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**OPINION OF THE
SCIENTIFIC COMMITTEE ON VETERINARY MEASURES RELATING TO
PUBLIC HEALTH**

ON

**STAPHYLOCOCCAL ENTEROTOXINS IN MILK PRODUCTS,
PARTICULARLY CHEESES**

(adopted on 26-27 March 2003)

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1. EXECUTIVE SUMMARY

Staphylococcal foodborne intoxication, in which major symptoms are vomiting and diarrhoea, occurs after ingestion of thermostable staphylococcal enterotoxins (SE) produced in food by enterotoxinogenic strains of coagulase-positive staphylococci mainly *S. aureus*. Staphylococcal foodborne intoxication is reported to be one of the most common bacterial foodborne outbreak in many countries. Milk and dairy products constitute 1 – 9 % (mean 4.8 %) of all *S. aureus* outbreaks in Europe.

SE are normally no or only slightly, inactivated during food processing, storage, distribution or during the preparation of the food in the kitchen. Therefore, if enterotoxinogenic staphylococci are able to grow in food to high numbers (more than 10^5 to 10^6 cfu/g or /ml) before they are killed there is still a risk for intoxication with consumption.

The currently available methods for SE detection have been developed according to three principles: ELISA (enzyme linked immunosorbent assay), ELFA (enzyme linked fluorescent assay) or RPLA (reverse passive latex agglutination). False-positive results may occur. One possible option is to screen food for thermostable thermonuclease (TNase) as an indicator of staphylococcal growth to high levels and after positive TNase result, to test food for SE. During early phase of cheese production *S. aureus* may grow and produce SE. However, the counts of *S. aureus* usually decrease in later stages of manufacturing and give little information on the possibility of presence of SE if cheese is then tested for *S. aureus* counts. Since SE are more stable compared to *S. aureus* bacterial cells, it is possible to test a product with negative results for *S. aureus* counts although SE exists in the products.

Based on the information on *S. aureus* growth and SE formation in the production of milk and different dairy products, on epidemiological evidence as well as on the methods currently available for *S. aureus* and SE detection, the microbiological criteria for *S. aureus* counts and/or SE in milk and in certain dairy products are essential and useful to protect public health. As far as the current criteria are concerned, the use of criteria for raw milk intended for direct human consumption, fresh cheese made from heat treated milk as well as for frozen milk-based products are valid.

The Committee recommends to establish a reporting system for foodborne staphylococcal intoxications in all Member States. Furthermore, SE risks in raw milk cheeses should be minimised by using GHP and proper starter cultures. The current criteria for cheeses made from raw and thermised milk and for soft cheese made from heat treated milk should be revised. In this, as well as for semi-hard and hard cheeses, the criteria should include both counts of *S. aureus* and detection of enterotoxin in cheese alone or after the detection of TNase. In addition, the current criteria for raw milk intended for processing as well as the criteria for powdered milk should be revised and the current criteria of fresh cheese applied also for whey cheese. The application of criteria for *S. aureus* or SE in cultured dairy products, pasta filata cheeses as well as in processed cheese is not needed. The Committee also suggests to reconsider the current criteria, which refer to *S. aureus*, to cover all the coagulase positive staphylococci.

2. BACKGROUND

Staphylococcal foodborne intoxication is, in many countries, a common cause of bacterial foodborne outbreaks. The hazard to public health is particularly linked to the ability of *Staphylococcus aureus* to produce thermostable enterotoxins. Milk products are involved in many of these outbreaks. The contamination of these products can be attributed to occurrence of coagulase-positive staphylococci in raw material or handling during the manufacturing process.

The Community legislation in force for milk and milk products (Council Directive 92/46/EEC) lays down criteria for *S. aureus* in raw milk, cheeses, milk powder and frozen milk products. However, the number of *S. aureus* may not always be a good indicator for the presence of staphylococcal enterotoxins in the product. Not all *S. aureus* strains produce enterotoxins, and the number of *S. aureus* cells may have already decreased although the product still contains enterotoxins.

In recent years the methodology for detection of staphylococcal enterotoxins has improved. Three types of methods can be used to detect such a contaminant in food: bioassays, molecular biology and immunological tools.

The Community legislation on food hygiene is currently under revision. Proposals for a recast of this hygiene legislation have been submitted to the Council and the European Parliament. In this context the Commission has also started a revision of the microbiological criteria in Community legislation and a comprehensive strategy to set these criteria is being prepared. This strategy would cover all foodstuffs as well as the whole production and distribution chain (including retail trade) in line with the proposed new hygiene legislation. Criteria would be set for food products on the market as well as for products at different stages of the manufacturing process.

3. TERMS OF REFERENCES

The Scientific Committee on Veterinary Measures relating to Public Health is asked, with regard to *Staphylococcus aureus* and its enterotoxin in milk products, particularly cheeses, to:

- review the epidemiology of *S. aureus* intoxications;
- describe the physico-chemical or other factors influencing the production of the enterotoxins by *S. aureus*;
- identify the points during the manufacturing process where conditions may favour enterotoxins production by *S. aureus*;
- examine the correlation between the production of enterotoxins and the *S. aureus* counts at different stages of the manufacturing processes;
- evaluate whether current criteria for *S. aureus* are valid and may be used as reliable indicators for the possible presence of the enterotoxins or for the risk of toxin production;

- identify if new criteria for *S. aureus* and/or tests for its enterotoxins are needed for milk products, especially cheeses.
- evaluate the analytical methods to detect enterotoxins in milk products.

4. INTRODUCTION

Unpasteurized milk and cheese are typical dairy products associated with foodborne outbreaks caused by staphylococcal enterotoxins (SE). The symptoms for SE intoxication include nausea, vomiting, abdominal pain and diarrhoea. Sometimes, headache and drop of blood pressure may also occur.

S. aureus are Gram positive, facultatively anaerobic, cocci with moderate nutritional requirements. During processing and storage, temperatures outside the range of 7-48° C prevent the growth of *S. aureus*. However, the strains are usually very tolerant to NaCl and grow well up to the 10% NaCl concentration and even up to 20% concentration the growth is possible, although retarded.

SE are heat-stable proteins produced by many strains of *S. aureus* and by some other coagulase positive staphylococci such as strains of *Staphylococcus intermedius* and *Staphylococcus hyicus*. In order to cause a foodborne intoxication, staphylococci must be able both to grow and produce enterotoxins. Enterotoxin production is possible over a slightly more limited range of conditions than growth. Depending on many factors, like type of food, pH, temperature, water activity, atmospheric conditions and presence of other microorganisms, different amounts of SE may be produced in food.

Currently, there are microbiological criteria for *S. aureus* in milk and dairy products in Community legislation. Depending on food category, the higher limits (M) range from 10² to 10⁴ colony forming unit (cfu)/g for cheeses made from raw milk (see Annex 1). Only two of the criteria (soft cheese and cheese made from raw milk and from thermised milk) take account of SE production of strains isolated from these products whereas no criteria are set on SE detection in milk or dairy products.

The following chapters review the current knowledge on staphylococcal enterotoxins in dairy products, particularly cheeses, focussing on epidemiology and enterotoxin production in processing systems. In addition, existing criteria and methods for *S. aureus* and its enterotoxins will be evaluated. The classification of cheese into different groups differs according to the purpose of classification. To identify processing conditions favouring SE production, the following groups of cheese have been evaluated: fresh, soft, semi-hard and hard cheeses, some divided into subcategories. In addition pasta filata, whey, processed and imitation cheeses, as well as liquid milk, non-fat dry milk, cultured dairy products, cream and butter and ice-cream) are discussed.

In the terms of reference, the Committee interprets “to identify if new criteria for *S. aureus* and/or tests for its enterotoxins are needed for milk products, especially cheeses”, either as whether the establishment of new criteria will contribute meaningfully to a reduction of the public health risk posed by the particular milk or dairy product or as how the current criteria should be changed in order to provide better protection to public health.

5. EPIDEMIOLOGY OF FOODBORNE *S. AUREUS* INTOXICATIONS

Staphylococcus aureus is a spherical bacterium (coccus) which on microscopic examination appears in pairs, short chains, grape-like clusters. These organisms are Gram-positive and catalase-positive. These bacteria are aero-anaerobic facultative, sensitive to antibiotics such as lysostaphine and furans and resistant to bacitracine. Today, 35 species of staphylococci have been described according to their potential to produce coagulase. Staphylococci are ubiquitous in the environment and exist in air, dust, sewage, water, environmental surfaces, humans and animals.

5.1. Foodborne intoxication

Staphylococcal foodborne intoxication is one of the most common form of bacterial foodborne disease in many countries (Balaban and Rasooly, 2000). This type of foodborne intoxication, which major symptoms are vomiting and diarrhoea, occurs within 30 min to 8 h after ingestion of heat stable staphylococcal enterotoxins (SEs) preformed in food by enterotoxinogenic strains of coagulase-positive staphylococci (CPS), mainly *S. aureus*.

As notification of foodborne diseases is based on spontaneous reporting by local authority, severe cases resulting in hospitalisation are more reported than milder diseases such as staphylococcal intoxications. Under-reporting of *S. aureus* family outbreaks could be even greater because they are not as severe as *Salmonella* spp outbreaks and most of the patients do not visit a physician.

Except for France and the USA no data are available for the staphylococcal foodborne intoxication hospitalisation rate. This rate is 15% and 18% in reported *S aureus* cases for France (Haeghebaert *et al.*, 2002) and the USA (Mead *et al.*, 1999) respectively. The hospitalisation rate for all cases has been estimated to be 1% in the USA (Mead *et al.*, 1999). However, there is an under reported numbers of cases as only 1% were the estimated cases in the USA (Mead *et. al.*, 1999).

Milk products, as well as other products with a high protein content, are a good substrate for growth of *S. aureus*. Such products are involved in foodborne diseases due to:

- the occurrence of coagulase-positive staphylococci in raw milk;
- cross-contamination during the process;
- the possible cross-contamination thereafter.

5.2. Outbreaks in the European Union

All the data, but one, were published in the WHO surveillance programme for control of foodborne infections and intoxications in Europe (1993-1998), 7th report (see Annex 2).

In France, *S. aureus* was the causative agent in 13.6 % of the foodborne disease outbreaks reported from 1993 to 1997. Milk products were involved in 26 % of the outbreaks due to a staphylococcal foodborne intoxication, and

in 5.0 % of all the incriminated foods. According to the law, confirmed and suspected cases of diseases and deaths, due to bacterial foodborne diseases, are notifiable in France.

In Germany, *S. aureus* was the causative agent in 2.8 % of foodborne disease outbreaks reported from 1993 to 1998. Milk products and cheese represented 3.9 % and 0.1 % of all the incriminated foods respectively. According to the law, confirmed and suspect cases of diseases and deaths due to bacterial foodborne diseases do not need notification in Germany.

In Italy, *S. aureus* was the causative agent in 1.8 % of foodborne disease outbreaks reported in 1998. Cheese and ice cream represented 3.6 % and 2.4 % of all the incriminated foods respectively. According to the law, confirmed and suspect cases of diseases and death due to bacterial foodborne diseases are notifiable in Italy.

In Spain, *S. aureus* was the causative agent in 4.1 % of foodborne disease outbreaks reported from 1993 to 1998. Milk products and cheese were both involved in 1.6 % of all the incriminated foods. *S. aureus* was the causative agent in 13.9 % of the foodborne outbreaks implicating cheeses and in 11.1 % implicating milk. The notification of foodborne intoxication is mandatory in Spain.

In Portugal, *S. aureus* was the causative agent in 9.9 % of foodborne disease outbreaks reported from 1993 to 1998. Cheeses were involved in 1.7 % of all the incriminated foods. Among foodborne diseases, staphylococcal foodborne intoxication does not need notification in Portugal.

In England and Wales, *S. aureus* was the causative agent in 1.0 % of foodborne disease outbreaks reported from 1993 to 1998. Milk products were involved in 3.0 % of all the incriminated foods. Foodborne intoxication is one of the infection notifiable in England and Wales.

In Scotland, *S. aureus* was the causative agent in 2.3 % of foodborne disease outbreaks reported from 1996 to 1998. Cheeses were constitute in 9.1 % of all the incriminated foods.

In Ireland, no case of foodborne disease due to *S. aureus* was reported from 1997 to 1998. Among foodborne diseases, staphylococcal foodborne intoxication is notifiable in Ireland.

In Belgium, *S. aureus* and *B. cereus* were the causative agents in 4.9 % of foodborne disease outbreaks reported from 1995 to 1998. Cheeses, milk and ice creams were involved in 2.5, 5.4 and 2.0 % of all the incriminated foods respectively. Milk products were not involved in outbreaks due to a staphylococcal foodborne intoxication. Staphylococcal foodborne intoxication are not notifiable.

In Luxembourg, no case of foodborne disease due to *S. aureus* was reported from 1993 to 1998.

In the Netherlands, *S. aureus* was the causative agent in 0.9 % of foodborne disease outbreaks reported from 1993 to 1998. Dairy products constituted in

5 % of all the incriminated foods. The notification of foodborne intoxication is mandatory in the Netherlands.

In Denmark, *S. aureus* was the causative agent in 3.2 % of foodborne disease outbreaks reported from 1993 to 1998. Milk products were not implicated in outbreaks due to a staphylococcal foodborne intoxication. The notification of foodborne intoxication is mandatory in Denmark.

In Sweden, *S. aureus* was the causative agents in 3.6 % of foodborne disease outbreaks reported from 1993 to 1998. Cheeses and milk products were involved in 1.7 and 1.1 % respectively of all the incriminated foods.

In Finland, *S. aureus* was the causative agents in 8.2 % of foodborne disease outbreaks reported from 1993 to 1998. Milk products were involved in 3.6 % of all the incriminated foods. Among milk products, 10 % of foodborne diseases were due to *S. aureus* intoxication. Among foodborne diseases, staphylococcal foodborne intoxication does not need notification in Finland.

In Austria, *S. aureus* have been found as the causative agents in 0.2 % of the foodborne disease outbreaks reported in 1998. According to the law, confirmed and suspect cases of diseases and deaths due to bacterial foodborne diseases are notifiable.

Statistical data concerning outbreaks of staphylococcal foodborne intoxication are lacking for Greece. However, 5 unconfirmed staphylococcal outbreaks during 1998-2000 were reported by relevant microbiological laboratories. Diagnosis was based on clinical symptoms and isolation of quite large members of coagulase positive staphylococci in the incriminated foods (2 salad dressings and 3 meat products). The only confirmed outbreak refers back to 1970 (Panetsos *et al.*, 1970), which describe an enterotoxin A, staphylococcal outbreak involving 35 persons who consumed a white brine cheese ("Teleme") produced from cow's milk. Among foodborne diseases, staphylococcal foodborne intoxication does not need notification in Greece.

This overview of staphylococcal foodborne disease reports from 16 European countries indicates that milk and milk products were involved in 1 – 9 % (mean 4.8 %) of all the incriminated foods. However, implication of one food category among others remains difficult to estimate due to the limitations of surveillance systems (Table 1).

Table 1: Implication of *S. aureus* in milk products and cheeses in foodborne diseases in the EU (WHO surveillance programme for control of foodborne infections and intoxications in Europe, 1993-1998).

Country	% of <i>S. aureus</i> as causative agent	Foodborne outbreaks due to		Notification
		Milk products	Cheeses	
Austria	0.2	no data	no data	yes
Belgium	4.9	5.4	2.5	no
Denmark	3.2	none	none	yes
England and Wales	1.0	3.0	no data	yes
Finland	8.2	1.1	1.7	no
France	13.6	26.0	no data	yes
Germany	2.8	3.9	0.1	no
Greece	no data	no data	no data	no
Ireland	none	none	none	yes
Italy	1.8	3.6*	2.4	yes
Luxembourg	none	none	none	not known
Portugal	9.9	no data	1.7	no
Scotland	2.3	no data	9.1	not known
Spain	4.1	1.6	1.6	yes
Sweden	3.6	1.1	1.7	not known
The Netherlands	0.9	5	no data	yes

*ice cream

5.3. Outbreaks outside European Union

In Norway, *S. aureus* was the causative agent in 24.2 % of foodborne disease outbreaks reported from 1993 to 1998. Milk products were involved in 6.7 % of all the incriminated foods.

In the United States of America, Olsen *et al.* (2000), reported that *S. aureus* was involved in 42 documented outbreaks of foodborne intoxication, with 1,413 notified cases and one death occurring from 1993 to 1997. *S. aureus* has been estimated to cause approximately 185,000 illnesses, 1,750 hospitalizations, 2 deaths per year, all from consumption of contaminated foods (Mead *et al.*, 1999).

One of the largest outbreak of staphylococcal foodborne intoxication involving a milk product occurred in July 2000 in Japan (Anonymous, 2001; Asao *et al.*, 2003). The Snow Brand incident had the following characteristics:

- a large scale incident involving 13,420 notified cases,

- the incriminated foodstuffs were e.g. processed milk such as low-fat milk and yogurt drink made from powdered skim milk,
- no viable organisms but enterotoxin (0.05 to 1.6 ng/ml) was detected in the incriminated milk products,
- from the skimmed milk powder, the source material for the incriminated milk products, enterotoxin (4 ng/g) was detected.

5.4. Outbreaks caused by *S. aureus* involving milk and milk products

An overview of outbreaks involving milk and milk products is summarised in Table 2 (De Buyser *et al.*, 2001).

Among 59 foodborne intoxication outbreaks due to *S. aureus* and involving milk and milk products from 1992 to 1997 in France, cheese and “fromage frais” were involved in 53 outbreaks (De Buyser *et al.*, 2001). It was specified that they were made from raw milk in 28 outbreaks and the heat treatment of the milk was unspecified for the 25 remaining outbreaks. Out of the 40 cheeses whose type was stated, 39 were semi-hard cheeses. For eight cheeses, *S. aureus* enumeration results and/or enterotoxin detection results were given - 6/6 had more than 10^5 cfu/g and 3/6, more than 10^6 cfu/g. Enterotoxin was detected in two cheeses made from raw milk and in two cheeses made from milk that was unspecified.

Table 2: *S. aureus* outbreaks involving milk and milk products in different countries (De Buyser *et al.*, 2001; Asao *et al.*, 2003)

Country	Year	No of cases	Food involved (SE type)	Type of milk	Reference
Canada	1980	62	Cheese curd (SEA, SEC)	Unspecified	Todd <i>et al.</i> , 1981
USA	1981	16	Cheese	Pasteurized	Altekruse <i>et al.</i> , 1998
England	1983	2	Cheese	Pasteurized	Barrett, 1986
France	1983	20	Ewe cheese (SEA, SED)	Raw	De Buyser <i>et al.</i> , 1985
Scotland	1984	27	Ewe cheese (SEA)	Raw	Bone <i>et al.</i> , 1989
Scotland	1985	2	Goat's milk	Unpasteurized	Sharp, 1989
USA	1985	860	Chocolate milk (SEA)	Pasteurized	Evenson <i>et al.</i> , 1988
Israel	1987	3	Goat's milk (strain SEB)	Raw	Gross <i>et al.</i> , 1988
England	1988	155	Stilton cheese	Unpasteurized	Maguire <i>et al.</i> , 1991
Brazil	1994	7	Cheese (SEH)	Unspecified	Pereira <i>et al.</i> , 1996
Japan	2000	13,420	Powdered skim milk used for low-fat milk and yogurt	unspecified	Asao <i>et al.</i> , 2003

5.5. Toxic dose

As staphylococcal enterotoxins are preformed in food by enterotoxinogenic strains of Coagulase Positive Staphylococci (CPS) strains (mainly *S. aureus*), all the data presented below referred to a toxic dose by ingestion route.

Notermans *et al.* (1991) demonstrated the feasibility of a reference material containing about 0.5 µg of staphylococcal enterotoxin A (SEA), as it had been suggested that this dose can cause symptoms such as vomiting (Bergdoll, 1970). Mossel *et al.* (1995) cited an emetic dose₅₀ value of about 0.2 µg SE /kg human body weight. They conclude that an adult has to ingest about 10-20 µg of SE to get symptoms. Other authors (Martin *et al.*, 2001) consider that less than 1 µg of SE may cause food-poisoning symptoms in sensitive individuals. Evenson *et al.* (1988) estimated that the amount of SEA needed to cause vomiting and diarrhoea was 0.144 µg, the amount recovered from a one-half pint (approx. 0.28 l) carton of contaminated 2 % chocolate milk. In staphylococcal intoxication in Japan, the total intake of SEA in low-fat milk per capita was estimated mostly at approximately 0.02-0.1 µg (Asao *et al.*, 2003). Today, no additional data are available to suggest a toxic dose.

5.6. Other *Staphylococcus* species producing enterotoxins

Among other Coagulase Positive Staphylococci (CPS), Becker *et al.* (2001) raised the enterotoxinogenic potential of *S. intermedius*. The enterotoxinogenic potential (particularly for SEC) of this species has been shown in strains isolated from dogs (Hirooka *et al.*, 1988). The presence in the environment of strains producing toxins raises a possible health hazard, especially when carried by animals such as dogs that come in close contact with humans. *S. intermedius* has been involved in one outbreak caused by butter blend and margarine involving over 265 cases in October 1991 in United States (Khambaty *et al.* 1994; Bennett, 1996).

S. hyicus, a coagulase-positive or -negative staphylococcus, has been shown to produce enterotoxins other than A to E, producing an emetic response in the monkey feeding test (Adesiyun *et al.*, 1984).

Today, no strain of coagulase negative staphylococci has been clearly involved in foodborne outbreaks.

6. FACTORS INFLUENCING THE PRODUCTION OF ENTEROTOXINS

6.1. Description of types of toxins

SE are primarily produced by *S. aureus* and by some of the coagulase positive staphylococci listed in Table 3 (for further details on SE producing non-*S. aureus* species we refer to chapter 5.6). According to Casman *et al.* (1963) they are sequentially assigned a letter of the alphabet in the order of their discovery (Table 4). SEF was produced by *S. aureus* strains involved in toxic shock syndrome (TSS) (Bergdoll *et al.*, 1981). Later it became clear that it was not an enterotoxin and not emetic. Thus, it has been removed

from the SE nomenclature system and is now referred to toxic shock syndrome toxin TSST-1 (Betley *et al.*, 1990).

Table 3: Genus *Staphylococcus*: coagulase positive species

Species	Main sources	References
<i>S. aureus</i> subsp. <i>aureus</i>	humans, animals	Rosenbach, (1884)
<i>S. aureus</i> subsp. <i>anaerobius</i>	sheep	De la Fuente <i>et al.</i> , (1985)
<i>S. intermedius</i>	dog, horse, mink, pigeon	Hajek, (1976)
<i>S. hyicus</i>	pig, chicken	Devriese <i>et al.</i> , (1978)
<i>S. delphini</i>	dolphin	Varaldo <i>et al.</i> , (1988)
<i>S. schleiferi</i> subsp. <i>coagulans</i>	dog (external ear)	Igimi <i>et al.</i> , (1990)
<i>S. lutrae</i>	otter	Foster <i>et al.</i> , (1997)

Table 4: Staphylococcal enterotoxins (SE)

SE type	Reference
A	Casman, 1960
B	Bergdoll <i>et al.</i> , 1959
C ₁	Bergdoll <i>et al.</i> , 1965; Borja and Bergdoll, 1967
C ₂	Bergdoll <i>et al.</i> , 1965; Avena and Bergdoll, 1967
C ₃	Reiser <i>et al.</i> , 1984
D	Casman <i>et al.</i> , 1967
E	Bergdoll <i>et al.</i> , 1971
G*	Betley <i>et al.</i> , 1992; Munson <i>et al.</i> , 1998
H*	Ren <i>et al.</i> , 1994; Su and Wong, 1995
I*	Munson <i>et al.</i> , 1998
J*	Zhang <i>et al.</i> , 1998
K*	Orwin <i>et al.</i> , 2001
L*	Fitzgerald <i>et al.</i> , 2001
M*	Jarraud <i>et al.</i> , 2001
N*	Jarraud <i>et al.</i> , 2001
O*	Jarraud <i>et al.</i> , 2001

*Not available in commercial test kits

The SE recognised to the present day (Table 3) form a group of serologically distinct extracellular proteins sharing a number of important properties, namely:

- (1) the ability to cause emesis and gastroenteritis in primates,
- (2) superantigenicity (unspecific activation of T lymphocytes followed by cytokine release and systemic shock) reviewed by Fraser *et al.* (2000); Papageorgiou and Acharya (2000); and Ulrich (2000),
- (3) resistance to heat and pepsin digestion, and
- (4) structural similarities, (two domains of the molecule and an intramolecular disulfide bond) were reviewed by Balaban and Rasooly (2000) and Dinges *et al.* (2000).

The SE's seem to be also similar in size. As far as is known, their molecular weights amount to 25 to 29 kD (Bergdoll, 1983; Betley *et al.*, 1992; Su and Wong, 1995; Munson *et al.*, 1998). SEs are encoded by prophages (e.g. SEA; Betley and Mekalanos, 1985), plasmids (SED; Bayles and Iandolo, 1989) or chromosomal pathogenicity islands (e.g. SEB) (Yarwood *et al.*, 2002). The role of pathogenicity islands was reviewed by Novick *et al.* (2001).

Commercially available test kits for the detection of SE in food or in cultures include SEA – SEE (Annex 3). On the other hand, the analysis of 120 strains involved in foodborne intoxication in the UK showed that 113 (94.2 %) of the outbreaks resulted from these enterotoxins, therefore, about 5 % did not. 88 (77.9 %) of the enterotoxinogenic strains (SEA – SEE) produced SEA and 48 (42.5 %) SED, either alone or together with another toxin. Interestingly, 30 strains (26.5 %) produced both SEA and SED (Wieneke, 1974). In a study examining 131 outbreaks in the USA from 1977 through 1981 of 29 successful attempts to identify enterotoxin, SEA was identified in 27. SED, however, was not found, but SEB was associated with two outbreaks (Holmberg and Blake, 1984). An outbreak in Brazil affecting seven members of a family was linked to the consumption of cheese. SEH was detected in the cheese and from a *S. aureus* strain isolated from it (Pereira *et al.*, 1996). In Annex 4 some data on SE produced by staphylococci from various sources are reviewed. It appears that *S. aureus* strains isolated from humans often produce SEA, whereas strains from cows produce SEC or SED and those from sheep SEC. Stephan *et al.* (2001), however, isolated strains from human nasal carriers and found 9 SE producers all positive for SED either alone or in combination with other toxins. Generally, a high percentage of isolates from healthy humans are enterotoxin producers (Bergdoll, 1989: about 40 – 50 %; Al Bustan *et al.*, 1996: 86.6 % of 133 strains isolated from nasal swabs of restaurant workers in Kuwait City; Mehrotra *et al.*, 2000: 34.6 % of healthy individuals). In healthy animals the percentage of enterotoxinogenic strains seems to be lower: 3.9 % – 6.0 % for strains isolated from normal cows' milk (Gilmour and Harvey, 1990). The incidence of toxin producing strains in mastitis animals depends on the species: 14.6 % – 41.3 % in those of mastitis cows

and 70.4 % and 83 %, respectively, in those of mastitic sheep (Gilmour and Harvey, 1990). This is in agreement with Bergdoll (1989) who reviewed data and found that the 70 – 80 % of strains isolated from mastitic sheep produced SEC.

6.2. Physical/chemical factors

Microorganisms in foods are affected by a multiplicity of parameters described as intrinsic and extrinsic factors, processing effects and implicit parameters. Some of these parameters will be discussed below. It should be stressed, however, that in complex media like foods these factors interact to a great extent. Therefore, many of the data presented here were derived from laboratory experiments in which all other conditions beside the factor to be tested were ideal. Table 5 summarises some of the factors affecting growth and SE production by *S. aureus*.

With regard to staphylococci the **water activity** (a_w) is of great importance because these bacteria are able to grow over a much wider a_w range than other food-associated pathogens. As it can be seen from Annex 5 the bacteria can grow at a minimum a_w of 0.86 (equivalent to about 20 % NaCl; Qi and Miller, 2000) provided that all other conditions are optimal. The optimum a_w is > 0.99 (Smith *et al.*, 1983). The a_w conditions for SE production are somewhat different than that for growth depending on the type of toxin (Annex 5). SEA and SED production occurs under nearly all a_w conditions allowing growth of *S. aureus* under otherwise optimal conditions. Production of SEB is very sensitive to reductions in a_w and it is hardly produced at a_w 0.93 despite extensive growth. The effect of a_w on SEC production follows the same patterns as SEB production (Ewald and Notermans, 1988; Qi and Miller, 2000). Thota *et al.* (1973) found SEE production in media containing 10 % NaCl (according to Troller, 1971 this concentration corresponds with a_w 0.92). Important factors affecting growth and SE production are also the humectant used to lower the a_w , the pH, the atmospheric composition as well as the incubation temperature (Annex 5). Thus, conditions for growth and SE production in laboratory media and in food, respectively, may differ to some extent.

Studies on the osmoadaptive strategies of *S. aureus* have revealed that when cells are grown in a low a_w medium, they respond by accumulating certain low molecular weight compounds termed compatible solutes. Glycine betaine, carnitine and proline have been shown to be principal compatible solutes accumulated within osmotically stressed *S. aureus* cells, and their accumulation results from sodium-dependent transport systems (Gutierrez *et al.*, 1995; Qi and Miller, 2000). There is strong evidence that compatible solutes not only stimulate growth but also toxin synthesis. SEB production, e.g., was significantly stimulated at low a_w when proline was available in broth (Qi and Miller, 2000).

Most staphylococcal strains grow at **pH values** between 4 and 10, with the optimum being 6 – 7 (Table 5). When the other cultural parameters became non-optimal, the pH range tolerated is reduced. For example, the lowest pH that permitted growth and SE production by aerobically cultured *S. aureus* strains was 4.0, while the lowest pH values that supported growth and SE

production in anaerobic cultures were 4.6 and 5.3, (Smith *et al.*, 1983). Other important parameters influencing the response of *S. aureus* to pH are the size of inoculum, the type of growth medium, the NaCl concentration (a_w), the temperature and the atmosphere (Genigeorgis, 1989). In Annex 6 the results of some studies on the minimum pH value for growth of *S. aureus* and SE production were compiled. As it can be seen from the first study mentioned the acidulant has an influence on the minimum pH allowing growth. It should be stressed that most experiments have been done by adjusting the medium to a specific pH value with no attempt to control the pH during incubation (Bergdoll, 1989). Results of a study by Barber and Deibel (1972) using a buffered BHI medium with controlled pH values are given in Annex 7. The majority of *S. aureus* strains tested produced detectable amounts of SE aerobically at a pH of 5.1. Anaerobically, however, most strains failed to produce detectable SE below pH 5.7.

Optimum **redox potential** and ranges for growth and SE formation are given in Table 5.

S. aureus is a facultative anaerobic bacterium which grows best in the presence of oxygen. Under anaerobic conditions, however, growth is much slower, and even after several days, cell numbers do not reach those attained under aerobic conditions. Thus, aerated cultures produced approximately 10-fold more SEB as compared to cultures incubated in an **atmosphere** of 95 % N₂ + 5 % CO₂. Similarly, greatly increased SEA, SEB and SEC production was observed in shaken as compared to static cultures. The level of dissolved oxygen plays a very important role (Bergdoll, 1989; Genigeorgis, 1989). Under strict anaerobic conditions the growth of *S. aureus* was slower than aerobically. In broth incubated at 37°C the anaerobic generation time was 80 min, compared with 35 min for aerobic culture. With slower anaerobic growth, relatively less SEA was produced than under aerobic conditions, but in both cases toxin was detected after 120 min of incubation (Belay and Rasooly, 2002). It has been already mentioned that minimum a_w and minimum pH for growth as well as for SE formation are influenced by the atmosphere (Annex 5, studies on precooked bacon; Annex 7).

Growth of *S. aureus* and SE production is also influenced by **nutritional factors**. Some data are given in Annex 8.

Milk contains some **natural antimicrobial substances** like the lactoperoxidase system and its components (lactoperoxidase, thiocyanate, H₂O₂), lysozyme, lactoferrin etc. (for review see IDF, 1991). The existing data suggest that these agents are not very effective against *S. aureus*.

S. aureus grows between 7 and 48°C, **temperature** being optimal at around 37°C (Table 5). The effect of temperature depends on the strain tested and on the type of the growth medium. In an extensive study (Schmitt *et al.*, 1990) using 77 strains isolated from different foods the optimum growth temperature was generally without much deviation within the range of 35 to 40°C. The minimum growth temperatures were irregularly distributed between 7 and 13°C, and the maximum between 40 and 48°C. The minimum temperatures for SE production varied quite irregularly over a broad range within 14 and 38°C, and the maximum temperatures from 35 to 38°C and

45°C. The results for the lower temperature limit for SE production are in good agreement with data from the literature compiled by Schmitt *et al.* (1990) according to which 15°C has been most frequently determined for production of low amounts of toxin after 3 to 4 days. SE formation at 10°C was reported by Tatini (1973) (Table 5) without indicating the detailed experimental conditions (Schmitt *et al.*, 1990).

One of the most effective measures for inactivating *S. aureus* in food is heating. The bacterium is killed in milk if proper **heat treatment** is applied. *S. aureus* was completely inactivated in milk after application of the following temperature/time conditions: 57.2°C/80 min, 60.0°C/24 min, 62.8°C/6.8 min, 65.6°C/ 1.9 min, 71.7°C/0.14 min (Bergdoll, 1989). In the case of heat inactivation in other dairy products, however, one should keep in mind that probably staphylococci become more heat resistant as the a_w is lowered until at an a_w level between 0.70 and 0.80, resistance begins to decline (Troller, 1986).

As mentioned above *S. aureus* is not tolerant of the **heat treatment** procedures applied in the dairy industry. SEs in food, however, are very heat resistant (Table 6). The thermal resistance is primarily dependent on relative purity of the SE preparation. Crude SEA in buffer was reduced from 21 µg/ml to < 1µg/ml after heating at 100°C for 130 min. Purified SEA (0.2 mg/ml), however, was completely inactivated in buffer after heating at 80°C for 3 min or 100°C for 1 min. Generally, crude SEB seems to be considerably more heat resistant than purified SEA. (Minor and Marth, 1976). The results of thermal inactivation of SE in milk and milk products are shown in Table 7. Generally, heat treatments commonly used in food processing are not effective for complete destruction of SE when present initially at levels expected to be found in food involved in foodborne intoxication outbreaks (0.5 – 10 µg /100 ml or g) (Bergdoll, 1989). Such levels are higher than those found in ten outbreaks involved in staphylococcal foodborne intoxication in France in 2002, ranging from 0.05 to 0.4 ng/g (Hennekinne, personal communication). It should, however, be borne in mind that thermal inactivation is often determined by loss of the serological reactivity of the SE. Biological activity may be lost before the serological activity. On the other hand, some outbreaks result from eating foods that have been heated after SE was produced (Bergdoll, 1989). Thermal stability of SE is influenced by the nature of the food, pH, presence of NaCl, etc., and the type of toxin. SEA, for instance, is relatively more stable to heat at pH 6.0 or higher than at pH 4.5 – 5.5. SED is relatively more stable at pH 4.5 – 5.5 than pH 6.0 or higher (Tatini, 1976). If SE is not completely inactivated by heat reactivation may occur under certain circumstances like cooking, storage or incubation (Tatini, 1976).

6.3. Bacterial antagonism

S. aureus does not grow well in the presence of a **competitive flora**. Its inhibition is mainly due to acidic products, lowering of the pH, production of H₂O₂ or other inhibitory substances like antibiotics, volatile compounds or nutritional competition (Genigeorgis, 1989). Important factors affecting the degree of inhibition are the ratio of the numbers of competitors to the

number of *S. aureus* as well as the temperature (Smith *et al.*, 1983 and Genigeorgis, 1989).

Starter cultures used in the production of fermented milk products like cheese, yoghurt, buttermilk, and others can effectively prevent growth of *S. aureus* and SE formation. In the case of a failure of these cultures, however, the pathogen will not be inhibited and the product may be hazardous. These aspects as well as the role of food additives used in the making of dairy products will be discussed in chapter 7.

**Table 5: Factors affecting growth and enterotoxin production by *S. aureus*
(mod. acc. to: Tatini, 1973; Crowther and Holbrook, 1980; Baird-Parker, 1990; ICMSF, 1996)**

Factor	Organism growth		SE production	
	Optimum	Range	Optimum	Range
Temperature	37	7 – 48	40 – 45	10 – 48
pH	6 – 7	4 – 10	7 – 8	4 – 9.6
Water activity (a_w)	0.98	0.83 – >0.99 ¹	0.98	0.85 – >0.99 ²
NaCl (%)	0	0 – 20	0	0 – 10
Redox potential (E_h)	> + 200 mV	< - 200 mV to > + 200 mV	> + 200 mV	< - 100 mV to > + 200 mV
Atmosphere	Aerobic	anaerobic – aerobic	Aerobic (5 – 20 % dissolved O ₂)	anaerobic – aerobic

¹ Aerobic (anaerobic 0.90 – > 0.99)

² Aerobic (anaerobic 0.92 – > 0.99)

6.4. Resistance to physical and chemical factors

Some processing factors affecting the destruction of *S. aureus* and its toxins are shown in Table 6.

Table 6: Factors affecting destruction of *S. aureus* and SE (Baird-Parker, 1990; Concon, 1988)

Factor	<i>S. aureus</i>	SE
Heat	broth (D _{60°C}) 0.43 – 8.0	Broth (D _{121°C}) 3 – 8
	milk (D _{60°C}) 3.16 – 3.29)	-
Irradiation (D – kGy)	0.1 – 0.6	> 30
Drying, chilling, freezing, ambient storage	resistant	resistant
Disinfection		
Alcohol (MIC – MBC)	20 – 40 %	NA
Chlorine (MBC)	0.1 µg/l	NA
QAC (MIC – MBC)	0.2 – 50 µ/l	NA

NA: not applicable

The effect of **γ-irradiation** on SEB in buffer and in milk was determined by Read and Bradshaw (1967). A dose of 50 kGy was required to reduce the SEB concentration in buffer from 31 µg/ml to 0.7 µg/ml. In milk a dose of 200 kGy was needed to inactivate a 30 µg/ml concentration to less than 0.5 µg/ml. The D-values (dose required to inactivate 90 % SEB) were 27 kGy with buffer and 97 kGy with milk. Inactivation of SEB in buffer was determined both with a serological and a biological test. No differences between the results could be observed.

SEA inactivation in buffer and in cooked minced beef slurries was investigated by Rose *et al.* (1988). In buffer the toxin was destroyed by irradiation at 8.0 kGy. In minced beef, however, 27 – 34 % of SEA remained. At 23.7 kGy 16 – 26 % of SEA still remained.

SE are resistant to **proteolytic enzymes** such as trypsin, chymotrypsin, rennin, papain. Pepsin does destroy the activity of SEB at a pH about 2 but is ineffective at higher values. The pH of the stomach would be this low only after starvation and is usually higher after food is ingested (Bergdoll, 1989).

Little is known about the **survival of SE during storage** of milk and milk products. In white cheese in brine stored at 20°C SE could be detected until the end of the experiments (62 d) (Krejaković-Miljković, 1960). Tatini *et al.*,

(1971a) studied the effect of Cheddar cheese ripening on SEA. They found that toxin persisted in normal starter cheese for over 3 years at 4.4°C.

Table 7: Thermal inactivation of SEA and SED in milk and milk products at 72°C for 15 sec¹ (Tatini, 1976)

Type of sample	Percent serological activity remaining ²	
	SEA	SED
Whole milk	36	30
Skim milk	56	30
Cream	24	15
Evaporated milk	56	NT
Reconstituted non-fat dry milk	45	NT

¹ 40 – 50 sec to reach 72°C

² 1µg/ml initial concentration

NT: not tested

7. PROCESSING CONDITIONS FAVOURING ENTEROTOXIN PRODUCTION

7.1. Cheeses

7.1.1. Introduction

According to Codex Alimentarius (FAO/WHO, 1973) "Cheese is the fresh or ripened product obtained by the curdling and draining of whole, or partly skimmed or skimmed milk, or buttermilk or mixture of all or some of the above products". Cheese is an important food commodity in the EU and the consumption is one of the highest in the world (See Annex 9).

This broad specification (FAO/WHO, 1973) reflects the existing variety of methods in cheese making practice. However as a general guideline milk pasteurized or not is coagulated by rennet or acid and the curd, scalded or not, is drained, pressed or not, salted and ripened for different periods of time (Scott, 1986). Ripening is achieved by the action of lactic acid bacteria, which in the beginning ferment lactose, and the curd becomes acid (pH = 5.0 – 5.2).

The presence of large numbers of lactic acid bacteria (LAB), the drop of pH, the concentration of salt and the temperature of processing, are cardinal factors for the fate of microorganisms in cheese during processing and ripening. Thus the effect of processing conditions on *Staphylococcus aureus* growth and enterotoxin production will be reviewed in connection with the grouping of cheeses in different categories according mainly to the texture of

their body (Fresh, Soft, Semi-hard, and Hard). However reference will be made in special categories of cheeses such as:

- a) Surface or internal mould ripened
- b) Surface smear ripened
- c) Pasta filata cheeses
- d) Feta and related white-brined cheeses.

7.1.2. Fresh cheeses

Fresh cheeses are prepared from raw or pasteurized milk, with or without the use of starter, although in the majority of the cases the curd is fermented by the action of added starter bacteria or by the natural LAB of the milk. The pH value usually drops rapidly to levels below 5.0 and their water content remains higher than 55% ($a_w = 0.95$ to 0.97). After their preparation they are consumed fresh, without a ripening period, and with a self-life of 5 to 30 days under refrigeration.

Representative types of these cheeses are, Cottage, Baker's, Cream cheese, Neufchatel, Quesco Blanco, Ymer, Quarg etc. Fresh cheeses are also the whey cheeses (Myzithra, Manouri, Ricottone), which are considered in chapter 7.1.7 as well as mozzarella (ch. 7.1.6), which is the representative type of the pasta filata cheeses (Kosikowski and Mistry, 1997, Anifantakis, 1991).

Zarate *et al.*, (1998) studied the fate of *S. aureus* in a Tenerife goat milk cheese, produced from raw milk, without the addition of starters, and consumed either fresh (within 2-3 days) or after 60 days of ripening. The authors observed a 2 to 3 logs/g increase of *S. aureus*, in the two days old cheese, but after that a rapid decrease was noted.

Hamana *et al.*, (2002) prepared Iben, a traditional Moroccan fresh cheese with or without addition of a nisin-producing *Lactococcus lactis*. Milk was inoculated with 10^3 to 10^5 cfu/ml with an enterotoxin C producing *S. aureus* strain and which was decreased rapidly in the cheese with *Lactococcus lactis*, while it survived for longer periods in the cheese prepared without nisin producing starter. However, when they used large initial inocula of *S. aureus* ($> 10^5$ cfu/ml), enterotoxin C was detected within 3 days. Daminelli (1999) could not detect enterotoxin, when prepared an Italia fresh cheese type "crescenza" from pasteurized milk with good starter activity, even when he used inocula of 10^6 cfu/ml of milk.

Lodi *et al.*, (1994) classified 32 cheeses of fresh type in 3 categories:

- a) Cheeses with high number of LAB;
- b) Cheeses with absence of LAB and a low number of natural flora, and
- c) Cheeses with absence of LAB but having high counts of natural flora.

They concluded that in category (a) the number of pathogenic bacteria (e.g. *S. aureus*) declines rapidly during processing while in the categories (b) and (c) pathogens survived for several weeks. The existing data suggest that growth of *S. aureus* is limited in fresh cheeses, if coagulation is mainly due to lactic fermentation. Enterotoxin A and more rarely enterotoxin D, have been detected in fresh cheeses prepared from raw or pasteurized milk when *S. aureus* could reach populations up to 10^6 cfu/g of cheese. Growth and toxin production are influenced by the initial concentration of enterotoxinogenic *S. aureus* in the milk, the activity of starter bacteria, the antagonistic effect, of the natural flora, the concentration of salt, the drop of pH and the temperature of processing and storage of the cheese. (Meyrand and Vernozy-Rozand, 1999).

S. aureus is a very salt tolerant bacterium capable of producing enterotoxin in substrates with salt concentration up to 10% ($a_w = 0.92$) while growth of many antagonistic to *S. aureus* bacteria of the natural flora of milk are inhibited (Letondeur-Lafarge and Lahellec, 1997).

In conclusion, in *fresh type* cheese enterotoxin can be produced when starter activity is reduced or the number of natural flora is low. Existing data suggest that in fresh cheeses with high number of lactic acid bacteria (LAB) staphylococci decline rapidly in numbers and production of enterotoxin is prevented even when large numbers (10^3 - 10^5 cfu/ml) of staphylococci are present in the beginning of the process.

7.1.3. *Soft cheeses*

The category of soft cheeses constitutes the largest family of cheeses with hundreds of cheese-types and names in Europe and worldwide. Many cheeses of Controlled Name of Origin, traditionally produced are included, each one being a unique environment, as far as growth of microorganisms concerns. Many of these cheeses are produced from raw milk and fermentation is based on the natural lactic flora, selected mainly by the processing conditions. However, due to hygienic problems the majority of soft cheeses are produced today from thermised ($65^\circ\text{C} - 68^\circ\text{C}/5$ - 15 min) or pasteurized ($68^\circ\text{C} - 72^\circ\text{C}/15$ sec) milk, with the use of selected starter cultures (Scott, 1986, Meyrand and Vernozy-Rozand, 1999). Within this large family we can differentiate several groups such as: (a) The lactic type soft cheeses as, Petit Suisse, Lactic, Bondon, Colwich, Carrick, Kingston, White cheese, and Mont d'Or; (b) Surface Mould ripened cheeses such as Camembert, Brie, Sainte Maure, Coulommiers, Carré de L'Est., etc.; (c) Smear surface ripened cheeses such as Limburger, Munster, Bel Paesa, Brick, Tilsit, etc.; (d) Feta and related white-brined cheeses such as traditional feta, Domiati, Brinza, Haloumi and related white-brined cheeses some of which are usually pickled.

a. Soft cheeses of lactic type

S. aureus growth and enterotoxin production was studied by Dengremont *et al.*, (1995) in experimentally produced soft cheeses. No enterotoxin was detected in the study even when enterotoxinogenic *S. aureus* reached counts

of 10^7 cfu/g. On the contrary after an increase of 1 - 3 logs in the beginning, *S. aureus* decreased during 30 days storage by 3 log cfu/g.

Tham *et al.*, (1990), observed no enterotoxin in goat's cheese produced from thermised milk with added starter bacteria. On the other hand, when cheese was produced from raw goat milk without starter culture, *S. aureus* was capable of growing in levels where enterotoxin was detected.

Vernozy-Rozand *et al.*, (1998), studied the growth of *S. aureus* in two goat's milk lactic cheeses, prepared from row milk, and observed enterotoxin production only when they used large inocula ($> 10^5$ cfu *S. aureus*/ml of milk) were used.

Similar results were obtained by Provent (1990) when Mont d'Or cheese was prepared using pasteurized milk and starter culture as well. The author concludes that the activity of starter culture is of paramount importance for controlling levels of *S. aureus* below those capable of enterotoxin formation.

Daminelli (1999) obtained parallel results, for an Italian soft cheese type "Italico", no enterotoxin being detected using inocula ranging from 10^3 to 10^6 cfu/ml of milk. On the other hand, enterotoxin A was detected by Anunciacao *et al.*, (1994) in a Brazilian white soft cheese when 10^4 cfu *S. aureus*/ml of milk were used. Santos and Genigeorgis (1981) studied the conditions of enterotoxin A, B and C production, in Minas cheese (semi-soft white cheese), using raw or pasteurized milk, with or without starter added in the milk. They observed that when starter were used only large inocula ($> 10^5$ cfu/ml of milk) could result in enterotoxin production. They concluded that pasteurization of milk and use of a starter culture was the best method to prevent growth of *S. aureus* and enterotoxin formation.

b. Surface mould ripened cheeses

S. aureus inoculated in Brie cheese increased in numbers during the first stages of production, reaching up to 10^5 cfu/g, after which populations started to decline. No enterotoxin was detected (De Buyser and Lapeyre, 1995). Growth of *S. aureus* during Camembert cheese production was studied by Mueller *et al.* (1996). Pasteurized milk with starter culture and inocula of *S. aureus* from 10^3 to 10^5 cfu/ml of milk were used. In 10 of 11 experiments enterotoxin was detected with 24 h, when *S. aureus* reached 10^6 cfu/g of cheese. Similar results were obtained by Meyrand *et al.* (1998) with enterotoxin A detectable in experimentally produced Camembert type cheese inoculated with 10^4 to 10^6 cfu/ml *S. aureus* of milk. The same authors were unable to detect any enterotoxin in lactic type soft cheeses, inoculated with the same numbers of *S. aureus*.

c. Surface smear ripened cheeses

Soft cheeses of this category are ripened not only with the action of LAB bacteria but also with the growth on their surface of a special strain of *Brevibacterium linens* or *Bacillus linens*, which produces a slimy smear. Further growth of *B. linens* alters the pH value of the surface of the cheese, raising it from acid zone (pH = 4.5) towards the neutral, giving thus the opportunity for pathogenic contaminants to multiply. This has been proved

for *Listeria monocytogenes*. Cheeses of this category have been responsible for some serious outbreaks of listeriosis (Ryser, 1998; Bula *et al.*, 1995).

Tatini *et al.* (1973) prepared Brick cheese (a U.S.A. surface smear ripened cheese) from pasteurized milk inoculated with different initial populations of *S. aureus*, and starter culture bacteria (*Streptococcus thermophilus* or *Streptococcus lactis*). In all lots but one, *S. aureus* numbers increased to levels $> 10^7$ cfu/g and enterotoxin was detected. The authors did not discuss on the effect of smear bacteria in the growth *S. aureus*.

d. Feta and related white-brined cheeses

Feta, a traditional Greek white-brined cheese, is prepared from sheep milk or mixture of sheep's and goat's milk (70:30). It is a fast fermented cheese reaching pH of 5.5 within 3-4 h and pH 4.6 within 72 h. Traditionally, raw milk was used but today pasteurized milk and lactic starter cultures are used.

Other representative types of these cheeses are Haloumi, Domiati, Briza etc.

In a study (Mantis, 1973) of the processing conditions under which enterotoxinogenic staphylococci can multiply and produce enterotoxins (A, B, C); it was observed that, with good starter activity, staphylococci can multiply only during the first 2-3 h of processing and only when large initial inocula ($> 10^3$ cfu/ml) were used, but enterotoxin was not detected. Staphylococci, after an initial increase of 2-3 logs/g of cheese, started to decline rapidly. Initial inocula of 5×10^5 cfu/ml of milk during storage reaching populations fell to < 10 cfu/g in less than 15 days.

In reduced starter activity or complete starter failure, even small inocula of staphylococci, (2×10^2 cells/ml), could multiply and produce enterotoxins. In the case of starter failure staphylococci decreased in numbers < 10 /g of cheese before the end of the ripening period (60 days), but enterotoxin persisted.

Finally inoculation of ripened feta cheese with *S. aureus* did not result in enterotoxin production, and *S. aureus* counts fell rapidly.

Similar results were obtained for Domiati cheese (an Egyptian white brined cheese) and a feta type cheese in Turkey (Ahmed *et al.*, 1983, Erkmén, 1995).

e. Conclusion

It can be concluded that, soft cheeses of the lactic type as well as white-brined cheese represent an unfavorable environment for *S. aureus* growth and enterotoxin production. According to available data enterotoxin was produced only when fermentation was retarded due to starter failure and large inocula ($> 10^4$ - 10^5 cfu/ml of milk) were used. This situation indicates failures in processing technology and does not represent normal processing conditions. Surface mould or smear-ripened cheeses represent a more favorable environment for *S. aureus* growth and enterotoxin if the initial numbers of staphylococci in the milk are higher than 10^3 cfu/ml.

7.1.4. *Semi – hard cheeses*

Cheeses of semi-hard texture compose, like the soft cheeses, a large family with many varieties. The majority are produced from pasteurized milk, with the addition of starter cultures and the curd scalded or not, is usually pressed. They are ripened during a storage period ranging from 4-5 weeks up to 3 months. A subcategory of this group includes cheeses with internal mould ripening.

a.) *Semi-hard cheeses with normal lactic bacteria*

Representative types of these cheeses are (e.g. Edam, Gouda, Danbo, Caerphilly, Lancashire, Reblochon, Cantal, Cremos, Port-Salut, Providence etc.)

Van Schouwenberg – Van Foeken *et al.* (1979) manufactured Gouda cheese with normal starter activity (acid pH) and inoculated *S. aureus* in different numbers in the milk. Enterotoxins A, B, and C were detected after 24 h in cheese lots containing from 10-100 millions of *S. aureus* cfu/g of cheese. Concentration of enterotoxins was estimated in the range of 0.5 µg to 2.0 µg/100 g of cheese.

The production of enterotoxin in Port-Salut cheese was studied by Degreumont *et al.*, (1995). The author inoculated 10^3 to 10^6 cfu *S. aureus*/ml of milk and observed growth of the pathogen for 1-3 logs, but failed to find any enterotoxin, even when *S. aureus* reached levels of 10^7 cfu/g of cheese. Growth of *S. aureus* in Reblochon and Cantal cheeses was studied by De Buyser and Lapeyre (1995). Initial populations of *S. aureus* ranging from 10^2 to 10^4 cfu/ml of milk increased by 1.0 to 2.0 logs early in processing, then remained almost stable in Reblochon but decreased slightly in Cantal. Hoffner (1996) obtained similar results for Reblochon cheese. Finally, Medina *et al.*, (1992) observed a 2 log increase of *S. aureus* in Cremos, a goat's raw milk cheese followed by a decrease and disappearance of *S. aureus* within 30 days of storage.

b.) *Internal mould ripened Semi-hard cheeses*

Representative types of these cheeses are Roquefort, Gorgonzola, Stilton, Danablu, Blue cheese, Adelost, Tiroler-Graukäse etc.

Internal mould ripened cheeses, are of great interest when considered as substrates for growth of pathogenic bacteria because of the growth of a mould in the cheese body (*Penicillium roqueforti* or *Penicillium glaucum*) which probably has an inhibitory effect.

Boer and Kuik, (1987) studied the microbiological quality of 256 samples of blue veined cheeses (Roquefort, Danablu, Gorgonzola) and found that *S. aureus* was always present at numbers less than 100 cfu/g. Two Spanish blue cheeses (Cabrale and Valdeon) were studied by Nunez and Medina (1980) and Lopez-Diaz *et al.* (1995), who found low numbers or absence of *S. aureus* in all cases. Finally, Tatini *et al.* (1973) studied the production of enterotoxin A in Blue cheese. They could not detect enterotoxin in all lots even when large inocula ($> 10^6$ cfu/ml) were used and *S. aureus* populations

reached of 10^7 cfu/g of cheese, or when a complete starter failure was induced by bacteriophage action.

The existing data suggest that cheeses ripened with internal mould activity are very hostile environment for *S. aureus*. This is probably due to the combined inhibitory effect of *Penicillium* spp. and starter bacteria (Tatini *et al.*, 1973, Meyrand and Vernozy-Rozand, 1999).

7.1.5. Hard cheeses

Cheeses of this category constitute also a large family. Representative types of these cheeses are: Cheddar, Colby, Manchego, Kefalotiri, Grana, Asiago, Pecorino, Emmental, Gruyère, Romano, Samsøe, Ras, Mazoero, Derby etc.).

They are produced mainly from pasteurized milk, with the use of starter cultures and the curd is heated (scalding) from 45°C to 55°C depending on the type of cheese. Some of them (Grana, Asiago, Romano) are very hard and they have a storage time for ripening up to 2 years.

Fonteca *et al.*, (1990) studied the changes in microbial flora in Mazorero (a Spanish hard cheese) produced from raw or pasteurized milk and observed that *S. aureus* survived for 90 days, although numbers were declining after the 35th day.

Manchego, another Spanish hard cheese was inoculated with 10^4 cfu of *S. aureus*/ml of milk. Enterotoxin A and D were detected in 5 of 16 lots (Gomez-Lucia *et al.*, 1992).

Naquib *et al.* (1979) found that *S. aureus* multiplied during the first days of the production of Ras cheese (an Egyptian hard cheese) but then declined to non-detectable levels within 38 days.

Tatini *et al.* (1971a) studied the production of enterotoxin A in Cheddar and Colby cheese prepared with normal starter activity. Enterotoxin was detected more frequently in Colby than in Cheddar, but only when populations of *S. aureus* reached 15×10^6 cfu/g. They reported the toxin remained in Cheddar cheese for 3 years. Enterotoxin A was also detected in Swiss cheese inoculated with different populations of *S. aureus* (Tanini *et al.*, 1973).

Koenig and Marth (1982) reported that salted Cheddar cheese (2.4%NaCl) was a more favourable environment for growth of *S. aureus* than unsalted cheese. Reiter *et al.*, (1964) observed that staphylococci multiplied more rapidly in Cheddar cheese if starters were inhibited by phage action.

The influence of starter culture on the growth of *S. aureus* and enterotoxin production of was also investigated in other studies (Tatini *et al.*, 1971a, Ibrahim *et al.*, 1981, 1981a), when a starter culture failure resulted in extensive growth of staphylococci and enterotoxin production. Salting during processing enhanced *S. aureus* growth and enterotoxin production.

In semi-hard and hard cheese of the lactic type the growth of *S. aureus* is always possible and enterotoxin can be produced in the majority of the cheese types if the initial population of the pathogen in the milk is higher

than 10^3 cfu/ml. Manufacturing processes allow multiplication of *S. aureus* from 3 to 5 logs cfu/g before pH drops to inhibitory levels. However cheeses with internal mould ripening do not represent a potential hazard for staphylococcus foodborne intoxication since in all experiments conducted no enterotoxin was produced whatever the number of *S. aureus* used as inoculum.

7.1.6. *Pasta filata* cheeses

Representative types of these cheeses are: Mozzarella, Provolone, Kaskaval, Kaseri etc.

Cheese of pasta filata types are characterized by the curd being heated initially at 45-48°C, then ripened at 13°C – 17°C until it becomes acid (pH = 5.0-5.2) and finally plasticised, either in whey or water at 78°C – 80°C, and manipulated by hand or mechanically (kneading), to become a plastic mass, which is salted and ripened for various length of time. Mozzarella is an exception to these general technological steps, because it does not ripen and is consumed fresh.

The acidity of the curd and the heating of cheese mass up to 80°C, affects the survival of pathogens in this type of cheeses. Tatini *et al.*, (1973) prepared Mozzarella cheese from milk inoculated with enterotoxin A producing *S. aureus* strain using *Streptococcus thermophilus/Lactobacillus bulgaricus* as starter culture. *S. aureus* reached maximum numbers by the end of the draining. Heating the cheese curd in hot water (82°C) along with stretching and molding of cheese pasta reduced *S. aureus* populations and no subsequent growth was observed. Enterotoxin was not detected in the cheese despite that *S. aureus* reached populations of 2×10^7 cfu/g.

7.1.7. *Whey* cheeses

Whey cheeses (fresh Myzithra, Manouri, Ricottone, Mysot, etc.) are produced by heating whey, to which 5-10% whole milk and 0-15% cream are added, to 90-95°C. Whey proteins (+ caseins) are coagulated and the coagulum, collected by floating, is drained, salted slightly (1.0-1.5% NaCl) and marketed fresh, with a shelf-life of 15-30 days under refrigeration (Scott, 1986). No lactic fermentation is involved.

Pathogenic bacteria multiply readily in this category of cheeses because of their high water activity (0.94-0.96) and high pH value (6.0-6.2). Karaioannoglou *et al.*, (1983) inoculated whey cheese "Manouri" with 10^2 to 10^5 cfu/g of *S. aureus* /g of cheese and stored it at 10°C, 20°C and 33°C. Enterotoxin B was produced in samples held at 20°C and 33°C, within 6 h to 24 h, depending on the initial inoculum and the temperature of storage. By the time enterotoxin was detected *S. aureus* counts were greater than 10^7 cfu/g.

7.1.8. *Processed* cheeses

Technology of processed cheese production involves addition of polyphosphates and heating (melting) of cheese mixture at 80-85°C. This is

the main reason that processed cheeses have an excellent safety record, not causing foodborne intoxication (Glass *et al.*, 1998, Johnson *et al.*, 1990).

Glass *et al.*, (1998) inoculated slices of processed cheese with 10^3 cfu of *S. aureus*/g of cheese and incubated at 30°C for 96 h. Populations of *S. aureus* remained constant and always below levels that support detectable enterotoxin levels.

7.1.9. Imitation cheeses

The ability of imitation (substitute) cheeses to support growth of *S. aureus* and enterotoxin production was studied by Bennett and Amos (1983). They tested eleven different types of imitation cheeses by inoculating 30 cfu of *S. aureus* /g of product and incubating them at 26°C. Enterotoxin was produced in seven of the eleven types of products within 4 days at 3×10^6 cfu *S. aureus*/g. However, the ability of some imitation cheeses to support growth and toxin production by *S. aureus* could not be correlated with pH, a_w , or product formula.

7.2. Other dairy products and milk

7.2.1. Liquid milk

Liquid milk, raw or pasteurized, is an excellent medium for the growth of *S. aureus* and enterotoxin production, if it is held at temperatures higher than 10°C, and the natural bacterial flora is low (Clark and Nelson, 1961).

The *S. aureus* counts in bulk milk are related to the mastitis situation of the herd, and may range from less than 10 to several thousands per ml of milk with occasional counts of 10^5 cfu/ml (Asperger and Zangerl, 2002).

Normally raw milk is pasteurized before consumption as liquid milk or when used for manufacturing purposes. Pasteurized milk is a better substrate for the growth of *S. aureus* and enterotoxin production than raw milk because the natural bacterial flora is competitive to *S. aureus* (Tatini *et al.*, 1971b). In the absence of a large background flora, Ikram and Luedecke, (1977) found that growth of *S. aureus* is enhanced, resulting in enterotoxin production in whole milk and in skim milk held at 37°C.

In raw or pasteurized milk, the storage temperature influences the enterotoxin production. With an initial inoculum of 10^6 cfu of *S. aureus* per ml of milk, enterotoxin was produced within 6 h at 35°C, 18 h at 25°C and 36 h at 20°C, while with an initial inoculum of 10^4 cfu/ml of milk enterotoxin was produced in 12 h at 35°C. In milk, with high counts of natural flora, only inocula of $> 10^6$ cfu/ml resulted in enterotoxin A production (Donnelly *et al.*, 1968).

7.2.2. Non-fat dry milk

Milk powder production technology includes pasteurization, evaporation and drying process. All heat treatments contribute to the overall lethality of the process. Staphylococci can multiply and produce enterotoxin in raw,

pasteurized, or concentrated milk, if they are present and the temperature is favorable (Asperger, 1994).

Spray drying decreases the number of staphylococci, if present in the milk concentrate, although some will survive, and multiplication and enterotoxin production is always possible after reconstitution (Chopin *et al.*, 1977, Galesloot and Stadhouders, 1968). However, if enterotoxin is produced before drying, it can persist for several years in the milk powder (Chopin, 1978).

7.2.3. *Cultured dairy products*

Cultured dairy products (yogurt, buttermilk etc.) have no record of causing staphylococcal poisoning, probably because production involves heating the milk at 90-95°C for at least 5 min, and subsequent inoculation of the heated milk with high inocula of starter culture bacteria. Actively growing starter bacteria inhibit growth of *S. aureus*, and in some cases killing of *S. aureus* is observed. Thus, if contamination takes place just before or during starter addition, *S. aureus* cannot multiply. *S. aureus* numbers are greatly reduced independent of the temperature of storage (Minor and Marth, 1970, 1972, 1972a).

7.2.4. *Cream and butter*

Butter has been implicated in staphylococcal poisoning and enterotoxin A was identified in the incriminated butter which caused an outbreak of 24 cases in 1970, in U.S.A. (CDC, 1970). Minor and Marth (1972b) studied the production of enterotoxin A in cream at 37°C and observed that enterotoxin was produced only at 37°C and only in artificially inoculated cream. In butter, initially containing 10⁵-10⁶ cfu of *S. aureus*/g and salted with 1.0% or 1.5% NaCl, some growth was observed in samples with 1% NaCl but not in those with 1.5% NaCl, kept at 23°C for 14 days. Enterotoxin production was not detected. If enterotoxin was produced in the cream, it appeared in the butter, with a ratio of buttermilk: butter 8:1 to 16:1.

The ability of cream containing up to 40% fat to support growth of *S. aureus* in levels producing detectable enterotoxin was confirmed also by Halpin and Marth (1986). Butter did not support growth of *S. aureus* at 23°C, while at 10°C numbers of staphylococci decreased (Minor and Marth, 1972b).

7.2.5. *Ice - Cream*

Ice cream and other frozen dairy desserts have been found to contain enterotoxinogenic strains of *S. aureus* (Batish and Chander, 1987, Tamminga *et al.*, 1980, Massa *et al.*, 1989) but no outbreaks have been attributed to this kind of dairy product. However, all authors agree that multiplication of *S. aureus* with enterotoxin production is always possible in the pre-freezing stages of processing and especially during the ripening of the ice-cream mixture. In this case enterotoxin would remain active for several months in frozen storage (Gogov *et al.*, 1984).

8. CORRELATION BETWEEN ENTEROTOXIN AND *S. AUREUS* COUNTS

It is generally considered that enterotoxinogenic staphylococci must reach levels of at least 10^5 to 10^6 cfu/g or ml to produce detectable amounts of SE. Under optimum conditions (incubation in Brain Heart Infusion broth in pure culture) Tatini *et al.* (1975) found SE when *S. aureus* was grown to populations of $\geq 5 \times 10^6$ /ml. Similar counts were needed to detect SEA or SED in cream, whole milk and skim milk (Tatini *et al.*, 1975). For the detection of SEA and SED in Gouda cheese whey, however, *S. aureus* had to reach levels of 1×10^7 / ml (Tatini *et al.*, 1975). In milk relatively free from competing microorganisms, a *S. aureus* population of 2 – 3 x 10^6 /ml was associated with detectable SEA (Tatini *et al.*, 1971b). In experiments by Becker (personal communication) using a commercially available ELISA test kit for the detection of SE even higher *S. aureus* population levels had to be reached to find SE in skim milk samples artificially contaminated with different toxin producing strains (Table 7). Thus, from those studies it can be concluded that a minimum of 10^6 cfu enterotoxinogenic *S. aureus*/g or ml are needed to produce detectable amounts of SE. Since SE are more stable compared to *S. aureus* bacterial cells, it is possible to test a product with negative results for *S. aureus* counts although SE exists in the products.

9. ANALYTICAL METHODS FOR DETECTION OF ENTEROTOXIN

9.1. Detection of enterotoxin

Three types of methods are usually used to detect a contaminant in food: bioassays, molecular biology and/or immunological tools.

The first, bioassays, are not sensitive. All the SE's were originally detected by their emetic activity in monkey-feeding and kitten-intraperitoneal tests (Surgalla *et al.* 1953 and Bergdoll, 1972). Symptoms of staphylococcal foodborne intoxication appear when the dose ingested by the animals is above 200 ng.

Recently, a super antigen bioassay to detect staphylococcal enterotoxin A type has been developed by Hawryluk and Hirshfield (2002) and is able to detect SEA at picomolar concentrations.

The second type involves molecular biology such as polymerase chain reaction (PCR). The problem is that this method gives information on the presence or absence of genes for staphylococcal enterotoxin and no information on the expression of these genes during food processing and storage. This method is therefore not applicable for staphylococcal enterotoxins detection in food. However, it allows characterisation of the *S. aureus* strains involved in food-poisoning outbreaks.

This is the reason that staphylococcal enterotoxins in food are detected by immunological tools. All available methods for detection of SE's are based on the use of specific antibodies to the enterotoxins (see table in Annex 3). Detection of SEs in food requires more sensitive methods than those used to determine enterotoxinogenicity of strains, as the amount of SEs present in foods may vary from less than 0.1 to greater than 20 ng per gram. The

infective dose for SEA is known to be as low as 144 ng (Evenson *et al.*, 1988).

Therefore methods have to be sensitive for assessing the safety of food.

Several types of immunological methods have been developed to detect SEs in food: immunodiffusion assay, Radio ImmunoAssay (RIA), Reversed Passive Latex Agglutination (RPLA) and Enzyme Immunoassay (EIA).

Three methods based on gel diffusion were used to detect SEs in food extracts: the first, micro slide technique (Casman *et al.*, 1969), was the first semi-quantitative method used to detect SEs in food with a limit of detection of 100 ng/ml. Other methods derived from gel diffusion e.g. Optimum Sensitivity Plate (OSP), (Robbins *et al.*, 1974) and Single Radial Immunodiffusion (SRD), (Meyer and Palmieri, 1980) were developed to screen production of enterotoxins from cultures of staphylococcal strains and were not sensitive enough to detect SEs in food outbreaks. These methods were left aside except in US where micro slide technique (Micro slide double gel diffusion kit) is still one of the AOAC official methods of analysis (AOAC, 1995a).

Radio Immunoassay was the first method sensitive enough to detect less than 1 ng per gram of food (Miller *et al.*, 1978; Janin *et al.*, 1983). The main problem with this method is the use of radionuclide, such as I^{125} . Because of safety concerns, long shelf-life, and therefore inconvenience, RIA gradually disappeared from most laboratories. Nevertheless, RIA was more sensitive (1 ng instead of 100 ng) than gel diffusion methods.

In the Reverse Passive Latex Agglutination (RPLA) method, specific antibodies are linked to latex particles. If SEs are present, a complex of SE-antibodies is formed and latex particles agglutinate. The limit of detection of this method is more than 1 ng per gram of food, which may be not be adequate in cases of small amounts of SEs.

An Enzyme-Linked ImmunoSorbent Assay (ELISA) method was developed (Freed *et al.*, 1982) to detect SEs in food and replaced RIA. Monoclonal antibodies against SEs were used to develop an ELISA sandwich type for an application in the official control of dairy products (Lapeyre *et al.*, 1987, 1988). This method is able to detect concentrations of SEs as low as 0.1 ng per gram of food.

The Community Reference Laboratory (CRL) of milk and milk products validated a reference method to detect staphylococcal enterotoxins in milk product. This method is based on a double sandwich type qualitative ELISA as detection step and a prior extraction step using dialysis concentration. The CRL asked the National Reference Laboratories (NRLs) to use this method in September 2000, moreover, this method was used by the CRL to organize an inter-laboratory proficiency trial in September 2001 for the NRLs. The outcome showed that the reference method could be satisfactorily used by the network of NRLs (Hennekinne *et al.*, 2003). Finally, all the NRLs except one, agreed at the workshop in October 2001 to use this method for official control of milk and milk products in the frame of the Directive 92/46.

Three methods have been adopted by AOAC as official methods for detection of staphylococcal enterotoxins in foods. The first one has already been described above using the micro slide technique (AOAC, 1995a). The second one (AOAC, 1995b) describes an extraction step using a concentration of the food extract by dialysis with poly ethylene glycol (PEG) as in the European reference method. The detection step is carried out by the micro slide technique. The third one (AOAC, 1995c) is based on the use of a commercially available test, the TECRA SET (see annex 3 and chapter 8.2).

As immunologically-based assays for the detection of staphylococcal enterotoxins require at minimum 1.5 hours, Schotte *et al.* (2002) developed a rapid immuno-chromatographic-based hand-held-assay to detect SEB in foods.

Today, no methods are commercially available to perform a quantitative detection of staphylococcal enterotoxins. In the European Union, according to the Council Directive 92/46, the Community Reference Laboratory developed (Lapeyre *et al.*, 1987, 1988) and validated (Macaluso *et al.*, 2000) a confirmatory method based on a quantitative ELISA SEA to SEE to confirm the results found positive by the NRLs.

Detection and characterisation of staphylococcal enterotoxins were performed by a quantitative indirect sandwich-type ELISA developed by Lapeyre *et al.* (1988). For the detection step specific monoclonal antibodies developed by Lapeyre *et al.* (1987) were used as coating antibodies and rabbit polyclonal antibodies as probing antibodies. The presence of staphylococcal enterotoxins is indicated by goat anti-rabbit immunoglobulins coupled to horseradish peroxidase, determined by a colorimetric measurement.

The test was performed after concentration by dialysis of staphylococcal enterotoxins as described in the reference method.

9.2. Evaluation of methods for detection of enterotoxins

Various commercially available kits can be used to detect staphylococcal enterotoxins in foods (see annex 3). All these kits use specific antibodies for staphylococcal enterotoxins. These kits have been developed according three principles: ELISA (enzyme linked immunosorbent assay), ELFA (enzyme linked fluorescent assay) and RPLA (reverse passive latex agglutination). Some of these tests can differentiate types of staphylococcal enterotoxins from A to E and other cannot. For all these tests, in order to detect low amounts of staphylococcal enterotoxins in food, a concentration of the food extract have to be carried out before the detection step.

Ridascreen SET A, B, C, D, E is an easy and rapid test to perform, able to differentiate staphylococcal enterotoxins types A to E. In raw milk products, some false positive results can be pointed out due to the presence of endogenous enzymes such as lactoperoxidase. According to Park *et al.* (1996), the Ridascreen kit is a very convenient, rapid, sensitive and specific test for detection and identification of SEs in foods.

TECRA staphylococcal enterotoxins SET identification and SET A-E are two kits able (TECRA SET identification) or not (TECRA SET A-E) to

differentiate staphylococcal enterotoxins types A to E. As described in the chapter 9.1, these detection methods are described in the official methods of the AOAC (AOAC, 1995c). These two kits are not suitable to detect low concentrations of SEs in foods due to their low sensitivity. Nonspecific SE reactions with the TECRA kit were previously observed with various types of seafood (Park *et al.*, 1993), or with food contaminated by microorganisms other than *Staphylococcus spp.* (Park *et al.*, 1992; Wu *et al.*, 1992).

SET RPLA is the only kit which cannot detect staphylococcal enterotoxin type E in the list presented in annex 3. Its use is time consuming. Due to the use of latex particles, no interference with endogenous enzymes has been observed but interpretation of results is not easy to perform in food extracts. Some false positive results have been observed using this kit especially in flour-based products (Wieneke, 1991).

Transia SET plate and tube are easy and rapid tests to perform but unable to differentiate staphylococcal enterotoxins types A to E. To detect low amounts of staphylococcal enterotoxins in food, a concentration of the food extract has to be carried out before the detection test, even if these tests have the best sensitivity (Lapeyre *et al.*, 1996) among the kits listed in annex 3. The Transia SET plate has been successfully used by the NRLs to carry out the first proficiency test (see chapter 9.1) on staphylococcal enterotoxins in milk products. As in all kits using enzymes such as alkaline phosphatase or lactoperoxidase, some false positive results have been observed in raw milk products due to presence of endogenous enzymes.

Vidas SET is a rapid and fully automatized kit unable to differentiate staphylococcal enterotoxins types A to E. Some false positive results have been obtained using this kit.

9.3. Detection of thermonuclease

The processing of certain foods may permit growth of *S. aureus* to numbers high enough (10^5 to 10^6 cfu/g or ml) to cause illness in the consumer. Subsequent steps, such as heating, can reduce numbers due to the death of *S. aureus*. In such foods enumeration of *S. aureus* is not a useful indication of the SE hazard because the toxins are much more heat tolerant than *S. aureus*. Such situations may necessitate testing for SE, which is expensive. While this may be justifiable in cases of foodborne intoxication, it may be not essential for routine quality control purposes. Screening food for thermonuclease (TNase) as an indicator of staphylococcal growth to high levels can be appropriate alternative.

TNase is an enzyme produced by all strains of *S. aureus* and by some other SE-producing staphylococci namely, *S. hyicus* spp. *hyicus*, *S. intermedius* and some enterococci and *Bacillus* spp. For several reasons TNase production by microorganisms other than *S. aureus* is not a problem: (1) *S. hyicus* spp. *hyicus* and *S. intermedius* are SE producers (Hoover *et al.*, 1983; Adesiyun *et al.*, 1984; Fukuda *et al.*, 1984; Alamzan *et al.*, 1987; Hirooka *et al.*, 1988; Becker *et al.*, 2001) and constitute a consumer risk as well as *S. aureus* itself; (2) the TNase assay is a screening test, and in the case of a

positive result tests for the presence of SE has to be carried out (see below); (3) TNases of enterococci and bacilli are less heat-stable than those of *S. aureus* (Park *et al.*, 1980); (4) very high counts of bacilli are needed to produce detectable amounts of TNase, such that complete spoilage of the food occurs (Becker *et al.*, 1984); (5) if necessary, a seroinhibition test using specific antibodies against *S. aureus* TNase can be carried out (Becker *et al.*, 1984).

Like SEs, TNase is very stable to processes such as heating ($D_{130\text{ }^{\circ}\text{C}} = 16.6$ min; Erickson and Deibel, 1973), fermentation, drying etc. and is detected in food using the assay described below if ca 10^5 to 10^6 cfu *S. aureus*/g are present. Some studies, however, indicate that detectable amounts of TNase are produced before detectable amounts of SE are formed (Tatini *et al.*, 1971b; Tatini *et al.*, 1975; Tatini *et al.*, 1976; Tatini, 1981; Table 8). These are hazardous levels provided the strain is an SE producer and able to form the toxins under the prevailing conditions of the food. Thus, TNase follows the patterns of synthesis and stability similar to SE in foods under all conditions of processing and storage (Tatini, 1981).

The TNase test is published as International IDF Standard since 1978. Collaborative studies were not carried out up to now but numerous intralaboratory evaluation studies from different countries are described in the literature.

The detection method as described in the International IDF Standard 83A:1998 "Milk and milk-based products - Detection of thermonuclease produced by coagulase-positive staphylococci in milk and milk products", a revision of IDF 83:1978 (IDF Standard 83A, 1998), is based on studies of Lachica *et al.* (1971); Tatini *et al.* (1976); Park *et al.*, (1979) and Becker *et al.* (1984). At the moment the IDF Standard is under revision again to become a joint ISO/IDF Standard.

Because it is well-known that false positive results, and results that cannot be clearly interpreted, may occur using the commercially available SE test kits described in section 9.2 and Annex 3 (Becker *et al.*, 1994), it is a further advantage of the TNase test that such results can be confirmed.

Table 8: *S. aureus* counts in skim milk compared to the results of TNase- and SE-test (Becker, personal communication)

SE type	Test positive at log ₁₀ cfu <i>S. aureus</i> /ml	
	TNase test	Ridascreen SET ¹
SEA	5.86	neg. at > 9.00
SEA	6.30	7.26
SEA	6.51	7.48
SEC	6.28	neg. at > 9.00
SEC	6.72	8.67
SEC	6.67	9.26
SED	5.58	7.57
SED	6.28	7.43
SED	6.04	7.26

¹see Annex 3

10. EVALUATION OF CURRENT CRITERIA

10.1. Microbiological criteria in general

The Codex document on Principles for the establishment of Microbiological Criteria (CAC, 1997a) used the following definition for microbiological criteria – “*a microbiological criterion for foodstuffs defines the acceptability of a product or food lot based on the absence or presence, or number of microorganisms including parasites and/or quantity of their toxins/metabolites, per unit(s) of mass, volume, area or lot*”.

The Scientific Committee on Veterinary Measure relating to Public Health also gave a definition for a microbiological criterion in its opinion of 1999 (SCVPH, 1999). The definition differs from the Codex definition only in that the word “*process*” is included before the word “*product*”.

The purpose of microbiological criteria is to protect the health of the consumer by providing safe, wholesome food products, and to meet the requirements of fair practices in trade. Thus the introduction and implementation of a criterion should not be an ad-hoc measure but rather should be the outcome of a deliberate process. Hence, a *microbiological criterion should be established and applied only where there is a definitive need for it and where it can be shown to be effective and practical*” (EC, 1997).

When applying criteria for assessing products, it is essential that only appropriate tests are applied to those foods and at those points in the food chain that offer maximum consumer benefits in terms of food safety (CAC, 1997b).

It appears that there is consensus that microbiological criteria should not be applied arbitrarily, but rather as the outcome of a deliberate process to achieve the optimal food safety (SCVPH, 2003).

Microbiological criteria can be applied differently, either as:

- Microbiological standards
- Microbiological guidelines, or
- Specifications

The SCVPH already stressed in a previous Opinion that the mere existence of microbiological criteria does not protect consumer health. The use of Good Hygienic Practice (GHP) and Hazard Analysis Critical Control Point (HACCP) systems will be of greater importance to ensure that pathogens are eliminated or minimized to the extent that they cannot cause harm to human health (SCVPH, 1999).

End product testing using microbiological criteria may have limited usefulness for food safety for a number of reasons, including low prevalence of the pathogen, or low diagnostic sensitivity of the testing procedure applied. While the finding of a pathogen in a foodstuff may indicate a problem for public health, necessitating appropriate risk management action; the failure on the other hand to detect a pathogen in a food product does not necessarily mean that the pathogen is absent from that food product, process or food lot (SCVPH, 2003).

Nevertheless, microbiological testing can be used in monitoring programmes along the food chain, for documentation purposes, HACCP, as an indicator of adherence to Good Hygienic Practices (GHP), on-the spot checks, monitoring the suitability of raw materials or food ingredients, and the hygienic status of the processing environment, all of which play an important role in maintaining food safety. Even if the application of a microbiological criterion does not result in a marked change in average prevalence of the pathogen, its implementation might facilitate official surveillance and inspection, and imposition of corrective action in the case of any unfavourable findings. Moreover, the use of a criterion can yield very useful results when collated and analysed on a national or regional scale, i.e., baseline prevalence studies that can be helpful in assessing risks associated with a particular pathogen. The use of equivalent methodologies is crucial for yielding comparable results (SCVPH, 2003).

10.2. Current criteria for *S. aureus* in milk and dairy products

Concerning *S. aureus* in milk and dairy products, it should be noted that only the enterotoxinogenic strains possess a risk to public health and the criteria applied should prevent the production of SE during processing and the occurrence of SE in the final product. It is generally considered that enterotoxinogenic staphylococci must reach levels of at least 10^5 - 10^6 cfu/g or ml to produce detectable amounts of SE.

The Committee is of the opinion that all of the milk and dairy products referred in the current criteria of *S. aureus* are important (Annex 1). However, based on scientific knowledge today, some of the criteria should be modified. In the following section, each of the current criteria is evaluated separately. The evaluation is based on the information on *S. aureus* growth and SE formation in the production of milk and different dairy products, on epidemiological evidence as well as on the methods currently available for *S. aureus* and SE detection.

10.2.1. Raw cow's milk intended for direct human consumption

Although fluid milk is less often associated with large staphylococcal foodborne intoxication outbreaks than dairy products, raw milk may still possess a risk for consumers. If a farm has a severe mastitis problem, the number of *S. aureus* may already be high in bulk milk. Liquid milk is an excellent medium for the growth and SE production of *S. aureus* (favourable pH, water activity and nutrients) and at temperatures over 10°C, numbers of enterotoxinogenic *S. aureus* may increase rapidly even when the initial levels are low.

In order to protect public health, a criterion for *S. aureus* counts is therefore needed. Since there often exist enterotoxinogenic strains among *S. aureus* strains in milk obtained from normal cows and at higher proportions from cows with mastitis, no special criteria for enterotoxinogenic strains are needed. Therefore, it appears to the Committee, the current criteria for counts of SA are needed and valid.

10.2.2. Raw milk intended for processing of raw products

If raw milk contains high levels of *S. aureus*, SE may be produced during processing of raw dairy products. Both butter and cheese made from raw milk have been reported to cause outbreaks. Therefore, the raw milk intended for the manufacturing of raw products should not contain high levels of *S. aureus*.

It appears to the Committee, that the use of microbiological criteria for raw milk intended for manufacturing of raw dairy products has a significant role in protecting public health. However, since there is a risk for the growth and SE production of *S. aureus* during manufacturing processes, there is now scientific basis why these criteria should be clearly higher than those for raw milk intended for direct human consumption (see 10.2.1 and Annex 1). Therefore, the Committee suggests that the similar criteria would be applied on raw milk both intended for direct human consumption and intended for processing.

10.2.3. Cheeses made from raw milk and from thermised milk

The Committee finds it very useful to have a criterion for cheese made from raw milk and from thermised milk. *S. aureus* may be present in raw milk and the temperature / time combination at thermization of milk does not guarantee elimination of *S. aureus* in every case. If abundant growth of enterotoxinogenic *S. aureus* is possible during first 48 hours of processing, enterotoxins will be formed. Although the *S. aureus* counts often decrease

during ripening and storage of cheese, the enterotoxins may persist over the whole shelf life of cheese.

However, the current criterion is not scientifically justified. First, the level of 10^4 cfu/g (M) is very close to the counts of *S. aureus* needed for the production of toxic dose of SE production. During cheese manufacturing, the levels of *S. aureus* often decrease, but the amounts of SE produced stay in the product. Therefore, acceptable results for this criterion do not tell whether the cheese has previously had higher levels of *S. aureus* and SE production and this criterion cannot be applied at any age of cheese.

Secondly, the pathogenicity of strain (i.e. ability to cause infection) is not relevant for milk products and it should be left out. Thirdly, the definition for enterotoxinogenic strain is not clear since some strains may possess the genes of SE production, but do not produce SE. Furthermore, the strain capable of producing SE in optimal conditions may not have been producing SE in cheese (see Chapter 11.1)

10.2.4. Soft cheese made from heat-treated milk

Pasteurisation of milk is effective in the elimination of *S. aureus* and therefore the counts reflect mainly the post-pasteurisation contamination. If counts are high in the first stage of production (48 hours), there is a risk of SE production. Although *S. aureus* counts often decrease during ripening and storage of soft cheeses, the enterotoxins may persist over the whole shelf life of cheese. Therefore, the current criterion is valid and useful when cheeses are analysed at 48 hours after start of the manufacturing. At later stages, other analytical methods should be used (see Chapter 11.2).

10.2.5. Fresh cheese from heat-treated milk

Pasteurisation of milk is effective in the elimination of *S. aureus* and therefore the counts reflect well the post-pasteurisation contamination. During the production and storage of fresh cheeses, *S. aureus* can grow and produce SE, especially if coagulation is not due to lactic fermentation. The criterion for *S. aureus* counts is therefore valid.

10.2.6. Powdered milk

S. aureus may grow and produce SE during processing of powdered milk (e.g. in storage tanks before spray drying). After processing *S. aureus* counts go down. Absence of *S. aureus* does not exclude the existence of SE since it could have been produced before in the manufacturing process. During storage of powdered milk, *S. aureus* cannot grow. However, if powdered milk is used for certain foodstuffs, *S. aureus* may grow and produce SE. Due to these reasons it is not surprising that powdered milk and foods containing powdered milk have been implicated in a number of staphylococcal foodborne intoxication outbreaks.

The criterion applied for this dairy product category is therefore needed. However, the current criterion is not valid (see Chapter 11.5).

10.2.7. Frozen milk-based products

S. aureus can grow during processing of frozen milk-based products but not during storage. If stored for longer period, *S. aureus* counts will gradually decrease. If *S. aureus* is detected at higher levels, SE may have been produced. Therefore, this criterion is valid.

11. IDENTIFICATION OF NEW CRITERIA

Based on the identification of processing conditions favouring enterotoxin production, analytical methods available and evaluation of current criteria presented in this report, the Committee has identified the need for the following new criteria.

11.1. Cheeses made from raw milk and from thermised milk

Depending on the type of cheese concerned the first 2 – 3 days in the beginning of manufacturing are critical period for SE production. The counts of *S. aureus* may increase and already start to decrease during this period. Since the exact timing for this growth / decrease –curve is not known, there should be a clear safety margin in order to avoid SE production.

The counts of *S. aureus* usually decrease in later stages of manufacturing and give little information on the possibility of SE production if cheese is tested for *S. aureus* counts. Therefore, if cheeses are tested for *S. aureus* counts, the age should not be over 48-72 hours.

Due to the extrinsic and intrinsic factors affecting the growth and toxin production of a *S. aureus* strain, the levels of SE produced in the optimal laboratory conditions compared to the suspected cheese may be much higher. Therefore, detecting of SE producing strains cannot be defined as a criterion for the presence of SE in a suspected cheese.

If cheese is analysed after 48-72 hours after the start of processing, the testing for SE instead of *S. aureus* counts is more valid. The cheese can be directly tested for enterotoxins. If enterotoxins are detected, the cheese is not acceptable for human consumption. Alternatively, TNase testing of the cheese can be made first. If it gives positive result, SE testing is necessary.

Using these kind of criteria instead of current ones, testing can either be applied on the beginning of processing or during later stages of the shelf-life of cheeses. The selection of analytical method therefore depends on the purpose of the use of criteria (e.g. controlling in processing or suspecting a cheese to have caused a foodborne outbreak).

11.2. Soft cheese made from heat-treated milk

The counts of *S. aureus* usually decrease in later stages of manufacturing and give little information on the possibility of SE production if cheese is tested for *S. aureus* counts (see Chapter 10.2.4). Therefore, if cheeses are tested for *S. aureus* counts, their age should not be over 48-72 hours.

If cheese is analysed after 48-72 hours after the start of processing, the testing for SE instead of *S. aureus* counts should be used. The cheese can be directly tested for enterotoxins. If enterotoxins are detected, the cheese is not acceptable for human consumption. Alternatively, TNase testing of the cheese can be made first. If it gives positive result, SE testing should be made.

Using these kind of criteria instead of current ones, testing can either be applied on the beginning of processing or during later stages of the shelf-life of cheeses. The selection of analytical method therefore depends on the purpose of the use of criteria (e.g. controlling in processing or suspecting a cheese to have caused a foodborne outbreak).

11.3. Semi-hard and hard cheese made from heat treated milk

Semi-hard and hard cheeses (e.g. Cheddar and Swiss cheese) have been involved in foodborne intoxications caused by SE. Therefore the Committee proposes that new criteria are set to these types of cheeses.

If cheese is analysed after 48-72 hours after the start of processing, the testing for SE instead of *S. aureus* counts should be used. The cheese can be directly tested for enterotoxins. If enterotoxins are detected, the cheese is not acceptable for human consumption. Alternatively, TNase testing of the cheese can be made first. If it gives positive result, SE testing is necessary.

Using these kind of criteria instead of current ones, testing can either be applied on the beginning of processing or during later stages of the shelf-life of cheeses. The selection of analytical method therefore depends on the purpose of the use of criteria (e.g. controlling in processing or suspecting a cheese to have caused a foodborne outbreak).

11.4. Fresh cheese made from heat-treated milk

S. aureus contamination is possible and it can easily multiply because of their high water activity (0.94-0.96) and high pH value (6.0-6.2) in whey cheese. Therefore, this criterion for fresh cheese should also be applied for whey cheeses.

11.5. Powdered milk

In order to quarantine the safety of powdered milk, the criteria applied should cover both the possibility that SE has been produced in powdered milk during manufacturing and the possibility that the product has been contaminated by *S. aureus* after processing. Therefore, the Committee finds it important, that powdered milk will be tested both for *S. aureus* counts and SE. If enterotoxins are detected, the product is not acceptable for human consumption. Alternatively, TNase testing of the products can be made first. If it gives positive result, SE testing should be made.

12. CONCLUSIONS

Epidemiology

- Staphylococcal foodborne intoxication is reported to be one of the most common form of bacterial foodborne outbreaks in many countries. The overview of outbreak reports from 15 European countries indicates that milk and dairy products were involved in 1 – 9 % (mean 4.8 %) of all the incriminated foods in staphylococcal outbreaks.
- Staphylococcal foodborne intoxication, in which major symptoms are vomiting and diarrhoea, occurs after ingestion of staphylococcal enterotoxins (SE) produced in food by enterotoxigenic strains of coagulase-positive staphylococci mainly *S. aureus*. Coagulase negative staphylococci have never been reported to cause foodborne outbreaks.
- *S. aureus* intoxications are not always notifiable in all Member States, therefore increasing the possibility of under reporting of cases.

Factors influencing SE in foods

- Coagulase positive staphylococci are facultative anaerobic bacteria which can grow over a relatively wide range of pH and temperature and at low water activity (minimum a_w is 0.83, provided that all other conditions are optimal). Ranges for SE production are somewhat narrower.
- However, not all coagulase positive staphylococci are SE producers and even so they will not produce toxin in every food. This is attributed to the fact that SE formation is influenced by numerous combinations of parameters of the food such as water activity, pH, redox potential and temperature, and to a great extent by bacterial antagonisms. In fact, starter cultures can effectively prevent growth of *S. aureus* and SE production.
- Growth and SE production of *S. aureus* in dairy products can be prevented by heat-treatment of milk, or inhibited by using starter cultures, antagonistic effect of natural flora, concentration of salt, drop of pH, low temperature of processing and storage of cheese and / or minimising the pressing time.
- SE are much more resistant to detrimental effects of the environment than the staphylococcal bacterial cells. SE are normally not or only slightly inactivated during food processing, storage, distribution or during the preparation of the food in the kitchen. Therefore, if enterotoxigenic staphylococci are able to grow in food to high numbers (more than 10^5 to 10^6 cfu/g or /ml) before they are killed there is still a risk for intoxication with consumption.
- In general, human strains are more often SE producers than strains isolated from bovines, and more toxinogenic strains are found in mastitis milk than in milk from the healthy udder. In strains isolated from food, production of SEA and SED either alone or together with other types of SE frequently dominate.

Conditions favouring SE production in milk and dairy products

- Liquid milk is an excellent medium for the growth and SE production (favourable pH, water activity and nutrients) and at temperatures over 7 °C,

numbers of enterotoxinogenic *S. aureus* may increase rapidly even when the initial levels are low.

- In cheese manufacturing enterotoxinogenic *S. aureus* can multiply and produce enterotoxin during the first stages of production when the pH of the curd is higher than 5.0 and the competing LAB bacteria have not reach high number. This favorable for multiplication of *S. aureus* period extends, in the different types of cheeses from several (5-10) to 48 hours at the maximum.
- Experiments for enterotoxins production in pasta filata cheeses, internal mould ripened cheeses and processed cheeses failed to prove the presence of enterotoxin whatever the size of the inoculum of enterotoxinogenic *S. aureus* was used.
- On the contrary, whey cheeses and imitation cheeses appears to be a favourable environment for growth of *S. aureus* and even small inoculum (10^2 - 10^3 cfu/g) can result in enterotoxin production.
- In cream production critical stage is considered the period of cream ripening if the process is conducted in favorable for *S. aureus* growth.
- In milk powder and ice cream production critical stages are the pre-drying and pre-freezing periods in which *S. aureus* can multiply if temperature is favorable.

Correlation between SE production and *S. aureus* counts

- In culture media, enterotoxin has been detected when *S. aureus* reached population greater than 10^5 cfu/ml. In cheese production, all experimental data agree that SE was detected when the number of enterotoxinogenic *S. aureus* was at least 10^6 cfu/g. No differences have been observed during the manufacture of different types of cheeses, concerning the above conclusions.
- In staphylococcal outbreaks, enterotoxinogenic *S. aureus* levels of 10^5 to 10^6 cfu/g or /ml or higher, have been reported.
- Therefore, the range of populations of enterotoxinogenic *S. aureus* in a food between to 10^5 and 10^6 cfu/ml or /gr or higher is consider to pose a risk for consumers. Since SE are more stable compared to *S. aureus* bacterial cells, it is possible to test a product with negative results for *S. aureus* counts although SE exists in the products.

Evaluation of the analytical methods for SE detection in milk products

- All methods available use specific antibodies against staphylococcal enterotoxins. These kits have been developed according to three principles: ELISA (enzyme linked immunosorbent assay), ELFA (enzyme linked fluorescent assay) or RPLA (reverse passive latex agglutination). Some of these tests can differentiate types of staphylococcal enterotoxins from A to E and others cannot. False-positive results may occur.
- One possible option is to screen food for thermonuclease (TNase) as an indicator of staphylococcal growth to high levels, especially when *S. aureus* bacteria have been killed after growth during processing. It gives information on the

past/present occurrence of high levels of *S. aureus* but not on SE production. Therefore, in case of a positive result confirmation is necessary by testing for the presence of SE.

Evaluation of current criteria

- Based on the information on *S. aureus* growth and SE formation in the production of milk and different dairy products, on epidemiological evidence as well as on the methods currently available for *S. aureus* and SE detection, the microbiological criteria for *S. aureus* counts and/or SE in milk and in certain dairy products are essential and useful to protect public health.
- The current criteria for raw milk intended for direct human consumption, fresh cheese made from heat treated milk as well as for frozen milk-based products, are valid.

Identification of new criteria

- During early phase of cheese production *S. aureus* may grow and produce SE. However, the counts of *S. aureus* usually decrease in later stages of manufacturing and give little information on the possibility of SE production if cheese is then tested for *S. aureus* counts.
- There is a need to establish criteria for semi-hard and hard cheeses.
- In whey cheeses, *S. aureus* can easily multiply because of their high water activity and pH. Therefore, there is a need for criteria.
- Criteria for *S. aureus* or SE in cultured dairy products, pasta filata cheeses and in processed cheese are not needed.

13. RECOMMENDATIONS

The Committee recommends

- (1) To establish and harmonise a reporting system for foodborne staphylococcal intoxications at European level.
- (2) To minimise the SE risks in raw milk cheeses by using GHP and effective starter cultures.
- (3) To apply the current criteria for fresh cheese also for whey cheese.
- (4) To revise the current criteria for cheeses made from raw and thermised milk and for soft cheese made from heat treated milk as well as to establish new criteria for semi-hard and hard cheeses, based on the same principles as for soft cheese. This should include two types of criteria:
 - In early phase (2 – 3 days after the beginning of production), the criteria should be based on counts of *S. aureus*;
 - In later stages, the analysis of enterotoxin in cheese alone or after the detection of TNase, should be applied.

- (5) To revise the current criteria for raw milk intended for processing as well as the criteria for powdered milk.
- (6) To reconsider the current criteria, which refer to *S. aureus*, to cover all the coagulase positive staphylococci.

14. ACKNOWLEDGEMENTS

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The working group was chaired by

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15. REFERENCES

- Adesiyun, A.A., Tatini, S.R., Hoover, D.G., (1984). Production of enterotoxins by *Staphylococcus hyicus*. Vet. Microbiol., 9, 487-495.
- Ahmed, A.A-H., Mustafa, M.K., Marth, E.H., (1983). Growth and survival of *Staphylococcus aureus* in Egyptian Domiati cheese. J. Food Prot. 46:412-415.
- Al Bustan, M.A., Udo, E.E., Chugh, T.D., (1996). Nasal carriage of enterotoxin-producing *Staphylococcus aureus* among restaurant workers in Kuwait City. Epidemiol. Infect., 116, 319-22.
- Almazan, J., De la Fuente, R., Gomez-Lucia, E., Suarez, G., (1987). Enterotoxin production by strains of *Staphylococcus intermedius* and *Staphylococcus aureus* isolated from dog infections. Zbl. Bakt. Hyg., A 264, 29-32.
- Altekruse, S.F., Timbo, B.B., Mowbray, J.C., Bean, N.H., Potter, M.E. (1998). Cheese-associated outbreaks of human illness in the United States, 1973 to 1992 : sanitary manufacturing practices protect consumers. J. Food. Prot. 61, 1405-1407.
- Anifantakis, E.M., (1991). Greek cheeses: A tradition of centuries. National Dairy Committee of Greece. Athens.
- Anonymous, 2001, *Staphylococcus* food poisoning in Japan. IARS, 22, 185-186. www.idsc.nih.go.jp
- Anunciacao, L.L.C., Linardi, W.R., Docarnco, L.S., Bergdoll, M.S., (1994). Production of Staphylococcal enterotoxin in a white cheese. Revista de Microbiologia 25:68-71.
- Asperger, H., (1994). *Staphylococcus aureus*. In "The significance of pathogenic microorganisms in raw milk" Int. Dairy Federation, Bulletin 9405 (S.I).
- Asperger, H., Zangerl, P., (2002). *Staphylococcus aureus*. In Encyclopedia of Dairy Sciences (eds. Roginski, H., Fuquay, J.W., Fox, P.) Academic press, Elsevier Science, London, p. 2563-2569.
- AOAC, (1995a). Staphylococcal enterotoxins in foods: Microslide gel double diffusion test. Sec. 17.5.03, method 976.31. In Official methods of AOAC international, 16th ed., P.A. Cunniff (Ed.), p. 34-37. AOAC international, Gaithersburg, MD.
- AOAC, (1995b). Staphylococcal enterotoxins in foods: extraction and separation methods. Sec. 17.5.03, method 980.32. In Official methods of AOAC international, 16th ed., P.A. Cunniff (Ed.), p. 40-41. AOAC international, Gaithersburg, MD.
- AOAC, (1995c). Staphylococcal enterotoxins in selected foods: Polyvalent enzyme immunoassay method (TECRA SET). Sec. 17.5.03, method 993.06. In Official methods of AOAC international, 16th ed., P.A. Cunniff (Ed.), p. 37-40. AOAC international, Gaithersburg, MD.

- Asao, T., Kumeda, Y., Kawai, T., Shibata, T., Oda, H., Haruki, K., Nakazawa, H. & Kozaki, S., (2003). An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. *Epidemiol. Infect.*, 130, 33-40.
- Avena, R.M., Bergdoll, M.S., (1967). Purification and some physicochemical properties of enterotoxin C, *Staphylococcus aureus* strain 361. *Biochemistry*, 6, 1474-80.
- Baird-Parker, A.C., (1990). The staphylococci: an introduction. *J. Appl. Bact. Symp. Suppl.*, 1S-8S.
- Balaban, N., Rasooly, A., (2000). Review - Staphylococcal enterotoxins, *Int. J. Food Microbiol.*, 61, 1-10.
- Barber, L.E., Deibel, H., (1972). Effect of pH and oxygen tension on staphylococcal growth and enterotoxin formation in fermented sausage. *Appl. Microbiol.*, 24, 891-8.
- Barrett, N.J., (1986). Communicable disease associated with milk and dairy products in England and Wales: 1983-1984. *J. Infect.*, 12, 265-272.
- Batish, V.K., Chander, H., (1987). Occurrence of *Staphylococcus aureus* and their preformed enterotoxins in frozen dairy desserts. *Aust. J. Dairy Technol.* 42:22-24.
- Bayles K.W., Iandolo J.J., (1989). Genetic and molecular analyses of the gene encoding staphylococcal enterotoxin D. *J. Bacteriol.*, 171, 4799-806.
- Belay, N., Rasooly, A., (2002). *Staphylococcus aureus* growth and enterotoxin A production in an anaerobic environment. *J. Food Prot.*, 65, 199-204.
- Becker, H., El-Bassiony, T.A., Terplan, G., (1984). Zur Abgrenzung der *Staphylococcus aureus*-Thermonuclease von hitzestabilen Nucleasen anderer Bakterien. *Arch. Lebensmittelhyg.*, 35, 114-118.
- Becker, H., Schaller, G., Märtlbauer, E., (1994). Nachweis von *Staphylococcus-aureus*-Enterotoxinen in Lebensmitteln mit kommerziellen Enzymimmuntests. *Arch. Lebensmittelhyg.*, 45, 27-32.
- Becker, K., Keller, B., von Eiff, C., Bruck, M., Lubritz, G., Etienne, J., Peters, G., (2001). Enterotoxigenic potential of *Staphylococcus intermedius*. *Appl Environ Microbiol* 67, 12, 5551-5557.
- Bennett, R.W., Amos, W.T., (1983). *Staphylococcus aureus* growth and toxin production in Imitation cheeses. *J. Food Sci.* 48:1670-1673.
- Bennett, R.W., 1996. Atypical toxigenic Staphylococcus and non-Staphylococcus aureus species on the horizon? An update. *J. Food Protect.*, Vol.59, 10, 1123-1126.
- Bergdoll, M.S., (1989). *Staphylococcus aureus*. In: Doyle, M.P. (ed.). Foodborne bacterial pathogens. Marcel Dekker, Inc., New York, Basel, pp. 463-523.

- Bergdoll, M.S., (1983). Enterotoxins. In: Easmon, C.S.F., Adlam, C. (eds). Staphylococci and staphylococcal infections. Vol. 2, pp. 560-98. Academic Press, London.
- Bergdoll, M.S., Crass, B.A., Reiser, R.F., Bobbins, R.N., Davis, P.D., (1981). A new staphylococcal enterotoxin, enterotoxin F, associated with toxic-shock-syndrome *Staphylococcus aureus* isolates. Lancet I, 1017-21, 1118.
- Bergdoll, M.S., Borja, C.R., Robbins, R., Weiss, K.F., (1971). Identification of enterotoxin E. Infect. Immun., 4, 593-5.
- Bergdoll, M. S., (1970). Enterotoxins in *Microbiol Toxins*, Vol. 3, Montie, T.C., Kadis, S., Ajl, S.J., (Eds.), Academic Press, New York, NY, pp. 265-326.
- Bergdoll, M.S., Borja, C.R., Avena, R.M., (1965). Identification of a new enterotoxin as enterotoxin C. J. Bacteriol., 90, 1481-5.
- Bergdoll, M.S., Surgala, J., Dack, G.M., (1959). Staphylococcal enterotoxin. Identification of a specific precipitating antibody with enterotoxin-neutralizing property. J. Immunol., 83, 334-8.
- Betley, M.J., Borst, D.W., Regassa, L.B., (1992). Staphylococcal enterotoxins, toxic shock syndrome toxin and streptococcal pyrogenic exotoxins: a comparative study of their molecular biology. Chem. Immunol., 55, 1-35.
- Betley, M.J., Schlievert, P.M., Bergdoll, M.S., Bohach, G.A., Iandolo, J.J., Khan, S.A., Pattee, P.A., Reiser, R.F., (1990). Staphylococcal gene nomenclature. ASM News, 56, 182.
- Betley M.J., Mekalanos J.J., (1985). Staphylococcal enterotoxin A is encoded by phage. Science, 229, 185-7.
- Boer, de E., Kuik, D., (1987). A survey of the microbiological quality of blue veined cheeses. Neth. Milk dairy J. 41:227-237.
- Bone, F.J., Bogie, D., Morgan-Jone, S.C., (1989). Staphylococcal food poisoning from sheep milk cheese. Epidemiol. Infect. 103, 449-458.
- Borja, C.R., Bergdoll, M.S., (1967). Purification and partial characterization of enterotoxin C produced by *Staphylococcus aureus* strain 137. Biochemistry, 6, 1467- 73.
- Bula, C.J., Bille, J., Glausez, M.P., (1995). An epidemic of foodborne listeriosis in Western Switzerland: description of 57 cases involving adults. Clin. Inf. Dis. 20-66.
- Casman, E. P., Bennett, R. W., Dorsey, A. E., Stone, J. E., (1969). The Micro-slide gel double diffusion test for the detection and assay of the staphylococcal enterotoxins. Health Lab. Sci. 6, 185-198.
- Casman, E.P., Bennett, R.W., Dorsey, A.E., Issa, J.A., (1967). Identification of a fourth staphylococcal enterotoxin, enterotoxin D. J. Bacteriol., 94, 1875-82.
- Casman, E.P., Bergdoll, M.S., Robinson, J., (1963). Designation of staphylococcal enterotoxin. J. Bacteriol., 85, 715-6.

- Casman, E.P., (1960). Further serological studies of staphylococcal enterotoxin. J. Bacteriol., 79, 849-56.
- CDC (Center for Disease Control), (1970). Staphylococcal food poisoning traced to butter. Alabama. Morb. Mort. Weekly Rep. 19:217.
- Chopin, A., Mocquot, G., Le Great, T., (1977). Destruction de *Microbacterium lacticum*, *Escherichia coli*, et *Staphylococcus aureus* au cours du lait par atomization. II. Influence des conditions de sechage. Can. J. Microbiol. 23:755-763.
- Chopin, A., Tesone, S., Vila J-P., Le Graet, Y., Mocquot, G., (1978). Survie de *Staphylococcus aureus* au cours de la preparation et de la conservation de lait ecreme en poudre. Problemes poses par le denombrement des survivants. Can J. Microbiol. 24:1371.
- Clark, W.S. and Nelson, F.E. (1961). Multiplication of coagulase positive staphylococci in grade A raw milk samples. J. Dairy Sci. 44:232-
- CAC (Codex Alimentarius Commission), 1997a. General Requirements (Food Hygiene). Supplement to volume 1B. Joint FAO/WHO Food Standards Programme, FAO, Rome.
- CAC (Codex Alimentarius Commission), 1997b. Principles for the establishment and application of microbiological criteria for foods. CAC/GL 21 – 1997, FAO, Rome.
- Concon, J.M., (1988). Food toxicology (in two parts). Part B: contaminants and additives, pp. 802-3. Marcel Dekker, Inc., New York, Basel.
- Crowther, J.S., Holbrook, R., (1980). Immunological detection of food-poisoning toxins. In: Goodfellow, M., Board, R.G. (eds), Microbiological classification and identification, pp. 339-357. Academic Press, London.
- Daminelli, P., (1999). Dinamica della sopravvivenza di *Staphylococcus aureus* in fromaggi tipo crescenza ed Italico artificialmente contaminati. Le Selezione Veterinaria, 11/815-829.
- De Buyser, M.L., Janin, F., Dilasser, F., (1985). Contamination of ewe cheese with *Staphylococcus aureus*: study of an outbreak of food poisoning. In : Jelkaszewicz, J., (Ed), The Staphylococci, Zbl. Bakt. Suppl. 14, Gustav Fisher Verlag, Stuttgart, pp. 677-678.
- De Buyser, M.L., Lapeyre, C., (1995). Inventaire des souches isolées de 5 types de fromages au lait cru: le méthodes d' analyse, le dénombrement de *Staphylococcus aureus*, le détection des toxines. In "Compte-rendu de la journée Thématique Staphylocoques". ARILAIT-Recherches, 30 Mars 1995. Paris. pp.67-72.
- De Buyser, M. L., Dufour, B., Maire, M., Lafarge, V., (2001). Implication of milk and milk products in foodborne diseases in France and in different industrialised countries, Int. J. Food Microbiol., 67, 1-17.
- De la Fuente, R., Suarez, G., Schleifer, K.H., (1985). *Staphylococcus aureus* subsp. *anaerobius* subsp. nov., the causal agent of abscess disease of sheep. Int. J. Syst. Bacteriol., 35, 99-102.

- Degremeont, E., Vincent, J.P., Catteau, M., (1995). Comportement de souches de *Staphylococcus aureus* en expérimentation planifiée. Croissance et production de toxins. In "Compte-rendu de la journée Thématique Staphylocoques". ARILAIT-Recherches, 30 Mars 1995. Paris. pp.73-87.
- Devriese, L.A., Hajek, V., Oeding, P., Meyer, S.A., Schleifer, K.H., (1978). *Staphylococcus hyicus* (Sompolinsky 1953) comb. nov. and *Staphylococcus hyicus* subsp. *chromogenes* subsp. nov. Int. J. Syst. Bacteriol., 28, 482-90.
- Dinges, M.M., Orwin, P.M., Schlievert, P.M., (2000). Exotoxins of *Staphylococcus aureus*. Clin. Microbiol. Rev., 13, 16-34.
- Donnelly, C.B., Lesli, J.E., Black, L.A., (1968). Production of enterotoxin A in milk. Appl. Microbiol. 16:917-924.
- EC (European Commission), 1997. Principles for the development of microbiological criteria for animal products and food of animal origin intended for human consumption. ISBN 92-828-1871-3. Brussels.
- Erickson, A., Deibel, R.H., (1973). Production and heat stability of staphylococcal nuclease. Appl. Microbiol., 40, 13-18.
- Erkmen, O., (1995). Behavior of *Staphylococcus aureus* in Turkish feta cheese during manufacturing and ripening. J. Food Prot. 58:1201-1205.
- Evenson, M.L., Hinds, M.W., Berstein, R.S., Bergdoll, M.S., (1988). Estimation of human dose of staphylococcal enterotoxin A from a large outbreak of staphylococcal food poisoning involving chocolate milk. Int. J. Food Microbiol., 7, 311-316.
- Ewald, S., Notermans, S., (1988). Effect of water activity on growth and enterotoxin D production of *Staphylococcus aureus*. Int. J. Food Microbiol., 6, 25-30.
- Fitzgerald, J.R., Monday, S.R., Foster, T.J., Bohach, G.A., Hartigan, P.J., Meaney, W.J., Smyth, C.J., (2001). Characterization of a putative pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. J. Bacteriol., 183, 63-70.
- FAO/WHO, (1973). Code of principles concerning milk and milk products. International standard and standard methods of sampling and analysis for milk products. 7th Edit. FAO, CAC/M-1, 1973. Rome.
- Fonteca, J., Pelaez, C., Juarem, M., Requena, T., Gomez, C., Ramos, M., (1990). Biochemical and microbiological characteristics of artisanal hard goat's cheese. J. Dairy Sci. 73:1150-1157.
- Foster, G., Ross, H.M., Hutson, R.A., Collins, M.D., (1997). *Staphylococcus lutrae* sp. nov., a new coagulase-positive species isolated from otters. Int. J. Syst. Bact., 47, 724-6.
- Fraser, J., Arcus, V., Kong, P., Baker, E., Proft, T., (2000). Superantigens – powerful modifiers of the immune system. Mol. Med. Today, 6, 125-32.

- Freed, R.C., Evenson, M.L., Reiser, R.F., Bergdoll, M.S., (1982). Enzyme-Linked immuno-sorbent assay for detection of staphylococcal enterotoxins in foods. *Appl. Environ. Microbiol.* 44, 1349-1355.
- Fukuda, S., Tokuno, H., Ogawa, H., Sasaki, M., Kishimoto, T., Kawano, J., Shimizu, A., Kimura, S., (1984). Enterotoxigenicity of *Staphylococcus intermedius* strains isolated from dogs. *Zbl. Bakt. Hyg., A* 258, 360-367.
- Galesloot, T.E., and Stadhouders, J., (1968). The microbiology of spray dried milk products with special reference to *Staphylococcus aureus* and *Salmonella*. *Neth. Milk. Dairy J.* 22:158-172.
- Genigeorgis, C.A., (1989). Present state of knowledge on staphylococcal intoxications. *Int. J. Food Microbiol.*, 9, 327-60.
- Gilmour, A., Harvey, J., (1990). Staphylococci in milk and milk products. *J. Appl. Bact. Symp. Suppl.*, 19, 147S-66S.
- Glass, K.A., Kaufman, K.M., Johnson, E.A., (1998). Survival of bacterial pathogens in pasteurized process cheese slices stored at 30°C. *J. Food Prot.* 61:290-294.
- Gogov, Y., Slavchev, G., Peeva, T. (1984). Cold resistance of *S. aureus* and A and C₂ staphylococcus enterotoxins in ice cream. *Vet. Sci. (Sofia)*, 21:46.
- Gomez-Lucia, E., Goyache, J., Orden, J.A., Domenech, A., Hernandez, F.J., Quiteria, J.A.R., Lopez, B., Blanco, J.L., Suarez, Q., (1992). Growth of *Staphylococcus aureus* and synthesis of enterotoxin during ripening of experimental Manchego-type cheese. *J. Dairy Sci.* 75:19-26.
- Gross, E.M., Weizman, Z., Picard, E., Mates, A., Sheiman, R., Platzner, N., Wolff, A., (1988). Milkborne gastroenteritis due to *Staphylococcus aureus* enterotoxin B from a goat with mastitis. *Am. J. Trop. Med. Hyg.* 39 (1), 103-104.
- Gutierrez, C., Abee, T., Booth, I.R., (1995). Physiology of the osmotic stress response in microorganisms. *Int. J. Food Microbiol.*, 28, 233-444.
- Hajek, V., (1976). *Staphylococcus intermedius*, a new species isolated from animals. *Int. J. Syst. Bacteriol.*, 26, 401-8.
- Halpin-Dohnalek, M.I., Marth, E.H., (1989). *Staphylococcus aureus*: production of extracellular compounds and behavior in foods – a review. *J. Food Prot.*, 52, 267-82.
- Halpin, M.I., Math, E.H., (1986). Growth and enterotoxin production by *Staphylococcus aureus* in cream with various amounts of milk fat. *J. Food Prot.* 49:851.
- Hamama, A., El Hankouri, N., El Ayadi, M., (2002). Fate of enterotoxigenic *Staphylococcus aureus* in the presence of nisin producing *Lactococcus lactis* strain during manufacture of Iben, Moroccan traditional fresh cheese. *Int. Dairy J.* 12:933-938.

- Haeghebaert, S., Le Querrec, F., Bouvet, P., Gallay, A., Espié, E., Vaillant, V. (2002). Les toxi-infections alimentaires en France en 2001. Bulletin d'épidémiologie hebdomadaire, 50, 249-253.
- Hawryluk, T., Hirshfield, I., (2002). A super antigen bioassay to detect staphylococcal enterotoxin A. J Food Prot, 65, 7, 1183-1187.
- Hennekinne, J-A., Gohier, M., Maire, T., Lapeyre, C., Lombard, B., Dragacci, S., (2003). First proficiency testing to evaluate the ability of National Reference Laboratories of the European Union to detect staphylococcal enterotoxins in milk products. J. AOAC Int. *in press*.
- Hirooka, E.Y., Muller, E.E., Freitas, J.C., Vicente, E., Yoshimoto, Y., Bergdoll, M.S., (1988). Enterotoxigenicity of *Staphylococcus intermedius* of canine origin. Int. J. Food Microbiol., 7, 185-191.
- Hoffner, F., (1996). *Staphylococcus aureus* en fabrication de Reblochon. Thèse de Doctorat Vétérinaire. Nantes.
- Holmberg, S.D., Blake; P.A., (1984). Staphylococcal food poisoning in the United States. New facts and old misconceptions. J. Am. Med. Assoc., 251, 487-9.
- Hoover, D.G., Tatini, S.R., Maltais, J.B., (1983). Characterization of staphylococci. Appl. Environ. Microbiol., 46, 649-60.
- Ibrahim, G.F., Baldock, A.K., Radford, D.R., Ireland, L.B., (1981). Inhibition of *Staphylococcus aureus* growth and enterotoxin A production in Cheddar cheese produced with variable starter activity. J. Food Prot. 44:263-267.
- Ibrahim, G.F., Radford, D.R., Baldock, A.K., Ireland, L.B., (1981a). Inhibition of growth of *Staphylococcus aureus* and enterotoxin A production in Cheddar cheese produced with induced starter failure. J. Food Prot. 44:189-193.
- ICMSF (International Commission on Microbiological Specifications for Foods), (1996). Microorganisms in Food 5: Characteristics of Microbial Pathogens. Blackie Academic and Professional, London. Kluwer Academic/Plenum Publishers, New York. *Staphylococcus aureus*, Ch. 17, pp. 299-333.
- IDF – International Dairy Federation, (1998). International IDF Standard 83A:1998. Milk and milk based products, detection of thermonuclease produced by coagulase-positive staphylococci in milk and milk based products.
- IDF – International Dairy Federation, (1991). Significance of the indigenous antimicrobial agents of milk to the dairy industry. Bull. IDF, 264, 2-19.
- Igimi, S., Takahashi, E., Mitsuoka, T., (1990). *Staphylococcus schleiferi* subsp. *coagulans* subsp. nov., isolated from the external auditory meatus of dogs with external ear otitis. Int. J. Syst. Bact., 40, 409-11.
- Ikram, M., Leudecke, O., (1977). Growth and enterotoxin A production by *Staphylococcus aureus* in fluid dairy products. J. Food Prot. 40:769-771.

- Janin, F., De Buyser, M.L., Lapeyre, C., Feinberg, M., (1983). Radio immunological quantitative determination of *Staphylococcus aureus* enterotoxin A in various foods. *Sci. Aliments* 3, 397-412.
- Jarraud, S., Peyrat, M.A., Lim, A., Tristan, A., Bes, M., Mougel, C., Etienne, J., Vandenesch, F., Bonneville, M., Linal, G., (2001). *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. *J. Immunol.*, 166, 669-77; erratum 4260.
- Jarvis, A.W., Lawrence, R.C., Pritchard, G.G., (1975). Glucose repression of enterotoxins A, B and C and other extracellular proteins in staphylococci in batch and continuous culture. *J. Gen. Microbiol.*, 86, 75-87.
- Johnson, E.A., Nelson, J.H., Johnson, M., (1990). Microbiological safety of cheese made from heat treated milk, part II. *Microbiology. J. Food Prot.* 53:519-540.
- Karaioannoglou, Pr., Koidis, P., Mantis, A., (1983). Growth and enterotoxin production by *Staphylococcus aureus* in whey cheese "Manouri". *Acta Microbiologica Hellenica* 28:86-95.
- Keller, G.M., Hanson, R.S., Bergdoll, M.S., (1978). Effect of minerals on staphylococcal enterotoxin B production. *Infect. Immun.*, 20, 158-160.
- Khambaty, F. M., Bennett, R. W., Shah, D. B., (1994). Application of pulse field gel electrophoresis to the epidemiological characterisation of *Staphylococcus intermedius* implicated in a food-related outbreak. *Epidemiol. Infect.*, 113, 75-81.
- Krejaković-Miljković, (1960). Die Erhaltung von Staphylokokken=Enterotoxin in weißem Lake=Käse. *Arch. Lebensmittelhyg.*, 11, 103-4.
- Koenig, S., Marth, E.H., (1982). Behavior of *staphylococcus aureus* in Cheddar cheese made with sodium chloride or mixture of sodium chloride and potassium chloride. *J. Food Prot.* 45:996-1002.
- Kosikowski, F.W., Mistry, V.V., (1997). Cheese and fermented milk foods (F.V. Kosikowski, L.L.C., Publs, Westport. Conn).
- Lachica, R.V.F., Genigeorgis, C., Hoeplich, P.D., (1971). Metachromatic agar-diffusion method for detecting staphylococcal nuclease in foods. *Appl. Microbiol.*, 21, 585-587.
- Lapeyre, C., Kaveri, S.V., Janin, F., Strosberg, A.D., (1987). Production and characterization of monoclonal antibodies to staphylococcal enterotoxins: use in immunodetection and immunopurification. *Mol. Immunol.*, 24, 1243-1254
- Lapeyre, C., Janin, F., Kaveri, S. V., (1988). Indirect double sandwich ELISA using monoclonal antibodies for detection of staphylococcal enterotoxins A, B, C1 and D in food samples. *Food Microbiol.* 5, 25-31.
- Lapeyre, C., de Solan, M.N., Drouet, X., (1996). Immunoenzymatic detection of staphylococcal enterotoxins : international interlaboratory study. *J. AOAC Int.* vol. 79, 5, 1095-1101.

- Letondeur-Lafarge, V., Lahellec, C., (1997). Aspects hygiénique. (In "Le Fromage". Techn. Et Doc. Lavoisier, Paris, pp. 741-755.
- Lodi, R., Malaspina, P., Brasca, M., (1994). Lactic acid bacteria: a quality parameter for fresh cheese. *Industria del latte*. 30:3-16.
- Lopez-Diaz, T.M., Santos, J.A., Gonzales, C.J., Moreno, B., Garcia, M., (1995). Bacteriological quality of a traditional Spanish Blue cheese. *Milchwissenschaft*, 50:503-505.
- Macaluso, L., Lapeyre, C., (2000). Characterisation of the staphylococcal enterotoxin research method in a dairy product. *Analisis*, 28, n°7, 610-615.
- Maguire, H.C.F., Boyle, M., Lexis, M.J., Pankhurst, J., Wieneke, A.A., Jacob, M., Bruce, J., O'Mahony, M., (1991). A large outbreak of food poisoning of unknown aetiologic associated with Stilton cheese. *Epidemiol. Infect.* 106, 497-505.
- Massa, S., Poda, G., Cesaroni, D., Trovatelli, L.D., (1989). A bacteriological survey of ice-cream. *Food Microbiol.* 6:129.
- Mantis, A., 1973: Production of staphylococcal enterotoxins in white-brined cheese feta. Docent-Doctoral Thesis. School of Vet. Med. Univ. Thessaloniki (In Greek).
- Martin, S.E., Myers, E.R., Iandolo, J.J., (2001). *Staphylococcus aureus* In : Hui Y. H., Pierson M. D., Gorham J. R. (eds) Foodborne disease handbook Vol. 1 – Bacterial pathogens. New York Basel : Marcel Dekker Inc., 345-381.
- Mead, P.S., Slutsker, L., Dietz, V., McCraig L.F., Bresee, J.S., Shapiro, C., Griffins, P.M., Tauxe, R.V. (1999). Food-related illness and death in the United States. *Emerg. Infect. Dis.*, 5, 605-625.
- Medina, M., Gaya, P., Nunez, M., (1992). Gredos goat's milk cheese: microbiological and chemical changes throughout ripening. *J. Dairy Res.* 59:563-566.
- Mehrotra, M., Wang, G., Johnson, W.M., (2000). Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1 and methicillin resistance. *J. Clin. Microbiol.*, 38, 1032-8.
- Meyer, R.F., Palmieri, M.J., (1980). Single radial immunodiffusion method for screening Staphylococcal isolates for enterotoxin. *Appl. Environ. Microbiol.*, 40, 6, 1080-1085.
- Meyrad, A., Boutrand-Loei, S., Ray-Gueniot, S., Mazuy, C., Gaspard, C.E., Jaubert, G., Perrin, G., Lapeyre, C., Vernory-Rozand, C., (1998). Growth and enterotoxin production of *Staphylococcus aureus* during the manufacture and ripening of Camembert type cheeses from raw goat's milk. *J. Appl. Microbiol.* 85:537-544.
- Meyrand, A., Vernozy-Rozand., (1999). Croissance et enterotoxinogenese de *Staphylococcus aureus* dans différents fromages. *Revue Med. Vet.* 150:601-616.
- Miller, B.A., Reiser, R.F., Bergdoll, M.S., (1978). Detection of staphylococcal enterotoxins A, B, C, D and E in foods by radioimmunoassay, using staphylococcal

- cells containing protein A as immuno-adsorbent. *Appl. Environ. Microbiol.* 36, 421-426.
- Minor, T.E., Marth, E.H., (1970). Growth of *Staphylococcus aureus* in acidified pasteurized milk. *J. Milk Food Technol.* 33:516.
- Minor, T.E., Marth, E.H., (1972). Fate of *Staphylococcus aureus* in cultured buttermilk, sour cream and yogurt during storage. *J. Milk and Food Technol.* 35:302-306.
- Minor, T.E., Marth, E.H., (1972a). Los of viability by *Staphylococcus aureus* in acidified media. Inactivation by several acids mixtures of acids, and salts of acids. *J. Milk Food Technol.* 35:191-196.
- Minor, T.E., Marth, E.H., (1972b). *Staphylococcus aureus* and enterotoxin A in cream and butter. *J. Dairy Sci.* 55:1410-1414.
- Minor, T.E., Marth, E.H., (1976). *Staphylococci and their significance in foods.* Elsevier Scientific Publishing Company, Amsterdam.
- Morita, T.N., Patterson, J.E., Woodburn, M.J., (1979). Magnesium and iron addition to casein hydrolysate medium for production of staphylococcal enterotoxins A, B, and C. *Appl. Environ. Microbiol.*, 38, 39-42.
- Morse, S.A., Mah, R.A., Dobrogosz, W.J., (1969). Regulation of staphylococcal enterotoxin B. *J. Bacteriol.*, 98, 4-9.
- Mossel, D.A.A., Corry, J.E.L., Struijk, C.B., Baird, R.M., (1995): *Essentials of the microbiology of foods. A textbook for advanced studies.* John Wiley and Sons (eds), Chichester, England, 146-150.
- Mueller, M., Hahn, G., Heeschen, W., (1996). Multiplication and production of enterotoxin by *Staphylococcus aureus* during the experimental manufacture of Camembert cheese. *Kieler Milchwirtschaftliche-forschungsberichte*, 48:195-207.
- Munson, S.H, Tremaine, M.T., Betley, M.J., Welch, R.A., (1998). Identification and characterization of staphylococcal enterotoxin types G and I from *Staphylococcus aureus*. *Infect. Immun.*, 66, 3337-48.
- Naquib, M.M., Nour, M.A., Noaman, A.A., (1979). Survival of *Staphylococcus aureus* in Ras cheese. *Arch. Lebensmittelhygiene*, 30:227-228.
- Notermans, S., Dufrenne, J., In't Veld, P., (1991). Feasibility of a reference material for staphylococcal enterotoxin A. *Int. J. Food Microbiol.*, 14, 325-331.
- Notermans, S., Heuvelman, C.J., (1983). Combined effect of water activity, pH and sub-optimal temperature on growth and enterotoxin production of *Staphylococcus aureus*. *J. Food Sci.*, 48, 1832-5, 1840.
- Novick, R.P, Schlievert, P., Ruzin, A., (2001). Pathogenicity and resistance islands of staphylococci. *Microbes Infect.*, 3, 585-94.
- Nunez, M., Medina, M., (1980). Les microcoques et les staphylocoques dans le fromage bleu de Cabrabes. *Le Lait*, 60:171-183.

- Olsen, S.J., MacKinen, L.C., Goulding, J.S., Bean, N.H., Stutsker, L., (2000). Surveillance for foodborne disease outbreaks – United States, 1993-1997. *Morb. Mort. Wkly. Rep.*, 49 (SS01), 1-51.
- Orwin, P.M., Leung, D.Y.M., Donahue, H.L., Novick, R.P., Schlievert, P.M., (2001). Biochemical and biological properties of staphylococcal enterotoxin K. *Infect. Immun.*, 69, 360-6.
- Panetsos, A., Mantis, A., Kilikidis, S., (1970). An outbreak of cheese poisoning due to staphylococcal enterotoxin A. *Hellenic Armed Forces Med. Rev. A*: 637-640 (In Greek).
- Papageorgiou, A.C., Acharya, K.R., (2000). Microbial superantigens: from structure to function. *Trends Microbiol.*, 8, 369-75.
- Park, C.E., El Derea, H.B., Rayman, M.K., (1979). Effect of non-fat dry milk on recovery of staphylococcal thermonuclease from foods. *Can. J. Microbiol.*, 25, 44-46.
- Park, C.E., De Melo Serrano, A., Landgraf, M., Huang, J.C., Stankiewicz, Z., Rayman, M.K., (1980). A survey for microorganisms for thermonuclease production. *Can. J. Microbiol.*, 26, 532-535.
- Park, C.E., Akhtar, M., Rayman, M.K., (1992). Nonspecific reactions of a commercial enzyme-linked immunosorbent assay kit (TECRA) for detection of staphylococcal enterotoxins in foods. *Appl. Environ. Microbiol.* 58, 2509-2512.
- Park, C.E., Akhtar, M., Rayman, M.K., (1993). Simple solutions to false-positive staphylococcal enterotoxin assay with sea food tested with an enzyme-linked immunosorbent assay kit (TECRA). *Appl. Environ. Microbiol.* 59, 2210-2213.
- Park, C.E., Warburton, D., Laffey, P.J., (1996). A collaborative study on the detection of staphylococcal enterotoxins in foods by an enzyme immunoassay kit (Ridascreen). *Int. J. Food Microbiol.*, 29, 281-295.
- Pereira, M.L., Simeao do Carmo, L., José dos Santos, E., Pereira, J.L., Bergdoll, M.S., (1996). Enterotoxin H in staphylococcal food poisoning. *J. Food. Prot.* 59, 559-61
- Provent, C., (1986). Contribution a l' étude de l' origine et de la maîtrise de la contamination du fromage Mont d' Or par *Staphylococcus aureus*. Thèse de Doctorat Vétérinaire, Lyon, 1986.
- Qi, Y., Miller, K.J., (2000). Effect of low water activity on staphylococcal enterotoxin A and B biosynthesis. *J. Food Prot.*, 63, 473-8.
- Read, R.B., Bradshaw, J.G., (1967). γ -irradiation of staphylococcal enterotoxin B. *Appl. Microbiol.*, 15, 603-5.
- Reiser, R.F., Robbins, R.N., Noletto, A.L., Khoe, G.P., Bergdoll M.S., (1984). Identification, purification, and some physicochemical properties of staphylococcal enterotoxin C₃. *Infect. Immun.*, 45, 625-30.

- Reiter, B., Fewins, B.G., Fryer, T.F., Sharpe, M.E., (1964). Factors affecting the multiplication and survival of coagulase positive staphylococci in Cheddar cheese. *J. Dairy Res.* 31:261-272.
- Ren, K., Bannan, J.D., Pancholi, V., Cheung, A.L, Robbins, J.C., Fischetti, V.A., Zabriskie, J.B., (1994). Characterization and biological properties of a new staphylococcal exotoxin. *J. Exp. Med.*, 180, 1675-83.
- Ryser, E.T., (1998). Public Health Concerns. In "Applied Dairy Microbiology". E.H. Marth and J.L. Steele, eds. Marcel Dekker. Inc. New York.
- Robbins, R., Gould, S., Bergdoll, M. S., (1974). Detecting the enterotoxigenicity of *Staphylococcus aureus* strains. *Appl. Microbiol.* 28, 946-950.
- Rose, S.A., Modi, N.K., Tranter, H.S., Bailey, N.E., Stringer, M.F., Hambleton, P., (1988). Studies on the irradiation of toxins of *Clostridium botulinum* and *Staphylococcus aureus*. *J. Appl. Bact.*, 65, 223-30.
- Rosenbach, F.J., (1884). Micro-organismen bei den Wund-Infektions-Krankheiten des Menschen. Bergmann, Wiesbaden, pp. 1-122.
- Santos, E.C., Genigeorgis, C., (1981). Survival and growth of *Staphylococcus aureus* in commercially manufactured Minas cheese. *J. Food Prot.* 44:177-184.
- SCVPH (Scientific Committee on Veterinary Measures relating to Public Health), 1999. The evaluation of microbiological criteria for food products of animal origin for human consumption, adopted on 23 September 1999, (http://europa.eu.int/comm/food/fs/sc/scv/out26_en.pdf).
- SCVPH (Scientific Committee on Veterinary Measures relating to Public Health), 2003. Opinion on Verotoxigenic *E. coli* (VTEC) in foodstuffs, adopted on 21-22 January 2003, (http://europa.eu.int/comm/food/fs/sc/scv/outcome_en.html#opinions).
- Schmitt, M., Schuler-Schmidt, U. Schmidt-Lorenz, W., (1990). Temperature limits of growth, TNase and enterotoxin production of *Staphylococcus aureus* strains isolated from foods. *Int. J. Food Microbiol.*, 11, 1-20.
- Schotte, U., Langfeldt, N., Peruski, A. Meyer, H., (2002). Detection of staphylococcal enterotoxin B (SEB) by enzyme-linked immunosorbent assay and by a rapid hand-held assay. *Clin. Lab.*, 48, 395-400.
- Scott, R., (1986). Cheesemaking practice. 2nd Edition. Elsevier Appl. Sci. Publisher. London, 1986.
- Sharp, J.C.M. (1989). Milk-borne infection. *J. Med. Microbiol.* 29, 239-242.
- Smith, J.L., Buchanan, R.L., Palumbo, S.A., (1983). Effect of food environment on staphylococcal enterotoxin synthesis: a review. *J. Food Prot.*, 46, 545-55.
- Stephan, R., Senczek, D., Dorigoni, V., (2001). Enterotoxin production and other characteristics of *Staphylococcus aureus* strains isolated from human nasal carriers. *Arch Lebensmittelhyg.*, 52, 7-9.

- Su, Y.C., Wong, A.C.L., (1995). Identification and purification of a new staphylococcal enterotoxin, H. *Appl. Environ. Microbiol.*, 61,1438-43.
- Surgalla, M., Bergdoll, M. S., Dack, G. M., (1953). Some observations on the assay of staphylococcal enterotoxin by the monkey feeding test. *J. Lab. Clin. Med.*, 41, 782-788.
- Tamminga, S.K., Beumer, R.R., Kampelmacher, E.H., (1980). Bacteriological examination of ice-cream in the Netherlands. Comparative studies on methods. *J. Appl. Bacteriol.* 49:239-253.
- Tanini, S.R., Jezeski, J.J., Olson, J.C.Jr., Casman, E.P., (1971). Factors influencing the production of staphylococcal enterotoxin A in milk. *J. Dairy Sci.* 54:312-320.
- Tatini, S.R., Jezeski, J.J., Morris, H.A., Olson, J.C., Casman, E.P., (1971a). Production of staphylococcal enterotoxin A in Cheddar and Colby cheeses. *J. Dairy Sci.*, 54, 815-25.
- Tatini, S.R., Jezeski, J.J., Olson, J.C., Casman, E.P., (1971b). Factors influencing the production of staphylococcal enterotoxin A in milk. *J. Dairy Sci.*, 54, 312-20.
- Tatini, S.R., Soo, H.M., Cords, B.R., Bennett, R.W., (1975). Heat-stable nuclease for assessment of staphylococcal growth and likely presence of enterotoxins in foods. *J. Food Sci.*, 40, 352-56.
- Tatini, S.R., Wesala, W.D., Jezeski, J.J., Morris, H.A., (1973). Production of Staphylococcal enterotoxin A in Blue, Brick, Mozzarella, and Swiss cheeses. *J. Dairy Sci.* 56:429-435.
- Tatini, S.R., (1973). Influence of food environments on growth of *Staphylococcus aureus* and production of various enterotoxins. *J. Milk Food Technol.*, 36, 559-63.
- Tatini, S.R., (1976). Thermal stability of enterotoxins in food. *J. Milk Food Technol.*, 39, 432-38.
- Tatini, S.R., Cords, B.R., Gramoli, J., (1976). Screening for staphylococcal enterotoxins in food. *Food. Technol.*, 30, 65-74.
- Tatini, S.R., (1981). Thermonuclease as an indicator of staphylococcal enterotoxins in food. In: Ory, R.L. (ed.) *Antinutrients and natural toxicants in foods*. Food and Nutrition Press, pp. 53 - 75, Westport, Connecticut.
- Tham, W.A., Hadju, L.J., and Danielson-Tham, V., (1990). Bacteriological quality of farm manufactured goat cheese. *Epidemiol. Infect.* 104:87-100.
- Thota, H., Tatini, S.R., Bennett, R.W., (1973). Effects of temperature, pH and NaCl on production of staphylococcal enterotoxins E and F. *Abstr. Ann. Meet. Am. Soc. Microbiol.*, E1, p. 1.
- Todd, E., Szabo, R., Gardiner, M.A., Aktar, M., Delorme, L., Tourillon, P., Rochefort, J., Roy, D., Loit, T., Lamontagne, Y., Gosselin, L., Martineau, G., Breton, J.P. (1981). Intoxication staphylococcique liée à du caillé de fromagerie – Québec. *Rapport hebdomadaire des maladies au Canada*, 7 (34), 171-172, 22 août.

- Troller, J.A., (1971). Effect of water activity on enterotoxin B production and growth of *Staphylococcus aureus*. Appl. Microbiol., 21, 435-9.
- Troller, J.A., (1973). The water relations of foodborne bacterial pathogens. A review. J. Milk Food Technol., 36, 276-288.
- Troller, J.A., (1986). The water relations of foodborne bacterial pathogens – an updated review. J. Food Prot., 49, 656-70.
- Troller, J.A., Stinson, J.V., (1975). Influence of water activity on growth and enterotoxin formation by *Staphylococcus aureus* in foods. J. Food Sci., 40, 802-4.
- Ulrich, R.G., (2000). Evolving superantigens of *Staphylococcus aureus*. FEMS Immunol. Med. Microbiol., 27, 1-7.
- Van Schouwenburg – Van Foeken, A.W., Stadhouders, J.J., Witsenburg, W.W., (1978). The number of enterotoxigenic *Staphylococcus aureus* reached in Gouda cheese made under normal acidification conditions and the amount of enterotoxin produced. Neth. Milk Dairy J. 33:49-59.
- Varaldo, P.E., Kilpper-Bälz, R., Biavasco, F., Satta, G., Schleifer, K.H., (1988). *Staphylococcus delphini* sp. nov., a coagulase-positive species isolated from dolphins. Int. J. Syst. Bact., 38, 436-9.
- Vernozy-Rozand, C., Meyrand, A., Mazuy, C., Delignette-Muller, M.L., Jaubert, G., Perrzin, G., Lapeyre, C., Richard, Y., (1998). Behaviour and enterotoxin production by *Staphylococcus aureus* during the manufacture and ripening of raw goat's milk lactic cheeses. J. Dairy Res. 65:273-281.
- WHO Surveillance programme for control of foodborne infections and intoxications in Europe 1993-1998. 7th report.
- Wieneke, A.A., (1974). Enterotoxin production by strains of *Staphylococcus aureus* isolated from foods and human beings. J. Hyg. Camb., 73, 255-62.
- Wieneke, A.A. (1991). Comparison of four kits for the detection of staphylococcal enterotoxin in foods from outbreaks of food poisoning. Int. J. Food Microbiol., 14, 305-312.
- Woodburn, M.J., Morita, T.N., Rowe, K., Park, S.S., (1978). Staphylococcal enterotoxin A and C production with various sugars as energy source. J. Food Prot., 41, 643-6.
- Wu, Z.L., Zhang, Z.C., Li, C.W., Li, S.S., Xi, Z.Y., Zhou, Y.N., Yin, C.R. (1992). Study of the non specific reaction problems in the procedure using TECRA kit for detection of staphylococcal enterotoxins. J. Assoc. Rapid Method Automation Microbiol. 4, 105-109.
- Yarwood, J.M., McCormick, J.K., Paustian, M.L., Orwin, P.M., Kapur, V., Schlievert, P.M., (2002). Characterization and expression analysis of *Staphylococcus aureus* pathogenicity island 3. J. Biol. Chem., 277, 13138-47.
- Zarate, V., Belda, F., Perez, C., Cardell, E., (1977). Changes in the microbial flora of Tenerife goats milk cheese during ripening. Int. Dairy J. 7:635-641.

Zehren, V., Zeheren, V., (1968). Relation of acid development during cheesemaking to development of staphylococcal enterotoxin A. *J. Dairy Sci.*, 51, 645-9.

Zhang, S., Iandolo, J.J., Stewart, G.C., (1998). The enterotoxin D plasmid of *Staphylococcus aureus* encodes a second enterotoxin determinant (*sej*). *FEMS Microbiol. Lett.* 168, 227-33.

16. GLOSSARY

AOAC : Association of Official Agricultural Chemists

cfu: colony forming unit

CPS : Coagulase Positive Staphylococci

CRL : Community Reference Laboratory

ELFA: Enzyme Linked Fluorescent Assay

ELISA : Enzyme Linked Immunosorbent Assay

LAB: Lactic Acid Bacteria

NRL : National Reference Laboratory

OSP : Optimum Sensitivity Plate

PCR : Polymerase Chain Reaction

PEG : Poly Ethylene Glycol

RIA : Radio ImmunoAssay

RPLA : Reverse Passive Latex Agglutination

SE : Staphylococcal Enterotoxin

SRD : Single Radial Diffusion

TNase : Thermonuclease

17. ANNEXES

Annex 1: Microbiological criteria for *Staphylococcus aureus* in milk and dairy products in community legislation in force (Council Directive 92/46/EEC¹)

Food category	Microorganisms	Sampling plan		Limits		Additional information
		n	C	M	M	
Raw cow's milk intended for direct human consumption	<i>S. aureus</i>	5	2	100 /ml	500 /ml	Milk for manufacturing of raw products
Raw milk intended for processing	<i>S. aureus</i>	5	2	500 cfu/ml	2000 cfu/ml	guideline*
Cheeses made from raw milk and from thermised milk	<i>S. aureus</i>	5	2	1000 cfu/g	10000 cfu/g	guideline*
Soft cheeses (made from heat-treated milk)	<i>S. aureus</i>	5	2	100 cfug	1000 cfu/g	guideline
Fresh cheeses	<i>S. aureus</i>	5	2	10 cfu/g	100 cfu/g	guideline
Powdered milk	<i>S. aureus</i>	5	2	10 cfu/g	100 cfu/g	guideline
Frozen milk – based products	<i>S. aureus</i>	5	2	10 cfu/g	100 cfu/g	guideline

*⁾ Mandatory if pathogenic or enterotoxigenic strains found

¹ O.J. N° L 268, 14.09. 1992, p 0001 - 0031

Annex 2: Foodborne outbreaks of staphylococcal intoxication between 1993 and 1998² (N/R: not reported)

Country	1993	1994	1995	1996	1997	1998	Total	As a % of total foodborne outbreaks
Denmark	3	0	0	3	2	4	12	3.2
England and Wales	1	2	3	5	2	0	13	1
Finland	0	5	1	1	4	2	13	8.2
France	37	60	32	34	32	N/R	195	13.6
Germany	8	5	5	4	2	2	26	2.8
Norway	7	12	2	10	2	7	40	24.2
Portugal	3	3	4	4	4	2	20	9.9
Spain	50	39	24	40	39	36	228	4.1
Sweden	1	2	6	3	4	3	19	3.6
Switzerland	1	1	0	2	3	1	8	5.8

² Source: WHO Surveillance Programme for control of foodborne infections and intoxications in Europe (http://www.bgvv.de/publikationen/who/7threport/7threp_fr.htm)

Annex 3: Test kits for detection of staphylococcal enterotoxins

Test kit	Principle of the test	Sensitivity ¹ (ng/ml)	Duration ^{1,2} (h)	Producer
Ridascreen SET A, B, C, D, E ³	Sandwich-ELISA in microtitre plates	0.2 – 0.7	2.5	R-Biopharm, Darmstadt
Staphylococcal Enterotoxin (SET) Identification TECRA ³	Sandwich-ELISA in microtitre plates	1	4	Bioenterprises, Australia
Staphylokokken Enterotoxin SET-RPLA	Reversed passive latex agglutination (RPLA)	0.5	20 – 24	Denka-Seiken, Japan
Staphylococcal Enterotoxin (SET A – E) TECRA	Sandwich-ELISA in microtitre plates	1	4	Bioenterprises, Australia
Transia Tube Staphylococcal Enterotoxins A, B, C, D, E	Sandwich-ELISA using tubes as solid phase	0.1	1	Diffchamb, France
Transia Plate Staphylococcal Enterotoxines A, B, C, D, E	Sandwich-ELISA in microtitre plates	0.1	2	Diffchamb, France
Vidas Staph enterotoxin (SET)	Automated Sandwich-ELFA using a pipette tip as solid phase	1	80 min.	BioMérieux, France

¹According to manufacturer's information

²Sample preparation not included

³ Capable of differentiating between different SE types

Annex 4: Enterotoxins produced by staphylococci from various sources (Genigeorgis, 1989)*

Country	Source	No. of strains	% positive	Type of SE produced (% of total strains)					
				A	B	C	D	E	other
Canada	Food poisoning	29	94	45	24	0	14		11
	Normal food	10	60	0	40	10	0		10
	Patients	40	45	20	8	0	0	0	17
Czechosl	Sheep	83	61	2	0	55	4	0	
	Sheep cheese	44	18	5	0	9	4	0	
	Food	103	40	18	5	6	0	0	11
	Food poisoning	57	73	43	6	4	4	2	12
	Human diarrhoea	205	29	18	2	7	1	<1	<1
	Clinical humans	585	17	7	<1	6			3
	Breast milk	43	37	0	0	37	0	0	
Denmark	Various foods	129	29	2	5	14	4	<1	2
France	Human clinical	374	51	13	15	3	nt	nt	11
Finland	Mastitic cases	171	42	15	2	10	8	0	13
	Foods	102	69	19	<1	11	9	0	28
Holland	Mastitic cows	24	4	0	0	4	0	0	0
	Human clinic infection	50	72 (?)	14	16	6	6	0	42
	Hospital carriers	24	63 (?)	29	6	6	3	0	30
Ireland	Chickens	139	25	1	0	0	23	0	<1
Japan	Chickens	386	3	<1	0	1	1	0	<1
Nigeria	Household dogs	309	17	3	5	7	nt	nt	
	Ready-to-eat foods	248	39	4	4	4	9	7	11
	Nose, restaurant worker	207	27	7	3	5	4	3	5
	Breast milk	216	40	19	10	4	nt	nt	5
Norway	Cows	46	70	0	0	35	nt	nt	34
	Humans	35	43	9	6	17	nt	nt	6
Spain	Bovine mastitis	57	7	0	0	5	2	0	0
	Ovine mastitis	71	72	3	0	65	1	0	3
Sri Lanka	Human clinical	293	39	5	17	7	7	<1	7
Sweden	Meat workers	86	51	40	5	0	nt	nt	2
UK	Turkeys	146	45	0	0	41	3	0	
URSS	Human patients	271	31	14	3	1	7	<1	5
URSS	Throat, healthy children	70	40	13	20	28	2	0	8

Annex 5: Minimum water activity (a_w) for growth of *S. aureus* and for SE production

Medium	Humectant	Conditions	Minimum a_w for growth	Minimum a_w for SE production	Reference
Broth	NaCl, KCl, Na ₂ SO ₄	Aerobic; 30°C	0.86	ND	Genigeorgis, 1989 (review)
Broth	NaCl/KCl/ Na ₂ SO ₄	Aerobic; 25°C Aerobic; 30°C	0.870 – 0.887 0.864 – 0.867	0.870 – 0.887 (SEA) 0.864 – 0.867 (SEA)	Smith <i>et al.</i> , 1983 (review)
Broth	NaCl	Aerobic; 12°C Aerobic; 18°C Aerobic; 24°C Aerobic; ≥12°C Aerobic; ≥18°C	ND ND ND ND ND	0.96 (SEA) 0.93 (SEA) 0.90 (SEA) 0.96 (SEB) 0.96 (SEC)	Genigeorgis, 1989 (review)
Broth	- - NaCl or sucrose NaCl or sucrose NaCl Sucrose NaCl or sucrose NaCl Sucrose NaCl NaCl NaCl Sucrose Sucrose	Aerobic 30/24°C; pH 4.6 pH 4.3 pH 4.6 pH 4.3 pH 4.6 pH 4.6 pH 4.3 pH 5.2 pH 5.2 Aerobic; 25°C pH 5.2 pH 4.9 pH 4.6 Aerobic; 30°C pH 7.0 pH 4.9 pH 7.0; 24/18°C pH 7.0; 24/18°C pH 7.0; 24/18°C	0.99: growth 0.99: no growth 0.96: growth 0.96: no growth 0.93: no growth 0.93: growth/no growth 0.93: no growth 0.87: growth/no growth 0.87: no growth	0.90 (SEA; after 19 d) 0.93 (SEA; after 12 d) 0.99 (SEA; after 12 d) 0.90 (SEB; after 10 d) 0.96 (SEB; after 4 d) 0.96 (SEC; after 6/13 d) 0.96 (SEC; not detected) 0.99 (SEC; after 6/7 d)	Notermans and Heuvelman, 1983
Broth	NaCl	Aerobic; 37°C	0.86 (within 6 d)	0.86 (within 6 d)	Ewald and Notermans, 1988

Annex 5 (cont. 1)

Medium	Humectant	Conditions	Minimum a _w for growth	Minimum a _w for SE production	Reference
ND	Glycerol NZ Amine NAK NaCl NaCl/KCl/Na ₂ SO ₄	Aerobic	ND ND ND ND	0.98 (SEB) 0.97 (SEB) 0.90 (SEB) 0.90 (SEB)	Genigeorgis, 1989 (review)
Broth, meat extract	ND	Aerobic	0.837 – 0.839	ND	Troller, 1986 (review)
Dried milk, dried soup, dried mutton	Reconstituted with water	Aerobic; 30°C	0.86 0.86 0.88	ND	Troller, 1973 (review)
Precooked bacon	ND	Aerobic; 37°C Anaerobic; 37°C Aerobic; 20°C Anaerobic; 20°C Microaerobic (5.5 % O ₂); 37°C Microaerobic (5.5 % O ₂); 20°C	0.84 0.90 0.88 0.91 0.87 0.91	0.84 (SEA) 0.90 (SEA) 0.88 (SEA) 0.94 (SEA) 0.87 (SEA) 0.91 (SEA)	Bergdoll, 1989 (review)
Pork slurry Pork slurry, freeze dried, rehydrated	Glycerol -	Aerobic	0.84 0.90	ND ND	Genigeorgis, 1989 (review)
Cured beef slurry Cured pork slurry	NaCl NaCl	Aerobic; 35°C; pH 5.5 Aerobic; 35 °C; pH 5.5/5.8	0.86, not 0.82 0.83	0.88, not 0.86 (SEA) 0.86, not 0.83	Tatini, 1973 (review)
Shrimp slurry	Glycerol	Aerobic; 37°C	Strain 196E: 0.93, not 0.89 Strain C-243:	Strain 196E: 0.95, not 0.93 (SEA) Strain C-243:	Troller and Stinson, 1975

			0.89 (lowest a_w tested)	0.93, not 0.91 (SEB)	
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Annex 5 (cont. 2)

Medium	Humectant	Conditions	Minimum a_w for growth	Minimum a_w for SE production	Reference
Potato dough	Glycerol	Aerobic; 37°C	Strain 196E: 0.88 (lowest a_w tested) Strain C-243: 0.88 (lowest a_w tested)	Strain 196E: 0.93, not 0.88 (SEA) Strain C-243: 0.97, not 0.93 (SEB)	Troller and Stinson, 1975
Processed cheese	-	ND	0.94	ND	Troller, 1986 (review)

ND: no data

Annex 6: Minimum pH for growth of *S. aureus* and SE production

Medium	Acidulant	Conditions	Minimum pH for growth	Minimum pH for SE production	Reference
Non fat dry milk	HCl or lactic acid	ND	5.0	5.0 (SEA)	Tatini, 1973 (review)
	Lactic acid		4.5: no growth	-	
Pork, ham	Lactic acid	Anaerobic; 30°C; 7 d	4.8 – 5.0: no growth	-	Tatini, 1973 (review)
Ham	Glucono-delta-lactone	Anaerobic; 22 and 30°C	ND	5.3 (SEB)	Tatini, 1973 (review) Bergdoll, 1989 (review)
Cured meat	ND	Aerobic	ND	4.7 (SEC)	Tatini, 1973 (review)
Different foods	ND	ND	5.0	5.0 (SEA, SEB, SEC, SED)	Tatini, 1973 (review) Smith <i>et al.</i> , 1993 (review)
Broth	ND	Aerobic	ND	5.1 (SEB)	Smith <i>et al.</i> , 1993 (review)
Broth	ND	Aerobic; 37°C	ND	5.02 (SEB) min. pH 9.08 (SEB) max. pH	Smith <i>et al.</i> , 1993 (review)
Cheddar cheese	-	-	ND	5.4 (SEA)	Zehren and Zehren, 1968
Mayonnaise	ND	Aerobic; 37°C; 7 d	ND	5.1 (SEA, SEB, SEC, SED)	Genigeorgis, 1989 (review)

ND: no data

**Annex 7: Lowest pH values for SE production of *S. aureus* in buffered BHI after 72 h at 37°C; acidulant: HCl
(Barber and Deibel, 1972)**

Initial pH value	Strain no. and type of SE produced												
	9 SEA	95 SEA	107 SEA	118 SEA	119 SEA	166 SEA	167 SEA	169 SEA	S-6 SEB	272 SEB	334 SEB	361 SEC ₂	326 SEE
Aerobic													
5.7	+	+	+	+	+	+	+	+	+	+	+	+	+
5.4	+	+	+	-	+	+	+	+	+	+	+	+	+
5.1	+	+	+	-	+	+	+	+	-	-	+	+	+
5.0	-	+	+	-	+	-	+	-	-	-	+	+	+
4.9	-	-	+	-	+	-	+	-	-	-	-	+	+
4.8	-	-	-	-	-	-	-	-	-	-	-	-	+
Anaerobic													
7.0	+	+	+	+	+	+	+	+	+	+	-	+	+
6.5	+	+	+	-	+	+	+	+	+	+	-	+	+
6.0	+	+	+	-	+	+	+	+	+	+	-	+	+
5.7	+	-	+	-	-	+	+	+	+	+	-	+	-
5.4	-	-	-	-	-	-	-	-	-	-	-	-	-

+: SE detected

-: SE not detected

Annex 8: Effect of nutritional factors on SE production of *S. aureus*

Factor	Medium	Enhancement	No effect	Repression	Reference
Magnesium ↑ Phosphate ↓ Potassium (30 mM) Ammonium Trace elements	Amino acid-salt-vitamins	SEB SEB	SEB SEB SEB		Keller <i>et al.</i> , 1978
Magnesium Iron	N-Z Amine NAK, suppl.	SEB, SEC SEB	SEA SEA, SEC		Morita <i>et al.</i> , 1979
Casein hydrolyzed	?	SEB			Bergdoll, 1989 (review)
Yeast	?	SEA, SED			Halpin-Dohnalek and Marth, 1989 (review)
Glucose (≥ 0.30 %)	Casein hydrolysate medium, suppl. (pH controlled)			SEB	Morse <i>et al.</i> , 1969
Glucose, glycerol	Amino acid medium (pH controlled)			SEA, SEB, SEC	Jarvis <i>et al.</i> , 1975
Lactose, maltose, sucrose, glucose, glucose + fructose (all 1 % and 5 %)	Casein hydrolysate medium		SEC		Woodburn <i>et al.</i> , 1978
Proline, histidine, alanine, serine,	Salts-vitamin-amino acid medium (amino acids individually added, 10 mM)	SEB (weak)			
Aspartate, glycine, threonine, glutamate	Salts-vitamin-amino acid medium (amino acids individually added, 10 mM)			SEB	Smith <i>et al.</i> , 1983 (review)
Pyruvate	Casein hydrolysate medium			SEB	Smith <i>et al.</i> , 1983 (review)

Annex 9: Cheese consumption (availability) for 2002 in the European Union and in third countries

Country	Consumption Kg/Cap/Year	Country or Geographical area	Consumption Kg/Cap/Year
Austria	19.2	Argentina	12.2
Belgium-Luxembourg	13.7	Australia	9.2
Denmark	15.1	Belarus	3.2
Finland	14.1	Brazil	0.3
France	23.6	Canada	11.8
Germany	18.9	China	0.2
Greece	25.4	Indonesia	0.0
Ireland	8.5	New Zealand	10.2
Italy	20.5	Poland	11.1
Netherlands	22.5	Russian Federation	2.9
Portugal	8.8	Turkey	2.0
Spain	6.3	United States	14.9
Sweden	17.4	Africa	1.0
United Kingdom	9.2	Asia	0.4
		Central America	1.8
European Union (15)	16.7	World	2.6