REVIEW ARTICLE

Staphylococcus aureus and its food poisoning toxins: characterization and outbreak investigation

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Abstract

Staphylococcal food poisoning (SFP) is one of the most common food-borne diseases and results from the ingestion of staphylococcal enterotoxins (SEs) preformed in food by enterotoxigenic strains of *Staphylococcus aureus*. To date, more than 20 SEs have been described: SEA to SElV. All of them have superantigenic activity whereas half of them have been proved to be emetic, representing a potential hazard for consumers. This review, divided into four parts, will focus on the following: (1) the worldwide story of SFP outbreaks, (2) the characteristics and behaviour of *S. aureus* in food environment, (3) the toxinogenic conditions and characteristics of SEs, and (4) SFP outbreaks including symptomatology, occurrence in the European Union and currently available methods used to characterize staphylococcal outbreaks.

A worldwide review of outbreaks related to coagulase-positive staphylococci and their toxins: the story

Staphylococcal food poisoning (SFP) is one of the most common food-borne diseases in the world following the ingestion of staphylococcal enterotoxins (SEs) that are produced by enterotoxigenic strains of coagulase-positive staphylococci (CPS), mainly *Staphylococcus aureus* (Jablonski & Bohach, 1997) and very occasionally by other staphylococci species such as *Staphylococcus intermedius* (Genigeorgis, 1989; Khambaty *et al.*, 1994).

When outbreaks occurred during large social events, chaotic situations resulted requiring the rapid implementation of medical care for a high number of cases (Bonnetain *et al.*, 2003; Do Carmo *et al.*, 2004).

Here are some examples of SFP outbreaks (SFPOs).

The first description of food-borne disease involving staphylococci was investigated in Michigan (USA) in 1884 by Vaughan and Sternberg. This food poisoning event was because of consumption of a cheese contaminated by staphylococci. The authors stated: '*It seems not improbable that the poisonous principle is a ptomaine developed in the cheese as a result of the vital activity of the above-mentioned Micrococcus or some other microorganisms which had preceded it, and had perhaps been killed by its own poisonous products*'.

Ten years later, Denys (1894) concluded that the illness of a family who had consumed meat from a cow that had died of vitullary fever was owing to the presence of pyogenic staphylococci.

In 1907, Owen recovered staphylococci from dried beef involved in an outbreak showing characteristics of SFP symptomatology (Dack *et al.*, 1930).

FEMS MICROBIOLOGY REVIEWS

Proof of the involvement of staphylococci in food poisoning was first brought by Barber in 1914. He demonstrated with certainty that staphylococci were able to cause poisoning by his consumption of unrefrigerated milk from a cow suffering from mastitis, an inflammation owing to staphylococci. However, the correlation between staphylococci-containing food and symptomatology was not recognized until other examples of food poisoning occurred later in the twentieth century. It was Baerthlein, when reporting on a huge outbreak involving 2000 soldiers of the German army during WWI, who established in 1922 the possible role of bacteria. '*I am going to report the case of an extended demonstration of poisoned sausages (approximately 2000 cases) held in the spring 1918 during the military campaign of Verdun, which would probably have catastrophic military consequences. Early in June 1918, sudden and massive demonstrations that have the appearance of an acute and in some cases severe gastroenteritis, similar to cholera, affected the troops around Verdun; entire companies were disabled except just a few people, and within two days about 2000 men had been affected. The symptoms were so severe that some troops (more than 200) had to be transferred to field hospitals. The suspicion of food poisoning has been mentioned because, according to reports of the sick, the disease occurred 2 or 3 hours (some of the symptoms appeared after 6 to 8 hours) after eating a dish of sausages. Only troops who did not eat the meal were spared, such as soldiers who had returned to headquarters to receive orders, soldiers who for other reasons had not eaten sausages, and soldiers who were on leave and/or following a different diet. However, it was surprising that among the troops that were not present at the front, such as butchers, who ate the same sausage two days earlier, we did not observe any cases of disease*'.

In 1930, Dack *et al.* found that a sponge cake was responsible for the intoxication of 11 individuals; he highlighted that the disease was probably linked to a toxin called 'enterotoxin' produced by yellow haemolytic *Staphylococcus*. Broth culture filtrates of this strain were administrated intravenously to a rabbit and orally to three human volunteers. The rabbit died, after first developing water diarrhoea, and the three volunteers developed nausea, chilliness and vomiting after 3 h. In the same year, Jordan showed that various strains of staphylococci exhibited cultural properties of generating a substance which was purified from broth and, when taken orally, produced gastrointestinal disturbance.

A few years later, in 1934, Jordan and Burrows observed nine outbreaks related to the presence of staphylococci in food remnants, whereas Dolman (1934) explained that '*the food poisoning substance is probably produced by only a few strains of staphylococci, and that it is a special metabolite whose formation and excretion are favored in the laboratory by such environmental conditions as a semi-fluid medium and atmosphere containing a high percentage of carbon dioxide, conditions which promote, respectively, abundant growth and increased cellular permeability with partial buffering*'.

One of the first well-documented SFP outbreaks was described by Denison in 1936. This outbreak occurred among high school students after they had eaten tainted cream puffs. He depicted the typical symptoms of 122 cases as follows: '*Within 2–4 hours after eating there was first noticed a feeling of nausea. Severe abdominal cramps developed and were quickly followed by vomiting which was severe and continued at 5–20 minute intervals for 1–8 hours [...] A diarrhea of 1–7 liquid stools usually began with the vomiting and continued for several hours after its onset [...] During the acute stage the temperature was normal or subnormal, the pulse noticeably increased, there were cold sweats, prostration was severe and the patients were very definitely in a state of shock. Headache was mild and of a short duration. Muscular cramping [...] was present in the majority. Dehydration was marked in some. While the acute symptoms usually lasted only 1–8 hours, complete recovery [...] was delayed for 1–2 days*'.

Staphylococcal food poisoning symptomatology has been extensively studied especially by the US Army: in a naturally occurring outbreak among US Army personnel, involving 400 of 600 men, DeLay (1944) reported that about 25% of cases were classified as severe or shock cases. Numerous SFP outbreaks have been described since the end of WWII. For example, Brink & Van Metter (1960) from the Institute for Cooperative Research of the University of Pennsylvania wrote a long report on an outbreak of SE food poisoning which happened in 1960: '*On a Saturday afternoon in the middle of summer, an epidemic of staphylococcal enterotoxin food poisoning occurred at a picnic held two miles from Gabriel, a small Midwestern town. (The name of the town and other names in this report are fictitious, in accordance with commitments to Task Surprise respondents.) About 1700 persons attended the picnic, which is an annual affair sponsored by the Johnson Co., of Croydon, some 60 miles away. Early in the morning, approximately seven hours before the picnic began, an unventilated, unrefrigerated truck containing a large supply of ham sandwiches was parked at the picnic grounds. The truck was exposed to the heat of direct sunlight, while the average ambient temperature for the day was close to 100 degrees Fahrenheit. In this environment, the staphylococcal organisms which elaborate the toxin multiplied rapidly. During the epidemic that followed, approximately 1100 persons became ill*'. A selection of 24 outbreaks involving SEs is presented in Table 1.

The main point highlighted by these reports is that any food that provides a convenient medium for CPS growth may be involved in a SFP outbreak (SFPO). The foods most

Table 1. Excerpt of food poisonings presented in the literature

Year	Location	Incriminated food	Number of cases	References
1968	School children, Texas	Chicken salad	1300	Anonymous (1968)
1971	UK army	Sausages rools, ham sandwiches	100	Morris *et al.* (1972)
1975	Flight from Japan to Denmark	Ham	197	Eisenberg *et al.* (1975)
1976	Flight from Rio to NYC	Chocolate eclairs	80	Anonymous (1976)
1980	Canada	Cheese curd	62	Todd *et al.* (1981)
1982	North Carolina and Pennsylvania	Ham and cheese sandwich; stuffed chicken	121	Anonymous (1983a)
1983	Caribbean cruise ship	Dessert cream pastry	215	Anonymous (1983b)
1984	Scotland	Sheep's milk cheese	27	Bone *et al.* (1989)
1985	France, UK, Italy, Luxembourg	Dried lasagna	50	Woolaway *et al.* (1986)
1985	School children, Kentucky	2% chocolate milk	> 1000	Evenson *et al.* (1988)
1986	Country Club, New Mexico	Turkey, poultry, gravy	67	Anonymous (1986)
1989	Various US states	Canned mushrooms	102	Anonymous (1989)
1990	Thailand	Eclairs	485	Thaikruea *et al.* (1995)
1992	Elementary school, Texas	Chicken salad	1364	Anonymous (1992)
1997	Retirement party, Florida	Precooked ham	18	Anonymous (1997)
1998	Minas Gerais, Brazil	Chicken, roasted beef, rice and beans	4000	Do Carmo *et al.* (2004)
2000	Osaka, Japan	Low-fat milk	13 420	Asao *et al.* (2003)
2006	Ile de France area, France	Coco nut pearls (Chinese dessert)	17	Hennekinne *et al.* (2009)
2007	Scouts' camp, Belgium	Hamburger	15	Fitz-James *et al.* (2008)
2007	Elementary school, Austria	Milk, cacao milk, vanilla milk	166	Schmid *et al.* (2009)
2008	Weeding dinner, Ile de France area, France	Carribean meals	47	De Buyser & Hennekinne, pers. commun.
2008	French district	Pasta salad	100	De Buyser & Hennekinne, pers. commun.
2009	Nagoya university festival, Japan	Crepes	75	Kitamoto *et al.* (2009)
2009	Various districts, France	Raw milk cheese	23	Ostyn *et al.* (2010)

frequently involved differ widely from one country to another, probably due to differing food habits (Le Loir *et al.*, 2003). For instance, in the UK or the United States, meat or meat-based products are the food vehicles mostly involved in SFP (Genigeorgis, 1989), although poultry, salads and cream-filled bakery items are other good examples of foods that have been involved (Minor & Marth, 1972). In France, various food types have been associated with SFPOs but as the consumption of unpasteurized milk cheeses is much more common than in Anglo-Saxon countries, milk-based products are more frequently involved than in other countries (De Buyser *et al.*, 2001).

To conclude this section, as SFP is a short-term disease and usually results in full recovery, doctors do not take it very seriously, especially when the outbreak affects only a few people. Although such outbreaks should be reported to the sanitary authorities, this situation leads to under-reporting (De Buyser *et al.*, 2001). However, many researchers consider that SFP is one of the most common food-borne diseases worldwide (Balaban & Rasooly, 2000).

Characteristics and behaviour of *S. aureus* in the food environment

Staphylococcus is a spherical, nonsporulating, nonmotile bacterium (coccus) that, when observed under the microscope, occurs in pairs, short chains or grape-like clusters. These facultative aero-anaerobic bacteria are Gram- and catalase positive. Staphylococci are ubiquitous in the environment and can be found in the air, dust, sewage, water, environmental surfaces, humans and animals.

To date, more than 50 species and subspecies of staphylococci have been described according to their potential to produce coagulase. Their classification thus distinguishes between coagulase-producing strains, designated as coagulase-positive staphylococci (CPS) and noncoagulase-producing strains, called coagulase-negative staphylococci (CNS). Among CNS, some species are known to play an important role in the fermentation of meat and milk-based products and are therefore considered as food grade. The enterotoxigenic potential of CNS has always been a subject of controversy. Several investigations failed to detect enterotoxin production or enterotoxin-like gene in CNS (Rosec *et al.*, 1997; Becker *et al.*, 2001). However, some studies found that certain CNS strains were able to produce enterotoxins which could lead to food poisoning (Vernozy-Rozand *et al.*, 1996; Zell *et al.*, 2008). More recently, another study demonstrated that, among 129 CNS strains isolated from fermented foodstuffs, only one carried SE genes (Even *et al.*, 2010). However, as only CPS strains have been evidenced in food poisoning incidents, this review will focus on these species.

Table 2. Genus *Staphylococcus*: coagulase-positive species

Species	Main sources	References
S. aureus ssp. *aureus*	Humans, animals	Rosenbach (1884)
S. aureus ssp. *anaerobius*	Sheep	De la Fuente *et al.* (1985)
*S. intermedius**	Dog, horse, mink, pigeon	Hajek (1976)
*S. pseudintermedius**	Dog, cat	Devriese *et al.* (2005)
*S. delphini**	Dolphin	Varaldo *et al.* (1988)
S. schleiferi ssp. *coagulans*	Dog (external ear)	Igimi *et al.* (1990)
S. lutrae	Otter	Foster *et al.* (1997)

**Staphylococcus intermedius*, *S. pseudintermedius* and *S. delphini* are very close species also called the *S. intermedius* group. *Staphylococcus pseudintermedius* is now considered as the main species isolated from dogs.

Among the seven described species belonging to the CPS group (Table 2), *S. aureus* ssp. *aureus* is the main causative agent described in SFPOs. Among other CPS, Becker *et al.* (2001) highlighted the enterotoxigenic potential of *S. intermedius*. The enterotoxigenic 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. *Staphylococcus intermedius* was involved in one outbreak caused by blended margarine and butter involving over 265 cases in October 1991 in the United States (Khambaty *et al.*, 1994; Bennett, 1996).

Reservoirs

Staphylococcus aureus belongs to the normal flora found on the skin and mucous membranes of mammals and birds. This bacterium can be disseminated in the environment of its hosts and survives for long periods in these areas. Several biotypes isolated from different hosts (human, poultry, cattle and sheep/goat) have been described within *S. aureus* species demonstrating the close adaptation of the bacterium to its host. They were identified according to four biochemical tests (staphylokinase, ß-haemolysin production, coagulation of bovine plasma and growth type on crystal violet agar) following the simplified biotyping scheme described by Devriese (1984). However, many strains cannot be assigned to these host-specific biotypes and belong to nonhost-specific (NHS) biotypes, i.e., those associated with several hosts. Later, a poultry-like biotype associated with meat products and meat workers was tentatively designated as a 'slaughterhouse' biotype by Isigidi *et al.* (1990). Indeed, introduction of an additional biochemical test, protein A production, and phage typing allowed researchers to differentiate the poultry biotype from this new biotype. However, as the protein A test is no longer commercially available, and as phage typing cannot be routinely used, these two biotypes cannot be easily distinguished. Several pitfalls were encountered when applying the biotyping method: discordant results owing to the variety of test parameters, lack of standardized reagents, problematic interpretation for 'haemolysin', 'bovine plasma coagulation' and 'crystal violet' tests when applied to some strains and, as previously mentioned, lack of commercially available tests to distinguish between the described biotypes. Despite these drawbacks, *S. aureus* biotyping has been useful in tracing or estimating the origin of this organism in various food products (Devriese *et al.*, 1985; De Buyser *et al.*, 1987; Rosec *et al.*, 1997), in the food industry (Isigidi *et al.*, 1990) and also for epidemiological investigations of food-poisoning outbreaks (Hennekinne *et al.*, 2003; Kerouanton *et al.*, 2007). In a recent study, Alves *et al.* (2009) performed pulsed-field gel electrophoresis (PFGE) typing of *S. aureus* strains isolated from small ($n = 88$) and large ruminants ($n = 65$). The authors carried out a molecular analysis and confirmed that ovine and caprine strains which could not be distinguished from one another were nonetheless different from bovine strains. To suggest the source of contamination (animal or human origins), molecular-based methods have been used by various authors to study the food poisoning outbreaks (Chiou *et al.*, 2000; Shimizu *et al.*, 2000; Wei & Chiou, 2002; Kerouanton *et al.*, 2007; Ostyn *et al.*, 2010). Among these methods, PFGE and the *Staphylococcus* protein A gene (*spa*) typing have been used alone or in association providing additional information to highlight the origin of the *S. aureus* contamination.

Means of contamination

The prerequisite of SFP is that food or one of its ingredients is contaminated with an enterotoxigenic strain of *Staphylococcus* spp. Moreover, to induce SFP, conditions for staphylococci growth and enterotoxin production are needed.

Five conditions are required to induce SFPOs: (1) a source containing enterotoxin-producing staphylococci: raw materials, healthy or infected carrier, (2) transfer of staphylococci from source to food, e.g., unclean food preparation tools because of poor hygiene practices, (3) food composition with favourable physicochemical characteristics for *S. aureus* growth and toxinogenesis, (4) favourable temperature and sufficient time for bacterial growth and toxin production, and (5) ingestion of food containing sufficient amounts of toxin to provoke symptoms. Most SFPOs arise because of poor hygiene practices during

processing (Asao *et al.*, 2003), cooking or distributing the food product (Pereira *et al.*, 1996). Moreover, after contamination, inadequate cooling of foods can induce *Staphylococcus* growth and/or stimulate toxin production, resulting in food poisoning (Barber, 1914; Anonymous, 1997).

Staphylococci are commonly found in a wide variety of mammals and birds, and transfer of *S. aureus* to food has two main sources: human carriage during food processing and dairy animals in case of mastitis. Human strains are mainly involved in SFPOs. However, animals are also known to be a potential source of primary contamination. For example, in the case of staphylococcal mastitis of ruminants such as cows, goats or ewes, *S. aureus* can be carried over from the udder into the milk. In a study conducted on 178 *S. aureus* strains associated with 31 SFPOs isolated, Kerouanton *et al.* (2007) demonstrated for the first time that animal strains were responsible for two outbreaks.

Numerous examples of SFPO are described in the literature of the last few decades (Table 1). Among these examples, the case which happened in 1997 in Florida (USA) during the course of a retirement party where precooked ham was served (Anonymous, 1997) provides an interesting demonstration of the five conditions needed to cause SFPO: on 27 September 1997, a community hospital in North-eastern Florida (USA) notified the Health Department about several persons who were treated in the emergency room because of gastrointestinal illnesses suspected of being associated with a common meal ingested on 26 September 1997.

On September 25, a food preparer had purchased a 16-pound precooked packaged ham, baked it at home at 204 °C for 1.5 h, and transported it to her workplace, a large institutional kitchen; finally, she sliced the ham while it was hot with the help of a commercial slicer. The food preparer declared that she had no cuts, sores or infected wounds on her hands. She reported that she routinely cleaned the slicer in place rather than dismantling it and cleaning it according to recommended procedures and that she did not use an approved sanitizer. All 16 pounds of sliced ham were placed in a 14-inch by 12-inch by 3-inch plastic container that was covered with foil and stored in a walk-in cooler for 6 h and then transported back to the preparer's home and refrigerated overnight. The ham was served cold at the party the next day. The leftover food was collected and submitted for laboratory analysis. Of the approximately 125 persons who attended the party, 98 completed and returned questionnaires. Of these, 31 persons attended the event but ate nothing, and none of them became ill; they were excluded from further analysis. A total of 18 (19%) persons had illnesses meeting the case definition, including 17 party attendees and one person who ate food brought home from the party. Eighteen persons reported nausea (94%), vomiting (89%), diarrhoea (72%), weakness (67%), sweating (61%), chills (44%), fatigue (39%), myalgia (28%), headache (11%) and fever (11%). Onset of illness occurred at a mean of 3.4 h after eating (range: 1–7 h); symptoms lasted a median of 24 h (range: 2–72 h). Seven persons sought medical treatment, and two of those were hospitalized overnight. Illness was strongly associated with the eaten ham (risk ratio = 26.8). Of the 18 ill persons, 17 (94%) had eaten ham. The ill person who had not attended the party had eaten only leftover ham. None of the other foods served at the party were significantly associated with illness. One sample of leftover cooked ham and one sample of leftover rice pilaf were analysed by reversed passive latex agglutination (RPLA) to identify SE and were positive for SEA.

Potential for methicillin-resistant *Staphylococcus aureus* (MRSA) contamination and transmission

Food is also an important factor for the transfer of antimicrobial resistance. Such transfer can occur by means of residues of antibiotics in food, through the transfer of resistant food-borne pathogens or through the ingestion of resistant strains of the original food microflora and resistance transfer to pathogenic microorganisms (Khan *et al.*, 2000; Pesavento *et al.*, 2007). Most animals may be colonized with *S. aureus*, but only recently MRSA strains were isolated from several food production animals, including pigs, cattle, chicken and other animals (Huijsdens *et al.*, 2006; de Neeling *et al.*, 2007). Pigs in particular, and also pig farmers and their families, were found colonized with MRSA, and in the Netherlands, contact with pigs is now recognized as a risk factor for MRSA carriage (Van Duijkeren *et al.*, 2007). An association between the emergence of MRSA strains in pigs and the use of antibiotics in pig farming has been suggested (de Neeling *et al.*, 2007; Wulf & Voss, 2008). During slaughtering of MRSA-positive animals, contamination of carcasses and the environment with MRSA may occur, and consequently, meat from these animals may become contaminated. MRSA strains have been detected in different foods, including bovine milk and cheese (Normanno *et al.*, 2007), meat products (Van Loo *et al.*, 2007; de Boer *et al.*, 2008) and raw chicken meat (Kitai *et al.*, 2005; Kwon *et al.*, 2006). These studies highlighted low isolation frequencies for MRSA in foods. Kitai *et al.* (2005) isolated two MRSA strains (0.5%) from 444 raw chicken meat products sampled in supermarkets in Japan. A study in Korea, including 930 slaughterhouse and retail meat samples, showed the presence of MRSA in two chicken meat samples (0.2%) but not in any pork or beef

samples (Kwon *et al.*, 2006). In an Italian survey of 1634 foodstuff samples, six (0.4%) MRSA strains were isolated from bovine milk and cheese (Normanno *et al.*, 2007) whereas Van Loo *et al.* (2007) found two (2.5%) MRSA strains in 79 samples of raw pork and beef.

Regarding the involvement of MRSA in SFP, Jones *et al.* (2002) reported for the first time an outbreak of gastrointestinal illness caused by community-acquired MRSA. In this outbreak, various *S. aureus* strains were isolated from food remnants, affected people and food handlers. Among these strains, one produced staphylococcal enterotoxin C and was identified as being MRSA. This isolate was resistant to penicillin and oxacillin but sensitive to all other antibiotics tested. To our knowledge, only few data are available on the occurrence of MRSA in SFP. In a study carried out in France on foods incriminated in SFPOs, Kerouanton *et al.* (2007) highlighted two MRSA strains of the 33 tested. They concluded that with reference to human clinical isolates, the SFPO strains were more susceptible to antibiotics (except for two that were resistant to methicillin).

Factors influencing the growth of CPS

Microorganisms in foods are affected by a multiplicity of parameters described as intrinsic and extrinsic factors and processing effects. Some of these parameters will be discussed later. However, it should be stressed that, in complex media such as foods, these factors interact with a great extent. 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 3 summarizes some of the factors affecting growth and SE production by *S. aureus*.

Water activity (a_w)

With regard to staphylococci, 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 can be seen from Table 3, the bacteria can grow at a minimum a_w of 0.83–0.86 (equivalent to about 20% NaCl; Troller & Stinson, 1975) 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 those for growth, depending on the type of toxin. SEA and SED production occurs under nearly all a_w conditions allowing growth of *S. aureus* as long as all other conditions are optimal. Production of SEB is very sensitive to reductions in a_w and hardly any is produced at a_w 0.93 despite extensive growth. The effect of a_w on SEC production follows the same pattern as SEB production (Ewald & Notermans, 1988; Qi & Miller, 2000). Thota *et al.* (1973) found SEE production in media containing 10% NaCl (according to Troller, 1971; this concentration corresponds to 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 and also the incubation temperature (Table 3). 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 the 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 & Miller, 2000). There is strong evidence that compatible solutes stimulate not only growth but also toxin synthesis. For example, SEB production was significantly stimulated at low a_w when proline was available in the broth (Qi & Miller, 2000).

pH

Most staphylococcal strains grow at pH values between 4 and 10, with the optimum being 6–7 (Table 3). When the other cultural parameters became nonoptimal, the pH range tolerated is reduced. For example, the lowest pH

Table 3. Factors affecting growth and enterotoxin production by *Staphylococcus aureus* (Tatini, 1973)

	Organism growth		Staphylococcal enterotoxin production	
Factor	Optimum	Range	Optimum	Range
Temperature	37	7–48	37–45	10–45
pH	6–7	4–10	7–8	4–9.6
Water activity (a_W)	0.98	0.83 →0.99*	0.98	0.85 →0.99†
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

*Aerobic (anaerobic 0.90 →0.99).
†Aerobic (anaerobic 0.92 → 0.99).

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). The majority of *S. aureus* strains tested produced detectable amounts of SE aerobically at a pH of 5.1. However, in anaerobic conditions, most strains failed to produce detectable SE below pH 5.7 (Tatini, 1973; Bergdoll, 1989; Smith *et al.*, 1983).

Redox potential

Optimum redox potential and ranges for growth and SE formation are given in Table 3. *Staphylococcus aureus* is a facultative anaerobic bacterium that 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 than cultures incubated in an atmosphere of 95% N_2 + 5% CO_2. 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 when cultivated 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 & Rasooly, 2002). It has already been mentioned that minimum a_w and minimum pH for growth as well as for SE formation are influenced by the atmosphere.

Temperature

Staphylococcus aureus grows between 7 and 48 °C, temperature being optimal at around 37 °C (Table 3). 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 generally did not vary much within the range of 35–40 °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 between 15 and 38 °C and the maximum temperatures from 35 to 45 °C. For the lower temperature limit for SE production, production of low amounts of toxin has been observed after 3–4 days. Moreover, SE formation at 10 °C was reported by Tatini (1973) (Table 3) without indicating the detailed experimental conditions.

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. *Staphylococcus 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 and 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 staphylococci probably become more heat resistant as the a_w is lowered until at an a_w between 0.70 and 0.80, and the resistance begins to decline (Troller, 1986).

Nutritional factors and bacterial antagonism

Growth of *S. aureus* and SE production is also influenced by nutritional factors. Some data are given in Table 4.

Staphylococcus aureus does not grow well in the presence of a competitive flora. Its inhibition is mainly because of acidic products, lowering of the pH, production of H_2O_2 or other inhibitory substances like antibiotics, volatile compounds or nutritional competition (Haines & Harmon, 1973; 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; Genigeorgis, 1989).

Starter cultures used in the production of fermented milk products such as cheese, yoghurt, buttermilk and others can effectively prevent the 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.

Toxinogenic conditions and characteristics of SEs

Nomenclature and structure

Since the first characterization of SEA and SEB in 1959 to 1960 by Casman and Bergdoll, 22 different SEs have been described (Table 5); they are designated SEA to SE*l*V2, in the chronological order of their discovery except for SEF which was later renamed TSST1 (Bergdoll *et al.*, 1959; Casman, 1960; Thomas *et al.*, 2007; Ono *et al.*, 2008): enterotoxins A (SEA), B (SEB), C_1 (SEC_1), C_2 (SEC_2), C_3 (SEC_3), D (SED), E (SEE), G (SEG), H (SEH), I (SEI), J (SE*l*J) (Balaban & Rasooly, 2000), K (SE*l*K) (Orwin

Table 4. Effect of nutritional factors on staphylococcal enterotoxin (SE) production by *Staphylococcus aureus*

Factor	Medium	Enhancement	No effect	Repression	References
Magnesium (0.4–1.5 mM)	Amino acid-salt-vitamins	SEB			Keller *et al.* (1978)
Phosphate (15–45 mM)			SEB		
Potassium (30 mM)		SEB			
Ammonium			SEB		
Trace elements			SEB		
Magnesium	BHI (or yeast extract) + N-Z Amine NAK	SEB, SEC	SEA		Morita *et al.* (1979)
Iron		SEB	SEA, SEC		
Hydrolyzed casein	Casein-based medium	SEB			Bergdoll (1989)
Yeast	Not defined	SEA, SED			Halpin-Dohnalek & Marth (1989)
Glucose ($\geq$ 0.30%)	Casein hydrolysate medium, suppl. (pH controlled)			SEB	Morse *et al.* (1969)
Glucose, glycerol	Amino acid medium (pH controlled)			SEA, SEB, SEC	Jarvis *et al.* (1975)
Lactose, maltose, sucrose, glucose, glucose + fructose (all 1% and 5%)	Casein hydrolysate medium		SEC		Woodburn *et al.* (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 *et al.* (1983)
Pyruvate	Casein hydrolysate medium			SEB	Smith *et al.* (1983)

et al., 2001), L (SE*l*L), M (SE*l*M), N (SE*l*N), O (SE*l*O) (Jarraud *et al.*, 2001), P (SE*l*P) (Omoe *et al.*, 2005), Q (SE*l*Q) (Orwin *et al.*, 2002), R (SER) (Omoe *et al.*, 2003), S (SES), T (SET) (Ono *et al.*, 2008), U (SE*l*U) (Letertre *et al.*, 2003), and U2 and V (Thomas *et al.*, 2006).

These toxins, (enterotoxin and enterotoxin like) are globular single-chain proteins with molecular weights ranging from 22 to 29 kDa. Moreover, their crystal structures, established for SEA, SEB, SEC, SED, SEH, SE*l*I and SE*l*K, reveal significant homology in their secondary and tertiary conformations (Mitchell *et al.*, 2000). However, SEs, SE*l*s and TSST-1 can be divided into four phylogenetic groups based on their primary amino acid sequences (Thomas *et al.*, 2007).

Properties

Staphylococcal enterotoxins are resistant to environmental conditions (freezing, drying, heat treatment and low pH) that easily destroy the enterotoxin-producing strain. They are also resistant to proteolytic enzymes retaining their activity in the digestive tract after ingestion (Bergdoll, 1989). Thermal resistance is dependent on the relative purity of the SE preparation. Crude SEA in buffer was reduced from 21 μg mL^{-1} to < 1 μg mL^{-1} after heating at 100 °C for 130 min. Purified SEA (0.2 mg mL^{-1}), 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 & Marth, 1972). The results of thermal inactivation of SEA and SED in milk and milk products are shown in Table 6 (Tatini, 1976). 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 food poisoning outbreaks (0.5–10 μg per 100 mL or 100 g) (Bergdoll, 1989). However, it should 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 also by 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 at 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).

These proteins have been named according to their emetic activity (Lina *et al.*, 2004) after oral administration in a primate model. Some were renamed SE-like toxins (SE*l*), because either no emetic properties were detected or they were not tested in primate models (Lina *et al.*, 2004; Thomas *et al.*, 2007). SEs belong to the broad family of pyrogenic toxin superantigens (Van den Bussche *et al.*, 1993). Superantigens (SAgs), unlike conventional

Table 5. Staphylococcal enterotoxin characteristics

	General characteristics		Mode of activity		
Toxin type	Molecular weight (Da)	Genetic basis of SE	Superantigenic action*	Emetic action†	References
SEA	27 100	Prophage	+	+	Betley & Mekalanos (1985) Borst & Betley (1994)
SEB	28 336	Chromosome, plasmid, pathogenicity island	+	+	Jones & Khan (1986) Shafer & Iandolo (1978) Shalita *et al.* (1977) Altboum *et al.* (1985)
$SEC_{1\text{-}2\text{-}3}$	≈ 27 500	Plasmid	+	+	Bohach & Schlievert (1987) Hovde *et al.* (1990) Altboum *et al.* (1985) Fitzgerald *et al.* (2001)
SED	26 360	Plasmid	+	+	Chang & Bergdoll (1979) Bayles & Iandolo (1989)
SEE	26 425	Prophage	+	+	Couch *et al.* (1988)
SEG	27 043	*enterotoxin gene cluster (egc)*, chromosome	+	+	Munson *et al.* (1998) Jarraud *et al.* (2001)
SEH	25 210	Transposon	+	+	Su & Wong (1996) Ren *et al.* (1994) Noto & Archer (2006)
SEI	24 928	*egc*, chromosome	+	(+)	Munson *et al.* (1998) Jarraud *et al.* (2001)
SElJ	28 565	Plasmids	+	nk	Zhang *et al.* (1998)
SEK	25 539	Pathogenicity island	+	nk	Orwin *et al.* (2001)
SElL	25 219	Pathogenicity island	+	−‡	Orwin *et al.* (2003)
SElM	24 842	*egc*, chromosome	+	nk	Jarraud *et al.* (2001)
SElN	26 067	*egc*, chromosome	+	nk	Jarraud *et al.* (2001)
SElO	26 777	*egc*, chromosome	+	nk	Jarraud *et al.* (2001)
SElP	26 608	Prophage	+	nk§	Kuroda *et al.* (2001) Omoe *et al.* (2005)
SElQ	25 076	Pathogenicity island	+	−	Jarraud *et al.* (2002) Diep *et al.* (2006)
SER	27 049	Plasmids	+	+	Omoe *et al.* (2003)
SES	26 217	Plasmid	+	+	Ono *et al.* (2008)
SET	22 614	Plasmid	+	(+)	Ono *et al.* (2008)
SElU	27 192	*egc*, chromosome	+	nk	Letertre *et al.* (2003)
$SElU_2$	26 672	*egc*, chromosome	+	nk	Thomas *et al.* (2006)
SElV	24 997	*egc*, chromosome	+	nk	Thomas *et al.* (2006)

*+, positive reaction.

†+, positive reaction; (+), weak reaction; −, negative reaction; nk, not known.

‡For SElL, emetic activity was not demonstrated in *Macaca nemestrina* monkey.

§For SElP, emetic activity was demonstrated in *Suncus murinus* but not in primate model.

antigens, do not need to be processed by antigen-presenting cells (APC) before being presented to T cells. They can directly stimulate T cells by cross-linking major histocompatibility complex (MHC) class II molecules on APC with the variable portion of the T-cell antigen receptor β chain (TCR Vβ) or the T-cell antigen receptor α chain for SE (TCR Vα), thereby inducing polyclonal cell proliferation (Li *et al.*, 1999; Pumphrey *et al.*, 2007; Thomas *et al.*, 2009). SAg-binding sites lie outside the peptide-binding groove and therefore do not depend on T-cell antigenic specificity but rather on the Vβ and/or Vα region of the TCR (Dellabona *et al.*, 1990; Li *et al.*, 1999; Pumphrey *et al.*, 2007). This leads to activation of a large number of T cells followed by proliferation and massive release of chemokines and proinflammatory cytokines that may lead to potentially lethal toxic shock syndrome

Table 6. Thermal inactivation of SEA and SED in milk and milk products at 72 °C for 15 s (Tatini, 1976)

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

NT, not tested.
*Initial concentration: 1 μg mL^{-1}.

(Balaban & Rasooly, 2000). The SAgs can interact with epithelial cells leading to their transepithelial transport, cell activation and induction of inflammatory state. First, most SAgs have dose-dependent capacity to cross the intestinal wall and produce a local and systemic action on the immune system. This transport is favoured by the production of pro-inflammatory cytokine-like elements (McKay & Singh, 1997). A protein motif (i.e. KKKVTAQELD) highly conserved among SE and located in the amino acids 120–130 and 144–161, for TSST1 and SE respectively, has been identified and is involved in this transcytosis (Shupp *et al.*, 2002). Stimulation of intestinal epithelial cells by SEA also induces an increase in the concentration of intracellular calcium *via* the release of cellular calcium reserves leading to their activation. This mechanism involves a nitric oxide synthase inducible by TNF-α (Hu *et al.*, 2005). Finally, superantigenic stimulation of intestinal epithelial cells induces an inflammatory response. The activation of T84 cells (a human epithelial cell line) by SAgs induces the production of (1) monocyte chemoattractant protein 1 (MCP-1) and (2) regulated on activation normal T-cell expressed and secreted protein (RANTES). These chemokines promote the recruitment of immune mononuclear cells, which may explain the role of the SAgs in the pathogenesis of inflammatory gastrointestinal diseases (Jedrzkiewicz *et al.*, 1999). In addition, both SEA and SEB bind to intestinal myofibroblasts with MHC class II molecules (Pinchuk *et al.*, 2007). However, only SEA induces an immune response with the release of MCP-1, interleukins 8 and 6 (IL-8 and IL-6), granulocyte macrophage colony–stimulating factor (GM-CSF), and granulocyte colony–stimulating factor (G-CSF).

Although the superantigenic activity of SEs has been well characterized, as previously presented, the mechanisms leading to the emetic activity are less documented. Despite the considerable efforts to identify specific amino acids and domains within SEs which may be important for emesis, results are still limited and controversial. For example, SE*l*L and SE*l*Q are nonemetic, whereas SEI displays weak emetic activity (Ono *et al.*, 2008). These toxins lack the disulphide loop characteristically found at the top of the *N*-terminal domain of other SEs. Nonetheless, the loop itself does not appear to be an absolute requirement for emesis, although it may stabilize a crucial conformation important for this activity (Hovde *et al.*, 1994). Harris *et al.* (1993) examined the correlation between emetic and T-cell stimulatory activities of SEA and SEB where the amino acids had been substituted. In most cases, genetic mutations resulting in a loss of superantigen activity also resulted in loss of emetic activity. However, as there was not a perfect correlation between immunological and emetic activities in all the mutants, this study suggested that these two activities could be dissociated.

In contrast to other bacterial enterotoxins, specific cells and receptors in the digestive system have not been clearly linked to oral intoxication by a SE. Sugiyama & Hayama (1965) suggested that SEs stimulate the vagus nerve in the abdominal viscera, which transmits the signal to the emetic centre. Supporting this idea, receptors on vagal afferent neurons are essential for SEA-triggered emesis (Hu *et al.*, 2007). In addition, SEs are able to penetrate the gut lining and activate local and systemic immune responses (Shupp *et al.*, 2002). The diarrhoea sometimes associated with SE intoxication could be attributed to the inhibition of water and electrolyte reabsorption in the small intestine (Sullivan, 1969; Sheehan *et al.*, 1970). In an attempt to link the two distinct activities of SEs, i.e., superantigenicity and emesis, it has been postulated that enterotoxin activity could facilitate transcytosis, enabling the toxin to enter the bloodstream and circulate through the body, thus allowing the interaction with APC and T cells that leads to superantigen activity (Hamad *et al.*, 1997; Balaban & Rasooly, 2000). In this way, circulation of SEs following ingestion of SEs, as well as their spread from an *S. aureus* infection site, could have more profound effects upon the host than if the toxin remains localized (Larkin *et al.*, 2009).

Genetic determinants and regulation of expression

Enterotoxin gene locations (Table 5) are numerous (Argudin *et al.*, 2010). They can be carried by plasmids (*seb*, *sed*, *sej*, *ser*, *ses*, *set*) (Shalita *et al.*, 1977; Bayles & Iandolo, 1989; Zhang *et al.*, 1998; Omoe *et al.*, 2003; Ono *et al.*, 2008), phage (temperate for *sea*, defective for *see*) (Betley & Mekalanos, 1985; Couch *et al.*, 1988; Coleman *et al.*, 1989) or by genomic islands (*seb*, *sec*, *seg*, *seh*, *sei*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep* and *seq*). Gene encoding for *sec* can be located on a plasmid or a pathogenicity island

depending on the origin of the isolate (Fitzgerald *et al.*, 2001). Jarraud *et al.* (2001) highlighted the existence of an operon, *egc* (enterotoxin gene cluster), encoding for several SEs such as SEG, SEI, SEM, SEN and SEO. The *egc* also contains two pseudogenes (ϕent1 and ϕent2). This locus probably plays the role of a nursery for *se* genes, as phenomena of duplication and recombination from a common ancestral gene could explain new forms of toxins. This was demonstrated by the identification of genes encoding for *seu*, *seu2* and *sev* within *egc* (Letertre *et al.*, 2003; Thomas *et al.*, 2006). The location of *se* genes on mobile genetic elements can result in horizontal gene transfer between the strains of *S. aureus*. For example, the *seb* gene is located on the chromosome in some clinical isolates (Shafer & Iandolo, 1978), whereas it has a plasmidic location in other strains of *S. aureus* (Shalita *et al.*, 1977).

A main regulatory system controlling the expression of virulence factors in *S. aureus* is the *agr* system (accessory gene regulator; Kornblum *et al.*, 1990). This system works in combination with the *sar* system (Staphylococcal accessory regulator; Cheung *et al.*, 1992; Novick *et al.*, 2001). Most but not all of the expression of SEs is controlled by the *agr* system. For example, expressions of *seb*, *sec* and *sed* genes are *agr*-dependent, whereas expressions of *sea* and *sej* are *agr*-independent (Tremaine *et al.*, 1993; Zhang *et al.*, 1998). Vojtov *et al.* (2002) demonstrated that SEB is a negative global regulator of exoprotein gene expression acting through the *agr* system. The expression of *agr* system is closely linked to quorum sensing (Novick *et al.*, 2001). In a recent study carried out on 28 enterotoxigenic strains of *S. aureus* isolated from food poisoning outbreaks or reference libraries to better understand *se* gene expression, Derzelle *et al.* (2009) demonstrated four different patterns of expression using quantitative reverse transcription PCR. The first pattern for *sea*, *see*, *sej*, *sek*, *sep* and *seq* indicated that the abundance of mRNAs was independent of the bacterial growth phases. In the second pattern, the transcript levels for *seg*, *sei*, *sem*, *sen*, *seo* and *seu* slightly decreased during bacterial growth. The third pattern indicated a huge and rapid induction of *seb*, *sec* and *seh* at the end of the exponential growth phase whereas the last highlighted a modest postexponential increase in *sed*, *ser* and *sel* expression.

To conclude this section, the currently known SEs form a group of serologically distinct, extracellular proteins that share important properties namely, (1) the ability to cause emesis in primate model; (2) superantigenicity through a noncomplete unspecific activation of T lymphocytes (as each SEs binds to a subset of Vβ chains) followed by cytokine release and systemic shock (Marrack & Kappler, 1990; Papageorgiou & Acharya, 2000); (3) resistance to heat and to digestion by pepsin; and (4) structural similarities (Dinges *et al.*, 2000).

SFPOs: symptomatology; reporting system including EU control; monitoring schemes; occurrence and analytical methods used

Symptomatology and toxic dose

The incubation period and severity of symptoms observed depend on the amount of enterotoxins ingested and the susceptibility of each person. Initial symptoms, nausea followed by incoercible characteristic vomiting (in spurts), appear within 30 min–8 h (3 h on average) after ingesting the contaminated food. Other commonly described symptoms are abdominal pain, diarrhoea, dizziness, shivering and general weakness, sometimes associated with a moderate fever. In the most severe cases, headaches, prostration and low blood pressure have been reported. In the majority of cases, recovery occurs within 24–48 h without specific treatment, while diarrhoea and general weakness can last 24 h or longer. Death is rare (0.02‰ according to Mead *et al.*, 1999), occurring in the most susceptible people to dehydration such as infants and the elderly (Do Carmo *et al.*, 2004) and people affected by an underlying illness.

Regarding the toxin dose, most of the studies referred to SEA. 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, 1989). Mossel *et al.* (1995) cited an emetic dose 50 value of about 0.2 μg SE per kg of human body weight. They concluded that an adult would need to ingest about 10–20 μg of SE to suffer symptoms. Other authors (Martin *et al.*, 2001) considered that < 1 μg of SE may cause food-poisoning symptoms in susceptible 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 half-pint (approximately 0.28 L) carton of a 2% chocolate milk. In SFP in Japan, the total intake of SEA in low-fat milk per capita was estimated mostly at approximately 20–100 ng (Asao *et al.*, 2003; Ikeda *et al.*, 2005). In an SFPO involving 'coconut pearls' (a Chinese dessert based on tapioca), Hennekinne *et al.* (2009) estimated the total intake of SEA per body at around 100 ng. Finally, Ostyn *et al.* (2010) investigated SFPOs owing to SEE and estimated that the total intake of SEE per body was 90 ng, a dose in accordance with those previously mentioned.

Reporting system, occurrence at European Union level

The reporting of food-borne outbreaks has been mandatory for the European Union Member States (EU MSs) since 2005. Moreover, since 2007, new harmonized specifications on the reporting of these outbreaks at Community level have come into force (Anonymous, 2007a). However, the food-borne outbreak investigation and reporting systems at national level are not harmonized within the EU. Therefore, differences in the number of reported outbreaks, the types of outbreaks and causative agents do not necessarily reflect different levels of food safety between EU MSs. The high number of reported outbreaks may reflect the increasing efficiency of the EU-MSs' systems in investigating and identifying the outbreaks.

The European Food Safety Authority (EFSA) is responsible for examining the data on zoonoses, antimicrobial resistance and food-borne outbreaks submitted by Member States in accordance with Directive 2003/99/EC (Anonymous, 2003) and for preparing the Community Summary Report from the results. Data were produced in collaboration with the European Centre for Disease Control (ECDC), which provides the information on zoonosis cases in humans. The Zoonoses Collaboration Centre (ZCC – contracted by EFSA) in the National Food Institute of the Technical University of Denmark assisted EFSA and ECDC in this task (Fig. 1).

In 2005, the first year for reporting of food-borne outbreaks in the European Union, only seven Member States reported food-borne outbreaks (n = 36) caused by SEs.

European data from 2006 to 2008 are presented in Fig. 2.

In 2006, EFSA (Anonymous, 2007c) reported that SEs were involved in 236 outbreaks of 5807 (4.1%) food poisoning outbreaks, corresponding to the fourth rank of causative agents after the ones associated with *Salmonella* spp. (59.3%), viruses (10.2%) and *Campylobacter* spp. (6.9%). Dairy products, red meat products and poultry were involved in 26 (11.0%), 19 (8.0%) and 16 (6.8%) of the 236 outbreaks respectively.

In 2007, EFSA (Anonymous, 2009) reported that bacterial toxins were involved in 458 of 5423 (8.1%) food poisoning outbreaks corresponding to the fourth rank of pathogenicity after those associated with *Salmonella* spp. (39.3%), viruses (11.8%) and *Campylobacter* spp. (8.1%). Among bacterial toxins, SEs were involved in 258 of the 458 notified outbreaks (56.3%). Thus, SEs were involved in 4.6% of all notified outbreaks in 2007.

Finally, in 2008, EFSA (Anonymous, 2010) reported that bacterial toxins were involved in 525 of 5332 (9.8%) food poisoning outbreaks, corresponding to the third

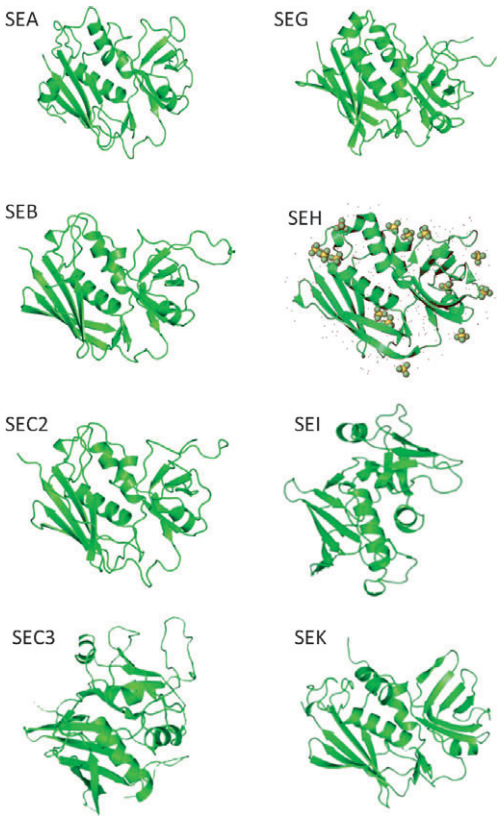

Fig. 1. 3D structure of various staphylococcal enterotoxins. SEA: Staphylococcal enterotoxin A (Schad *et al.*, 1995); SEB: Staphylococcal enterotoxin B (Papageorgiou *et al.*, 1998); SEC2: Staphylococcal enterotoxin C2 (Swaminathan *et al.*, 1995); SEC3: Staphylococcal enterotoxin C3 (Chi *et al.*, 2002); SEG: Staphylococcal enterotoxin G (Fernandez *et al.*, 2011); SEH: Staphylococcal enterotoxin H (green) with sulfate ions and water (Hâkansson *et al.*, 2000); SEI: Staphylococcal enterotoxin I (Fernandez *et al.*, 2006); SEK: Staphylococcal enterotoxin K (Gunther *et al.*, 2007). All structures obtained from http://www.ebi.ac.uk/ebisearch/search.ebi?db=macromolecularStructures&t=%22staphylococcal+enterotoxin%22&requestFrom=navigateYouResults) (Adapted by L. Bandounas).

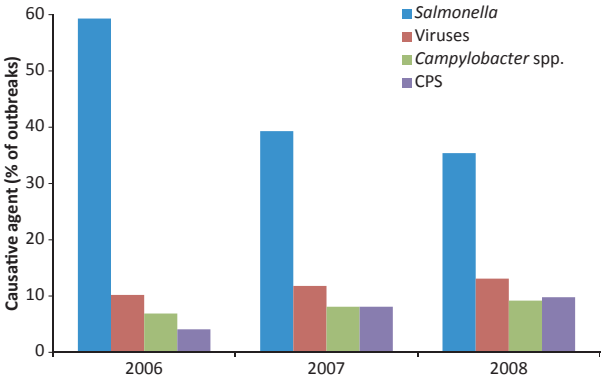

Fig. 2. Causative agents involved in European Union outbreaks 2006–2008 (data extracted from The Community Summary Reports 2006, 2007 and 2008).

rank of pathogenicity after those associated with *Salmonella* spp. (35.4%) and viruses (13.1%). Among bacterial toxins, SEs were involved in 291 of the 525 notified outbreaks (55.4%). Thus, SEs were involved in 5.5% of all notified food poisoning outbreaks in 2008.

Analytical methods for SE detection (Fig.3)

Diagnosis of SFP is generally confirmed either by the recovery of at least 10^5 *S. aureus* g^{-1} from food remnants or by the detection of SEs in food remnants. In some cases, confirmation of SFP is difficult because *S. aureus* is heat sensitive, whereas SEs are not. Thus, in heat-treated food matrices, *S. aureus* may be eliminated without inactivating SEs. In such cases, it is not possible to characterize a food poisoning outbreak by enumerating CPS in food remnants or detecting *se* genes in isolated strains.

While *S. aureus* is usually enumerated using microbiological techniques with dedicated media such as Baird Parker or rabbit plasma fibrinogen agar, three types of methods are used to detect bacterial toxins in food: bioassays, molecular biology and/or immunological techniques.

Bioassays

Bioassays are based on the capacity of an extract of the suspected food to induce symptoms such as vomiting, gastrointestinal symptoms in animals and/or superantigenic action in cell cultures. Historically, SEs have been detected based on their emetic activity in monkey-feeding and kitten-intraperitoneal tests (Surgalla *et al.*, 1953; Bergdoll, 1989) and, more recently, using animal models such as house musk shrews *Suncus murinus* (Hu *et al.*, 2003; Ono *et al.*, 2008). Symptoms of SFP appear if the dose of SEA ingested by the animals is above 2.3 μg, a considerably higher amount than those involved in human food poisoning (Asao *et al.*, 2003; Ostyn *et al.*, 2010). Thus, this technique is not appropriate for characterizing SFPOs.

Molecular methods

Molecular biology methods often involve the polymerase chain reaction (PCR). These methods usually detect genes encoding enterotoxins in strains of *S. aureus* isolated from contaminated foods. However, these methods have two major limitations: first, staphylococcal strains must be isolated from food, and second, the results inform as to the presence or absence of genes encoding SEs, but do not provide any information on the expression of these genes in food. This method therefore cannot be the sole method for confirming *S. aureus* as causative agent in an outbreak. However, the PCR approach is a specific, highly sensitive and rapid method that can characterize the *S. aureus* strains involved in SFPOs, thereby providing highly valuable information. In outbreaks described by Ostyn *et al.* (2010), SEE has been found in the common source vehicle and the *see* gene was present in the tested *S. aureus* isolates. In such a case, *se* gene determination

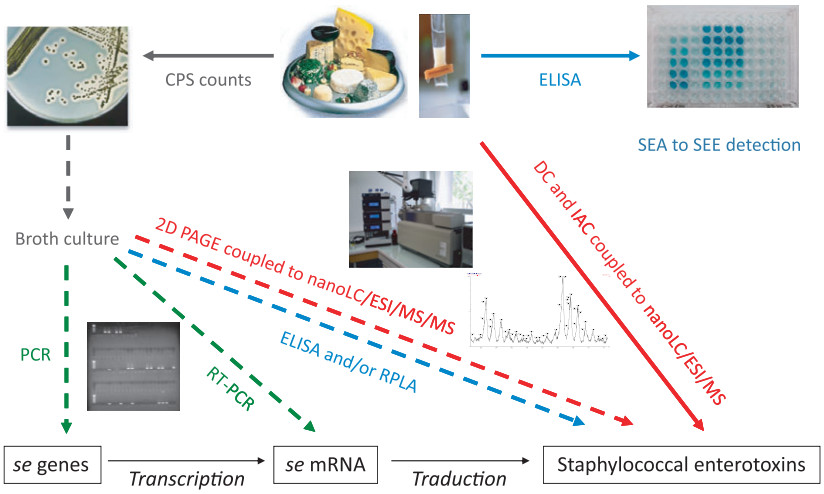

Fig. 3. General overview of analytical methods used to improve SFPO characterization. 2D PAGE; two dimension gel electrophoresis; CPS, coagulase positive staphylococci; DC, dialysis concentration; IAC, immunoaffinity chromatography; anoLC/ESI/MS, nano-liquid chromatrography/electrospray-ionization/mass spectrometry; RPLA, reversed passive latex agglutination; RT-PCR, reverse transcriptase PCR; SE, staphylococcal enterotoxin; SFPO, staphylococcal food poisoning outbreak. Continuous line: analysis performed from food sample; discontinuous line: analysis performed from strains or culture supernatant. Brown: microbiological methods; green: molecular methods; blue: immunological methods; red: mass spectrometry-based methods.

helps to confirm the role of an SE rarely encountered. Very recent efforts have been directed to determining directly which *se* genes are found in suspected foods. Following the huge SFP event which occurred in Japan in July 2000 (more than 13 000 people were intoxicated by powdered or liquid milk), Ikeda *et al.* (2005) developed a PCR-based methodology whereby *sea*, *seg*, *seh* and *sei* genes could be detected in the incriminated powdered skim milk, although cultivable *S. aureus* were not recovered from the sample.

Moreover, to evaluate the toxic potential of strains isolated from SFPOs, various authors (Lee *et al.*, 2007; Akineden *et al.*, 2008; Derzelle *et al.*, 2009) have recently designed primers to perform PCR and reverse transcription PCR (RT-PCR) for *se* genes.

Finally, Duquenne *et al.* (2010) developed an efficient method for extracting bacterial RNA accessible for RT-quantitative PCR (RT-qPCR) from cheese and adapted a simple, sensitive and reproducible, method for quantifying relative transcript levels to evaluate *S. aureus* enterotoxin gene expression during cheese manufacture. These approaches demonstrate possible transcription of mRNA from those genes, but do not indicate whether those strains were able to produce detectable or poisonous levels of toxins in food.

Immunological methods

The third and most commonly used method for detecting SEs in food is based on the use of anti-enterotoxin polyclonal or monoclonal antibodies. Commercially available kits have been developed according to two different principles: (1) enzyme immunoassay (EIA) comprising ELISA and enzyme-linked fluorescent assay (ELFA); and (2) RPLA. It is widely recognized that the use of immunological methods to detect contaminants in food matrices is a difficult task, mainly because of the lack of specificity and sensitivity of the assay. Many drawbacks impair the development and use of these techniques for detecting SEs. First, highly purified toxins are needed to raise specific antibodies to develop an EIA; purified toxins are difficult and expensive to obtain. Moreover, and until very recently, only antibodies against SEA to SEE, SEG, SEH and SElQ were available (Schlievert & Case, 2007). The ELISA test will not detect the other SEs, which could partly explain why some outbreaks remained uncharacterized without a known aetiological agent. Another drawback is the low specificity of some commercial kits, where false positives may occur depending on food components (Wieneke, 1991) as it is well known that some proteins, such as protein A, can interfere with binding to the Fc fragment (and, to a lesser extent, Fab fragments) in immunoglobulin G from several animal species, such as mouse or rabbit, but not rat or goat. Other interferences are associated with endogenous enzymes, such as alkaline phosphatase or lactoperoxidase.

Whatever the detection method used and owing to the low amount of SEs present in food, it is crucial to concentrate the extract before performing detection assays. For this purpose, various methodologies have been tested (Macaluso *et al.*, 1998; Meyrand *et al.*, 1999; Lapeyre *et al.*, 2001). Among them, only extraction followed by dialysis concentration has been approved by the European Union for extracting SEs from food (Anonymous, 2007b).

However, up to now, after enumerating CPS strains, conclusive diagnosis of SFPs has mainly been based on demonstrating the presence of SEs in food using commercial EIA kits designed to detect SEA to SEE (Bennett, 2005) or using a confirmatory in-house ELISA method (Lapeyre *et al.*, 1988) to differentiate and quantify these types of SEs.

Mass spectrometry-based methods

Owing to the drawbacks with currently available detection methods and the lack of available antibodies against the newly described SEs, other strategies based on physicochemical techniques have been developed very recently. Among these, mass spectrometry (MS) has newly emerged as a very promising and suitable technique for analysing protein and peptide mixtures (Mamone *et al.*, 2009). It is among the most sensitive techniques currently available because it provides specific, rapid and reliable analytical quantification of the amount of enterotoxins (Brun *et al.*, 2007). The development of two soft ionization methods, such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), and the use of appropriate mass analyzers such as time-of flight (TOF) have revolutionized the analysis of biomolecules. Given the wide range of methodologies available, a single MS technique cannot be used for all proteins. The MS method thus requires the development of a series of techniques, individually suited for each particular case.

In the case of food analysis, the situation is complex because the matrix can contain many proteins, lipids and many other molecular species that interfere with the detection of the targeted toxin and may distort quantification. Sample preparation remains the critical step of the analysis. Several authors have tried to improve this step, by, for example, optimizing digestion parameters (Norrgran *et al.*, 2009) or by adding a purification step (Oeljeklaus *et al.*, 2009). The strategy of incorporating an isotopically labelled internal standard into the samples has also been developed. In the case of SE detection, some authors have developed MS tools to detect these

toxins in culture supernatants and in spiked samples, such as water or apple juice. For example, Bernardo *et al.* (2002) developed a MALDI-TOF method for detecting *S. aureus* virulence factors such as enterotoxins and demonstrated that this technique was suitable for detecting SEs other than SEA to SEE in culture supernatants. Callahan *et al.* (2006) detected and quantified SEB using liquid chromatography coupled to ESI/MS detection in apple juice used as a model food matrix. In this study, enterotoxin types SEA and SEB were detected in spiked cheese. More recently, Brun *et al.* (2007) developed an MS approach able to perform absolute quantification of SEA and TSST1 in spiked water or urine samples. To improve characterization and quantification of SEs, this latter methodology was successfully used to carry out absolute quantification of SEA in a naturally contaminated cheese sample (Dupuis *et al.*, 2008) and applied to a recent case of food poisoning outbreak (Hennekinne *et al.*, 2009). In this outbreak, MS tools in combination with tools presented earlier were used by the European Union Reference Laboratory for CPS. This MS-based method overcame specific technical limitations of existing ELISA for SE characterization but its throughput and cost per analysis compared unfavourably with ELISA (€650 vs. €280). This last method was no doubt the gold standard of low-cost and high-throughput techniques for the detection and quantification of protein compounds down to subnanomolar concentrations in large sample cohorts. However, the timescale for ELISA assay development was of the order of 1 year and high developmental costs precluded systematic ELISA optimization. This cost also made ELISA less suitable for the characterization of small panels such as SFP elucidation. In this regard, the versatility and low development cost of the absolute quantification methodology positioned it as a good alternative to ELISA for these specific applications, keeping in mind that purified SEs standards were also needed to establish the accuracy and specificity of MS-based methods.

Thus, combining classical microbiology for enumerating CPS strains with immunological techniques, molecular biology and mass spectrometry-based methods, the diagnosis was reinforced and these outbreaks could be attributed to the presence of SEs.

Concluding remarks

Staphylococcal food poisoning is one of the most common food-borne diseases, resulting from ingestion of SEs produced in food by enterotoxigenic strains of staphylococci, mainly CPS and only occasionally CNS. From 2006 to 2008, the European Food Safety Authority reported that SEs were involved in 5% of food poisoning outbreaks, but this percentage is certainly underestimated owing to poor analytical performance in the detection and identification of SEs in food remnants.

Prevention of staphylococcal food-borne poisoning is based on hygiene measures to avoid or reduce contamination of food by *S. aureus*. These procedures must include control of raw materials, proper handling, cleaning and disinfection of equipment from farm to fork. However, as these requirements are usually not sufficient, it is necessary to destroy staphylococci through appropriate treatment, thermal or otherwise, to prevent their growth under refrigerated conditions. Respect for the cold chain is critical in regard to staphylococci especially for foods served at large gatherings such as social events.

To improve SFPO characterization, various techniques, such as immunological and molecular-based methodologies, have been integrated in the diagnosis strategy. The PCR approach is known to provide information on the presence or absence of genes encoding SEs, but not their expression. To complete SFPO characterization, MS tools have also been used in combination with those presented earlier. Thus, an overall approach combining classical microbiology to enumerate CPS strains with immunological techniques, molecular biology and mass spectrometry-based methods offers an interesting alternative for attributing outbreaks to SEs (Fig. 3). While the quantitative MS method overcomes specific technical limitations of existing ELISA methods for detecting and quantifying SEs, its throughput and cost per analysis compare unfavourably with ELISA. For this reason, when the MS-based method becomes available for all SEs involved in SFPOs, it will not be employed for routine analysis, but only in special cases to confirm outbreaks because of SEs.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Reporting scheme for food poisoning outbreaks at European Union level.

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