Modelling production of *S. aureus* enterotoxin C_{bovine} in milk, and its production during cheesemaking

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Abstract Staphylococcal enterotoxin type C (SEC) is one of the classical enterotoxins implicated in staphylococcal food poisoning. *Staphylococcus aureus* strains isolated from bovine unpasteurised milk and associated cheese samples produced the SEC_{bovine} sub-type. The objectives of this study were to determine the cell numbers required for SEC_{bovine} production in milk and to determine if SEC_{bovine} was produced by *S. aureus* during cheesemaking. To predict the point at which toxin production begins, SEC production was modelled against cell numbers of *S. aureus* (at different controlled pH/temperature combinations) in sterile reconstituted milk using a biphasic model fitted using the Solver routine in Excel. Under the conditions tested, the average cell number required for SEC_{bovine} production was $\log 8.43 \pm 0.39$ in sterile reconstituted milk. A semi-soft cheese was manufactured using milk inoculated with either washed (to remove any pre-formed toxin) or unwashed (containing toxin) *S. aureus* at 10^5 and 10^7 cfu.mL⁻¹. An enzyme-linked immunosorbent assay was used to quantify toxin production and Baird-Parker agar to quantify *S. aureus*. Cheese made with washed and unwashed cells showed a one log increase in *S. aureus* cell numbers during cheesemaking. In cheese made with washed cells, no SEC was detected. With unwashed cells, the SEC concentration remained constant throughout cheesemaking. The

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results show that SEC_{bovine} is not formed in milk or cheese at $<10^8$ cfu.mL⁻¹, and therefore, the risk-associated with SEC_{bovine} in cheese is low.

Keywords *Staphylococcus aureus* · Enterotoxin · Enterotoxin C production · Cheese · Milk · Modelling

1 Introduction

Coagulase positive *S. aureus* are indicators of staphylococcal enterotoxin (SE)-producing strains that cause staphylococcal food poisoning (SFP). Pasteurisation kills *S. aureus*; however, it will not destroy SEs (Le Loir et al. 2003). SEs are large, preformed, secreted virulent peptides produced by certain *S. aureus* strains. According to the official European Union data from 2011 (EFSA 2014), 346 foodborne outbreaks were attributed to *Staphylococcus* spp. This represented 6.4% of all reported foodborne outbreaks. The amount of SE required to cause illness has been reported at between 20–100 ng, although this can vary depending on type of SE (Pinchuk et al. 2010; Asao et al. 2003). However, these concentrations are taken from a limited number of food outbreaks where the SE concentration was determined.

Enterotoxin-producing *S. aureus* strains can be found in the milk of animals suffering from mastitis (Zadoks et al. 2001) or when poor hygiene in the handling and processing environments of dairy products occurs (Gutiérrez et al. 2012). A wide variety of SE toxins can be produced; presently, 21 SEs or enterotoxin-like proteins have been identified (Schlievert and Case 2007; Thomas et al. 2006). Well-known classical types SEA, SEB, SEC, SED and SEE are most frequently implicated in food contamination, with outbreaks of SFP, for example, from chocolate milk and sheep's milk cheese (Asao et al. 2003; Vitale et al. 2015). SEC and SEA are the most commonly occurring SEs in milk (Lindqvist et al. 2002; Scherrer et al. 2004; Aragon-Alegro et al. 2007). The presence of SEs, including SEC, is not permitted in cheese, according to Commission Regulation 2073/2005 (EC 2005). In food-related outbreaks of SFP, SEC is amongst the least SE types implicated (Pinchuk et al. 2010).

SEC is sub-classified to six subtypes, SEC1, SEC2, SEC3, SEC_{ovine}, SEC_{bovine} and SEC_{goat} (Argudín et al. 2010), which are structurally different (Metzger et al. 1975) and therefore produce different toxins and have different toxin production characteristics. Enterotoxin production, even with high bacterial growth, will not always occur in a cheese environment (Schelin et al. 2011). SEC production in cheese depends on several factors, such as the cheese type, the processing environment, the *S. aureus* cell numbers achieved during production (Valihrach et al. 2013; Janštová et al. 2012) and the SEC sub-type implicated (Valihrach et al. 2013).

From a survey of unpasteurised milk used in farmhouse cheese production in Ireland, two different strains of SEC_{bovine} were identified (Hunt et al. 2012), and the factors affecting toxin production were studied (Hunt et al. 2014). The objectives of this study were to establish (using a modelling approach) the *S. aureus* numbers needed to produce SEC_{bovine} in milk and to assess the potential for SEC_{bovine} production during cheesemaking of a semi-soft cow's pasteurised milk cheese.

2 Materials and methods

2.1 Modelling SEC_{bovine} production against cell numbers in sterile reconstituted milk

S. aureus and SEC data were modelled to determine the minimum cell numbers required for SEC production in sterile milk and to predict the point at which toxin detection began. A biphasic linear model (Buchanan et al. 1997) was fitted to the SEC concentration produced at a particular log bacterial number. The model was fitted using the Solver routine in Excel (Microsoft, WA, USA). The Solver routine minimised the sum of squares difference between predicted and actual values of toxin by optimising the values of the inflection point and the slope of the line representing toxin concentration.

2.2 *S. aureus* cultures

S. aureus strains 178 and 211 (Hunt et al. 2012) with SEC_{bovine}-producing ability were isolated from unpasteurised milk and unpasteurised milk cheese, respectively, and maintained at -80°C in cryovials (Pro-Lab Microbank®). The strains were transferred from cryovials to tryptic soya agar (TSA) and incubated at 37°C for 18 h. One colony was transferred to 100 mL tryptic soya broth (TSB) and incubated at 37°C for 18 h prior to preparation of the inoculum.

2.3 *S. aureus* inoculum preparation for cheesemaking

Fresh pasteurised and homogenised milk (Avonmore, Dublin, Ireland), acquired locally on the day of the experiment, was used for cheesemaking. Prior to inoculation of the milk, the inoculum was grown in TSB (as above) and washed twice in Maximum Recovery Diluent (MRD) to remove the SEC that resulted from growth in TSB; the bacterial cells were resuspended in 10% reconstituted skim milk (RSM; Kerry Ingredients, Charleville, Ireland) and added to 10 L of pasteurised milk, resulting in $\log 5$ and $\log 7$ cfu.mL⁻¹ in the milk. Unwashed inoculum (100 mL in TSB) was added to 10 L of milk to a final concentration of $\log 7$ cfu.mL⁻¹. Each experiment was undertaken in independent triplicate for each strain.

2.4 Laboratory scale cheesemaking

The pasteurised milk used for cheesemaking was analysed for total bacterial count, coliforms and coagulase positive *S. aureus* (as described below). The temperature of the milk (inoculated with washed or unwashed cells of a SEC_{bovine}-producing strain of *S. aureus*) was raised to 20°C , and a liquid starter culture (*Lactococcus lactis* 303, Chr. Hansens, Cork, Ireland), pre-incubated for 16 h at 30°C in sterile 10% RSM, was added at 1.5%. The temperature of the milk was increased to 32°C , and stirred until the pH reached 6.55 (approximately 30 min). Double strength rennet (CHY-MAX PLUS Fermentation-produced Chymosin, diluted 10-fold in sterile distilled water; CHR HANSEN, Horsholm, Denmark) was added at a concentration of 0.18 mL.L⁻¹. After testing the gelled milk for firmness (approximately 45 min after the addition of the rennet), the curd

was cut into cubes approximately 1 cm^3 . The curd-whey mixture was cooked from 32 to 36 °C at a rate of 1 °C every 5 min, stirring continuously. The curd was then put into moulds (top diameter 89 mm, bottom diameter 82 mm and height 83 mm; Moorlands Cheesemakers Limited, UK) and turned every 30 min until a pH of 5.30 was reached. During cheesemaking, milk and cheese were analysed (as described below) after starter culture addition, and at 1, 2 and 6 h. After manufacture (approximately 6 h), the cheese was brined in 23% NaCl/0.02% calcium chloride solution for 1 h, and ripened for 1 week (unwashed inoculum cheese only) at 8 °C/ 75% relative humidity (RH), 8 °C/85% RH and 14 °C/ 85%RH, with daily turning. Samples were analysed (as described below) after brining and after one, 3 and 7 days of ripening. Three independent cheesemaking experiments were undertaken at each condition.

2.5 Microbiological and physico-chemical analyses

Samples were enumerated for total bacterial count on milk plate count agar (Merck KGaA, Darmstadt, Germany), coliform on Violet Red Bile Agar (LAB031, United Kingdom), *S. aureus* on Baird Parker Rabbit Plasma Fibrinogen selective agar (CM0961, Oxoid Limited, United Kingdom) and starter bacteria on lactose modified *Lactococcus* species agar with 0.5% lactose (Merck, Germany), using the methods ISO 6610:1992, ISO 4831:2006, ISO 688-2:1999 and ISO 27205:2010, respectively. All cheese samples were tested for moisture (IDF4A and IDF21B), pH (BS770:5:1975) and the temperature recorded. The salt (chloride) content of brined cheese was tested using the IDF 88:2006 method.

2.6 SEC_{bovine} quantification in milk, cheese and curd

An enzyme-linked immunosorbent assay (ELISA) method described by Wallin-Carlquist et al. (2010) was used for quantification of SEC_{bovine} in milk, curd and cheese. The antibodies used were as described by Valihrach et al. (2013). SEC in 10% RSM was used to construct a standard curve to obtain a quantification range of 0.16–10 ng.mL⁻¹. For cheese and curd samples, the standard curve was constructed in phosphate buffered saline (PBS), as the toxin from the cheese was concentrated by dialysis to PBS by the method described by Hennekinne et al. (2003), except that a pH of 5.5 (instead of pH 3.5) was used, as better recovery was obtained. A recovery rate of 69% was obtained with cheese deliberately inoculated with SEC (Toxin Technologies® 7165 Curtiss Ave Sarasota, FL 34231, USA), and this was accounted for in experimental cheeses. The limit of quantification for SEC_{bovine} in the milk samples was 0.16 ng.mL⁻¹ and in the curd and cheese samples was 0.32 ng.mL⁻¹. The concentration of SEC_{bovine} was determined in triplicate in all samples.

To determine the stability of toxin in frozen cheese, SEC (Toxin Technologies), dissolved in sterile RSM, was added to the milk prior to cheesemaking to obtain cheese samples at 0.32, 5, and 10 ng.mL⁻¹ final concentration. Independent triplicate cheeses were manufactured at each SEC concentration. The cheeses were frozen at -20 °C, and the toxin concentration determined after 6 months, using the method described above.

2.7 Pre-incubation of SEC-producing *S. aureus* with added starter culture in milk

In order to determine the effect of pre-incubation of *S. aureus*-contaminated milk with starter culture, washed cells of strains 178 and 211 were inoculated into pasteurised milk at $\log 6 \text{ cfu.mL}^{-1}$. This culture was split into three parts, no starter culture was added to one, *L. lactis* 303 was added at a concentration of 1.5% to the second which was incubated at 36 °C for 5 h and *L. lactis* 303 was added at a concentration of 1.5% to the third which was incubated at 4 °C for 24 h, followed by further incubation for 5 h at 36 °C. The milk was sampled in duplicate for enumeration of *S. aureus* using Baird Parker Rabbit Plasma Fibrinogen selective agar.

2.8 Statistical analysis

Statistical analysis was performed in Microsoft Excel (Microsoft) and Graph Pad InStat (Graph Pad Software, La Jolla, CA., USA). For analysis of the data in RSM, Combase DMFit (IFR web access DMFit; Baranyi and Roberts no lag model) was fitted, where different growth rates were compared by multiple regression analysis at the conditions tested.

3 Results

3.1 *S. aureus* growth and modelling SEC_{bovine} production in sterile RSM

A full factorial experiment of four temperatures (25, 30, 37 and 40°C) and four pH values (5.5, 6.0, 6.5 and 7.0) in batch cultures of 10% sterile RSM inoculated with $\log 4.5 \text{ cfu.mL}^{-1}$ was undertaken (Hunt et al. 2014). In a modelling approach, the data from that study was further analysed using the Baranyi and Roberts (no lag) model in DMFit (ComBase DMFit 2015). For all fitted conditions, there was good agreement between predicted and actual values, see for example Fig. 1, except in 3 of 16 conditions tested where there was a decline in cell numbers during the stationary phase (30°C/pH 7,

Fig. 1 Representative fitting of *S. aureus* concentration ($\log \text{ cfu.mL}^{-1}$) using the Baranyi and Roberts (no lag) model at 25°C/pH 5.5 (time in min)

Fig. 2 Representative biphasic linear model of SEC_{bovine} formation with *S. aureus* concentration at 30°C/pH 6.5. The dotted line represents the best fit biphasic linear model to the experimental data (square)

40°C/pH 7 and 40°C/pH 6.5). The growth rate of *S. aureus* was not correlated with the pH or temperature ($p > 0.05$). The average *S. aureus* maximum specific growth rate was $0.22 \pm 0.04 \log \text{ cell number. h}^{-1}$. At each pH/temperature combination used, the concentration of SEC_{bovine} was plotted against the *S. aureus* cell number (see example in Fig. 2). The inflection point of the fitted biphasic model is the cell number at which toxin production was detected and represents the minimum bacterial numbers required for formation of detectable SEC_{bovine}. The inflection point from the modelled data for SEC production generally fitted the experimental data obtained, except at conditions of pH 6.5/40 °C and all temperatures at pH 7.0. Over the range of pH and temperature conditions, the average cell number required for SEC production was $\log 8.43 \pm 0.39 \text{ cfu.mL}^{-1}$. There was no correlation between the inflection point and the temperature/pH combinations tested.

3.2 SEC_{bovine} production in cheese with washed and unwashed cells of *S. aureus*

The total bacterial count for all pasteurised milk used was $< \log 4 \text{ cfu.mL}^{-1}$. No coagulase positive *S. aureus* or coliforms were detected in the pasteurised milk prior to inoculation. In cheese made with washed/unwashed cells of both strains 178 and 211, lactic acid bacterial numbers increased to approximately $\log 9.9 \text{ cfu.g}^{-1}$ whilst *S. aureus* numbers increased to approximately $\log 7.3 \text{ cfu.mL}^{-1}$. The pH reduced from 6.70 to 5.21 after 6 h. At all sampling times up to the end of manufacture, the SEC concentration was $< 0.32 \text{ ng.mL}^{-1}$ in cheese made with washed cells. In cheese made with unwashed cells, equivalent to added SEC_{bovine} at a concentration of approximately 3 ng mL^{-1} , SEC_{bovine} concentrations were similar throughout ripening at 8 °C/75% RH and 14 °C/85% RH and were reduced at ripening conditions of 8 °C/85% RH (Fig. 3).

3.3 Effect of freezing on measurement of SEC_{bovine}

After freezing the cheese samples for 6 months, the concentration of SEC in the spiked cheese samples was $\pm 15\%$ of the initial concentration at 10 and 5 ng.mL^{-1} and $\pm 20\%$ at 0.32 ng.mL^{-1} .

Fig. 3 SEC_{bovine} stability in cheese during 7 days of ripening at three different conditions: 8 °C/75% RH (closed circle); 8 °C/85% RH (open circle); 14 °C/85% RH (closed triangle). RH relative humidity

3.4 Effect of pre-incubation of starter culture with *S. aureus*

In the milk with no added starter culture, the numbers of *S. aureus* reached approximately $\log 8 \text{ cfu.mL}^{-1}$ at 36 °C for 5 h. When starter culture was added at 1.5% prior to incubation, the growth of *S. aureus* was inhibited. There was further inhibition of *S. aureus* if the culture was incubated at 4 °C for 24 h prior to incubation at 36 °C for 5 h (Fig. 4).

Fig. 4 *S. aureus* growth in pasteurised milk incubated at 36 °C for 5 h with different treatments: A *S. aureus* culture only, B *S. aureus* with starter culture and incubated immediately, C *S. aureus* with starter culture and chilled for 24 h prior to incubation

4 Discussion

Production of SEC in cheese is considered a risk factor, particularly with respect to raw milk cheese. Therefore, it is important to understand the factors affecting SEC production in cheese. Hunt et al. (2014) showed that pH and temperature influenced SEC_{bovine} production in milk. Modelling this data with a biphasic linear model demonstrated that at combinations of controlled pH and temperature values in the range of 25–40 °C and pH 5.5–7, the required levels of *S. aureus* cell numbers for SEC_{bovine} detection were $\log 8.43 \pm 0.39$ cfu.mL⁻¹. At cell numbers below $\log 8.43 \pm 0.39$ cfu.mL⁻¹, no detectable SEC_{bovine} was produced.

To test this model, SEC_{bovine} production was studied during cheesemaking. During cheesemaking with washed cells of *S. aureus*, the SEC_{bovine}-producing *S. aureus* cell numbers never reached levels above log 8 and no SEC_{bovine} was detected. Initial concentrations of log 5 and log 7 cfu.mL⁻¹ SEC_{bovine}-producing *S. aureus* culture inoculated into pasteurised milk prior to manufacture of cheese resulted in no detectable SEC_{bovine} in the cheese, with an increase in *S. aureus* and lactic acid bacteria numbers to approximately log 7.3 cfu.mL⁻¹ and log 9.9 cfu.g⁻¹, respectively. It has been reported that SEA production occurred in cheese at cell numbers of *S. aureus* at log 5 and log 6 cfu.g⁻¹ (Vernozy-Rozand et al., 1996), whilst Rola et al. (2013) found that no SED was produced in cheese with numbers of *S. aureus* reaching less than log 7 cfu.g⁻¹. Even though SEC can cause SFP, based on the results of this study, there is a low risk of SEC_{bovine} production in this type of cheese. The study indicates that the results from the milk-based model were valid in cheese.

Alternatively, the cheese matrix may have inhibited toxin production or inactivated the toxin. When SEC_{bovine} was deliberately added to the cheese at approximately 3 ng.mL⁻¹, along with the *S. aureus* producing strain (unwashed cells), after cheesemaking, the SEC_{bovine} remained at a similar concentration, even when the bacterial cell numbers increased by over one log. During the ripening of cheese, the SEC_{bovine} concentration remained constant for 7 days at 8 °C/75% RH and 14 °C/85% RH. The SEC concentration decreased after 7 days of ripening at 8 °C/85% RH, indicating that the cheese matrix may have an effect on pre-formed toxin. In boiled ham, Wallin-Carlquist et al. (2010) also reported a reduction in SEA concentration during storage at room temperature. The reason for such a reduction requires further investigation, but may be due to conformational changes in the enterotoxin structure, altering the antibody/antigen complex binding during the ELISA quantification.

The results from the experiments on the effect of freezing on toxin measurement are acceptable under the guidance for Industry of Bioanalytical Method, FDA, (FDA 2001), indicating that freezing had no significant impact on SEC measurement in cheese.

The starter culture inoculated to the milk 24 h prior to cheesemaking with refrigeration at 4 °C showed a reduction in *S. aureus* growth (Fig. 4) compared to milk inoculated immediately prior to cheesemaking and milk not inoculated with starter culture. Cretenet et al. (2011), Le Marc et al. (2009) and Pacheco and Galindo (2010) predicted that starter cultures inhibited SEA production in cheese. The effect of the starter culture *L. lactis* inhibition on *S. aureus* is thought to be partially attributable to a reduction in the pH of the milk (Alomar et al. 2008) whereas Charlier et al. (2008) discussed that acidification was not involved.

It has been shown previously that *L. lactis* and other lactic acid bacteria (LAB) can influence the behaviour of *S. aureus* under different conditions (Nouaille et al. 2014). In particular, it has been shown that in a cheese matrix, the accessory gene regulator (*agr*),

the main regulatory system controlling *S. aureus* virulence factors such as enterotoxins, is affected by LAB; metabolism of *S. aureus* is also affected by LAB (Cretenet et al. 2011). The presence of the *L. lactis* cells, even at concentration of $\log 3 \text{ cfu.g}^{-1}$ (Charlier et al., 2008; Le Marc et al., 2009) can affect *S. aureus* in a multifactorial way, leading to difficulty in studying the effect of individual parameters in a mixed culture context (Even et al., 2009). Regardless of the mode of action, using *L. lactis* as a biocontrol agent in food to control virulence of *S. aureus* seems to be a realistic possibility, offering a mechanism of improving food safety with respect to SEs.

5 Conclusion

There is little risk of $\text{SEC}_{\text{bovine}}$ formation in this cheese type even from milk containing high bacterial cell numbers. When $\text{SEC}_{\text{bovine}}$ is preformed (unwashed cells added) in the milk before cheesemaking, there is a risk of $\text{SEC}_{\text{bovine}}$ persisting during cheesemaking in this cheese type, and during ripening for 1 week at 8 °C/75% RH and 14 °C/85% RH. The addition of starter culture to the milk prior to cheesemaking reduced the *S. aureus* growth in milk; growth was further reduced by pre-incubating the starter culture at 4 °C for 24 h. As *S. aureus* contamination of milk at high bacterial numbers would be visually identified to the manufacturer as the milk coagulates at levels required for detectable toxin formation, the risk to public health from enterotoxin-producing *S. aureus* in cheese during cheesemaking can be quantified to some extent and can be managed by physical control.

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Conflict of interest All authors declare that they have no conflict of interest.

References

- Alomar J, Loubiere P, Delbes C, Nouaille S, Montel MC (2008) Effect of *Lactococcus garvieae*, *Lactococcus lactis* and *Enterococcus faecalis* on the behaviour of *Staphylococcus aureus* in microfiltered milk. Food Microbiol 25:502–508
- Aragon-Alegro LCEM, Konta K, Suzuki MG, Silva AF, Júnior Rall R, Rall VLM (2007) Occurrence of coagulase-positive *Staphylococcus* in various food products commercialized in Botucatu, SP, Brazil and detection of toxins from food and isolated strains. Food control 18:630–634
- Argudín MÁ, Mendoza MC, Rodicio MR (2010) Food poisoning and *Staphylococcus aureus* enterotoxins. Toxins 2:1751–1773
- Asao T, Kumeda Y, Kawai T, Shibata T, Oda H, Haruki K, Nakazawa H, Kozaki S (2003) An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. Epidemiol Infect 130:33–40
- Buchanan RL, Whiting RC, Damert WC (1997) When is simple good enough: a comparison of the Gompertz, Baranyi, and three-phase linear models for fitting bacterial growth curves. Food Microbiol 14:313–326
- Charlier C, Even S, Gautier M, Le Loir Y (2008) Acidification is not involved in the early inhibition of *Staphylococcus aureus* growth by *Lactococcus lactis* in milk. Int Dairy J 18:197–203
- Combase DMFit (2015) web edition. <http://www.combase.cc/index.php/en/about-combase/2-uncategorised/136-dmfit-web-edition>. Downloaded [10/1/15]

- Cretenet M, Even S, Le Loir Y (2011) Unveiling *Staphylococcus aureus* enterotoxin production in dairy products: a review of recent advances to face new challenges. *Dairy Sci Technol* 91:127–150
- EC (2005) Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union* 338.22 (2005): 1–26.
- EFSA – European Food Safety Authority (2014) The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012 *EFSA Journal* 12:3547.
- Even S, Charlier C, Nouaille S, Ben Zakour NL, Cretenet M, Cousin FJ, Gautier M, Coccagn-Bousquet M, Loubiere P, Le Loir Y (2009) *Staphylococcus aureus* virulence expression is impaired by *Lactococcus lactis* in mixed cultures. *Appl Environ Microbiol* 75:4459–4472
- FDA Guidance for Industry of Bioanalytical Method (2001) (<http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf>) Downloaded [10/1/15].
- Gutiérrez D, Delgado S, Vázquez-Sánchez D, Martínez B, Cabo ML, Rodríguez A, Herrera JJ, García P (2012) Incidence of *Staphylococcus aureus* and analysis of associated bacterial communities on food industry surfaces. *Appl Environ Microbiol* 78:8547–8554
- Hennekinne JA, Gohier M, Maire T, Lapeyre C, Lombard B, Dragacci S (2003) First proficiency testing to evaluate the ability of European Union National Reference Laboratories to detect staphylococcal enterotoxins in milk products. *J AOAC Int* 86:332–339
- Hunt K, Butler F, Jordan K (2014) Factors affecting staphylococcal enterotoxin C bovine production in milk. *Int Dairy J* Volume 39:41–46
- Hunt K, Schelin J, Rådström P, Butler F, Jordan K (2012) Classical enterotoxins of coagulase-positive *Staphylococcus aureus* isolates from raw milk and products for raw milk cheese production in Ireland. *Dairy Sci Technol* 92:487–499
- Janštová B, Necidová L, Janštová B (2012) Comparing the growth of *S. aureus* and production of Staphylococcal enterotoxin C in sheep's and goat's milk. *J Micro Biotech Food Sci* 1:758–768
- Le Loir Y, Baron F, Gautier M (2003) *Staphylococcus aureus* and food poisoning. *Genet Mol Res* 2:63–76
- Le Marc Y, Valík L, Medvedová A (2009) Modelling the effect of the starter culture on the growth of *Staphylococcus aureus* in milk. *Int J Food Microbiol* 129:306–311
- Lindqvist R, Sylvén S, Vågsholm I (2002) Quantitative microbial risk assessment exemplified by *Staphylococcus aureus* in unripened cheese made from raw milk. *Int J Food Microbiol* 78:155–170
- Metzger JF, Johnson AD, Spero L (1975) Intrinsic and chemically produced microheterogeneity of *Staphylococcus aureus* enterotoxin type C. *Infect Immun* 12:93–97
- Nouaille S, Rault L, Jeanson S, Loubiere P, Le Loir Y, Even S (2014) Contribution of *Lactococcus lactis* reducing properties to the downregulation of a major virulence regulator in *Staphylococcus aureus*, the agr system. *Appl Environ Microbiol* 80:7028–7035
- Pacheco FP, Galindo AB (2010) Microbial safety of raw milk cheeses traditionally made at a pH below 4.7 and with other hurdles limiting pathogens growth. *Curr Res Technol Educ Top Appl Microbiol Microbial Biotechnol* 2:1205–1216
- Pinchuk IV, Beswick EJ, Reyes VE (2010) Staphylococcal enterotoxins. *Toxins* 2:2177–2197
- Rola JG, Korpysa-Dzirba W, Osek J (2013) Prevalence of *Staphylococcus aureus* and staphylococcal enterotoxins at different stages of production of raw milk cheeses-preliminary results. *Bull Vet Inst Pulawy* 57:341–345
- Schelin J, Wallin-Carlquist N, Cohn MT, Lindqvist R, Barker GC, Rådström P (2011) The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment. *Virulence* 2: 580
- Scherrer D, Corti S, Muehlherr JE, Zweifel C, Stephan R (2004) Phenotypic and genotypic characteristics of *Staphylococcus aureus* isolates from raw bulk-tank milk samples of goats and sheep. *Vet Microbiol* 101: 101–107
- Schlievert PM, Case LC (2007) Molecular analysis of staphylococcal superantigens. *Methicillin-Resistant Staphylococcus aureus* (MRSA) protocols, Springer: 113–126.
- Thomas DY, Jarraud S, Lemercier B, Cozon G, Echasserieau K, Etienne J, Gougeon ML, Lina G, Vandenesch F (2006) Staphylococcal enterotoxin-like toxins U2 and V, two new staphylococcal superantigens arising from recombination within the enterotoxin gene cluster. *Infect Immun* 74:4724–4734
- Valihrach L, Alibayov B, Demnerova K (2013) Production of staphylococcal enterotoxin C in milk. *Int Dairy J* 30:103–107
- Vernozy-Rozand C, Mazuy C, Prevost G, Lapeyre C, Bes M, Brun Y, Fleurette J (1996) Enterotoxin production by coagulase-negative staphylococci isolated from goats' milk and cheese. *Int J Food Microbiol* 30:271–280

- Vitale M, Scatassa ML, Cardamone C, Oliveri G, Piraino C, Alduina R, Napoli C (2015) Staphylococcal food poisoning case and molecular analysis of toxin genes in *Staphylococcus aureus* strains isolated from food in Sicily Italy. *Foodborne Pathog Dis* 12:21–3
- Wallin-Carlquist N, Márta D, Borch E, Rådström P (2010) Prolonged expression and production of *Staphylococcus aureus* enterotoxin A in processed pork meat. *Int J Food Microbiol* 141:S69–S74
- Zadoks RN, Allore HG, Barkema HW, Sampimon OC, Wellenberg GJ, Gröhn YT, Schukken YH (2001) Cow-and quarter-level risk factors for *Streptococcus uberis* and *Staphylococcus aureus* mastitis. *J Dairy Sci* 84:2649–2663