



Factors influencing staphylococcal enterotoxin production in Dairy Products

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Factors Influencing Staphylococcal Enterotoxin Production in Dairy Products

The objective of this report was to inform regulators about available scientific information on prevalence and **concentration of Staphylococcus aureus in raw milk and raw milk cheeses, and factors affecting growth and** inactivation of, and toxin production by, *S. aureus* during the raw milk cheese making process.

While the report is based on an extensive literature search, quoting over 200 scientific papers published during the last 50 years, readers should be aware that the definition of raw milk products in the report differs from the definition in MPI's Animal Products (Raw Milk Products Specifications) Notice 2009:

http://www.foodsafety.govt.nz/elibrary/industry/Animal_Products-Sets_Requirements.pdf

In addition, the report refers to a recent study (2007-2008) of the non-spore forming pathogens in raw milk in New Zealand. A scientific report of this study has subsequently been accepted for publication in the International Journal for food Microbiology:

Hill B, Smythe B, Lindsay D and J Shepherd (2012) *Microbiology of raw milk in New Zealand*. Int J Food Microbiol. (In Press; available online April 2012)

Clearer identification of the growth/no growth and toxin production/no production and may be helpful for regulators and cheese makers. Data describing the conditions required for enterotoxin production are far fewer than those defining growth rate boundaries.



**FACTORS INFLUENCING STAPHYLOCOCCAL
ENTEROTOXIN PRODUCTION
IN DAIRY PRODUCTS**

Prepared for the Ministry of Agriculture and Forestry under project MFS/10/10 – Factors influencing staphylococcal enterotoxin production in dairy products, as part of overall contract for scientific services

Client report no. FW 11034

by

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June 2011

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SUMMARY

Recent regulatory changes have opened the way for domestic production of cheeses made from raw milk where manufacturers are able to assure product safety through a validated process, and demonstrate compliance with regulations specified in the Animal Products (Raw Milk Products Notice) 2009 under an approved Risk Management Programme. Clause 23 of this Notice outlines the Food Safety Criteria applicable to such products including a requirement for absence of staphylococcal enterotoxin in 25g of product

The objective of this report was to identify conditions and physico-chemical parameters during the raw milk cheese making process that may influence growth and enterotoxin production by enterotoxigenic strains of *Staphylococcus aureus*.

Coagulase positive staphylococci (CPS), the group of bacteria to which enterotoxigenic *S. aureus* belong, can be readily isolated from raw milk sometimes at high concentrations ($>10^6$ CFU ml⁻¹). While only a proportion of CPS are enterotoxigenic *S. aureus*, as measured by the possession of enterotoxin genes and/or production of enterotoxin in pure culture, this proportion is reported to be as high as 77%.

The initial stages of cheese production involve the incubation of raw milk at warm temperatures for varying periods such that *S. aureus* can commence growth. While the pH of raw milk will allow growth of *S. aureus*, the growth of competing bacteria will reduce the milk pH. In time the pH may decrease to a point (approximately pH 5) where *S. aureus* cannot grow. The literature suggests that there may also be other mechanisms by which the starter culture is able to inhibit or retard the growth of *S. aureus*, although some of these mechanisms are poorly defined. The initial concentration of *S. aureus* and the degree to which it can grow during fermentation will determine the final *S. aureus* concentration.

If enterotoxigenic *S. aureus* do grow to a sufficiently high concentration, or are initially present at a very high concentration, they may be able to produce enterotoxin in the curd/cheese. Further treatment of the cheese may reduce the concentration of viable cells, but the enterotoxin is likely to remain and may cause illness in consumers. There is little specific literature detailing the contribution of raw milk cheeses to the burden of disease attributable to enterotoxigenic *S. aureus*. Outbreak data suggest that such disease may be more prevalent in countries such as France, where raw milk cheeses are readily available, than in countries where cheese is predominantly produced from pasteurised milk.

To assess the potential for enterotoxigenic *S. aureus* to produce enterotoxin during raw milk cheese production it is necessary to understand both bacterial growth and enterotoxin production boundaries. It is generally accepted that the minimum temperature for growth is 7°C, but the minimum temperature for enterotoxin production is 10°C. Both of these temperatures are well below those used in the initial stages of cheese production. The minimum pH for growth in cheese appears to be close to 5.0, with raw milk having a pH of about 6.5. Growth is likely to occur in the early stages of manufacture. However, there are still data gaps surrounding combinations of temperature and pH (with associated lactic acid concentration) and other physico-chemical parameters which define the growth/no growth and enterotoxin/no enterotoxin boundaries. Clearer identification of these boundaries may be

helpful for cheese makers. Data concerning the conditions required for enterotoxin production are far fewer than those defining growth rates and boundaries.

1 INTRODUCTION

Staphylococcal food poisoning is caused by the ingestion of one or more types of staphylococcal enterotoxin (SE), usually produced by the species *Staphylococcus aureus*. These enterotoxins pass through the stomach into the intestinal tract where they stimulate emesis and diarrhoea. The most common symptoms are nausea, vomiting, retching, abdominal cramping and diarrhoea. Symptoms typically start 1-6 hours after consuming food containing the enterotoxins and resolve within 1-3 days without the need for treatment. While staphylococcal food poisoning is not usually fatal, there are occasional reports of fatalities in very young or old people (Bergdoll and Lee Wong 2006, Stewart 2003).

The organism responsible for causing the illness is referred to in a number of ways. The overarching group is known as the “coagulase-positive staphylococci” (CPS). However, some coagulase-producing strains do not produce enterotoxins and some non coagulase producers can produce enterotoxins. Food testing protocols generally report results in terms CPS. While other staphylococci are able to produce enterotoxins, foodborne intoxications are usually caused by *S. aureus* (Seo and Bohach 2007). *S. intermedius* is the only non-*S. aureus* species which has been associated with food poisoning (Le Loir *et al.* 2003) with a proportion of isolates harbouring and expressing the *sec* enterotoxin gene (Becker *et al.* 2001). Another characteristic of enterotoxigenic *S. aureus* is the production of a thermonuclease, or thermostable nuclease (Ibrahim *et al.* 1981a). In general staphylococci that produce enterotoxins are coagulase and/or thermonuclease positive.

A study of foodborne disease in the USA concluded that over 185,000 cases of staphylococcal food poisoning occur annually, comprising 1.3% of the total number of foodborne illnesses (Mead *et al.* 1999). The disease contributed 2.9% of foodborne hospitalisations and 0.1% of the deaths. A similar, but more recent, study estimated more than 241,000 domestically acquired cases of staphylococcal intoxication in the USA annually (Scallan *et al.* 2011). Given an estimated 6.4% hospitalisation rate for laboratory confirmed cases, a mean 1,064 cases were hospitalised, and 6 deaths resulted.

A review of cheese-associated outbreaks in the USA from 1973 to 1992 concluded that outbreaks associated with this food were rare (Altekruse *et al.* 1998). Of the 11 outbreaks identified only one was caused by *S. aureus*, although the proportion of cases hospitalised (68%) was high. The outbreak was caused through improper pasteurisation. A more recent analysis concluded that 1-9% (mean 4.8%) of all *S. aureus* outbreaks in Europe could be attributed to milk and dairy products (Scientific committee on veterinary measures relating to public health 2003), although attribution of the proportion of cases to these food types was not attempted. A recent review lists reports of staphylococcal food poisonings associated with milk and cheese consumption (Cretenet *et al.* 2011).

A systematic review of disease resulting from the consumption of raw dairy products failed to identify a strong association between *S. aureus* intoxications and these foods (Jaros *et al.* 2008). However, this was a consequence of the design of the studies on which the assessment was based rather than, necessarily, the absence of a link between the pathogen, the food and the disease. A more recent survey of the literature identified only one outbreak reported in the literature between 2000 and 2010 (Hall and French 2011).

SE types A, B, C, D, E and H (referred to as SEA, SEB, SEC, SED, SEE and SEH) have all been associated with raw milk product outbreaks (milk powder, Japan 2000; Halloumi, 1981; Ewes milk cheese, UK 1984/85 and France 2009; semi cured cheese, Brazil 1996). SEA is the most common enterotoxin found in food related outbreaks and is most prevalent in the milk product outbreaks in the literature. Milk is a common source of SED producing strains. The number of recorded outbreaks associated with SED and milk products has reduced with the introduction of milk pasteurisation (Wieneke *et al.* 1993). Other enterotoxin types have been identified but evidence of emetic activity is scarce.

An attempt to analyse foodborne disease involving dairy products as the vehicle has been carried out for France and other industrialised countries (Buyser *et al.* 2001). Four pathogens were considered; *Salmonella*, *S. aureus*, *Listeria monocytogenes* and pathogenic *Escherichia coli*. Examination of data from 60 published outbreaks and four single cases indicated that 32.8% of the food vehicles were made from pasteurised milk, 37.5% from raw milk, 10.9% from “unpasteurised” (heat treated but at conditions less bactericidal than standard pasteurisation) milk and 18.8% from milk whose provenance was unspecified. Overall 2-6% of outbreaks in the countries examined could be attributed to dairy products. *S. aureus* was the organism most often associated with 69 French outbreaks involving dairy products (87% cheese) made from raw or unspecified milk. For 51% of the food vehicles, the heat treatment applied to milk was unspecified. The data also need to be interpreted in relation to the volumes of foods produced using the different types of heat treatment. For example, at least 48.4% of the outbreaks were from foods made from milk that had received a lesser heat treatment than pasteurisation, yet most dairy products are produced from pasteurised milk.

An analysis of 31 staphylococcal foodborne disease outbreaks in two areas of France, Ile de France and Aquitaine, showed that nine (29%) outbreaks were either confirmed or suspected as involving raw milk cheese (Kérouanton *et al.* 2007). A further four outbreaks were associated with cheese consumption but the type of milk used to make the cheeses is not listed.

Raw milk cheese consumption appears to be a significant cause of staphylococcal intoxications in France but not in the USA. This may reflect the relatively abundant supply and consumption of raw milk cheeses in France when compared to the USA.

Staphylococcus aureus may occur in the milk of animals with clinical or sub-clinical mastitis or as the result of poor hygienic practices during milk collection. This can result in *S. aureus* and SE being present in cheese made from either raw or pasteurised milk. The ability of *S. aureus* to produce detectable amounts of enterotoxins in food depends on whether or not the strain is enterotoxigenic and whether the environmental conditions necessary for enterotoxin synthesis exist. Concentration around 10^5 - 10^6 CFU g⁻¹ are considered to be the minimum necessary to allow detection of SE in foods (Bisping and Amtsborg 1988). Several studies have shown that *S. aureus* can reach high concentrations during the early stages of cheese making even when present at low concentrations in the milk (Meyrand *et al.* 1998). If abundant growth of enterotoxigenic *S. aureus* occurs during the early stages of manufacture, enterotoxins could be formed. Despite the possibility of staphylococcal counts decreasing during ripening and storage of cheese, the enterotoxins may persist and be consumed. For cheeses tested two days or more post-manufacture it is

more valid to assay for the presence of SE rather than determining the concentration of *S. aureus* (EFSA 2003).

While *S. aureus* is eliminated by pasteurisation, raw milk cheeses have no such pathogen elimination step. For this reason, safety cannot be guaranteed and assurances rely upon application and of good agricultural and manufacturing practices including the monitoring of herds, temperature control of milk, and the cheese production and maturation steps themselves.

Recent regulatory changes have opened the way for domestic production of cheeses made from raw milk where manufacturers are able to assure product safety through a validated process, and demonstrate compliance with regulations specified in the Animal Products (Raw Milk Products Notice) 2009 under an approved Risk Management Programme. Clause 23 of this Notice outlines the Food Safety Criteria applicable to such products including a requirement for absence of staphylococcal enterotoxin in 25g of product

MAF commissioned this project to identify conditions and physico-chemical parameters during the raw milk cheese making process that influence growth and enterotoxin production by enterotoxigenic strains of *S. aureus*.

2 METHODS

2.1 Definitions

Raw milk means milk produced in accordance with a registered risk management programme and that has not been subjected to any processing intended to alter the quality or composition characteristics of the milk. Raw milk products are defined as “milk products that have not undergone pasteurisation, ultra high temperature treatment, or ice-cream treatment” ([http://www.foodsafety.govt.nz/elibrary/industry/Imported Food Requirement-Sets_Clearance.pdf](http://www.foodsafety.govt.nz/elibrary/industry/Imported_Food_Requirement-Sets_Clearance.pdf)).

Thermisation is a “heat treatment applied to raw milk aimed at reducing the number of microorganisms in the milk and permitting longer storage of milk prior to further processing. The heating conditions are 62 to 65/C for 15 to 20 seconds. Thermized milk must be phosphatase positive.” (ftp://ftp.fao.org/codex/ccmmp4/mm00_15e.pdf)

Pasteurisation is defined in the Food (Milk and Milk Products Processing) Standard 2007 as being achieved by the following methods:

- (i) The holding method, by which milk or milk product is rapidly heated to a temperature of not less than 63 degrees Celsius, retained at that temperature for 30 minutes and then-
 - (A) Immediately and rapidly reduced to 5 degrees Celsius or less in the case of milk or milk products other than cream, or to 7 degrees Celsius or less in the case of cream; and
 - (B) Maintained at or below that temperature until the milk or milk product is removed from the premises for delivery;
- (ii) The high-temperature short-time method, by which the milk or milk product is rapidly heated to a temperature of not less than 72 degrees Celsius, retained at that temperature for not less than 15 seconds, and then treated in accordance with subparagraphs (A) and (B) of the method in paragraph (i);
- (iii) Any other heat treatment that is as effective in terms of bacterial reduction as methods (i) and (ii).

2.2 Literature Review

Three principal databases were used in searching for information. Scopus (1960-2011), Web of Science (1990-2011) and Pub Med (all years). These three databases provide a wide coverage of the topic despite some overlap in results, each provided enough unique material to warrant inclusion. The search strategy used was exactly the same for all three databases, although some slight variations between Scopus and Web of Science were necessary because of the way they treat certain aspects of the search. However this did not reduce the number of results that were obtained from the databases.

The search strategy used in all of the databases was as follows

Database	Products	Keywords
Web of Knowledge	Milk and Cheese	((raw OR unprocessed OR untreated OR unpasteur*) AND (milk OR cheese) AND (diseas* OR epidemi* OR infec* OR fatal OR "dose response") AND staphyl*)
Scopus	Milk and Cheese	((raw OR unprocessed OR untreated OR unpasteur*) AND (milk OR cheese) AND (diseas* OR epidemi* OR infec* OR fatal OR "dose response") AND staphyl*)
PubMed	Milk and Cheese	((raw OR unprocessed OR untreated OR unpasteur*) AND (milk OR cheese) AND (diseas* OR epidemi* OR infec* OR fatal OR "dose response") AND staphyl*)

A more specific search was made for growth boundary models:

Scopus TITLE (staph* AND bound* AND grow*)

The results from all three searches were entered into a bibliographic database (Endnote) database and all duplicates removed.

3 PREVALENCE, CONCENTRATION OF *S. AUREUS* AND CARRIAGE OF ENTEROTOXIN GENES IN RAW MILK/CHEESE

3.1 New Zealand

New Zealand national data on the incidence of *S. aureus* food poisoning associated with raw milk, dairy products or cheese are not available and only limited information is available about the prevalence, concentration and carriage of enterotoxin genes in these foods. Raw milk and raw milk products are not produced in New Zealand, but imported raw milk cheeses are widely available.

In a recent study information was collected on the prevalence of several pathogens, including *S. aureus*, in New Zealand's raw milk supply between 2006 and 2007 (Denise Lindsay Pers. Comm.). A total of 297 samples was collected on a monthly basis over the period of a year. Samples were obtained from five randomly selected farm vats in five major dairying regions of the country. The distribution of *S. aureus* counts was:

- not detected (< 1 CFU ml⁻¹) in 21% of samples;
- concentrations between 1 and 10² CFU ml⁻¹ in 39% of samples
- between 10² and 10³ CFU ml⁻¹ in 30% of samples
- on one occasion *S. aureus* exceeded 10⁴ CFU ml⁻¹ (0.34%).

The authors concluded that raw milk sampled from New Zealand farm vats does contain pathogens that can result in food poisoning and pasteurisation of raw milk remains an important and effective preventative measure. None of the raw milk samples tested in this study contained concentrations of *S. aureus* nearing 10⁵ CFU ml⁻¹, which is conventionally considered to be the minimum required to cause human illness (see Section 7).

It is possible that these data do not reflect the concentrations and prevalence rates that might occur in milk used by potential artisan cheese makers. The herd sizes in the cited study are likely to be larger and the contribution from a single animal (for example, a sub-clinical but mastitic animal) will be diluted.

Another study reported that 17% of raw milk samples from bulk tank milk, sampled monthly between August and December 2004, from seven dairy herds in the Waikato region of New Zealand, contained $> 5 \times 10^2$ CFU ml⁻¹ of *S. aureus* (Howard 2006).

3.2 Overseas

There have been many general surveys of raw milk and cheeses for the presence of *S. aureus*. Because of this, and the likelihood that hygiene measures applied on the farm have improved over time, only data for papers published since 2005 have been considered. The full table is presented in Appendix 1. These studies primarily considered milk from bovine, ovine or caprine sources. The prevalence of *S. aureus* in raw milk samples varied widely. However, data may be reported on different bases (staphylococci, CPS or *S. aureus*) and so do not allow straightforward comparisons to be made. One study reported 100% of Brazilian

raw caprine milk sampled was contaminated with staphylococci (Oliveira *et al.* In Press). In contrast, only 1% of Brazilian bovine milk was positive for the presence of enterotoxigenic *S. aureus* (Fagundes *et al.* 2010). It is clear that enterotoxigenic *S. aureus* represent a sub-population of the CPS population present in raw milk. Concentrations are also difficult to compare between studies but vary widely from $<10^1$ to $>10^6$ CFU ml⁻¹. A wide variety of enterotoxin genes were detected among the isolates obtained, and the proportion of isolates expressing or containing enterotoxin genes varied from 12% (Shuiep *et al.* 2009) to 77% (Hwang *et al.* 2010).

A similar variation in the prevalence of staphylococci was found with data for cheese. The concentration varied from being below the limit of detection (Ramsey and Funk 2009) to a maximum in excess of 10^6 CFU ml⁻¹ (Rosengren *et al.* 2010). In a survey of Italian Monte Veronese cheese, 37 of 46 (80.4%) of cheese samples contained enterotoxigenic *S. aureus* and all 37 isolates recovered produced enterotoxin (Poli *et al.* 2007). The presence of enterotoxin in cheese is often not reported, but isolates are occasionally tested for both the production of enterotoxin and possession of enterotoxin genes. For example, of 60 *S. aureus* isolates from sheep cheese only seven produced enterotoxin (4 SEA, 2 SEB and 1 SED) as determined by ELISA testing (Ertas *et al.* 2010). From the same 60 isolate set, only eight isolates possessed the respective genes indicating that one isolate contained the gene *sea* but did not express SEA.

4 FACTORS AFFECTING GROWTH AND INACTIVATION OF *S. AUREUS* AND ENTEROTOXIN PRODUCTION

This section describes some factors influencing the growth and enterotoxin production for *S. aureus* and those more relevant to cheese making are explored in greater detail in section 5. Table 1 shows the generally accepted boundaries for this organism.

Table 1. Limits for growth and enterotoxin production (International Commission on Microbiological Specifications for Foods 1996).

Factor	Growth		Toxin production	
	Optimum	Range	Optimum	Range
Temperature °C	37	7 - 48	40 - 45	10 - 48
pH	6-7	4-10	7-8	4.5 - 9.6 Aerobic 5.0 - 9.6 Anaerobic
Water activity	0.98	0.83 - > 0.99 Aerobic 0.9 - >0.99 Anaerobic	0.98	0.87 - >0.99 Aerobic 0.92 - >0.99 Anaerobic

4.1 Growth and Inactivation

4.1.1 Low Temperature

The minimum recorded growth temperature for *S. aureus* on a food is 5°C on bacon (Farrell and Upton 1978). Growth was also measured at 6.7°C in chicken à la king (Angelotti *et al.* 1961). Although there are some caveats with respect to the amount of growth measured at 6.7°C, growth occurred unequivocally at 7.8°C on that food. Growth at 7°C has also been reported in UHT milk (Medved'ova *et al.* 2009). In broth no growth occurred in a cocktail of five enterotoxigenic isolates at 7.5°C. At 8°C growth was only measured when the conditions were otherwise optimal (Valero *et al.* 2009). Incubation of a wide range of isolates at closely spaced temperature intervals determined that the lowest temperature for growth was “about 7°C” (Schmitt *et al.* 1990). Further information is given in the report “Minimum growth temperatures of foodborne pathogens and recommended chiller temperatures” prepared by ESR for MAF (Hudson 2011a).

4.1.2 High Temperature

The maximum reported temperature allowing growth of *S. aureus* in food is 48.9°C in skim milk (Stiles and Witter 1965). Other studies report growth at around 45°C (Angelotti *et al.* 1961, Vandenbosch *et al.* 1973). A further study claimed that the organism can only grow at 46°C when protected by 1 M NaCl (El-Banna and Hurst 1983), although growth at 47.8°C in skim milk has been reported (George *et al.* 1959). No growth was recorded for one isolate at 51°C, but it did grow at 46°C (Medved'ova *et al.* 2009). Further information is given in the

report “Maximum growth temperatures of foodborne pathogens and appropriate hot holding temperatures” prepared by ESR for MAF (Hudson 2011b).

4.1.3 pH

Most isolates grow over a range from 4.5 to 9.3 (Bergdoll and Lee Wong 2006).

For a cocktail of five enterotoxigenic isolates growth has been recorded at pH 4.5 (Valero *et al.* 2009). A similar limit has been recorded in milk where the pH was adjusted with lactic acid (Charlier *et al.* 2008). Prior exposure to pH 4.5 for two hours and pH 9.5 for 30 minutes increased survival on exposure to pH 2.5 and 12.0 respectively (Cebrián *et al.* 2010). The magnitude of the acquired resistance was 1.6 times the control at low pH and around two fold at high pH.

4.1.4 Salt and water activity (a_w)

For a cocktail of five enterotoxigenic isolates growth has been recorded at a_w 0.867 when salt was used as the humectant (Valero *et al.* 2009). In pasta, growth of a single strain appeared to occur until an a_w of below 0.86 was achieved (Valik and Görner 1993) but the increase in concentration was very small. Depending on the broth and temperature used, growth was recorded for a single isolate at a_w 0.893 (15.25% added NaCl) but not at 0.869 (18.17% added NaCl) (Medved'ova *et al.* 2009).

By using water and propylene glycol to adjust the a_w of intermediate moisture cheeses of around pH 5.7 at room temperature, it was determined that *S. aureus* could grow on cheese made with an a_w of 0.94, but not at 0.91 (Kreisman and Labuza 1978). Lowering the a_w from 0.993 to 0.95 was shown to increase the lag time, and reduce the growth rate and maximum concentration achieved. The effects became more marked as the temperature was decreased.

A review reports that the organism may grow in meat at NaCl concentrations of up to 20% (Tatini 1973).

4.1.5 Atmosphere

Growth in broth is more rapid and the maximum population density higher under aerobic than anaerobic conditions (Belay and Rasooly 2002). Anaerobic conditions were found to be more restrictive to growth of a *S. aureus* isolate in fermented sausage as the pH of the sausage was reduced using glucono delta lactone (Barber and Biebel 1972).

The organism was inactivated using a pressure of 9 MPa carbon dioxide in skim milk at 25°C over 2 hours (Erkmen 1997). However, under the same conditions there were still surviving cells in whole milk, although an approximate 4-5 log₁₀ reduction was recorded.

4.1.6 Organic acids

The Minimum Inhibitory Concentrations (MIC) of acetic and lactic acids for *S. aureus* are reported to be 0.6 and 2.5 µl ml⁻¹, equivalent to 3 g l⁻¹ (de Oliveira *et al.* 2010). Lactic acid was found to be more effective than acetic acid in controlling the pathogen in meat broth

when added at the MIC. Inhibitory activity was also demonstrated when organic acids were used at sub-MIC concentrations in combination with carvacrol and thymol. In meat, the reductions resulting from these antimicrobials were less pronounced.

Growth in reconstituted milk occurred at pH 4.5 at 37°C when the pH was adjusted with HCl, but inactivation occurred at the same pH after adjustment with lactic acid (Tatini *et al.* 1971). In broth incubated at 40°C and pH 6 the growth rate of *S. aureus* was largely unaffected by the presence of up to 63 mM lactic acid (5.6 g l⁻¹), but the addition of 125 mM (11.25 g l⁻¹) caused a significant increase to the lag time (Aoyama *et al.* 2008). These concentrations are higher than the MIC reported above, but this may be because the pH was adjusted to 6, while no pH adjustment seems to have been made in the study of de Oliveira *et al.* (2010).

When milk was reduced in pH by the addition of lactic acid to simulate the reduction in pH that would occur during fermentation, and then held for 24 hours, there was considerable inhibition. For example when the pH was reduced to 4.87 the concentration was approximately 4 log₁₀ CFU g⁻¹ less than the control (Ibrahim 1978).

4.1.7 Competitive microorganisms

The growth kinetics of *S. aureus* in raw and pasteurised milk at 37°C were the same over the first 16 hours of incubation. However, the decline in concentration after the maximum population density had been reached was more rapid in raw milk, and the concentration was 2 log₁₀ CFU ml⁻¹ lower than in pasteurised milk after 72 hours incubation (Pitt *et al.* 2000). With respect to the early stages of cheese making these data suggest that growth would be the same in raw and pasteurised milk. Growth of *S. aureus* inoculated into mastitic milk (from a forequarter infected with *Streptococcus*) proceeded at a slightly higher rate than that in normal raw milk (Fang *et al.* 1993).

In raw milk cheeses the competing organisms likely to inhibit pathogens are the lactic acid bacteria (LAB). These are present in raw milk (Ortolani *et al.* 2010) and are also added as cheese starter cultures.

The potential for using bacteriocin-producing starter cultures to reduce the growth of *S. aureus* during the manufacture of cheese has been shown (Rodríguez *et al.* 2005). The best result was a reduction in concentration of about 1 log₁₀ CFU/g compared to the control after 30 days incubation. Pediocin-producing LAB produced a greater inhibition than those producing nisin. An examination of the competition between *Lactococcus lactis* and *S. aureus* showed that the *Lactococcus* was capable of inhibiting the growth of the pathogen in milk by mechanisms in addition to the lowering of pH, but not by the production of a bacteriocin or hydrogen peroxide (Charlier *et al.* 2008). Nisin producing *L. lactis* inhibited the growth of *S. aureus* during the fermentation stage of semi-hard cheese production (Rodríguez *et al.* 2000).

A mixed, but unquantified, starter culture of LAB was shown to prevent a significant increase, and then result in a slow decrease in the concentration of *S. aureus* in fermenting yoghurt even when the pathogen was added in excess of 10⁵ CFU ml⁻¹ (Pazakova *et al.* 1997). Control of a low inoculum of *S. aureus* was achieved in a fresh cheese when the

starter produced nisin. No control was observed at a higher *S. aureus* inoculum concentration (Hamama *et al.* 2002).

The effects of the addition of single isolates of three species of LAB were examined in Portuguese cheese production (Pereira *et al.* 2009). *Lactococcus lactis* effectively inhibited *S. aureus*, while *Lactobacillus brevis* and *Lb. plantarum* were much less effective. LAB were also shown to produce a 1 log₁₀ CFU g⁻¹ difference in *S. aureus* concentration in Manchego cheese when compared to a starter-free control (Gaya *et al.* 1988). A mixture of *L. lactis* and *Lb. plantarum* was also shown to retard the growth of *S. aureus* in skim milk at 30°C (Radovanovic and Katic 2009).

There are numerous reports of the inhibition of *S. aureus* by LAB including, in addition to the above, *Lb. paracasei* subsp. *paracasei* and *Lb. rhamnosus* (Anas *et al.* 2008), *Streptococcus thermophilus* (Buriti *et al.* 2007) and *L. garvieae* and *Enterococcus faecalis* (Alomar *et al.* 2008).

A review has been produced summarising current views on the mechanisms of the inhibition of *S. aureus* by LAB (Charlier *et al.* 2009). Acidification of the medium was considered to be one of the main factors involved. A further mechanism is the production of bacteriocins by LAB. An example of this is the inhibitory effect of, for example, a lantibiotic produced by *L. lactis* on both *S. aureus* (Rilla *et al.* 2004) and CPS (Kim *et al.* 2010). The production of hydrogen peroxide is another potentially inhibitory mechanism, especially in the early stages of fermentation (Delbes-Paus *et al.* 2010). Competition for nutrients was identified as an inhibitory mechanism in another study (Iandolo *et al.* 1965).

Moulds may be added to some cheeses such as Brie, Camembert and Blue. The mould used in the production of Camembert (*Penicillium camemberti*) has been shown not to be inhibitory to *S. aureus* (Larsen and Knøchel 1997).

4.1.8 Flavourings and Additives

When added at 100 and 500 IU ml⁻¹ during the production of Minas Serro cheese the bacteriocin nisin reduced the concentration of *S. aureus* by 1.2 and 2.0 log₁₀ CFU g⁻¹ respectively compared to the controls after seven days of ripening (Pinto *et al.* 2011). Annatto, which is a colouring used in cheese and butter, has an inhibitory effect on *S. aureus* when present at 0.16% (Galindo-Cuspinera *et al.* 2003) although the concentration needed to achieve a bactericidal effect was ten times higher. These concentrations are higher than those used in cheeses in the US.

An extract of *Camellia japonica* petals was found to retard the growth of *S. aureus* in milk incubated at 25°C (Kim *et al.* 2001). Growth in the presence or absence of the extract did not occur within seven days at 7°C.

Zinc oxide nanoparticles were shown to reduce the concentration of *S. aureus* by more than 90% in three hours of exposure. Electron microscopy showed that cell lysis occurred in the presence of the nanoparticles (Tayel *et al.* 2011)

4.1.9 High Pressure (HP)

The effects of HP (100-300 MPa) homogenisation and temperature (5-50°C) have been modelled (Diels *et al.* 2003). At low temperatures (<40°C) there was almost no inactivation of the organism, but inactivation occurred at higher temperatures. At a slightly higher pressure (330 MPa) a 2-3 log₁₀ CFU ml⁻¹ reduction was shown in milk (López-Pedemonte *et al.* 2006). Further experiments at a higher pressure (450 MPa) and 25°C revealed nonlinear inactivation kinetics with tails and shoulders present (Cebrian *et al.* 2010). An approximate 3-5 log₁₀ decrease in concentration was achieved over 60 minutes depending on the strain. At 600 MPa rapid inactivation occurred, for example greater than 8 log₁₀ reduction in 8 minutes at 45°C (Guan *et al.* 2006). Significant survivor tails were also observed.

Inactivation of naturally occurring CPS has been demonstrated in La Serena cheese treated at 300 and 400 MPa (Arqués *et al.* 2006). Reductions were also reported for *S. aureus* in soft curd cheeses inoculated with 7.5 log₁₀ CFU g⁻¹ cheese. SE was detected in cheeses before and after high pressure treatment (López-Pedemonte *et al.* 2007).

A study has been published which investigated the use of HP in conjunction with a bacteriocin-producing starter culture (Arqués *et al.* 2005). The results suggested a synergistic effect, and a 4 log₁₀ CFU g⁻¹ reduction compared to the control was achieved using a bacteriocin-producing culture and HP treatment at 500 MPa.

The application of HP treatments to dairy products was recently reviewed (Datta and Deeth 2011). Various technological aspects are described, for example beneficial effects from the pressure-treatment of curds. Microbiologically, low numbers of coliforms, or none at all, are isolated from pressurised milk cheese.

4.1.10 Pulsed Electric Field (PEF)

S. aureus normally produces a golden yellow colour when grown on agar, but non-pigmented colonies can also occur. The production of these pigments is under control of the general stress factor, Sigma B. Pigmented and non-pigmented isolates exhibited similar inactivation curves when subject to PEF at 26 kV/cm (Cebrián *et al.* 2007). In another study (using 22 kV/cm) most strains behaved similarly, although one isolate was notably more resistant than the others (Rodríguez-Calleja *et al.* 2006). The application of PEF to milk has been described, with reductions of 4-5 log₁₀ for non-spore forming bacteria reported (Deeth and Datta 2011, Molina *et al.* 2002).

4.1.11 Ultrasound

Only a four-fold difference was found in the inactivation rate of 15 strains subject to ultrasound (117 µm of amplitude and 200 KPa), with D values greater than those for most other vegetative species (Rodríguez-Calleja *et al.* 2006). Ultrasonication of dairy products can both reduce bacterial concentrations and benefit product quality (Deeth and Datta 2011).

4.1.12 Fat concentration

There is some evidence that *S. aureus* grows to lower maximum population density in dairy foods with high fat concentrations, although this was very strain dependent (Halpin-Dohnalek and Marth 1989). This phenomenon may be linked to the production of lipases by *S. aureus* and the consequent inhibition of its growth by the free fatty acids formed.

4.1.13 Bacteriophages (phages)

Phages can contaminate cheese making resulting in the failure of the starter culture. It is possible that raw milk might also contain phages capable of infecting *S. aureus* that could lead to death of the pathogen in conditions where it might otherwise have grown. A cocktail of two lytic phages isolated from raw milk was used at a concentration of around 10^8 PFU ml^{-1} to control of *S. aureus* present at 10^6 CFU ml^{-1} (García *et al.* 2007). Good control was shown in Ultra Heat-Treated (UHT) milk at 37°C and during the formation of soft acid curd under industrial conditions. The same group showed poorer control of *S. aureus* in both pasteurised and raw milks at 37°C (García *et al.* 2009) but the phages were added at a much lower concentration (10^4 - 10^5 PFU ml^{-1}) to a low concentration ($<10^3$ CFU ml^{-1}) of host cells. Significant control was only demonstrated in pasteurised milk when the concentration of host cells grew to exceed 10^6 CFU ml^{-1} . A synergistic effect has been described when phages were combined with nisin (Martínez *et al.* 2008). In other publications, problems have been reported when phage K was used to control *S. aureus* in raw milk (Gill *et al.* 2006, O'Flaherty *et al.* 2005), possibly because of the binding of whey proteins to the cell surface (Gill *et al.* 2006). Models have been produced to predict the inactivation of *S. aureus* in pasteurised milk with only temperature and phage concentration found to be significantly involved with predicted host inactivation (Obeso *et al.* 2010).

4.1.14 Thermal Inactivation

The organism is not abnormally heat resistant, with a D value (time taken to reduce the population by 90%) of approximately 6 minutes at 60°C . However, in salty foods thermal resistance is much greater. For example the D value at 60°C increased from 6 minutes to 25 minutes when the NaCl content of meat macerate was increased from 0% (w/v) NaCl to 8.4%. At 58°C D values varied from 0.93 to 0.20 min in buffer (pH 7.0). Stationary phase cells showed increased heat resistance compared to those from mid-log phase, and pigmented cells were more heat resistant than those without pigment (Cebrián *et al.* 2007). Differences in thermal stability were also shown among strains when heated at 58°C , with D values varying from 0.11 to 1.1 minute (Rodríguez-Calleja *et al.* 2006). Examples of D values are given in Table 2.

A comparison of thermal resistance in phosphate buffer, whey and milk showed that the strains used were more resistant in skim milk and whey than in buffer by a factor of up to two-fold (Walker and Harmon 1966). Thermal resistance increased in parallel with the age of the culture. Non log-linear inactivation curves were produced at treatment temperatures from 58 to 62°C . The thermal inactivation rate at 72.5°C was not significantly different in various liquid dairy products of differing protein (3.5-16.0%), fat (0.28-10.0%) and lactose (4.5-15%) concentrations (Kornacki and Marth 1989).

Exposure to a sub-lethal heat shock has been shown to increase thermal resistance at 58°C (Cebrián *et al.* 2010) and 60°C (El-Banna and Hurst 1983). Adaptation at 45°C for 2 hours

resulted in a six fold increase in the time taken for the population to reduce ten-fold. Prior thermal exposure also resulted in cross protection to acidic pH and hydrogen peroxide exposure. In contrast pre-chilling did not affect the heat resistance of *S. aureus* in broth, with D values ranging over 94.3 - 127.9, 13 - 21.7 and 4.8 - 6.5 minutes at 50, 55 and 60°C respectively (Kennedy *et al.* 2005). Similar results have been noted elsewhere, and it was concluded that *S. aureus* growing in foods stored at lower temperatures should be more susceptible to thermal inactivation than if they were previously stored at higher temperatures (Smith and Marmer 1991). The presence of glucose and galactose has been associated with the recovery of thermally-injured cells (Stiles and Witter 1965).

Table 2: Some D values for inactivation of *S. aureus*

Temperature (°C)	D value (min)		
	(El-Banna and Hurst 1983)	(Firstenberg-Eden <i>et al.</i> 1977)	(Kornacki and Marth 1989)
50	2.7 ¹ /12.6 ²	10.0	
55		3.1	
60		0.9	
63			0.50-0.85
65		0.2	0.50-0.69
70		0.1	
72			0.047-0.071
75		0.01	
77			0.028-0.059
79			0.033-0.051

¹ Pre-incubation at 37°C ² Pre-incubation at 46°C

Experiments with milk showed that between 50 and 60°C the injury rate for a single strain was more rapid than the death rate, but at temperatures >60°C the opposite was true (Firstenberg-Eden *et al.* 1977).

A protective effect has been shown when the organism was heated in fish and oil with D₆₀ values of 3.9 to 12.7 minutes being recorded, dependent on culture age (Gaze 1985). Additionally, under conditions designed to optimise thermal resistance and when heated in oil D₁₂₀ values of 3-6 min were measured.

D values for two isolates were significantly higher when both heating and enumeration were performed under anaerobic conditions (Ugborogho and Ingham 1994). The size of this effect was 3-15 fold in broth, but a maximum of approximately three fold in skim milk.

The presence of the lactoperoxidase system has been shown to enhance the thermal inactivation of *S. aureus* in milk, producing up to 15 fold decrease in the D value (Kamau *et al.* 1990).

In Grana Padano cheese production the curds are cooked at 55°C and then cooled to 25°C. The centre of this cheese maintains a temperature > 50°C for over four hours, but the surface does not. Experiments showed that the concentration of *S. aureus* on the surface of the cheese did not change markedly during production (Ercolini *et al.* 2005).

4.1.15 Naturally-occurring components of raw milk and biopreservatives

Lactoperoxidase is a naturally-occurring component of raw milk, and has been shown to improve the keeping quality of milk inoculated with *S. aureus* (Marks *et al.* 2001). A study of nisin, reuterin and the lactoperoxidase system on the inhibition of *S. aureus* in the curdled milk dairy product Cuajada showed that nisin was the most inhibitory when the inhibitors were compared individually. The effect was better when they were used pairwise, and when all three were present in the food an approximate 5 log₁₀ reduction was measured compared to the control, biopreservative-free, food (Arques *et al.* 2008).

4.2 Enterotoxin Production

4.2.1 Characteristics of the enterotoxins and their production

Reference to the various types of enterotoxin is conventionally abbreviated to SE and then the type, such that SEA is staphylococcal enterotoxin type A. The gene responsible for the production of SEA, for example, is written as *sea*.

The staphylococcal enterotoxins are short proteins secreted into the medium by staphylococci carrying the required genes. The SEs are highly stable, resisting the activity of proteolytic enzymes occurring in the digestive tract. They are also thermally stable.

Fourteen enterotoxins were reported in a review published eight years ago (Le Loir *et al.* 2003), but the number has continued to increase to at least 21 (Ostyn *et al.* 2010). However, only a sub-set (SEA to SEI, SER, SES and SET) of the SEs known are proven to be emetic. Given that many SEs have now been identified and that no rapid method is able to detect them all, it is fortunate that only a few are known to be of significance. These “classically described” enterotoxins are SEA, SEB, SEC, SED, and SEE, but most foodborne intoxications occur following the consumption of staphylococcal enterotoxin SEA (Stewart 2003). Some very large outbreaks have been attributed to SEA consumption (Asao *et al.* 2003).

Enterotoxin genes are present on a variety of mobile genetic elements such as prophages, and control of their expression is complex (Cretenet *et al.* 2011). Production of some SEs (SEB, SEC, SED) is linked to quorum sensing via the *agr* (accessory gene regulator) system (Ortega *et al.* 2010) and so cells need to be present at a high concentration for significant enterotoxin production to occur. Another system involved is *sarA*, the staphylococcal accessory regulator. The concentration of enterotoxin produced varies between SE types, and different strains expressing the same SE type may also produce different concentrations

(Cretenet *et al.* 2011). The alternative sigma factor (*sigB*) is a negative regulator of *sed*, and *sec* is under the control of *saeRS*, another regulator of virulence expression. SEA is produced constitutively.

Examination of the expression of enterotoxin genes by reverse transcription PCR demonstrated that the isolates they tested which possessed enterotoxin genes had the potential to produce SEs (Derzelle *et al.* 2009). Four distinct patterns of SE expression were observed; 1) unchanged transcription throughout growth 2) slight decrease in transcript after exponential growth phase 3) significant induction of expression at the end of exponential phase or 4) modest induction of expression at the end of exponential phase.

The prevalence of the genes responsible for enterotoxin production and the ability to produce enterotoxins are listed in detail in Appendix 1 and described in sections that follow.

4.2.2 Temperature

The minimum and maximum temperatures for enterotoxin production are 10 and 45°C respectively (Bergdoll and Lee Wong 2006), with the optimum between 35 and 40°C (Vandenbosch *et al.* 1973). In Brain Heart Infusion broth enterotoxin was detected at 10.8°C after five days, but not at 8.7°C after 10 days incubation (Aoyama *et al.* 2008). However, growth at temperatures above 10°C does not necessarily mean that enterotoxin production will occur. No enterotoxin was produced by two enterotoxigenic isolates when incubated up to 36 hours at 18°C. The final concentration of *S. aureus* was almost 10^9 CFU g⁻¹ (Yang *et al.* 2001).

4.2.3 pH

With respect to pH, the limits are reported to be 4.5-9.6 (International Commission on Microbiological Specifications for Foods 1996). When *S. aureus* was inoculated at a high concentration (10^8 CFU ml⁻¹) without added salt, SEC was produced in the pH range 4.00 to 9.83 (Genigeorgis *et al.* 1971). The optimum pH for production was approximately 5. Growth, but not enterotoxin production, occurred in the presence of 12% NaCl. Given the high concentration used it is not clear how these results might translate to more realistic inoculum concentrations. Optimum SEA production has been reported at pH 6.5-7.0 in pH and oxygen tension controlled culture (Carpenter and Silverman 1976). A difference in pH minima was reported when cultures were incubated aerobically or anaerobically (Barber and Biebel 1972). For eight SEA producing strains the pH minimum ranged between pH 5.7 and 4.9 under aerobic conditions. It was noted that growth occurred in the absence of enterotoxin production under some conditions. No culture was able to produce enterotoxin at a pH less than 5.7 under anaerobic conditions. Similar results were obtained for three SEB, and one SEC-producing strains. However a strain producing SEE formed enterotoxin weakly at pH 4.8.

4.2.4 Water activity (a_w)

Low a_w conditions were shown to restrict the production of SEB more than SEA (Qi and Miller 2000). Growth of three strains at 37°C and enterotoxin production (SEA and SEB) occurred down to an a_w of 0.95. The concentration of SEA produced was similar between a_w

0.996 and 0.95, while SEB production reduced (two isolates) as the a_w decreased. The addition of proline as a compatible solute was found to stimulate SEB production at a_w 0.95. SEA was produced at 35°C in pork at an a_w of 0.86, but not at 0.83. On beef, enterotoxin production occurred only at an a_w of 0.88 (Tatini 1973). Growth occurred over a broader range of a_w values than that allowing enterotoxin production.

4.2.5 Competing organisms

The effects of competing bacteria on enterotoxin production have been reviewed (Smith *et al.* 1983). Enterotoxin production was found to vary according to the species competing with the pathogen. Partially purified SEA was degraded by certain LAB (*Lactobacillus*, *Streptococcus* and *Leuconostoc*), but not by a limited range of non-LAB organisms (*Bacillus*, *Pseudomonas*, *Escherichia* and *Saccharomyces*). It has been suggested that enterotoxin production is reduced when the pathogen is in competition, and that a higher concentration of *S. aureus* cells may be needed for enterotoxin formation when the organism is competing (Tatini 1973). For example, it was reported that a concentration of 3×10^6 CFU ml⁻¹ yielded enterotoxin in broth culture, while a concentration of $7-8 \times 10^6$ CFU ml⁻¹ did not yield enterotoxin when grown in commercial cheese milk.

Some inhibition of enterotoxin formation during co-culture with coagulase negative staphylococci has been reported (Gonzalez-Fandos *et al.* 1996). A high concentration of *S. carnosus* was able to inhibit enterotoxin production in two *S. aureus* strains such that it was non-detectable up to 72 hours incubation. For two further strains the amount of enterotoxin detected was very low. Enterotoxins were detectable after 16 hours incubation in the absence of *S. carnosus*. In milk, a non-enterotoxigenic *S. aureus* strain was able to prevent the production of enterotoxin by an enterotoxigenic strain when added at 100-1000 times the initial concentration of one enterotoxigenic strain, but this was not the case for a second enterotoxigenic isolate (Noleto and Bergdoll 1980).

4.2.6 Atmosphere

At 37°C, an anaerobic atmosphere did not prevent production of SEA, but less enterotoxin was produced than under aerobic conditions (Belay and Rasooly 2002, Carpenter and Silverman 1976). An inhibitory effect on SEA production was measured in fermented sausage incubated in 5% oxygen compared to incubation at higher oxygen concentrations (Barber and Biebel 1972). No SEA was measured when incubated under oxygen-free conditions. A similar effect was shown in broth cultures.

4.2.7 Concentration of cells in food

It is possible for staphylococci to grow to high concentrations without enterotoxin formation. For example, enterotoxigenic *S. aureus* reached 4.2×10^8 CFU g⁻¹ in defrosting pies yet no enterotoxin was detected (Scheusner *et al.* 1973).

SEA was detected in pasta dough incubated at 25°C when the concentration of *S. aureus* reached around 10^7 CFU g⁻¹ (Lee *et al.* 1975). SEA was detected in cream, whole milk, and skim milk incubated at 37°C when the concentration of *S. aureus* exceeded 10^5 CFU g⁻¹ (Tatini *et al.* 1975), but not in cheese whey. In milk predominantly free of competing

bacteria, $2\text{--}3 \times 10^6$ CFU ml⁻¹ *S. aureus* were associated with the production of SEA at 37°C, but this did not occur in commercial (high count) raw milk (Tatini *et al.* 1971). Enterotoxin was produced in sterile reconstituted milk of initial pH 4.5 to 6.5.

Spontaneous creaming is a process involved in the production of various Italian cheeses (Carminati *et al.* 2008). The process involves holding the milk at temperatures which can be as high as 29°C to allow the cream to rise. Growth occurred under in this situation at 20°C and 25°C and enterotoxin was detected in the cream fraction ($8.4 \log_{10}$ CFU g⁻¹), but not in the skimmed milk ($6.9 \log_{10}$ CFU g⁻¹).

4.2.8 Nutrients

The available carbon sources also influence the production of enterotoxins (Smith *et al.* 1983). For example, the addition of glucose inhibits the production of SEA, SEB and SEC, although this may be caused by the lowering of the pH which results from metabolism of the sugar. Addition of amino acids to a minimal medium influenced SEB production, but the effects were neutral or repressive. The addition of various ions is reported to have differential effects on enterotoxin production, but it is not clear whether the stimulatory effects are simply caused by increased bacterial growth (Smith *et al.* 1983).

In contrast, SEA production in a fermentor was found to occur under all conditions in which the producing strain grew (Carpenter and Silverman 1976). The effects of gas flow rate, pH and dissolved oxygen were evaluated. However, the conditions tested were not extreme. For example the pH range used was 6.0 – 8.0 and enterotoxin production would be expected in this range.

4.3 Behaviour in Cheese

Appendix 2 summarises the behaviour of *S. aureus* in a variety of cheeses. Note that an increase does not necessarily equate to bacterial growth as cells are concentrated in the curd in the absence of growth.

There is usually an increase in *S. aureus* concentration during cheese making because of the warm temperatures at which the milk is held and the absence inhibitory activity arising from the production of lactic acid or other antimicrobial compounds. Various other processes are then applied to the curd that will reduce the pH and a_w . In many cheeses this results in conditions under which the organism is unable to grow. When this occurs it is likely that temperature is the main driver of the rate of inactivation of the pathogen (Ross *et al.* 2008). The likelihood of enterotoxin production during the process will depend on factors which include 1) the initial concentration of toxigenic *S. aureus* 2) the time and temperature profile of the initial fermentation which will influence the growth rate of the pathogen and any starter culture present 3) the concentration of the starter 4) the rate of change of pH and 5) the relationship between the concentrations of pathogen and enterotoxin and 6) the nature of the microflora in the raw milk. Other factors, such as the degree of anaerobiosis will also influence the quantity of enterotoxin produced.

Subsequent treatments of the cheese (e.g. high pH, brining) are unlikely to reduce the concentration of enterotoxin present.

5 FACTORS AFFECTING ENTEROTOXIN PRODUCTION DURING CHEESE MAKING

This section is subdivided into the different parts of the cheese making process and factors affecting *S. aureus* survival, proliferation and enterotoxin production at each stage will be discussed. There is relatively little information on the production of enterotoxins compared to that available describing the growth of the pathogen.

5.1 Milk

The Animal Products Act (Raw Milk Products Specifications) Notice 2009 (http://www.foodsafety.govt.nz/elibrary/industry/Animal_Products-Sets_Requirements.pdf) states that raw milk or raw milk products must comply with specific microbiological limits, including the absence of staphylococcal enterotoxins from the raw milk product. *S. aureus* is one of the causative agents of mastitis in dairy herds. As such, monitoring of herds also includes the stockman's obligation to exclude the milk from infected (or potentially infected) animals from the bulk storage tank. Other factors important in meeting the criteria include temperature control of milk, good hygienic conditions on the farm and the cheese production and maturation steps themselves. Key risk factors that may affect raw milk quality on the farm include: animal health, age/production status, housing, faecal contamination of the udder, effluent, feed, water (stock drinking), milking, water (use during milking), storage and transport (FSANZ 2006). If a farm has a sub-clinical mastitis problem, the concentration of *S. aureus* may already be high in the milk and, as milk is an excellent medium for pathogen growth, *S. aureus* may multiply rapidly during cheese making (Scientific committee on veterinary measures relating to public health 2003).

The thermal stability of SEs in food is one of the most important properties in respect to staphylococcal food poisoning. SEA exposed at least twice to heat treatment, at 130°C for 4 or 2 seconds, has been shown to retain at least partial immunological and biological activities. The amount of SEA in inoculated sterile milk increased linearly with time at temperatures between 14 and 32°C once the cell population reached 3.2×10^6 CFU ml⁻¹ (Fujikawa and Morozumi 2006).

Tatini *et al.* (1970) demonstrated that SEA production was less likely to occur in milk containing high numbers of competing microorganisms. They only detected enterotoxin in high count raw milk at a *S. aureus* population of 1.3×10^7 CFU ml⁻¹. Competing bacteria may alter the rate and extent of SE production by *S. aureus*. It is possible that the competing microorganisms present in milk change the oxidation-reduction potential during growth. As microorganisms differ in their ability to alter this potential, the number and type, of organisms present could influence the extent of this change (Tatini *et al.*, 1970). When raw milk was used to make the Moroccan fresh cheese Jben, initial concentrations of *S. aureus* in the milk of $\leq 10^3$ CFU ml⁻¹ did not result in enterotoxin production (Hamama *et al.* 2002). Lactic acid production resulted in rapid reduction in pH and inhibited growth of *Staphylococcus*. However enterotoxin was rapidly formed when higher concentrations of *S. aureus* ($\geq 10^5$ CFU ml⁻¹) were added to the milk despite the use of nisin-producing lactic culture for fermentation.

A quantitative microbial risk assessment for *S. aureus* and SEA in raw milk has recently been conducted (Heidinger *et al.* 2009). The authors determined the consumer risk from both pathogen (at the 99.9th and 99.99th percentile of servings) and enterotoxin (at the 99.99th percentile of servings) in raw milk. They also highlighted the need to address certain knowledge gaps, in particular, to establish further the relationship between growth of *S. aureus* and enterotoxin production; to study the expression pattern of enterotoxin genes present in milk and to gain an understanding of the relationship between dose and response following consumption of individual enterotoxins.

5.2 Starter Culture

Starter cultures, such as those containing LAB which convert lactose to lactic acid, are frequently used in the production of cheese and other fermented milk products. During fermentation the lactic acid produced decreases the pH and inhibits growth of *S. aureus*. It has been shown that production of SEA, SEB and SEC was optimal between pH 6.5 and 7.3 (Jarvis *et al.* 1973). The selection of a starter culture that is a fast acid producer is important in cheese making and is crucial to the microbiological safety of the final product. If the starter culture dominates the population of microorganisms in milk there is less opportunity for pathogen growth because of the reduction in nutrient availability, declining pH and increasing levels of organic acids. Failure of starter cultures can result in high concentrations of pathogen and enterotoxin being potentially present in the final product (Cogan 2003). Starter cultures may fail from a lack of viability or the presence of antibiotics and/or phages in the milk. The type of starter culture used will depend largely on the nature of the cheese being manufactured and the rate of pH reduction is characteristic of the cheese. The time taken for the pH to change can vary from 5-6 hours for Cheddar and cottage-type cheeses to 10-12 hours for Dutch and Swiss types (Fox *et al.* 2000d).

Several studies have compared the growth and survival of *S. aureus* in raw milk cheese with and without the presence of starter culture. A reduction in the final concentration of *S. aureus* in cheeses manufactured with various starters compared to starter-free controls is caused by a retardation of the growth rate. Gaya *et al.* (1988) demonstrated a 5.8-fold reduction in the concentration of *S. aureus* in the Spanish ewe's milk cheese Manchego after 60 days of ripening following inoculation of milk with 1% *Streptococcus lactis* (now *Lactococcus lactis*). A further 2.0-fold reduction occurred following the addition of 0.1% *Lb. plantarum*. Similarly, Stecchini *et al.* (1991) used the same starter culture (*Lb. plantarum* but at 0.2%) and observed a marked reduction in the concentration of *S. aureus* present in the Italian cows milk cheese Montasio from 0 to 45 days of ripening. Gomez-Lucia *et al.*, (1986) found that *S. aureus* growth in Manchego cheese was faster following the addition of a 0.1% commercial starter compared to a 1% starter. These authors detected SEA, but not SEB, with concentrations reaching as high as 7.69 $\mu\text{g kg}^{-1}$ in cheese from the 0.1% starter batches following the addition of an SEA and SEB-producing strain of *S. aureus* at 2.5×10^4 CFU ml⁻¹.

Arques *et al.* (2005) demonstrated that adding bacteriocin-producing LAB (BP-LAB) as a starter lowered *S. aureus* concentrations in cheese at day 3 by up to 0.46 log₁₀ CFU g⁻¹ compared to control cheese. When the BP-LAB were combined with the use of high-

pressure treatment of raw milk as an alternative to pasteurisation, the *S. aureus* concentrations (at 500 MPa) were reduced by 10^4 CFU g⁻¹ compared to control cheese. The growth and survival of *S. aureus* in the Spanish raw milk cheese Burgos was inhibited in cheeses manufactured with 1% starter culture (*L. lactis*) compared with those made with 0.1%, 0.01% or no starter culture (Nunez *et al.* 1986). Ibrahim *et al.* (1981a) detected enterotoxin in cheese at the end of cheddaring in batches made with a large initial inoculum of *S. aureus* and/or low starter activities. During cheese production *S. aureus* grew abundantly. However, following the addition of a *Streptococcus* starter culture (0.4%) and an initial *S. aureus* inoculum of 2×10^5 CFU ml⁻¹ the concentrations of SEA detected at the end of cheddaring (4.0 µg kg⁻¹) were generally less than those detected in Cheddar cheese produced with induced starter failure. Enterotoxin-containing Cheddar cheese can result, in the absence of starter activity, from as low an initial inoculum of enterotoxigenic *S. aureus* as 5 CFU ml⁻¹ (Ibrahim *et al.* 1981b).

By contrast to the above studies, it has been demonstrated that the use of a nisin-producing *L. lactis* strain as a starter culture for the Moroccan raw milk cheese Jben were of little help in preventing *S. aureus* growth and subsequent formation of enterotoxin (Hamama *et al.* 2002). This was particularly so when the initial milk contamination with *S. aureus* was greater than 10^5 CFU ml⁻¹. Nisin added to milk in white pickled cheese manufacture (Abdalla *et al.* 1993) or produced *in situ* by a *L. lactis* subsp. *lactis* strain in a semi-hard cheese variety (Rodríguez *et al.* 2000) showed little bactericidal effect on *S. aureus*, but a complete elimination of the pathogen was achieved when nisin was added to cheese spreads (Zottola *et al.* 1994).

5.3 Coagulation and the Formation of Curds and Whey

During coagulation, milk proteins (casein) form a gel which entraps milk fat. Coagulation may be achieved by a variety of methods including the addition of rennet (enzymatic), acidification to pH 4.6, or less, or acidification in combination with heating to approximately 90°C (Fox *et al.* 2000d). Most cheeses are rennet-coagulated at a temperature which is optimum for growth of mesophilic starter cultures. If there are problems with the viability or vigour of the starter culture, a rapid increase in the population of *S. aureus*, and resulting enterotoxin production, may occur during coagulation. Bacteria become immobilised in the curd during coagulation and it is generally accepted that 90% of the bacteria present in the milk are retained in the curd while only 10% are lost in the whey during draining (Hannon *et al.* 2006). Some cheese making processes incorporate a curd cooking step which may stop starter growth, increase curd contraction and increase whey expulsion. While curd cooking temperatures can vary according to the type of cheese being manufactured, temperatures may allow pathogen growth until curd acidity increases.

5.3.1 Rennet coagulation experiments in which enterotoxin production was measured

There are several reports on *S. aureus* concentration and enterotoxin production during the coagulation stages of cheese making. Physical entrapment of bacteria by curd particles has been reported by Stecchini *et al.* (1991). They observed an increase in the concentration of toxigenic *S. aureus* in Italian Montasio cheese between the addition of the test organisms

and cutting the curds in the absence of a starter culture. Despite the increase in bacterial concentration (to a maximum of 1.1×10^7 CFU ml⁻¹) no SEA was detected.

SEA-D-producing *S. aureus* isolates were inoculated into raw and pasteurised bovine milk to determine growth and enterotoxin production in Anatolian herby cheese (Akkaya and Sancak 2007). All *S. aureus* counts increased rapidly during the coagulation and curd stages, yet no enterotoxin was detected in cheese made from raw milk despite the *S. aureus* concentration reaching 10^7 CFU g⁻¹. In contrast, SEA was detected during curdling in cheeses made from pasteurised milk. The significantly reduced microflora present in pasteurised milk could have allowed the production of enterotoxin in the herby cheese.

The growth of *S. aureus*, and the production of SEA during the manufacture and ripening of raw goats' milk lactic cheese has been explored. Following rennet coagulation (20-22 hours at 24°C) and draining (20-22 hours 22°C) bacterial counts were higher in the curds than in the whey (Vernozy-Rozand *et al.* 1998). *S. aureus* grew and reached a maximum concentration of around 3.2×10^5 CFU g⁻¹ 24 hours after inoculation of the milk, an increase of approximately 1 log₁₀ CFU g⁻¹. Bacterial concentrations decreased during draining and the subsequent ripening steps by more than 5 log₁₀ CFU g⁻¹. No enterotoxin was detected until day 5 of ripening.

Meyrand *et al.* (1998) determined the growth kinetics of *S. aureus* and enterotoxin production during the manufacture (rennet coagulation and holding at 34°C for 30-40 minutes) and ripening of Camembert-type raw goats' milk cheeses. Growth of *S. aureus* and bacterial entrapment in the curds after whey drainage accounted for an increase in pathogen concentration from milk to curd of approximately 1.5 log₁₀. SEA was detected at the first sampling point post curd draining, 22 hours from the start of manufacture, in the high inoculum cheeses only. *Staphylococcus aureus* concentrations increased considerably during this stage of manufacture and 0.6 and 1.2 µg SEA kg⁻¹ were found in the cheeses inoculated with 10^5 and 10^6 CFU ml⁻¹ of milk respectively. No SEA was detected, at this stage, in cheeses made with the addition of lower inocula as lower concentrations were reached. In contrast, high concentrations of SEA have been found in Colby and Cheddar cheeses with an initial *S. aureus* population of $3 \times 10^4 - 2 \times 10^5$ CFU ml⁻¹ of milk (Tatini *et al.* 1971) and in Manchego-type cheese with an initial inoculum of 2×10^4 CFU ml⁻¹ (Gomez-Lucia *et al.* 1986). These cheeses were manufactured using higher temperatures than the Camembert-type cheeses during the initial production stages, allowing more growth of *S. aureus*.

5.3.2 Rennet coagulation experiments in which enterotoxin was not measured

Tuckey *et al.* (1964) determined the survival and growth of *S. aureus* (added to milk) at different stages in cheese making to using different varieties of cheese. The authors concluded that the pathogens which were concentrated in the curds increased in concentration until salting. During these early stages of manufacture, staphylococci were recovered from Cheddar, Colby and Swiss-type cheese, but not from fresh Cottage cheese that underwent a cooking step (54°C for 40 minutes at pH 4.5).

5.3.3 Heat coagulation

Manufacture of the hard Italian ewes' milk cheese Canestrato Puliese includes a step where curds are heated in hot whey (80°C for 30 seconds). This has been shown to have a killing

effect similar to pasteurisation, with the reduction in pathogen concentration being maintained throughout ripening (Albenzio *et al.* 2001). No naturally-occurring *S. aureus* were detected in the cheeses sampled. Similarly, high cooking temperatures ($>50^{\circ}\text{C}$) for a long period (up to 1 hour) used in the manufacture of Emmental and some other cheeses result in a reduction in bacterial counts (Fox *et al.* 2000b). By contrast, Ozer *et al.* (2004) demonstrated that scalding in whey (95°C for 3 minutes) had no effect on the concentration of *S. aureus* in experimentally-manufactured white-brined Turkish cheese. Enterotoxin was not assayed for in these experiments. The discrepancy between these results is most likely because the Turkish cheese is heated as a block. The temperature gradient between the outer and inner layers would be significant, with the centre of the block being cooler than the outside. During the manufacture of the Italian cheese Grana Padano the curds are cooked at 55°C for 20 minutes and held under whey for 40 minutes. A curd cooling model estimated that the inside of the cheese remains above 50°C for at least 4 hours during cooling whereas the crust cools to 30°C during the first hour. The fate of *S. aureus* will depend on its location within the cheese (Ercolini *et al.* 2005).

5.4 Salting and Brining

Cheeses are typically salted by immersion in brine or by surface application of dry salt. Some cheeses may require the addition of salt to the curd while others, such as Cheddar, are salted by mixing dry salt with the cut curd towards the end of the manufacturing process. Salt added to the curds will retard the growth of starter bacteria and reduce the rate at which the pH is lowered although metabolic activity of the starter can continue (Fox *et al.* 2000a). In brine-salted cheeses, the salt concentration is influenced directly by the size of the cheese. As salt diffusion into the centre of cheeses is a relatively slow process, the pH is able to reduce for longer meaning that the centre of large cheeses may be more acidic than the outside surfaces of the cheese. As such, there may be longer periods before the inhibitory effects of pH alone can influence growth inhibition of pathogens, including *S. aureus*. The concentration of salt added to cheeses typically ranges from 0.7-6% (Guinee and Fox 2004). Salt also plays an important role in reducing the growth of pathogens in cheese, but *S. aureus* is relatively salt-tolerant and can grow in the presence of 6.5% NaCl (Cogan 2003). Furthermore, during the initial stages of cheese making, salt is not distributed evenly throughout the curd mass. Dry salt applied to the surface of the cheese requires time to diffuse, resulting in the possibility of continued pathogen growth.

Several studies have examined the effects of salting on *S. aureus* growth and enterotoxin production. Many report a reduction in *S. aureus* concentrations following the salting step (Otera *et al.* 1993; Necidova *et al.* 2009; Vernozy-Rozand *et al.* 1998). Ahmed, (1983) enumerated SEA-producing *S. aureus* in Egyptian Domiati cheeses prepared from raw unsalted milk and from raw milk with added NaCl (5% or 10%). All cheeses were then stored in whey containing 15% NaCl at 30°C prior to sampling. The authors observed a rapid decrease in concentrations of viable *S. aureus* during the storage of cheese made from unsalted milk and cheese made from milk with 5% added salt. However, in the cheeses made with 10% added NaCl, *S. aureus* survived until the fourth week post manufacture at a concentration of $5 \times 10^4 \text{ CFU g}^{-1}$ (from a maximum of $3.2 \times 10^7 \text{ CFU g}^{-1}$). Despite the high concentration of *S. aureus* in the cheese, no SEA was detected. The authors concluded that the combination of high salt concentration and slow decrease in pH permitted survival. In

contrast, in samples with 5% added NaCl the pH decreased more rapidly during storage thereby inactivating the pathogen more rapidly.

Vernozy-Roland *et al.* (1998) measured the concentrations of SEA recovered following salting of raw goats milk lactic cheese (saturated brine solution at 13°C for 7 minutes). At *S. aureus* concentrations of 10^5 or 10^6 CFU ml⁻¹ milk, the quantity of SEA detected after the salting step was low (1 µg kg⁻¹), but was even lower when 10^4 CFU ml⁻¹ milk was used as an inoculum. In the Turkish cheese, Urfa, a dense brining solution of 20-23% NaCl (wt/vol) is typically used (Ozer *et al.* 2004). As a high salt concentration can affect the activities of the starter organisms and natural microflora, the authors aimed to determine whether a reduction in brine concentration (to 12-13% NaCl wt/vol, stored for 90 days at 6°C) would prevent the survival of *S. aureus*. While bacterial counts declined during the first seven days, *S. aureus* began multiplying again and remained viable to the end of the ripening period. Enterotoxin production was not measured. The authors concluded that the rapid initial salt penetration of the cheese and the subsequent stable NaCl concentration resulted in an environment selective for salt-tolerant organisms.

NaCl was inhibitory to *S. aureus* inoculated into sterile milk but higher populations were achieved in pasteurised milk (Ibrahim 1978). The higher pH in salted simulated Cheddar cheese samples (3.5-5% salt) compared to unsalted samples indicated differences in the microflora present. The higher pH values of these cheeses could represent a potentially conducive environment for SE production. Further experiments (Ibrahim *et al.* 1981a; 1981b) confirmed that concentrations of *S. aureus*, enterotoxin, and pH, were always lower in the batches of unsalted Cheddar cheese compared with the batches of curd-salted cheese at the end of pressing. The suppression of salt-sensitive bacteria in the salted Cheddar allowed better growth and enterotoxin production by the salt-tolerant *S. aureus*. From an average initial inoculum of 7.8×10^3 CFU ml⁻¹, the counts of *S. aureus* and concentration of enterotoxin at the end of cheddaring were 7.9×10^6 CFU ml⁻¹ and 5.8 µg kg⁻¹ (detected in 1 out of 6 cheeses only) respectively. At the end of pressing, the bacterial counts in the unsalted cheese and salted cheese were 1.3×10^7 CFU ml⁻¹ and 5×10^7 CFU ml⁻¹ while the concentrations of enterotoxin in the two cheeses were 4.6 µg kg⁻¹ (detected in 3 out of 6 cheeses only) and 8.4 µg kg⁻¹ (all 6 cheeses).

5.5 Ripening or Maturation

The unique characteristics of individual cheeses develop during the ripening or maturation stages, under specific time and temperature conditions. The combined effects of pH, salt, a_w and storage temperature aim to limit pathogen growth during this stage of cheese making (FSANZ, 2006). The length of the ripening period can range from a short period (approximately three weeks) for soft cheeses such as Mozzarella, to more than two years for harder cheeses such as mature Cheddar or Parmigiano Reggiano. The a_w is lowered during ripening through moisture loss, while added salt binds free moisture making it unavailable for bacterial growth. The hydrolysis of proteins to peptides and amino acids and of lipids to glycerol and fatty acids during ripening further reduces the availability of water (FSANZ, 2006). Various parameters can also differ throughout the mass of larger cheeses. For example, the a_w can be affected by the temperature gradient in the cheese during the early stages of fermentation, the loss of moisture during ripening, the NaCl gradient in the cheese and the microbial activity on the rind (Fox *et al.* 2000c). Pathogens are susceptible to

reduced water activity, meaning that cheeses with a relatively high a_w may support the growth of pathogenic bacteria while those with a water activity less than 0.92 will typically inhibit the growth of pathogens. *S. aureus* can grow when the a_w exceeds 0.86 (International Commission on Microbiological Specifications for Foods 1996). The temperature of ripening is another balancing factor important in controlling pathogen growth. Higher temperatures promote faster ripening by the starter but may also allow more rapid growth of pathogens. Typically ripening temperatures are sub-optimal for pathogens, but may not be inhibitory.

5.5.1 Soft cheeses

Meyrand *et al.* (1998) found that maximum *S. aureus* concentrations were detected in Camembert-type cheeses made from raw goats' milk at 22 hours post manufacture. Pathogens were not eliminated during ripening and there were no differences between counts at the surface and the centre of the cheeses. SEA was detected in some cheeses made with an initial inoculum over 10^3 CFU g⁻¹ *S. aureus*, but only once concentrations reached 10^6 CFU g⁻¹ or higher. A few studies have looked at the behaviour of *S. aureus* and enterotoxin production during the ripening stages of the traditional Spanish cheese Manchego (Gomez-Lucia *et al.* 1986, Otero *et al.* 1988, Otero *et al.* 1993). Otero *et al.* (1993) observed that the concentration of *S. aureus* decreased during the ripening process. Despite pathogen growth during manufacture (up to a maximum of 10^7 CFU g⁻¹), no enterotoxin (C₁ or C₂) was detected. A similar finding has been reported for Manchego cheese inoculated with two *S. aureus* strains producing SEA, B, C and D and SEC respectively (Nuñez *et al.* 1988). It has been suggested that the influence of LAB on SEC₁ and C₂ concentration by inhibition of enterotoxin synthesis and their destruction may explain the lack of detectable enterotoxins in this type of cheese.

In a similar study (Gomez-Lucia *et al.* 1986) lower initial *S. aureus* concentrations (2×10^4 - 2×10^5 CFU ml⁻¹) resulted in the production of high levels of SEA, but not SEB during the ripening of Manchego cheese. Despite marked reduction in staphylococcal concentrations after 35 days of ripening, SEA was detected at a peak of 7.69 µg kg⁻¹ of cheese six weeks post manufacture. These cheeses were manufactured using temperatures (32-29°C) close to optimum for *S. aureus*. It has previously been suggested that SEA production may be less affected by adverse environmental conditions than SEC production (Notermans and van Otterdijk 1985). Furthermore, there were differences in the *S. aureus* strain, the milk origin and condition (raw vs pasteurised), the type of starter culture, and the manufacturing and ripening temperatures used by the different authors which could all have contributed to the differing results. Failure to detect staphylococci in cheese is no guarantee of the absence of enterotoxins. Gomez-Lucia *et al.* (1992) detected SEA and SED in high levels at the end of ripening in some cheeses despite the fact that no staphylococci were detected. Necidova *et al* (2009) and Vernozy-Roland *et al* (1998) observed that *S. aureus* counts in soft cheese plateaued or decreased during ripening, yet enterotoxin was still detected. The amount of enterotoxin produced clearly depends on many factors including the starting inoculum and the temperature of manufacture or ripening.

5.5.2 Hard cheeses

Hard cheeses are less likely to support the survival and growth of pathogenic bacteria during ripening, largely due to their low a_w and longer maturation periods (Bautista and Kroll 1988, Tuckey *et al.* 1964). Enterotoxin production was not detected in Italian Montasio cheese during 90 days of ripening (Stecchini *et al.* 1991), despite persistence of a relatively high *S. aureus* concentration of approximately 10^6 CFU ml⁻¹.

Ibrahim *et al* (1981a and 1981b) determined the growth of *S. aureus* and SEA production in Cheddar cheese with both induced starter failure and variable starter activity. Despite a reduction in *S. aureus* concentration after six weeks of ripening, SEA could still be detected in unsalted cheese made with inhibited starter activity. The rate of inactivation at a storage temperature of 11°C exceeded that at 4°C. Increases in *S. aureus* and enterotoxin concentrations occurred in some batches of salted cheddar stored at 11°C, whereas a slight decrease in *S. aureus* concentration and no change in enterotoxin concentration occurred in all of these cheeses stored at 4°C (Ibrahim *et al.*, 1981a).

In a study examining growth and enterotoxin production in Turkish herby cheese (Akkaya *et al.*, 2007), it was found that *S. aureus* decreased from day 15 in cheese made from pasteurised milk and was only present in low numbers (10^2 CFU g⁻¹) by day 90. In cheese made from raw milk, *S. aureus* counts increased rapidly at the start of ripening and then decreased to approximately 10^5 CFU g⁻¹ by the 90th day of ripening. Enterotoxin (SEA, SEC or a mix of SEA, SEB, SEC, SED) was detected in the pasteurised milk cheese samples throughout the ripening stage. No enterotoxin was detected in the cheeses made from raw milk. The natural microflora present in raw milk is known to have a partial inhibitory effect on the growth of *S. aureus*, but the concentrations of pathogen present in these samples were certainly conducive to the production of enterotoxin.

6 DEFINING THE GROWTH BOUNDARIES WITH SPECIAL REFERENCE TO DAIRY PRODUCTS

The pH, temperature and a_w ranges for growth under optimal conditions were described in Section 4. Combinations of these factors may provide hurdles to growth. Additional inhibitory factors associated with cheese, such as lactic acid concentration, were discussed in Section 5.

While there are many reports on the factors that prevent growth of CPS (Troller 1986), the complexity of their interactions is best summarised in the form of a model. Growth/no growth boundaries for a mixture of five enterotoxigenic strains of *S. aureus* in a broth were defined over a temperature range of 8 to 19°C, pH range of 4.5 to 7.5 and water activity 0.88 to 0.999 (Valero *et al* 2009). The lowest temperature where growth was observed was 8°C, but this only occurred at optimum values of pH and a_w . For example, at an a_w of 0.96 (a possible value for cheddar (Marcos *et al.* 1981)) growth was only observed in broth with a pH of greater than 5.5 and at a temperature of 8°C.

6.1 Growth Boundaries in the pre-Ripening Phase of Manufacture

The initial stages of cheese production, warming the milk, coagulation, curd cutting, draining and pressing provide good conditions for the growth of *S. aureus* (Arques *et al.* 2008, Eckner and Zottola 1991, Le Marc *et al.* 2009, Reiter *et al.* 1964). If cheese making does not include an early cook step capable of killing *S. aureus*, it is highly likely growth will occur.

The question becomes not if the pathogen will grow, but by how much it will grow. This, taken into account with the likely initial starting concentration, will determine whether a sufficiently high concentration is achieved to enable potential enterotoxin production.

6.2 Growth Boundaries in the Ripening Phase of Manufacture

There is some evidence that growth of *S. aureus* can occur during the ripening or storage phase of cheese manufacture. Figure 1 shows observed growth or no growth data, as described for cheeses in the literature, given cheese pH and storage temperature. The same information is given in Appendix 3.

The boxed area in Figure 1 (<7°C) represents the area where no growth is predicted to occur (section 4). Also plotted in Figure 1 are the predicted growth/no growth boundaries (probability of growth = 0.5) predicted by Valero *et al.* (2009) for two a_w values for *S. aureus* in broth. In these experiments HCl and NaOH were used to adjust the pH. The boundary lines cover the experimental range of pH values over which the growth/no growth boundary model was fitted.

A water activity of 0.98 is typical for a Camembert cheese and 0.91 is typical of hard cheeses such as Parmesan or Provolone. Most papers (Appendix 3) do not provide

information on the a_w of the cheeses they described, so the data could not be directly compared to the predicted growth boundaries with respect to this variable.

Figure 1. Observed growth and no growth for assorted cheeses during ripening or storage, compared to the broth growth no growth boundaries (Valero *et al.* 2009).

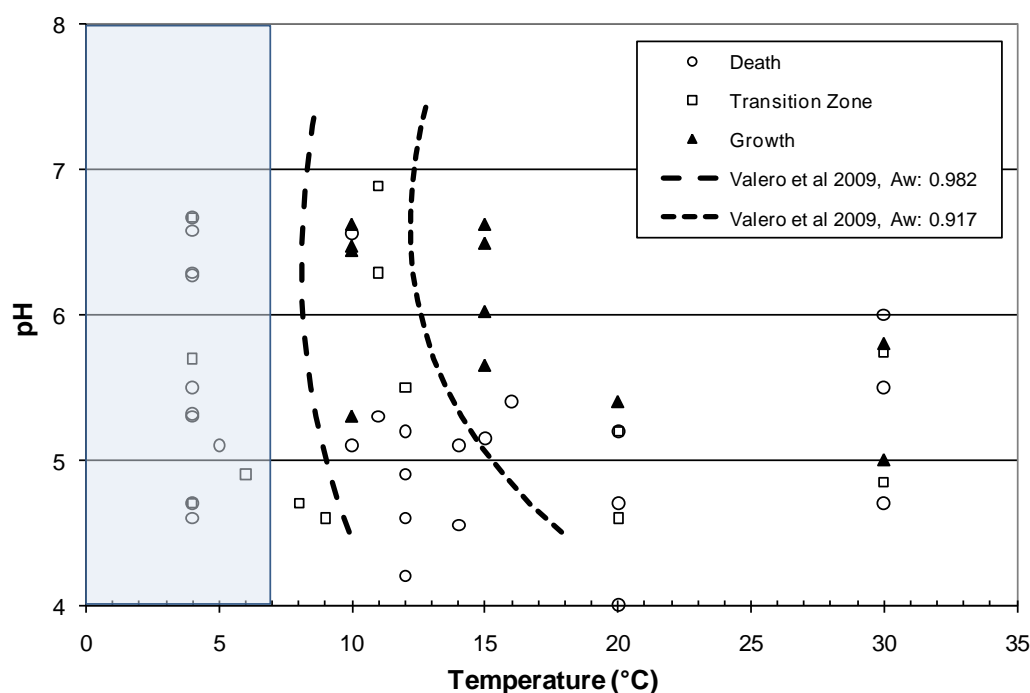


Figure 1 shows that there is an overlap of conditions reported to result in growth and death of *S. aureus* when described by the pH and temperature of the cheeses. The data points indicated as “transition zone” are those where no appreciable growth or inactivation occurred. From the data displayed in Figure 1, no growth was observed in cheeses with pH < 5 or at a temperature below 10°C. Growth has been reported in broth under conditions outside these limits (Valero *et al.* 2009). This discrepancy could be the result of the lack of comprehensive growth response data in cheese. There are data gaps across the temperature ranges that would be expected to be used for ripening or storage of cheese. Given that these data are for many strains in many cheese then it seems likely that other factors, such as strain effects, also influence the growth/no growth boundary in cheeses.

To provide a better understanding of the growth boundary in a raw milk cheese matrix, a more systematic approach involving sampling across pH, temperature and a_w would need to be undertaken. Such a systematic approach would be limited by the combinations possible in cheese.

7 CORRELATION BETWEEN CELL CONCENTRATION AND ENTEROTOXIN IN FINAL PRODUCT, DOSE RESPONSE RELATIONSHIP FOR STAPHYLOCOCCAL ENTEROTOXIN IN FOODS

Staphylococcal cells can be introduced into raw milk cheeses through one of three pathways:

1. The presence of staphylococcal cells in the raw milk used to produce the cheese.
2. Cross contamination from an infected cheese maker or food handler during or after cheese making.
3. Cross contamination of contaminated surfaces/machinery during cheese making.

For most cheese-related incidents and outbreaks reported in the literature it is unclear which pathway resulted in contamination of the raw milk cheese. Table 3 provides examples from the literature of products that have been found to contain staphylococcal enterotoxins.

Table 3. Examples of intoxications where both enterotoxin and viable cells were detected in cheese

Product	Staphylococcal cells and enterotoxin detected.	Reference
Home made Minas cheese from Brazil	SEA, SEB and SEC producing staphylococcal strains were detected in cheese samples at concentrations from 1×10^5 CFU g ⁻¹ to 2×10^8 CFU g ⁻¹ . It was not known if milk was pasteurised before cheese making started. Raw milk tested from the same source following the outbreak contained SEA and SEB producing strains.	(do Carmo <i>et al.</i> 2002)
Semi cured cheese from Brazil	SEH enterotoxin was detected in cheese extract and SEH a producing strain of <i>S. aureus</i> detected at a concentration of 2.9×10^8 CFU g ⁻¹ .	(Pereira <i>et al.</i> 1996)
Raw milk soft red smear cheese	SEA detected at 0.01 to 0.04 µg kg ⁻¹ cheese and SEC detected at 0.04 to 0.05 µg kg ⁻¹ cheese. <i>S. aureus</i> was detected at concentration $<1 \times 10^4$ CFU g ⁻¹ .	(de Reu <i>et al.</i> 2002)

The high concentration of staphylococcal cells which produced the enterotoxins in a product may not necessarily be present in the final product. The cheese may undergo a cooking step after enterotoxins have been produced which will kill or reduce the detectable staphylococcal cells to concentrations not considered to be likely to produce enterotoxins. Table 4 gives examples of this scenario given in the literature.

Alternatively, the cheese making process may eliminate or reduce the staphylococcal cells from initial concentrations in the milk or introduced by cross contamination. For example, SEA was found in the absence of *S. aureus* in raw ewes' milk cheese implicated in three outbreaks. A SEA producing staphylococcal strain was isolated in subsequent samples of milk from the dairy that made the cheese (Bone *et al.* 1989). Data presented in the paper indicate that the concentration of *S. aureus* in cheese decreased during ripening.

Table 4. Examples of intoxications where only enterotoxin was detected because the cheese was heat-treated

Product	Staphylococcal cells and enterotoxin detected.	Reference
Soft raw milk cheese	From one batch of cheese; $>1.8 \times 10^7$ CFU g ⁻¹ of CPS was detected in cheese sampled from one outbreak, while $<10^2$ CFU g ⁻¹ was detected in another outbreak where the cheese had been cooked before eating. SEE was detected in samples from both outbreaks.	(Ostyn <i>et al.</i> 2010)
Halloumi cheese	SEA was detected in cheese and brine, but <i>S. aureus</i> could not be found. During manufacture, Halloumi has a cook step following the pressing of curds which would kill staphylococcal cells.	(Wieneke <i>et al.</i> 1993)
Cooked (non dairy) produce from food outlets	Eight outbreaks associated with food outlets tested positive for staphylococcal enterotoxins, with <10 CFU g ⁻¹ CPS detected.	(Wong 1996)

7.1 Dose Response Information

The small amount of dose response information available for humans is from volunteer trials or foodborne disease outbreaks. Concentrations of enterotoxin in food samples taken from outbreak investigations will only be indicative, as the concentration of enterotoxin is likely to vary throughout the food. Relevant examples are discussed in section 7.2.1.

7.1.1 Dose dependency on enterotoxin type

Before the development of reliable laboratory methods for detection of SE, monkeys were one of a number of animals used to test for its presence in foods. A difference in dose response between the enterotoxin types was observed for the monkeys, with 5 µg of SEA per 3 kg monkey required to produce an emetic response, while SEB, SEC and SEE required 10 µg per 3 kg monkey and SED required 20 µg per 3 kg monkey. It should be noted that humans are several times more sensitive than monkeys to enterotoxins (Bergdoll and Lee Wong 2006).

7.1.2 Foodborne outbreaks

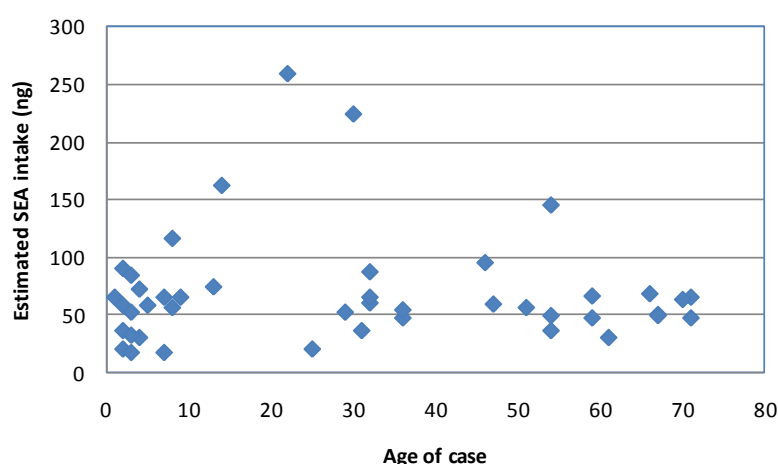
SEE was detected in soft cheese made with raw cows' milk in France (Ostyn *et al.* 2010). In one of the six outbreaks reported 200g portions of cheese were consumed, with enterotoxin detected at 0.45 µg kg⁻¹ of cheese. In this outbreak, five out of six people who ate the cheese became ill (83% attack rate). A different cheese sample from the same batch associated with another outbreak had enterotoxin detected at >0.92 µg kg⁻¹. In this second outbreak three out of four people became ill (75% attack rate), but no information is given about portion sizes. This implies the enterotoxin dose for the first outbreak could have been in the range 90 to >180 ng per person.

SEA was detected in chocolate milk associated with an outbreak among American school students (850 cases aged 5 to 19) (Evenson *et al.* 1988). Twelve 280 ml cartons of chocolate milk were analysed and found to contain SEA at concentrations of 94 to 184 ng per carton

(0.34-0.66 $\mu\text{g kg}^{-1}$ milk). The attack rate among those who consumed one carton was 31.5% compared to 37.6% for those drinking more than one and 44.4% for those children who consumed three or more cartons.

In Japan, an outbreak was caused by skim milk powder which, when reconstituted contained 0.38 to 0.8 $\mu\text{g l}^{-1}$ of SEA in positive samples (Asao *et al.* 2003). SEA intake estimates were calculated for 44 cases who provided left over milk samples for testing and milk consumption information. A plot of age against intake of SEA is given in Figure 2. This figure gives the estimated dose that people consumed and is correlated to the portion size of milk consumed by these people. From this plot it can be seen that some people from all ages became symptomatic given doses less than 50 ng of SEA. Children became sick with doses as low as 17 ng (0.017 μg) of SEA. A second paper (Ikeda *et al.* 2005) describes further testing of the milk samples, resulting in detection of SEH in similar quantities to the amounts of SEA.

Figure 2. Dose of SEA consumed plotted against consumer age for the Japanese outbreak associated with milk consumption.



7.1.3 Volunteer studies

Volunteers who consumed purified enterotoxin experienced signs and symptoms similar to those observed in food poisoning outbreaks (Bergdoll 1989).

Vomiting and diarrhoea were exhibited by healthy adult males who were given SEA, SEB, SEC at a dose rate of 50 ng kg^{-1} body weight (3,500 ng per 70 kg man). Milder symptoms were observed in two of four volunteers at a dose of 10 ng SEA kg^{-1} body weight (700 ng per 70 kg man). These are unpublished data and no extra information has been given.

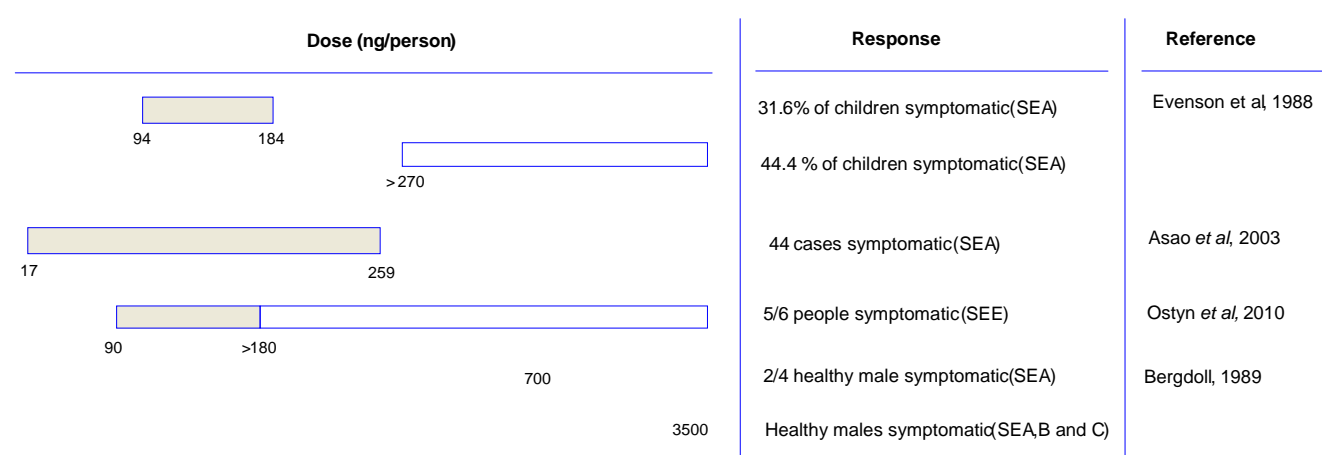
In a paper describing the consumption of 50,000 ng of SEB in distilled water by two people (19 and 26 years old) and 900,000 ng by one person (35 year old), all three became sick (Raj and Bergdoll 1969). The paper reports unpublished work where ingestion of either 1,000 ng or 10,000 ng of SEB in two volunteers failed to make them sick (Sugiyama, unpublished

data), whereas it was estimated that ingestion of <1,000 ng of SEA in cheese consumed by volunteers could cause illness (Bergdoll, unpublished data).

7.1.4 Summary of dose response data

Figure 3 summarises the available dose response data.

Figure 3. Summary of dose response data for staphylococcal enterotoxins



The white parts of the bars indicate that the upper bound is not known (> value)

7.1.5 Correlation between cell concentration and presence of enterotoxin

A previous report described the available data linking the concentration of *S. aureus* and the production of enterotoxin (Horn and Hudson 2007). It was discussed in that report that, as counts are measured at discrete steps in the manufacturing process, the concentrations of *S. aureus* measured may be lower than those actually present when the enterotoxin was produced. Given that caveat, inspection of the literature showed that the majority of occasions where enterotoxin was detected involved *S. aureus* concentrations in excess of 10^5 CFU g⁻¹.

However there are reports of concentrations of the pathogen in excess of this where enterotoxin was not detected. In contrast there are reports of the presence of enterotoxin in cheeses in the absence or presence of very low concentrations of *S. aureus*. See Appendix 2 for examples of both of these situations.

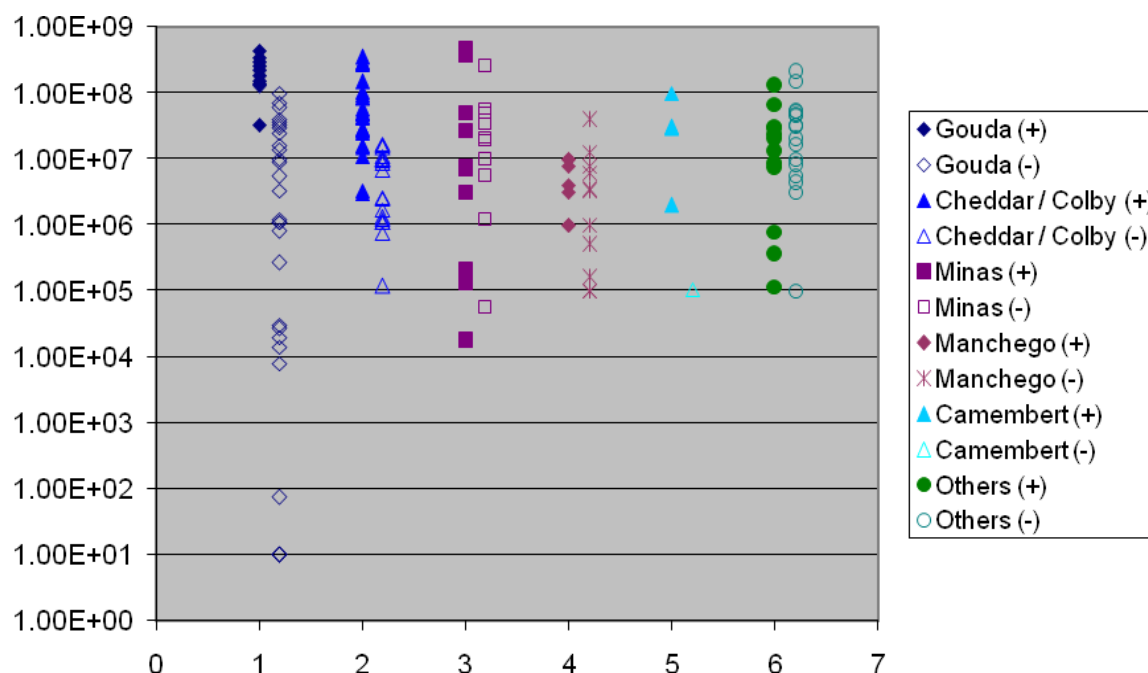
Precise information is lacking on the concentrations required to produce enterotoxin and whether there is any variability between strains producing different enterotoxin types. A summary of some data for cheeses is shown in Figure 4 below.

Figure 4 reflects the observation that there may be high concentrations of the pathogen in the absence of enterotoxin production (although the organisms enumerated may not have been

toxigenic). Fewer data are shown representing enterotoxin at low concentrations of *S. aureus*, but there are few data at lower concentrations of the pathogen.

With respect to situations where high concentrations of *S. aureus* may be present in the absence of enterotoxin the following discussion focuses on Cheddar and Colby cheeses as these are commonly consumed cheeses. In the production of unsalted Cheddar cheese inoculated with a SEA-producing *S. aureus*, no enterotoxin was detected during manufacture and storage of the cheese despite the bacterial concentration reaching 7.8×10^6 CFU g⁻¹ after two weeks of ripening at 11°C (Ibrahim *et al.* 1981a). Salted cheeses, which supported higher concentrations of the pathogen, were positive for SEA. In an examination of Colby and Cheddar cheeses (Ibrahim 1978) enterotoxin was absent from cheeses when the maximum concentration of *S. aureus* reached was between 1.2×10^5 and 2.3×10^7 CFU g⁻¹. Enterotoxin was detected in samples where the maximum concentration was between 3.3×10^6 and 3.5×10^8 CFU g⁻¹. So, while enterotoxin tended to be detected when the organism was at the highest concentrations (Figure 4), a high concentration of the pathogen was no guarantee of the presence of enterotoxin.

Figure 4. Concentration of *S. aureus* and the detection of enterotoxin in cheese.



Filled symbols: enterotoxin detected, empty symbols: enterotoxin not detected

The highest reported concentration of *S. aureus* at which enterotoxin was not detected was $>10^8$ CFU g⁻¹ in Minas cheese (dos Santos and Genigeorgis 1981). This cheese was produced from raw milk inoculated with three enterotoxigenic strains and none of the raw milk cheeses were positive for enterotoxin even though the maximum concentration of *S. aureus* was always $> 10^7$ CFU g⁻¹. In cheese made from pasteurised milk, enterotoxin was detected at lower *S. aureus* concentrations. When a starter was used enterotoxin was not detected in three cheeses containing $>10^7$ CFU g⁻¹ *S. aureus*. Similarly Burgos cheese manufactured from pasteurised ewes' milk supported growth of enterotoxigenic *S. aureus* to

$>10^8$ CFU g⁻¹ without the production of detectable enterotoxin (Otero *et al.* 1988) and, in most cases, thermonuclease. Similar results have been presented for Domiati cheese made from raw milk (Ahmed *et al.* 1983). However, enterotoxin is formed in other raw milk cheeses (Delbes *et al.* 2006, Meyrand *et al.* 1998, Vernozzy-Rozand *et al.* 1998). Enterotoxin formation requires a high concentration of enterotoxigenic *S. aureus* to be present, but other factors such as pH, a_w and the presence of oxygen are also important in determining the extent to which enterotoxin is produced (Cretenet *et al.* 2011).

In cases where enterotoxin is present, yet there are few/no *S. aureus* (and assuming no cooking of the cheese has occurred) this appears to be caused by production of enterotoxin followed by non-thermal reduction in the concentration of *S. aureus*. Such events are suggested by the wide variation in the concentration of *S. aureus* in Cheddar cheese samples associated with outbreaks (Donnelly *et al.* 1964). Of 13 outbreak-related samples two contained *S. aureus* at concentrations <50 CFU g⁻¹. The concentrations in the remaining samples varied from 50 to 3.8×10^6 CFU g⁻¹. By the method used (a bioassay), only three of 13 samples were positive for enterotoxin while the concentrations of *S. aureus* in these three samples ranged from 50-100 CFU g⁻¹ (depending on the medium used) to $>1.5 \times 10^5$ and 3.8×10^6 CFU g⁻¹.

The production and persistence of enterotoxin alongside the growth and then decline in concentration has been shown in Manchego-type cheese (Gomez-Lucia *et al.* 1992). While this was strain-dependent, one strain reduced from a concentration of around 10^6 CFU g⁻¹ to below the level of enumeration while the concentration of enterotoxin remained static.

8 DISCUSSION AND PRELIMINARY RECOMMENDATIONS

It is clear that there is considerable potential for growth of *S. aureus* to occur during cheese production, mainly at the early stages where the pH is relatively high and the temperature permissive for the growth of the organism. This growth will continue until such time as the physic-chemical parameters are altered to prevent growth. After this point death will occur at a rate largely determined by the temperature. Variables such as pH, a_w and lactic acid concentration, which determine whether growth will occur or not, will differ widely between varieties of cheese.

The potential for enterotoxin to be produced in cheese will depend on;

- The initial concentration of *S. aureus* in the cheese milk prior to cheese making
- The possession, or otherwise, of genes encoding SE production in the cheese milk *S. aureus*
- The period for which the pH, temperature and other factors permit growth and enterotoxin production, and how enterotoxin is produced under these conditions

While subsequent treatments such as brining and cooking may inactivate *S. aureus*, any enterotoxin which has been formed is unlikely to be removed from the cheese.

For raw milk cheeses to be produced safely with respect to enterotoxigenic *S. aureus*, the period for which growth and enterotoxin production can occur should be minimised. A good quality milk supply with a low concentration of the pathogen should be used. The most significant factor in cheese making that will retard and/or prevent growth of *S. aureus* is the activity of the starter culture. This effect is mediated through several mechanisms including pH reduction/lactic acid production, production of bacteriocins, production of hydrogen peroxide, competition for nutrients and other factors which have not yet have been identified. Of these, only the pH can be measured by the cheese maker, although they will also control the quality and quantity of the starter culture added.

While the pH minimum for growth of the organism is quite low (pH 4.5) examination of the growth/no growth boundary indicates that the minimum is closer to pH 5 in cheese, possibly because of the nature of the acidulant (i.e. lactic acid). No data were discovered which reported growth at pH <5. Inspection of Figure 1 shows that there are few datapoints for pH values at around 5 over the temperature range 15 to 30°C, and this is the temperature range in which most of the initial stages of cheese production are conducted. Additionally there are few data in the pH 5-6, temperature 7-10°C range. It is recommended that further laboratory studies concentrate on producing data to cover these regions, especially when considering that the one datapoint at pH 5 where growth did occur was at 30°C. Data obtained in this region should be supplemented with some other combinations within the relatively defined region (pH <5.5, 10-15°C) as a benchmark against existing data. The duration of the experiments should be sufficient to allow the construction of a growth curve but within reasonable bounds of the time normally passing before a pH of 5 is reached during cheese production.

Salt concentration/ a_w is unlikely to be a significant consideration if the potential for growth occurs at stages of cheese production prior to brining, but will be if there is the possibility of enterotoxin production occurring at subsequent stages. Although pressing will reduce a_w and increase the water phase salt concentration, and pH reduction will continue, *S. aureus* is able to grow over a wide range of values for these parameters.

There is some evidence that the presence of background flora (non-starter bacteria) may also influence growth rate and enterotoxin, and the poor competitive ability of *S. aureus* is a widely accepted property of the organism. It is therefore possible that growth and enterotoxin production in pasteurised milk will be faster than in raw milk which contains a significant competing flora. This could be investigated by adding different doses of a defined artificial raw milk microflora to sterile coagulum samples.

It is difficult to define a concentration of cells at which enterotoxin production may occur. *Staphylococcus aureus* may grow, produce enterotoxin and then die, giving the appearance of enterotoxin production at a low concentration of cells. Alternatively, the detection of enterotoxin may occur at a time after that at which a detectable concentration was reached, giving the appearance that a higher concentration of cells is needed for enterotoxin production than is actually the case. Better information on the concentrations of cells required to produce enterotoxin at biologically active concentrations, and whether the degree of variability between strains producing different enterotoxin types could be obtained through targeted experimentation. It may also be of use to study the expression of enterotoxin genes present in milkborne *S. aureus* to assess potential differences between SE types.

The literature does not provide enough information to be able to provide a clear dose response relationship for staphylococcal intoxications. However, the following comments can be made:

- Different strain types may have different dose response relationships
- There is a trend of increasing numbers of people becoming sick with higher doses consumed for doses above 90 ng of SEA.
- Estimated doses as low as 17 ng of SEA can cause symptoms in children and estimated doses of 50 ng can cause symptoms in all age groups. It is not known what proportion of exposed people is likely to get sick at these doses.

APPENDIX 1. DATA CONCERNING PREVALENCE AND CONCENTRATION OF CPS, ENTEROTOXIN GENES AND TOXIGENIC ISOLATES IN RAW MILK AND CHEESES

Type of milk/ cheese (country)	Raw milk product (Y/N)	Animal source of milk/cheese (number)	Sample tested (number)	Prevalence of enterotoxin /toxin genes	% isolates positive for at least one enterotoxin gene	Which enterotoxin genes/toxins	Toxin genes expressed in product (Y/N)	Concentration of coagulase-positive staphylococci (log ₁₀ CFU g ⁻¹ or ml ⁻¹)	Toxin produced	Reference
Raw Milk										
Raw milk (Brazil)	Y	Bovine	Bulk milk (54 samples from 5 farms)		68.4%	<i>sea</i> , <i>sec</i> , <i>sed</i> , <i>seb</i> , <i>see</i> , <i>seg</i> , <i>sei</i> , <i>seh</i> , <i>sej</i>	NT	70.4% samples tested (38/54) Up to 5.94.	NT	(Rall <i>et al.</i> 2008)
Raw milk (Brazil)	Y	Bovine	Milk from mastitic cows (208) Bulk tank milk (37)	NT	NT	NT	NT	6.7% <i>S. aureus</i> +ve, 1% toxigenic <i>S. aureus</i> +ve 10.8% <i>S. aureus</i> +ve, 5.4% toxigenic <i>S. aureus</i> +ve	NT	(Fagundes <i>et al.</i> 2010)
Raw milk (Brazil)	Y	Caprine	Bulk tank milk (96)	NT	NT	NT	NT	100% +ve for staphylococci, 2.7-7.5, mean 6.3	NT	(Oliveira <i>et al.</i> In Press)
Raw milk (Brazil)	Y	Not stated	Raw milk (140)	NT	NT	NT	NT	NT	19% of isolates, of those SEA (12.8%), SEB (7.3%), SEC (2.8%),	(Oliveira <i>et al.</i> 2011)

Type of milk/ cheese (country)	Raw milk product (Y/N)	Animal source of milk/cheese (number)	Sample tested (number)	Prevalence of enterotoxin /toxin genes	% isolates positive for at least one enterotoxin gene	Which enterotoxin genes/toxins	Toxin genes expressed in product (Y/N)	Concentration of coagulase-positive staphylococci (log ₁₀ CFU g ⁻¹ or ml ⁻¹)	Toxin produced	Reference
									SED (68.8%)	
Raw milk (Czech Republic)	Y	Bovine (298 dairy herds)	Bulk milk (440)		55.7% (39 isolates)	<i>sei</i> (38.6%), <i>seg</i> (31.4%), <i>sea</i> (27.1%), <i>seb</i> (10%), <i>seh</i> (4.3%), <i>sed</i> (2.9%), <i>sej</i> (2.9%), <i>sec</i> (1.4%)	NT	15.9% (70/440) SA +ve	12.9% of isolates (9/70): SEB (10%); SED (2.9%)	(Zouharova and Rysanek 2008)
Raw milk (Ethiopia)	Y	Camel	Milking vessel (35)	ND	NT	NT	NT	7.14% isolates tested (4/56)	NT	(Hadush <i>et al.</i> 2008)
Raw milk (Hungary)	Y	Bovine	Bulk milk tanks (20 farms)		27.1% isolates tested (16/59)	<i>sea</i> (6.8%) <i>seb</i> (8.5%) <i>sec</i> (6.8%) <i>sed</i> (3.3%) <i>seg</i> & <i>sei</i> (1.7%)	NT	0.9 –1.74 (mean) 70% farms positive (14/20)	NT	(Peles <i>et al.</i> 2007)
Raw milk (Italy)	Y	Not stated	Cheese milk (54)	NT	38.7% isolates tested (31/80)	<i>sea, sec, sed</i>	NT	45/54 (83%) <i>S. aureus</i> +ve	NT	(Bartolomeo li <i>et al.</i> 2009)
Raw milk (Italy)	Y	Not stated	Farm milk (13)	NT	NT	NT	NT	Mean 4.94 presumptive CPS, 54% <i>S. aureus</i> +ve, mean 3.67	NT	(Fusco <i>et al.</i> 2011)
Raw milk (Italy)	Y	No stated	Retail samples	NT	55.9 %	SEA (17), SEB (2), SEC (40), SED (6), SEA+SEB	NT	168/437 <i>S. aureus</i> +ve	NT	(Normanno <i>et al.</i> 2005)

Type of milk/ cheese (country)	Raw milk product (Y/N)	Animal source of milk/cheese (number)	Sample tested (number)	Prevalence of enterotoxin /toxin genes	% isolates positive for at least one enterotoxin gene	Which enterotoxin genes/toxins	Toxin genes expressed in product (Y/N)	Concentration of coagulase-positive staphylococci (log ₁₀ CFU g ⁻¹ or ml ⁻¹)	Toxin produced	Reference
						(2), SEA+SED (26), SEA+SEC (1)				
Raw milk (Korea)	Y	Bovine	Mastitic milk (not stated)	Not stated	77.7% isolates tested (34/44)	<i>seg, sei, selm, seln, selo</i> 36.4%, <i>seh</i> 34%, <i>sea, seh, selk, selq</i> 2.3%, <i>seb, selk, selq</i> 2.3%, <i>sea, seg, sei, selm, seln, selo</i> 2.3%, none 22.7%	NT	-	NT	(Hwang <i>et al.</i> 2010)
Raw milk (Korea)	Y	Bovine	Mastitic milk (714)		32% of isolates tested (57/179)	SEA, SEB, SEC, SED	NT	179 (25%) +ve for <i>S. aureus</i>	NT	(Moon <i>et al.</i> 2007)
Raw milk (Korea)	Y	Bovine	Milk collected on farm (30,019)	NT	NT	NT	NT	181 (0.6%) +ve CPS + <i>S. aureus</i> (NB enrichment not used)	NT	(Park <i>et al.</i> 2007)
Raw milk (Mongolia)	Y	Bovine (cattle and yak)	Milk (97)	23% (5/22 <i>S. aureus</i> isolates)	NT	SEC	NT	7/65 (10.7%) yak +ve, 15/32 (46.9%) cattle +ve	Y	(Tsegmed <i>et al.</i> 2007)
Raw milk (Poland)	Y	Bovine	Not stated	NT	35% of isolates tested (24/68)	<i>seh</i> (12.5%) , <i>seb/sek</i> (4%),	NT	NT	NT	(Bystron <i>et al.</i> 2009)

Type of milk/ cheese (country)	Raw milk product (Y/N)	Animal source of milk/cheese (number)	Sample tested (number)	Prevalence of enterotoxin /toxin genes	% isolates positive for at least one enterotoxin gene	Which enterotoxin genes/toxins	Toxin genes expressed in product (Y/N)	Concentration of coagulase-positive staphylococci (log ₁₀ CFU g ⁻¹ or ml ⁻¹)	Toxin produced	Reference
						<i>seg, sei, selm, seln, selo</i> (83.5%)				
Raw milk (Sudan)	Y	Cameline	Milk (320)	NT	3/25 (12%)	<i>sec, seg, sei, sem, sen, seo</i>	NT	28 (8.8%) <i>S. aureus</i> +ve	NT	(Shuiep <i>et al.</i> 2009)
Raw milk (Dar Es Salaam, Tanzania)	Y	Bovine	Raw milk (128)	ND	Not tested	NT	NT	<i>S. aureus</i> : 6.10 (mean). 6.3% samples positive (8/128)	NT	(Kivaria <i>et al.</i> 2006)
Raw milk (Trinidad)	Y	Bovine	Raw milk (322 samples)		14.4% isolates tested (24/168)	SEA, SEB, SEC, SED. SEA only was the most frequent (33% of isolates)	NT	37.2% isolates positive (340/915)	NT	(Adesiyun <i>et al.</i> 2007)
Farmstead (Vermont, USA)	Y	Bovine	Bulk milk (67)	27.4% <i>S. aureus</i> positive 18.4% <i>S. aureus</i> positive 85.7% <i>S. aureus</i> positive	NT	NT	NT	≤ 0 – 4.3	NT	(D' Amico <i>et al.</i> 2008)
		Caprine	Bulk milk (49)							
		Ovine	Bulk milk (22)							

Type of milk/ cheese (country)	Raw milk product (Y/N)	Animal source of milk/cheese (number)	Sample tested (number)	Prevalence of enterotoxin /toxin genes	% isolates positive for at least one enterotoxin gene	Which enterotoxin genes/toxins	Toxin genes expressed in product (Y/N)	Concentration of coagulase-positive staphylococci (log ₁₀ CFU g ⁻¹ or ml ⁻¹)	Toxin produced	Reference
Artisan cheeses (Vermont, USA)	Y	Bovine Caprine Ovine	Farm milk (45) Farm milk (25) Farm milk (15)	NT	NT	NT	NT	29% (13) <i>S. aureus</i> +ve 48% (12) <i>S. aureus</i> +ve 47% (7) <i>S. aureus</i> +ve	NT	(D'Amico and Donnelly 2010)
Cheese										
Arzua-Ulloa (Spain)	Y N N	Bovine	Conventional (57) Conventional (67) Organic (61)	NT	NT	NT	NT	83.9% <4, 10.7% 4-5, 5.4% >5 74.6% <2, 16.4% 2-3, 9.0% >3 82.0% <2, 4.9% 2-3, 13.1% >3	NT	(Miranda <i>et al.</i> 2009)
Carra (Turkey)	Y	Caprine	50 cheese samples	NT	NT	NT	NT	3.40 (mean), 80% <2, 12% 2-3, 8% 4-5	NT	(Aygün <i>et al.</i> 2005)
Colonial and American cheeses (Brazil)	Not stated	Not stated	72	NT	11/116 (12%)	<i>sed, see</i>	NT	19/45 (42%) cheese samples CPS +ve. 28% CPS +ve >3, 13% >5	NT	(Pelisser <i>et al.</i> 2009)

Type of milk/ cheese (country)	Raw milk product (Y/N)	Animal source of milk/cheese (number)	Sample tested (number)	Prevalence of enterotoxin /toxin genes	% isolates positive for at least one enterotoxin gene	Which enterotoxin genes/toxins	Toxin genes expressed in product (Y/N)	Concentration of coagulase-positive staphylococci (log ₁₀ CFU g ⁻¹ or ml ⁻¹)	Toxin produced	Reference
Cream, soft, semi-hard and hard cheese (various countries)	Y (raw and pasteurised)	Caprine (181)	Curd (cream cheese) (50) Soft cheese (62) Semi-hard cheese (56) Hard cheese (13)		9% (9/181) pasteurised enterotoxigenic (A-E production).	<i>sea</i> (0.5%) <i>sec</i> (2.2%) <i>seg</i> / <i>sei</i> (2.2%)	NT	2.95 – 5.11 (raw) 1.48 – 3.49 (past) 8.0 % SA +ve (raw and past) 1.72 – 2.68 (raw) 2.20 – 4.47 (past) 8.1% SA +ve (raw and past) 1.9 (raw) 4.51-5.93 (past) 5.4% SA +ve (raw and past) 1.70 (raw) 1.58 (past) 15.4% SA +ve (raw and past)	SEA, SEC (pasteurised milk)	(Akineden <i>et al.</i> 2008)
Farmstead cheeses (USA)	Y	Bovine (30) Caprine (2)	32 cheeses	NT	NT	NT	NT	2.0–4.2. 15% <i>S. aureus</i> +ve <2.0. 0% <i>S. aureus</i> +ve	NT	(Ramsey and Funk 2009)
Fresh and short time ripened (Sweden)	Y	Bovine (49) Caprine (94) Bovine + caprine (4)	55 raw milk cheese 96	ND	70%	<i>sea</i> , <i>sec</i> , <i>seg</i> , <i>sei</i> , <i>seh</i>	Yes	CPS found in 69% of raw milk cheese and 6% of cheeses made	NT	(Rosengren <i>et al.</i> 2010)

Type of milk/ cheese (country)	Raw milk product (Y/N)	Animal source of milk/cheese (number)	Sample tested (number)	Prevalence of enterotoxin /toxin genes	% isolates positive for at least one enterotoxin gene	Which enterotoxin genes/toxins	Toxin genes expressed in product (Y/N)	Concentration of coagulase-positive staphylococci (log ₁₀ CFU g ⁻¹ or ml ⁻¹)	Toxin produced	Reference
		Ovine (4)	pastuerised milk cheese					from pasteurised milk. Max 6.57. 38% SA in raw milk cheeses 6% SA in pasteurised milk cheeses		
Monte Veronese (Italy)	Y	Bovine	Mature curd (21) Ripened 1 month (16) Ripened 3 months (9)	95% 69% 78%	100% 100% 100%	<i>sea, seb, sec, sed, seg, seh, sei, sej, sek, sel, sem, seo, sep, ser</i>	ND	< 2 -5.6 <i>S. aureus</i>	4/4 (100%) <i>sea</i> +ve samples , 3/5 (60%) <i>seb</i> +ve samples, 3/5 (60%) <i>seb</i> +ve samples, 0/1 (0%) <i>sec</i> +ve sample	(Poli <i>et al.</i> 2007)
Portuguese	Y	Bovine (5) Ovine (49) Caprine (4)	Finished cheeses (70)	NT	NT	NT	NT	Unsatisfactory (2) 3.3-3.8, unacceptable (2) 5.30->5.21 Unsatisfactory (25) <0-3.9, unacceptable (9) 0->5.2 Unsatisfactory	NT	(Almeida <i>et al.</i> 2007)

Type of milk/ cheese (country)	Raw milk product (Y/N)	Animal source of milk/cheese (number)	Sample tested (number)	Prevalence of enterotoxin /toxin genes	% isolates positive for at least one enterotoxin gene	Which enterotoxin genes/toxins	Toxin genes expressed in product (Y/N)	Concentration of coagulase-positive staphylococci (log ₁₀ CFU g ⁻¹ or ml ⁻¹)	Toxin produced	Reference
		Caprine/ovine (10) Caprine/ovine/bovine (2)						(2) <0 Unsatisfactory (6) <0-3.9 Unsatisfactory (2) <0-3.0		
Semi hard and soft cheese from FP outbreaks (France)	Y	Bovine Ovine	13 cheese samples	85%	85%	Yes	NT	4.4 – 8.5	NT	16
Sheep milk cheeses	Y	Ovine	Cheese wheels (12) collected 6 h after moulding	NT	NT	NT	NT	3.9-5.8	NT	(Spanu <i>et al.</i> 2010)
Sheep milk cheeses	Not stated	Ovine	100 cheese samples	NT	11.7% (7/60) CPS isolates	<i>sea</i> (5), <i>seb</i> (2), <i>sed</i> (1) encoded by 7 CPS isolates	NT	60% <i>S. aureus</i> +ve; 35% 2-4, 25% 5-6	SEA (4), SEB (2) and SED (1) produced by 7 CPS isolates	(Ertas <i>et al.</i> 2010)
Soft cheese (Brazil)	Y	Not stated	50 cheese samples	NT	NT	NT	NT	17 (30.9%) CPS>4	NT	(Moraes <i>et al.</i> 2009)

Type of milk/ cheese (country)	Raw milk product (Y/N)	Animal source of milk/cheese (number)	Sample tested (number)	Prevalence of enterotoxin /toxin genes	% isolates positive for at least one enterotoxin gene	Which enterotoxin genes/toxins	Toxin genes expressed in product (Y/N)	Concentration of coagulase-positive staphylococci (log ₁₀ CFU g ⁻¹ or ml ⁻¹)	Toxin produced	Reference
Soft cheese (Italy)	Y	Not stated	9 cheese samples	33% by direct DNA isolation 88.9% by DNA isolated from first dilution	NT	<i>sed</i> (67%), <i>sej</i> (67%), <i>sea</i> (100%) of positives <i>sea</i> (86%), <i>sed</i> (57%), <i>sej</i> (57%) <i>sec</i> (43%), <i>sel</i> (43%), <i>seg</i> (43%), <i>sea</i> (29%) of positives	NT	NT	NT	(Bernini <i>et al.</i> 2010)
Soft and semi-hard cheese (various countries)	Y (raw and thermised)	Bovine (1071), ovine (346), caprine (156), other or unknown (246)	Fresh (soft) unripened (62) Ripened soft (806) Semi-hard (951)		31% (4/13)	<i>sed</i> , <i>seg</i> , <i>seh</i> , <i>sei</i>	NT	13 samples were deemed unsatisfactory : 5.2 –7.0	NT	(Little <i>et al.</i> 2008)
Terrincho cheese (Portugal)	Y	Ovine (5 batches)	10 cheeses (2 per batch)	ND	NT	NT	NT	4.13 –5.44 (not specified as <i>S. aureus</i>)	NT	(Pintado <i>et al.</i> 2008)
Urfa cheese (Turkey)	Y	Bovine, ovine or a mixture of both (11 cheeses)	Cheese (11)	NT	NT	NT	NT	27% (3/11) cheeses <i>S. aureus</i> +ve	NT	(Uraz <i>et al.</i> 2008)

Type of milk/ cheese (country)	Raw milk product (Y/N)	Animal source of milk/cheese (number)	Sample tested (number)	Prevalence of enterotoxin /toxin genes	% isolates positive for at least one enterotoxin gene	Which enterotoxin genes/toxins	Toxin genes expressed in product (Y/N)	Concentration of coagulase-positive staphylococci (log ₁₀ CFU g ⁻¹ or ml ⁻¹)	Toxin produced	Reference
Mixed Products										
Canastra cheese (Brazil)	Y	Bovine (10 farms)	Raw milk Natural starter Curds Cheese 5 days ripening Udder (34)	100% (3/3) 100% (5/5) 92% (12/13) 90% (9/10)	93.3% of isolates tested (70/75)	SEA, SEB, SEC, SED	NT	<2 –4.6 <2 –5.7 4.3 –6.3 <2 –6.3 31.82% samples tested (7/56)	NT	(Borelli <i>et al.</i> 2006)
Dairy products (Brazil)	Not stated	Dairy product isolates (30)	CNS (15), CPS (15)	NT	21/30 (70%)	38% <i>sea</i> , 29% <i>seb</i> , 24% both	NT	NT	Y, in most cases according to the genetic composition	(Veras <i>et al.</i> 2008)
Dairy products (Turkey)	Y Not stated	Not stated Not stated	Milk (36) Cheese (118)	NT	NT	NT	NT	27.9% +ve <i>S. aureus</i> , 26.3% +ve toxigenic <i>S. aureus</i> 33.3% +ve <i>S. aureus</i> , 25% +ve toxigenic <i>S.</i>	SEA, SED, SEC/D SED, SEC/D	(Güven <i>et al.</i> 2010)

Type of milk/ cheese (country)	Raw milk product (Y/N)	Animal source of milk/cheese (number)	Sample tested (number)	Prevalence of enterotoxin /toxin genes	% isolates positive for at least one enterotoxin gene	Which enterotoxin genes/toxins	Toxin genes expressed in product (Y/N)	Concentration of coagulase-positive staphylococci (log ₁₀ CFU g ⁻¹ or ml ⁻¹)	Toxin produced	Reference
								<i>aureus</i>		
Dairy products (Turkey)	Y	Not stated	Raw milk	NT	31/63 (49%)	<i>sea</i> (9.7%), <i>sec</i> 22.6%), <i>seh</i> (3.2%), <i>seo</i> (3.2%), <i>sep</i> (13%), <i>sea/sec</i> (3.2%), <i>sea/seh</i> (3.2%), <i>sel/sep</i> (6.5%), <i>sem/seo</i> (3.2%), >2 genes 29.%	NT	Not stated	SEA, SEB, SEC, SED	(Aydin <i>et al.</i> In Press)
	Not stated	Not stated	Dairy products		36/54 (67%)	<i>sea</i> (11.1%), <i>sec</i> 8.3%), <i>seh</i> (8.3%), <i>sen</i> (2.8%), <i>seo</i> (5.5%), <i>sep</i> (16.6%), <i>seu</i> (2.8%) <i>sea/sec</i>				

Type of milk/ cheese (country)	Raw milk product (Y/N)	Animal source of milk/cheese (number)	Sample tested (number)	Prevalence of enterotoxin /toxin genes	% isolates positive for at least one enterotoxin gene	Which enterotoxin genes/toxins	Toxin genes expressed in product (Y/N)	Concentration of coagulase-positive staphylococci (log ₁₀ CFU g ⁻¹ or ml ⁻¹)	Toxin produced	Reference
						(2.8%), <i>sea/seq</i> (2.8%), <i>seb/sec</i> (2.8%), <i>seb/sep</i> (2.8%), <i>seg, sei</i> (2.8%), >2 genes 30.5%				
Fiore Sardo hard cheese (Sardinia)	Y	Ovine (12 batches)	Milk 48h 1 month 3 months 6 months 9 months	NT	NT	NT	NT	4.18 4.94 3.21 1.23 <D <D	NT	(Pisano <i>et al.</i> 2006)
Iben and Jben (Morocco)	Y	Not stated	Milk and milk product CPS isolates (81)	NT	39% (18/46) enterotoxin +ve, 65% (30/47) SE gene +ve	<i>sea, seb, sec, sed, seh</i> SEA, SEB, SEC, SED	NT	NT	NT	(Bendahou <i>et al.</i> 2009)

Type of milk/ cheese (country)	Raw milk product (Y/N)	Animal source of milk/cheese (number)	Sample tested (number)	Prevalence of enterotoxin /toxin genes	% isolates positive for at least one enterotoxin gene	Which enterotoxin genes/toxins	Toxin genes expressed in product (Y/N)	Concentration of coagulase-positive staphylococci (log ₁₀ CFU g ⁻¹ or ml ⁻¹)	Toxin produced	Reference
Raw milk, Minas Frescal soft cheese (Brazil)	Y	Bovine	Milk (24) Cheese (24)	NT	NT	NT	NT	<1.0 –5.8. 66.7% (16/24) SA +ve <1.0 –5.5. 70.8% (17/24) SA +ve	NT	(Andre <i>et al.</i> 2008)
Minas Frescal (Brazil)	N	Bovine (70)	Mastitic cow's milk (125) Bulk tank milk (96) Cheese (70)	13.6% 41.7% 72.9%	37.5% (109/291)	<i>sea, seb, sec, sed, seg, seh, sei, selj, sell</i>	NT	NT	NT	(Arcuri <i>et al.</i> 2010)

Type of milk/ cheese (country)	Raw milk product (Y/N)	Animal source of milk/cheese (number)	Sample tested (number)	Prevalence of enterotoxin /toxin genes	% isolates positive for at least one enterotoxin gene	Which enterotoxin genes/toxins	Toxin genes expressed in product (Y/N)	Concentration of coagulase-positive staphylococci (log ₁₀ CFU g ⁻¹ or ml ⁻¹)	Toxin produced	Reference
Norwegian cheese	Y	Bovine (73)	Bovine bulk milk	ND	NT	NT	NT	0.1 –2.02. 47.2% SA +ve	NT	(Jakobsen <i>et al.</i> 2011)
			Bovine curd 2-3 hr					0.74 –3.53. 73.6% SA +ve		
			Bovine press 24 hr					0.85 –3.44. 76.7% SA +ve		
			Bovine press 30 days					0.1 –1.58. 24.7% SA +ve		
		Caprine (49)	Caprine bulk milk					1.46 –3.75. 91.8% SA +ve		
			Caprine curd 2-3 hr					1.66 –5.41. 91.8% SA +ve		
			Caprine press 24 hr					2.34 –5.12. 95.9% SA +ve		
			Caprine press 30 days					0.1 –2.77. 42.9% SA +ve		
Soft, semi-hard and hard (Norway)	Y	Bovine (10), Caprine (14), Cervine (2)	Bovine bulk milk (10)	40% (4/10)	8.8% (34/386)	<i>sec, seb. seg, sei</i>	NT	NT	NT	(Loncarevic <i>et al.</i> 2005)
			Caprine bulk milk (8)	75% (6/8)						

Type of milk/ cheese (country)	Raw milk product (Y/N)	Animal source of milk/cheese (number)	Sample tested (number)	Prevalence of enterotoxin /toxin genes	% isolates positive for at least one enterotoxin gene	Which enterotoxin genes/toxins	Toxin genes expressed in product (Y/N)	Concentration of coagulase-positive staphylococci (log ₁₀ CFU g ⁻¹ or ml ⁻¹)	Toxin produced	Reference
			Cheese (26)	92% (24/26)						
White cheese (Brazil)	Not stated	Not stated	10	0%	0 (1 isolate of CNS <i>S. warneri</i>)	-	NT	6.1 (CNS)	NT	(Da Cunha <i>et al.</i> 2006)

NT = Not tested. <D Below the limit of detection. CNS coagulase negative staphylococci.

APPENDIX 2. BEHAVIOUR OF *S. AUREUS* IN VARIOUS CHEESES DURING MANUFACTURE AND RIPENING.

Type of cheese	Temperature/other characteristics	Change in concentration (log ₁₀)		Enterotoxin production	Reference
		Manufacture	Ripening		
Afuega'l Pitu	pH 4.2-4.3, NaCl <0.5%, 12°C	-	↓ in 14 d with nisin + starter, ↑ 1 to day 1 then ↓ 3 to day 14 with nisin – starter	NT	(Rilla <i>et al.</i> 2004)
Blue	pH 5.2-5.4 at end of hooping	↑ 1-3 to 24 h	-	Not detected	(Tatini <i>et al.</i> 1973)
Brick	pH 4.9 at end of hooping	↑ 1-2 to 24 h	-	Detected in some samples	(Tatini <i>et al.</i> 1973)
Burgos	pH 6.7 (curd) reducing little.	↑ 0.5 to curd	4°C: Little change days 4-7 10°C: ↑ 0.1-1.2 days 1-7 15°C: ↑ 1.8-3.7 days 1-7	Not detected even at >8	(Otero <i>et al.</i> 1988)
Camembert	pH surface 6.35 (curd) to 7.33 day 41. pH interior 6.35 (curd) to 6.75 day 41. Ripened at 4°C.	↑ 3 in 22 h then	↓ 1 max to 42 days	None when inoculum ≤ 3, present when inoculum >3	(Meyrand <i>et al.</i> 1998)
Camembert	-	-	-	Toxin detected when >6	(Muller <i>et al.</i> 1996)
Cheddar	12°C	"No significant multiplication"	↓ 4 over 112 days of ripening	NT	(Bautista and Kroll 1988)
Cheddar	pH 5.0, temperature not stated.	↑ 3 to curd at milling stage	↓ 3-6 over next 24 weeks	NT	(Reiter <i>et al.</i> 1964)
Cheddar	4 and 11°C, pH started at 6.2 and reduced to 5.6.	↑ 3-4 from inoculum to end of cheddaring	No real change to ↓ 2 over 42 depending on temperatures and starter	Toxin detected in salted cheeses stored at 11°C	(Ibrahim <i>et al.</i> 1981a)
Cheddar	Cheese making conditions not supplied in detail. Ripening at: 7.2 10 12.8°C	↑ >1 in 26 h (curd after pressing)	↑ <1 2-3 weeks, ↓ 1 weeks 3 to 26 ↓ 1 weeks 2 to 26 ↓ 1 weeks 2 to 26	NT	(Tuckey <i>et al.</i> 1964)
Cream cheese, fresh	pH 5.2 to 4.8 over 21 days, 4°C.	-	↑ 0.6 to day 1 then static	NT	(Buriti <i>et al.</i> 2007)

Type of cheese	Temperature/other characteristics	Change in concentration (log ₁₀)		Enterotoxin production	Reference
			(<i>Staphylococcus</i> sp.)		
Cottage (long set)	Involves 22°C x 11 h and cooking at 54°C for 30 min steps. pH 4.68 in curd after washing.	↓ 1	static during storage to 12 days	NT	(Tuckey <i>et al.</i> 1964)
Cottage (short set)	Involves 30°C x 4 h and cooking at 54°C for 30 min steps. pH 4.60 in curd after washing.	↓ >2 during manufacture	↑ > 2 during storage to 10 days	NT	(Tuckey <i>et al.</i> 1964)
Cuajada	10°C	-	↑ 2.7 days 0 to 3, ↑ 1.6 day 3 to day 9, ↓ 0.3 to day 12	NT	(Arques <i>et al.</i> 2008)
Domiat	Stored at 30°C for 28 d. NaCl increased to up to 7.4% (WP).	↑ 3 raw milk to finished cheese	↓ 6 day 0 to day 28, except in highly salted cheese where ↑ 3 to finished cheese then ↓ 3 to day 28	Not detected	(Ahmed <i>et al.</i> 1983)
Emmentaler	Curds cooked 53°C for 45 min. Ripened 90 days at 11-13°C. pH 5.3-5.6.	↓ 0.5 milk to curd after cooking ↓ 5 from curd after cooking to 1 day	-	Not detected	(Bachmann and Spahr 1995)
Feta	pH 6.4, around 6.5% salt on day 0; pH 5.23-6.99, 6.98-7.84% salt at day 75 depending on use of starter and brining conditions. Ripened at 4°C.	Initial ↑ 2 in 7 h (milk to curd)	↓ 3 max 7 h to 75 days	NT	(Erkmen 1995)
Goats' milk lactic	21 h at 24°C during coagulation, 21 h at 22°C during draining, chilled 4°C, brined 13-14°C for 3d, matured 13-14°C for 12 d, packed and stored at 4°C. pH curd 4.5 rising to >5.5, WPS 1.2% rising to 2.2%	Initial ↑ 1.4 in 24 h	↓ 5 over 21-35 d	ND with inoculum = 4, +ve at day 12 with inoculum = 5, +ve day 5 with inoculum = 6	(Vernozy-Rozand <i>et al.</i> 1998)
Gouda	Cheesemaking conditions not given.	3-7 generations in pasteurised	-	Tested at 24 h, detected in	(van Schouwenburg-

Type of cheese	Temperature/other characteristics	Change in concentration (log ₁₀)		Enterotoxin production	Reference
	pH at 24 h 5.1-5.2.	milk, 2-11.2 generations in raw milk		1 sample at 7.5, in others at >8	van Foeken <i>et al.</i> 1979)
Herby	Ripened at 9°C, initial temperature 32°C Pasteurised milk: pH reduced 6.5 to 4.4 to curd stage, stable to 90d ripening. NaCl increased to 6% Raw milk: pH reduced 5.1 to 4.8 to curd stage, increased to 5.2-6.2 to 90 d ripening. NaCl increased to 6-6.5%	↑ 1 milk to curd, ↑ 1.5-2 curd to day 1 ↑ 2 milk to curd, static to day 1	↓ 4 day 1 to day 90 ↓ 1.5 day 1 to day 90	Toxin detected from coagulum onwards for SEA, others at later stages Not detected	(Akkaya and Sancak 2007)
High moisture Jack	Ripened at 4.5°C	Initial ↑ up to 9 to salting	Static then to 183 days	NT	(Eckner and Zottola 1991)
Imitation	26°C, a _w 0.942-0.973, pH 5.53-6.14	-	↑ up to 3 in 12 days, but NG in one sample	Detected in some but not others.	(Bennett and Amos 1983)
Jben	Room temperature, pH 6.1 to 4.1 in 96 h	Initial ↑ 1.7 in 24 h (milk to coagulum). ↓ 3.3->5.4 to 96 h (coagulum to cheese)	-	SEC detected at inoculum 10 ⁵ /g 24 h after inoculation, not at 10 ³	(Hamama <i>et al.</i> 2002)
Limburger	Cheese making conditions not supplied in detail. pH 5.2-5.3 after manufacture rising to 7.6 (exterior) after 3 weeks at 10°C	↑ >1 to end of manufacture	↓ 1 weeks 3 to 15	NT	(Tuckey <i>et al.</i> 1964)
Manchego	Ripening at 5 10 15 20°C	-	day 60 vs day 1 ↓ 3 ↓ 4 ↓ 4 ↓ 5	NT	(Gaya <i>et al.</i> 1988)
Manchego	Ripened at 15°C, pH 5	-	Varied between strains. Some no change others ↓ 6 to 60 days	Some strains no enterotoxin, others from day 24 onwards	(Gomez-Lucia <i>et al.</i> 1992)

Type of cheese	Temperature/other characteristics	Change in concentration (log ₁₀)		Enterotoxin production	Reference
Manchego	Ripened at 15°C, pH 5-5.5.	-	Varied according to starter conc ⁿ . 0.1% NG weeks 1-7 then ↓ 6 over next 10d. 1% starter NG weeks 1-3 then ↓ 6 over next 5 weeks	SEB not detected, SEA detected at 3.5 weeks	(Gomez-Lucia <i>et al.</i> 1986)
Manchego	Ripened at 10-12°C. pH 6.74 in curd, 5.2 after 90 days ripening	↑ 1.5 curd to pressing	↓ 2.8-→4.8 to day 90 depending on starter	Not detected	(Otero <i>et al.</i> 1993)
Minas	Ripened at 14-16°C. pH reduced around 0.2 over 21 d to 5.2 – 5.6, salt increased from 8.5 to 9.5-10% over 21 d	Varied by isolate, some no growth, others ↑ 2 to day 0 of ripening	Generally ↑ from day 0 to day 14 then static	SEA, B and C detected in some cheeses made from pasteurised milk, not raw milk	(dos Santos and Genigeorgis 1981)
Minas Serro	At day 14, pH = 5.01, a _w = 0.92, temp 30°C At day 60, pH = 5.22, a _w = 0.83, temp 30°C	↑ slightly from milk to curd	↑ 0.5 between days 0 and 14, ↓ 4 between days 14 and 60	NT	(Pinto <i>et al.</i> 2011)
Montiaso	Ripened 90 days at 12°C. pH 6.2 to 5.2 in 3 days then back to 5.4-5.6 for up to 90d.	↑ 1 to curd cutting (entrapment?)	Static	Not detected	(Stecchini <i>et al.</i> 1991)
Mozzarella	pH 5.2 out of brine	↑ 1 to 24 h	-	Not detected	(Tatini <i>et al.</i> 1973)
Not specified	pH stable at 4.9-5.0 at 12°C	-	↓ 0.18 between days 4 and 30	NT	(Rodríguez <i>et al.</i> 2005)
Prato	pH 6.8, 12°C	-	<2 throughout storage	NT	(Vianna <i>et al.</i> 2008)
Processed	30°C, pH 5.6-5.7, 6.0-6.5% salt (WP)	↓ <1 in 96 h		NT	(Glass <i>et al.</i> 1998)
Semi-hard	Ripened 12°C for 60 d. pH reduced from 6 to 5.	↑ 1 in 8 h	↓ 1 between days 1 and 60	NT	(Rodríguez <i>et al.</i> 2000)
Semi-hard (Saint Nectaire and Salers)	pH 6.7 reducing to 5.2 on day 1.	Similar for all. ↑ 4 in first 6 h	↓ 4 between days 1 and 150	Detected in two Salers cheeses below quantification limit at 5.55 and 5.06.	(Delbes <i>et al.</i> 2006)
Smear ripened cheeses	pH 6.7-4.7 during manufacture. Surface pH increased to 6.5 during 42 d ripening. NaCl 4.8% (WP) after	↑ 2-3 during manufacture	Reduced in core, static on surface	NT	(Maher and Murphy 2000)

Type of cheese	Temperature/other characteristics	Change in concentration (log ₁₀)		Enterotoxin production	Reference
	ripening. Ripened 12-15°C for 12-12 days then stored at 4°C.				
Soft	pH 4.6-4.8, a _w = 0.98-0.99, NaCl = 2%	↓ 3 by pasteurisation (72°C x 15 sec), ↑ 2 from post-pasteurisation to salting, ↓ 1 after 24h at 4°C	Static	SEA produced with a high inoculum and when reaching 5.6	(Necidová <i>et al.</i> 2009)
Swiss	Cheese making conditions not supplied in detail. Final pH 5.3 Ripening at 10°C	↑ >1 during manufacture,	↑ >2 to week 2, ↓ 3 to week 30	NT	(Tuckey <i>et al.</i> 1964)
Swiss	pH 5.2-5.4 at end of hooping	↑ 1-2 to 24 h	-	Detected in some samples	(Tatini <i>et al.</i> 1973)
Sürk	pH 4.1-4.2, a _w 0.98 over 30 d at room temperature, aerobic pH 4.1-5.4, a _w 0.98-0.96 over 30d at room temperature, aerobic	-	↓ 4 between days 0 and 5 then increase and further reduction to ↓ 6 by day 30 ↓ 4 between days 0 and 5, stayed low	NT	(Masatcioğlu and Avşar 2005)
Tilsiter	Curds cooked 42°C for 15 min. Ripened 90 days at 11-13°C. pH 5.3-5.6.	↑ 1 from raw milk to cheese at 1 d	Static to 7d then ↓ 5.5 to day 60	Not detected	(Bachmann and Spahr 1995)
Urfa	Ripened in brine 90 days, 6°C. Final salt conc ⁿ 5-8%.	-	↓ 1-2 between days 0 and 7 then ↑ 1 7-30 days then static to day 90	NT	(Ozer <i>et al.</i> 2004)
White pickled cheese	Rapid pH reduction 6.5 to 4.5 over 4 days then stable	-	↓ 5 within 30 days	NT	(Abdalla <i>et al.</i> 1993)

NT= Not tested

APPENDIX 3. RAW DATA USED TO ASSESS THE GROWTH/NO GROWTH BOUNDARY IN CHEESE

Cheese	Reference	T (°C)	pH	NaCl (WPS)	Growth/no growth
Pickled white	Abdalla et al 1993	4	4.6	4	Death
Goats	Vernozy-Rozand 1998	4	4.7	2.3	Death
Cheddar	Ibrahim et al., 1981	4	5.3	0.5	Death
Feta	Erkman 1995	4	5.32	6.5	Death
Goats	Vernozy-Rozand 1998	4	5.5	2.4	Death
Pickled white	Abdalla et al 1993	4	5.7	4	No growth
Feta	Erkman 1995	4	6.27	7.4	Death
Cheddar	Ibrahim et al., 1981	4	6.29	4.4	Death
Burgos	Otero et al 1988	4	6.58	0.15	Death
Burgos	Otero et al 1988	4	6.67	0.15	Death
Burgos	Otero et al 1988	4	6.67	0.15	No growth
Soft cheese	Necidova et al 2009	4	4.6-4.8	NA	No growth
Manchego	Gaya et al., 1988	5	5.1	0.5	Death
Pickled white	Ozer et al., 2004	6	4.9	6	No growth
Soft cheese	Necidova et al 2009	8	4.6-4.8	NA	No growth
Herby	Akkaya and Sancak 2007	9	4.6	NA	No growth
Herby	Akkaya and Sancak 2007	9	4.3-5	NA	No growth then decline
Manchego	Gaya et al., 1988	10	5.1	0.5	Death
Swiss	Tuckey et al 1964	10	5.3	NA	Growth then death
Burgos	Otero et al 1988	10	6.44	0.15	Growth
Burgos	Otero et al 1988	10	6.47	0.15	Growth
Burgos	Otero et al 1988	10	6.56	0.15	Death
Burgos	Otero et al 1988	10	6.62	0.15	Growth
Cheddar	Ibrahim et al., 1981	11	5.3	0.5	Death
Cheddar	Ibrahim et al., 1981	11	6.29	4.4	No growth
Camembert	Meyrand et al 1998	11	6.89	1.7	No growth
Afuega'l Pitu	Rilla et al 2004	12	4.2	1.06	Death
cows milk semi hard	Arques et al 2005	12	4.6	NA	slow death
cows	Roderiguez et al 2000	12	4.9	NA	Death
generic cheese	Roderiguez et al 2005	12	4.9	NA	slow death
Manchego	Otero et al., 1993	12	5.2	0.5	Death
Montasio	Stecchini et al 1991	12	5.5	NA	No growth
Goats	Vernozy-Rozand 1998	14	4.55	1.9	Death
Goats	Vernozy-Rozand 1998	14	5.1	1.9	Death
Manchego	Gaya et al., 1988	15	5.15	0.5	Death
Burgos	Otero et al 1988	15	5.65	0.15	Growth
Burgos	Otero et al 1988	15	6.02	0.15	Growth
Burgos	Otero et al 1988	15	6.49	0.15	Growth
Burgos	Otero et al 1988	15	6.62	0.15	Growth
Manchego	Gomez-Lucia et al 1986	15	5 – 5.5	NA	Initial growth
Minas	Genigeorgis, 1981	16	5.4	7.5	Death
Surk	Masatcioglu et al 2005	20	3.9	1	Death
Surk	Masatcioglu et al 2005	20	4	1	Death
Surk	Masatcioglu et al 2005	20	4.6	1	No growth
Jben	Hamama et al 2002	20	4.7	1	Death

Manchego semi hard cows	Gaya et al., 1988	20	5.2	0.5	Death
Surk	Rodriguez et al 2000	20	5.2	NA	No growth
Domati	Masatcioglu et al 2005	20	5.4	1.9	Growth
Minas Serro	Ahmed et al., 1983	30	4.7	4	death
Domati	Pinto et al 2011	30	4.85	NA	No growth
Domati	Ahmed et al., 1983	30	5	1.1	Growth
Domati	Ahmed et al., 1983	30	5.5	6.2	Death
Processed cheese	Glass et al 1998	30	5.74	6.29	No growth
Domati	Ahmed et al., 1983	30	5.8	2.4	Growth
Domati	Ahmed et al., 1983	30	6	4.8	Death

WPS = water phase salt

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