pH of the product (pH = 6.29). In the challenge assay, we observed that the starter culture was able to outcompete *Staph. aureus* (Table 4), with a maximum increase of CPS counts of 1 log₁₀. This is particularly remarkable, as physical concentration effects alone can result in an increase of

Table 3. Growth of the starter culture evaluated by CNS counts using a model cheese production¹

Time point	Batch A		Batch B (+ <i>Staphylococcus aureus</i>)
	CNS (cfu/g)	pH	CNS (cfu/g)
After coagulation	1.4 × 10 ⁶	—	1.3 × 10 ⁶
After pressing	2.2 × 10 ⁵	—	2.5 × 10 ⁵
Next morning	2.9 × 10 ⁷	6.29	6.0 × 10 ⁷

¹Growth in presence of *Staph. aureus* was determined using an initial milk contamination level of 10³ cfu *Staph. aureus* per milliliter of milk.

Table 4. Challenge test assay assessing inhibition of *Staphylococcus aureus* growth by the new starter culture in a model cheese production¹

Batch	Coagulase-positive staphylococci (CPS)		CNS
	Artificial contamination in milk (cfu/mL)	After 24 h (cfu/g)	After 24 h (cfu/g)
C	10 ⁶	2.0 × 10 ⁷	2.4 × 10 ⁸
D	10 ⁵	6.0 × 10 ⁵	2.1 × 10 ⁸

¹Growth of *Staph. aureus* and the new starter culture was quantified using CPS and CNS counts, respectively.

1 log₁₀ during the cheese-making process (Peng et al., 2013).

CONCLUSIONS

We conclude that although the pasteurization process of the raw milk used for barbecue cheese production was successful, the risk of SFP persists due to *Staph. aureus* contamination by colonized cheesemakers. As decolonization of food handlers cannot ensure long-term freedom from nasal or skin carriage, the most promising strategy to increase food safety in the traditional manual production process is the use of a suitable starter culture. We were able to show that the new *Staphylococcus vitulinus* starter culture inhibited growth of *Staph. aureus* while meeting the sensory and technological requirements of barbecue cheese production. The dairy has now successfully implemented the new starter culture in the production process of the barbecue cheese and resumed regular production.

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