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REVIEW



## *Staphylococcus aureus* enterotoxin in food of animal origin and staphylococcal food poisoning risk assessment from farm to table

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### ABSTRACT

*Staphylococcus aureus* is a gram-positive bacterium, commonly found in the nostrils, on the skin and on the hair of warm-blooded animals, including humans. It can produce a wide variety of virulence factors, including staphylococcal enterotoxins (SEs). In literature, 24 different SEs and many variants have been identified; among these, only five (the so-called classic enterotoxins) have been well-defined. Due to their emetic activity, SEs are frequently responsible for staphylococcal food poisoning, when consumers ingest contaminated food. SEs are proteins with a high tolerance of denaturing and can maintain their activity, even when the vegetative form of the bacteria is inactivated during food processing. The enterotoxin encoding genes are found in a variety of different genetic elements and, as a result, enterotoxin production varies widely between different populations of *S. aureus*. SEs production is modulated by multiple, and often overlapping, regulatory pathways, which are influenced by environmental factors. Furthermore, complex food matrices possess many characteristics (storage temperature, pH, sugar or salt concentration, presence of competitive microorganisms, etc.) that have a high impact on *S. aureus* behaviour. The multiple factors influencing *S. aureus* growth in food matrices and the production of SE complicates risk assessment procedures. In this review, we focus on enterotoxin production by *S. aureus* in food of animal origin, its regulation and detection and on the most recent developments in predictive microbiology and risk assessment models.

### HIGHLIGHTS

- *Staphylococcus aureus* produces several virulence factors that contribute to the pathogenesis of several serious human diseases: among these staphylococcal enterotoxins (SEs) have emetic activity, and are responsible for staphylococcal food poisoning (SFP).
- SEs are proteins that maintain their activity even though the vegetative form of the bacteria is inactivated during food processing.
- Enterotoxin encoding genes are found in a variety of genetic elements and enterotoxin production varies and is regulated by multiple regulatory pathways, which are influenced by environmental factors.

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## Introduction

Bacterial toxins are considered the third most significant cause of food-borne outbreaks worldwide. In 2015, over half of all the food-borne outbreaks ( $n = 434$ ) associated with bacterial toxins were caused by staphylococcal enterotoxins (SEs; EFSA 2016). In the annual summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks, the European Food Safety Authority (EFSA) reported that

in 2017, approximately 1800 samples (cheeses and dairy products) were tested within the context of national monitoring and surveillance and/or surveys by Cyprus, the Czech Republic, Italy, Romania and Spain. In total, 23 samples tested positive (1.2%), mainly from Italy (cheese and pasteurised milk) and Spain (milk). Data on SEs in other food was submitted by five member states. Out of the 645 samples tested, 40 were positive. These included two samples from Bulgaria (potato-based dishes and ready-to-eat (RTE)

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pig meat), seven from Italy (meat preparation, other processed food), 12 from Slovakia (from sandwiches, RTE food, confectionery products and frozen desserts) and 19 from Spain (in bakery products, sauces and dressings, meat-based dishes, vegetable-based dishes, RTE salads, other prepared dishes). Suspect samples were collected by Ireland (ice-cream, pre-cut vegetables, prepared dishes) and Hungary (noodles and fermented sausages; EFSA 2018). The Centres for Disease Control in the United States estimated that 240,000 cases of staphylococcal food poisoning (SFP) occur each year, leading to 1000 hospitalisations and six fatalities (Scallan et al. 2011). Food contamination often occurs either as a direct result of the presence of the bacteria in food producing animals, or is due to poor hygiene during the production, storage or sale of food products (Aydin et al. 2011; Kotzekidou 2013). Humans and livestock are considered major reservoirs for the transmission of enterotoxin-producing *S. aureus* (Fisher et al. 2018). The severity of the illness depends on the amount of toxin ingested and the general health of the consumer. Patients report symptoms, e.g. nausea and vomiting, shortly after the oral intake of the toxins, often accompanied by watery diarrhoea, prostration and fever. Symptoms usually subside within 24 hours, but in higher risk categories, e.g. children or the elderly, a fatality rate of 4.4% was reported (Doyle and Beuchat 2007). The amount of enterotoxin required to cause the illness can be as little as 20–100 ng (Asao et al. 2003). Of 24 SEs identified and reported in literature, only five (SEA, SEB, SEC, SED and SEE, the so-called classic enterotoxins) have been well-defined, and are detectable using commercially available assays or in-house developed methods (Nia et al. 2016). SEs are highly stable proteins, resistant to the environmental conditions that can easily inactivate the *S. aureus* vegetative form. SEs are stable at high temperatures: e.g. they are not completely destroyed during pasteurisation (15 s at 72 °C) and crude enterotoxin A remains active at 100 °C for 2 h in broth and at 121 °C for 28 min in mushrooms (Hennekinne et al. 2012), and high or low pH. They are also resistant to proteolytic enzymes; hence they retain their activity in the digestive tract after ingestion (Argudín et al. 2010). As a result of these characteristics, SEs are considered a serious threat to public health and the notification of food poisoning outbreaks caused by them has been mandatory since 2005 [Commission Regulation (EC) No. 1441/2007 of 5 December 2007].

The prevention of the growth of *S. aureus* and consequent enterotoxin production is of major

importance to minimise the risk of food-poisoning. However, studies have demonstrated that assessing the SFP risk by *in vitro* models (liquid cultures with bacteria in a planktonic state) or based solely on the count of colony-forming units is unreliable (Zeaki et al. 2014). In fact, food-related factors (temperature, pH, sugar or salt concentration, presence of competitive microorganisms, etc.) have a high impact on the growth rate, lag phase duration and virulence of the gene expression of *S. aureus* (Schelin et al. 2011). Understanding the behaviour of enterotoxin producing *S. aureus* in authentic, complex food matrices and the effect of stressor factors on enterotoxin production is crucial for improving risk assessment models, consumer safety and food product quality. In this review, we aim to present and discuss the current knowledge along with novel findings regarding the growth and production of enterotoxins by *S. aureus* in foods and the advances made in risk assessment.

### The organism and enterotoxins

*S. aureus* is a Gram-positive coccus, occurring in single, paired or grape-like clusters (*staphylé* means grape in Greek). Due to the production of a carotenoid pigment, colonies of these bacteria appear gold in colour, giving rise to the species name *aureus* (meaning golden). They are facultative, anaerobic, non-motile, non-sporing, catalase and coagulase positive bacteria (Willey and Prescott 2008). *S. aureus* can be found in the nostrils, on the skin and on the hair of warm-blooded animals, including humans (Le Loir et al. 2003). It is able to grow in a wide range of temperatures (7–48.5 °C with optimum 30–37 °C), pH (from 4.2 to 9.3 with an optimum of 7 to 7.5) and sodium chloride concentrations (NaCl concentrations up to 15%; Schmitt et al. 1990). These characteristics, combined with its particular ecological niche, can easily explain the relevance of these bacteria for the safety of foods, and, more specifically, those that require manipulation during processing. *S. aureus* can produce many virulence factors, e.g. toxins, invasiveness and antibiotic resistance, and can produce a wide variety of symptoms ranging from furuncles to toxic shock syndrome, based on different combinations of those virulence factors. On the other hand, SFP relies on one single type of virulence factor, the SEs.

In literature, 24 different SEs and many variants have been identified, based on their antigenicity, and named from SEA to SE/Y in chronological order of discovery (Ono et al. 2015; Denayer et al. 2017; Table 1). This superfamily of proteins shares many common

**Table 1.** Characteristics of staphylococcal enterotoxins.

Enterotoxin	Genetic base	ORF length, bp	Molecular weight, Da	Emetic activity		Phylogenetic group
				Monkey	Shrew	
SEA (Betley and Mekalanos 1985)	Prophage	774	27,100	(Bergdoll et al. 1965)	(Hu et al. 2003)	SEA
SEB (Novick et al. 2001)	Chromosome, SaPI, plasmid (pZA10)	801	28,336	(Bergdoll et al. 1965)	(Hu et al. 2003)	SEB
SEC1 (Fitzgerald et al. 2001)	SaPI	801	27,531	(Bergdoll et al. 1965; Schlievert et al. 2000)	nd	SEB
SEC2 (Fitzgerald et al. 2001)	SaPI	801	27,531	(Bergdoll et al. 1965)	(Hu et al. 2003)	SEB
SEC3 (Fitzgerald et al. 2001)	SaPI	801	27,563	(Reiser et al. 1984)	nd	SEB
SED (Bayles and landolo 1989)	Plasmid (pIB485)	777	26,360	(Igarashi 1972)	(Hu et al. 2003)	SEA
SEE (Couch et al. 1988)	Prophage	774	26,425	(Bergdoll et al. 1971)	(Hu et al. 2003)	SEA
SEG (Jarraud et al. 2001)	<i>egc1, egc2, egc3, egc4</i>	777	27,043	(Munson et al. 1998)	(Hu et al. 2003)	SEB
SEH (Le Loir et al. 2003)	Transposon (MGE <sub>mw2</sub> /mssa476 <i>seh</i> /D <sub>seo</sub> )	726	25,210	(Su and Wong 1995)	(Hu et al. 2003)	SEA
SEI (Jarraud et al. 2001)	<i>egc1, egc2, egc3</i>	729	24,298	<100 µg/kg (Munson et al. 1998)	(Hu et al. 2003)	SEI
SEJ (Omoe et al. 2003)	Plasmid (pIB485, pF5)	806	28,565	nd	nd	SEA
SEK (Yarwood et al. 2002)	Prophages, SaPI1, SaPI3, SaPI5, SaPI <sub>bov1</sub>	729	25,539	(Omoe et al. 2013)	(Ono et al. 2017)	SEI
SEL (Fitzgerald et al. 2001)	Prophages, SaPI <sub>n1</sub> , SaPI <sub>m1</sub> , SaPI <sub>mw2</sub> , SaPI <sub>bov1</sub>	723	24,593	(Omoe et al. 2013)	(Ono et al. 2017)	SEI
SEM (Jarraud et al. 2001)	<i>egc1, egc2</i>	722	24,842	(Omoe et al. 2013)	(Ono et al. 2017)	SEI
SEN (Jarraud et al. 2001)	<i>egc1, egc2, egc3, egc4</i>	720	26,067	(Omoe et al. 2013)	(Ono et al. 2017)	SEA
SEO (Jarraud et al. 2001)	<i>egc1, egc2, egc3, egc4</i> , transposon	783	26,777	(Omoe et al. 2013)	(Ono et al. 2017)	SEA
SEP (Kuroda et al. 2001)	Prophage (Sa3n)	783	27,000	(Omoe et al. 2013)	(Omoe et al. 2005)	SEA
SEQ (Yarwood et al. 2002)	Prophage, SaPI1, SaPI3, SaPI5	729	25,207	(Omoe et al. 2013)	(Hu et al. 2017)	SEI
SER (Omoe et al. 2003)	Plasmid (pIB485, pF5)	600	27,049	<100 µg/kg (Ono et al. 2008)	<100 µg/kg (Ono et al. 2008)	SEB
SES (Ono et al. 2008)	Plasmid (pF5)	774	26,217	<100 µg/kg (Ono et al. 2008)	<100 µg/kg (Ono et al. 2008)	SEA
SET (Ono et al. 2008)	Plasmid (pF5)	651	22,614	<100 µg/kg (Ono et al. 2008)	<100 µg/kg (Ono et al. 2008)	SE/X
SEU (Letertre et al. 2003)	<i>egc2, egc3</i>	786	27,100	nd	nd	SEB
SEW (SE/U2) (Thomas et al. 2006)	<i>egc4</i>	771	nd	nd	nd	SEB
SEV (Thomas et al. 2006)	<i>egc4</i>	720	nd	nd	nd	SEI
SE/X (Wilson et al. 2011)	Chromosome	nd	nd	nd	nd	SE/X
SE/Y (Ono et al. 2015)	Chromosome	nd	nd	nd	(Ono et al. 2015)	SE/X

ORF: open reading frame obtained from the National Centre for Biotechnology Information; NCBI: [www.ncbi.nlm.nih.gov/ludwig/lub.lu.se/gene](http://www.ncbi.nlm.nih.gov/ludwig/lub.lu.se/gene); nd: not demonstrated or unavailable at the time of writing; monkey: cynomolgus monkey (*Macaca fascicularis*), oral administration; shrew: house musk shrew (*Suncus murinus*), intraperitoneal administration; SaPI, *S. aureus* pathogenicity island; egc, enterotoxin gene cluster; MGE, mobile genetic element.

characteristics: they are non-glycosylated, single-chain proteins with a homologous, globular structure and a low molecular weight (19–29 kDa; Thomas et al. 2006). They can be divided into two groups: the true SEs, which comprise the toxins with demonstrated emetic potential in monkeys, and the enterotoxin-like toxins

(SE/-), which comprise those lacking emetic activity or which have not been tested in non-human primates (Fisher et al. 2018). Based on their nucleotide and amino acid sequences, SEs and SEIs can be divided into several groups: the SEA group (SEA, SED, SEE, SEJ, SEH, SEN, SEO, SEP, SES), the SEB group (SEB,

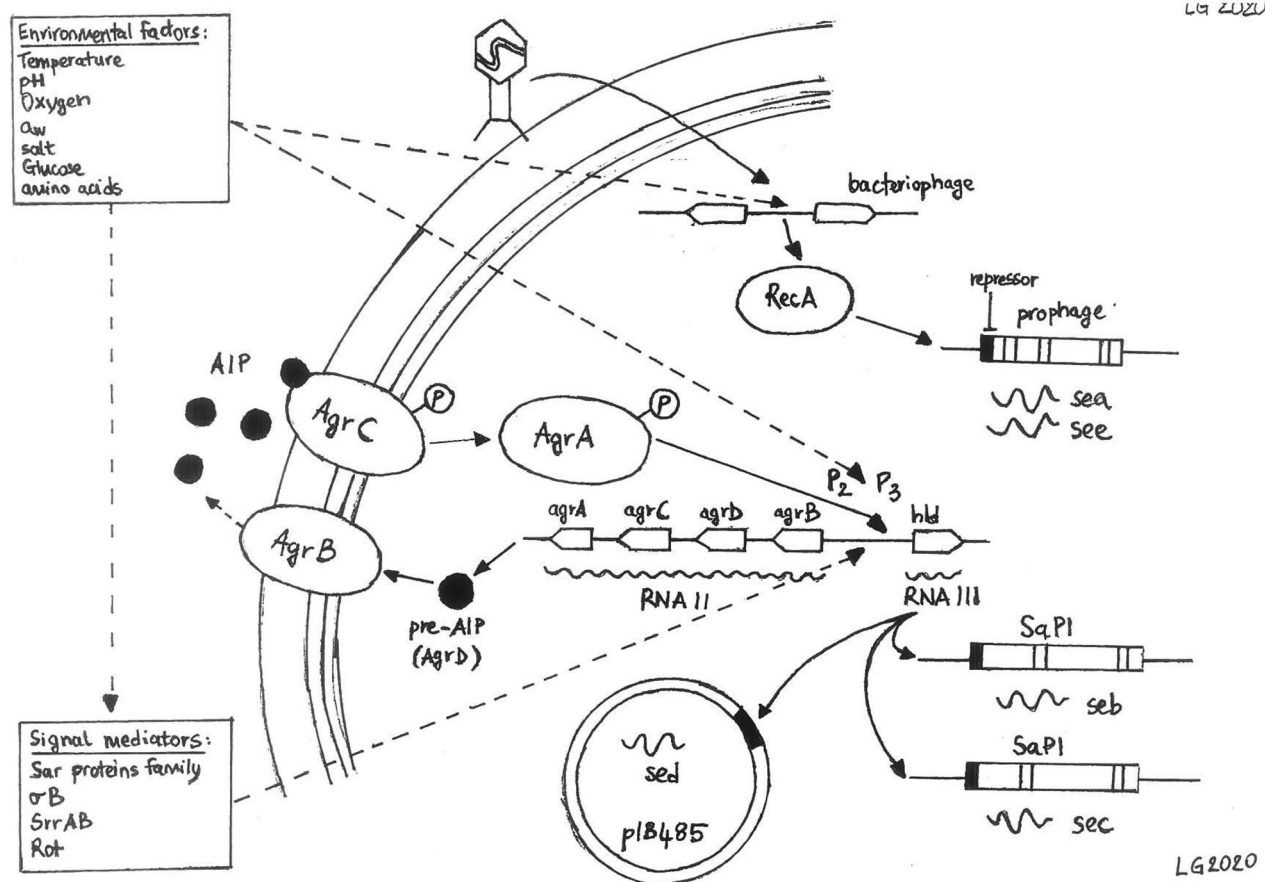
SECs, SEG, SER, SE/U, SEM, previously known as SE/U2), the SEI group (SEI, SEK, SEL, SEQ, SEM, SEV) and the SE/X group (TSST-1, SET, SE/X, SE/Y and members of another group of staphylococcal exotoxins, called superantigen-like (SSL) toxins; Ono et al. 2015).

Most of the enterotoxin genes are found, alone or in groups, on a wide variety of mobile segments of DNA (mobile genetic elements, MGEs), including prophages, plasmids, transposons, *S. aureus* pathogenicity islands (SaPIs) and enterotoxin gene clusters (*egc*; Malachowa and DeLeo 2010). For example, among the classic SEs, *sea* is carried by a family of temperate phages (Wallin-Carlquist et al. 2010), *seb* and *sec* are located on SaPIs (Novick et al. 2001; Novick 2003), *sed* is carried by a plasmid and *see* is carried by a defective phage (Le Loir et al. 2003). The *egc* contains an operon of genes encoding SEG, SEI, SEM, SEN, SEO and two pseudogenes,  $\phi$ ent1 and  $\phi$ ent2 (Jarraud et al. 2001). The deletion, duplication and recombination in this tract of genomes often lead to the generation of new types of enterotoxins and variants (Thomas et al. 2006). Two toxin genes, *selx* (Wilson et al. 2011) and

*sely* (Ono et al. 2015) are found exclusively on the genome. Considering the wide spectrum of different genetic bases for the production of enterotoxins, it is easy to understand that *S. aureus* isolates vary considerably: 80% of isolates carry an average of 5–6 SE genes (Umeda et al. 2017).

### The regulation of enterotoxin production

Current knowledge of the regulation of enterotoxin production is still incomplete: whereas it is known that *S. aureus* is able to respond to environmental changes, using a combination of quorum-sensing and at least another 16 two-component systems and many trans-acting regulatory proteins (Haag and Bagnoli 2016). All those systems allow the bacteria to quickly adapt to stress factors by regulating the expression of genes associated with important physiological features, including enterotoxin production. Each system can directly or indirectly control the transcription of specific sets of genes and a single gene can be



**Figure 1.** Direct and potential regulatory pathways of the regulation of SEs transcription. The individual regulators and pathways are described in the text. AIP, autoinducing peptide; agr, accessory gene regulator; SaPI, *S. aureus* pathogenicity island.

influenced by multiple systems, leading to many layers of regulation (Fisher et al. 2018; Figure 1).

### Regulation of classic enterotoxins (SEA-SEE)

The production of bacteriophage-associated enterotoxins (SEA and SEE) is generally constitutive, even though strains with distinct low and high levels of SEA production have been described in literature (Thomas et al. 2007; Wallin-Carlquist et al. 2010). The *sea* gene is carried by a polymorphic family of temperate bacteriophages inserted in the bacterial genome, which usually behave as prophages (Betley and Mekalanos 1985). It has been demonstrated that the transcription of *sea* is tied to the life-cycle of the encoding prophage (Cao et al. 2012) and is inducible by bacterial stress (Zeaki et al. 2015b). Six different *sea*-carrying prophages ( $\phi$ 252B,  $\phi$ Mu3,  $\phi$ Mu50A,  $\phi$ NM3,  $\phi$ Sa3ms and  $\phi$ Sa3mw) have been identified and they all often possess the genes for enterotoxin A, staphylokinase and the complement inhibitor (Goerke et al. 2009). The different prophages lead to different levels of SEA production (Borst and Betley 1994). The analysis of the nucleotide sequence and of the neighbouring regions has indicated that SEA-producing strains can be divided into two major groups, SEA<sub>1</sub> and SEA<sub>2</sub> (Wallin-Carlquist et al. 2010) and that there are two promoter regions (P<sub>1</sub> and P<sub>2</sub>). P<sub>1</sub> is found immediately upstream of *sea* in both groups (Borst and Betley 1994), whereas the phage-related, latent promoter P<sub>2</sub> appears to be linked to a stress-induced boost in SEA production (Sumby and Waldor 2003). The *see* gene is, on the contrary, situated on a defective prophage and its expression does not appear to be linked to bacterial growth (Derzelle et al. 2009).

Despite the different genetic backbones, the production of the three non-phage-associated, classic enterotoxins (SEB, SEC and SED) usually occurs between the exponential and the stationary phase of bacterial growth (Zhang and Stewart 2000) and is regulated by the accessory gene regulator (Agr) system (Betley and Mekalanos 1985; Regassa et al. 1991; Derzelle et al. 2009). The *seb* gene is found on the SaPI3 pathogenicity island of *S. aureus* (Novick et al. 2001), whereas *sec* exists in multiple variants (C1, C3, Cbov), found on different pathogenicity islands (SaPI4, SaPI<sub>n1/m1</sub> and SaPI<sub>bov</sub>, respectively; Novick 2003). The *sed* gene, on the other hand, is situated on a 27.6 kb penicillinase plasmid (pIB485; Bayles and landolo 1989). The Agr system is a quorum sensing system, activated at high cell densities, and consists of two different transcripts: RNAII, which encodes for the

structural genes *agrA*, *agrB*, *agrC* and *agrD*, and RNAIII, a regulatory RNA. These two transcriptional units are driven by the promoters P<sub>2</sub> and P<sub>3</sub>, respectively, and are transcribed in opposite directions (Novick et al. 1995). *agrD* contains the sequence of the autoinducing peptide (AIP), which is transported out of the cell by *agrB*, a membrane-associated export protein (Kavanaugh et al. 2007). AIP is the ligand of *agrC*, a membrane-bound, histidine kinase, which leads to the phosphorylation of *agrA*. The activated form of *agrA* up-regulates the promoters P<sub>2</sub> and P<sub>3</sub>, leading to the generation of more RNAII and RNAIII, which is the intracellular effector of gene regulation in *S. aureus*, resulting in a positive feedback loop (Koenig et al. 2004). The expression of *agr* is modulated by many trans-activating modulators, e.g. the Sar family of proteins,  $\sigma$ B, SrrAB (Staphylococcal respiratory response AB) and Rot (repressor of toxins, a member of the Sar family; Pragman et al. 2004). When the Agr system is induced, RNAIII mediates the translational repression of *rot* (Boisset et al. 2007). Importantly, all these regulatory elements respond to various environmental stresses and stimuli, e.g. high temperature, alkaline pH, high salinity, catabolites, anaerobic and hypoxic conditions (Mashruwala and Boyd 2017). All these mechanisms lead to an increased transcription of secreted virulence factors, such as enterotoxins, and to a reduced transcription of a set of genes encoding for cell wall proteins (Dunman et al. 2001). It must be noted that SEB, SEC and SED are only partially regulated by the Agr system and can also be produced independently (Yarwood and Schlievert 2003).

### Regulation of non-classic enterotoxins

Our knowledge of the regulation of non-classic enterotoxins is only beginning to emerge. It appears that, unlike the majority of classic enterotoxins, regulation of the newer enterotoxins is often Agr-independent, with the exception of SEH and SEJ (Derzelle et al. 2009). The production of the enterotoxins encoded in the *ecg* operon (SEG, SEI, SEM, SEN, SEO and SEU) is highest in the initial stages of the exponential growth phase and is dependent on  $\sigma$ B (Kusch et al. 2011). On the other hand, the production of SEH is predominant in the late exponential phase of bacterial growth and appears to be positively regulated by Rot, via direct binding to the *seh* promoter,  $\sigma$ B, several Sar homologs and SaeR (Sato'o et al. 2015). SaeRS also appears to have a positive impact on the production of SEI (Langley et al. 2017).

## Impact of food-related factors on enterotoxin production

SFP is usually associated with the consumption of protein-rich, manipulated foods, e.g. meat preparations, creams and dairy products (De Buyser et al. 2001) that are vehicles of amino acids and low-molecular-weight peptides that support the survival and growth of *S. aureus* (Peles et al. 2007). All these products possess highly complex matrices in terms of microbial content, storage temperature, pH, salt, oxygen and nutrient availability compared with broth (Valero et al. 2009). *S. aureus* survivability and growth in every environment are linked to the presence of several nutrients (e.g. branched-chain amino acids; Grispoldi et al. 2019a). In foods, *S. aureus* often forms biofilm, where molecular signalling and communication between cells are highly relevant compared to the planktonic state found in laboratory culture broths. All these parameters can influence enterotoxin production by *S. aureus* in foods.

Temperature appears to affect both prophage-related and non-prophage-related enterotoxin synthesis more than bacterial growth in milk, ham and egg products (Schelin et al. 2011). It has been demonstrated that the effect of temperature on SEA production can vary, depending on the prophage variant present. In general, SEA production in foods was detected between 10 °C and 45 °C, with an increase of toxin production linked to an increase in temperature (Tsutsuura et al. 2013).

*S. aureus* appears to have a higher tolerance to pH variation under aerobic compared to anaerobic growth conditions. The pH range for SEs production in foods is 5–9.6, with an optimum between 7 and 8. Lactic acid appears to inhibit the formation of Agr-dependent toxins (SEB, SEC and SED) in particular (Schelin et al. 2011). It has been demonstrated that treating pork sausage with lactic acid solution (1–2%) can reduce SEA formation (Zeaki et al. 2014), whereas the use of non-dissociated lactic acid was reported to increase SEA production in Brain Heart Infusion broth (Rosengren et al. 2013). One study has demonstrated that mild, acetic acid stress (pH 5.5–7) can lead to prophage induction and a consequent increase of *sea* expression in ham products, whereas no or very low SEA was detected at lower pH levels (4.5–5; Wallin-Carlquist et al. 2010). Another study reported little or no effect of sorbic acid (0.15%, pH5) on phage induction (Zeaki et al. 2015a). Sihto et al. (2016) reported that lactic acid stress had no significant effect on *sed* expression.

The lower limit of water activity for the production of enterotoxins in food by *S. aureus* reported in literature is 0.86. SEB and SEC appear to be the more sensitive to this parameter. On the other hand, the upper limit of NaCl concentration is 12% (Schelin et al. 2011). Mild NaCl stress (2%) was able to lead to phage induction and a consequent increase in SEA production in food. Sihto et al. (2017) showed that mild stress conditions, similar to those encountered by *S. aureus* in many foods (NaCl 4.5%, nitrite 150 mg/L, glucose 30%, pH 6), can strongly influence *seb* expression, with glucose and NaCl reducing *seb* promoter activity. The expression of *sec* was also reported to be influenced by glucose and NaCl stress: a 1.2 M concentration of NaCl led to a 16-fold reduction of both *sec* mRNA and SEC protein (Regassa and Betley 1993). Another study demonstrated that NaCl (4.5%) and glucose stress can decrease *sed* expression (Sihto et al. 2015). It has been reported that nitrites (food additives often used as a preservative and colour fixative in cured meat) have little to no inhibitory effect on the growth and enterotoxin production by *S. aureus* at the concentration permitted by food regulations (Grispoldi et al. 2019c).

The production of SEs is optimal under aerobic conditions. However, it has been demonstrated that 10% dissolved oxygen in vacuum-packed ham and sausages can lead to a 10-fold increase in SEB production (Schelin et al. 2011).

The effect of co-culturing of *Lactococcus lactis* and *S. aureus* has been investigated in different environments. A mixed culture with *L. lactis* at a constant pH of 6.6 showed no effect on *S. aureus* growth, but strongly reduced the expression of *sec*. The same study reported a slight increase in *sea* expression during the stationary growth phase. On the other hand, expression of other SEs was impaired (*sel*) or unaffected (*sek*, *seg*, *seh*; Even et al. 2009). Studies have demonstrated the inhibition potential against *S. aureus* growth and SEs production by selected starter cultures (*Lactococcus* spp., *Lactobacillus* spp. and *Enterococcus faecalis*; De-Xian et al. 2020) in Italian dry sausage (Cenci-Goga et al. 2012; Cenci-Goga et al. 2015; Cenci-Goga et al. 2016; Cenci-Goga et al. 2018) and fresh cheeses (Nogueira Viçosa et al. 2019).

## Enterotoxigenic *Staphylococcus aureus* in farm animals

Most of the data available in literature on the prevalence and characterisation of enterotoxins' producing *S. aureus* in farm animals are focussed on milk producing animals and are often associated with the

diagnosis of mastitis. *S. aureus* is a common causative agent of inflammation of the mammary gland, leading to risk of culling or death of the affected animals, increased antimicrobial treatments and severe economic losses for the dairy industries due to decreased milk production and additional costs for medical treatments (Francoz et al. 2017). There are many studies that analyse the presence of SEs' producing *S. aureus* in milk samples from mastitic cows, ewes and goats around the world. Among the most recent ones, De-Xian et al. (2020) reported the presence of *S. aureus* in 12% of the milk samples (56/462) obtained from clinical and subclinical mastitic cows in the Lianoning province of China. Twenty-three out of 56 isolates (41%) were found to have one or more genes that encode for enterotoxins. Isolates with *seg* (27%) were the most prevalent followed by those with *sea* (16%), *seb* (7%) and *sec* (7%). Algammal et al. (2020) reported a prevalence in Ismailia province (Egypt) of *S. aureus* in milk samples from individual quarters of 36% (53/146) in cattle and 35% (31/88) in buffaloes. Thirty-six % of the isolates were enterotoxigenic, with 27% of them positive for *sea* gene, 7% positive for *sec* gene and none positive for *seb* and *sed* genes. Fursova et al. (2020) investigated 21 *S. aureus* isolates from bovine mastitis in seven regions of Western Russia using whole-genome sequence analysis. None of the investigated strains contained genes encoding the classic enterotoxins from *sea* to *see*. However, 38% of the isolates were classified as toxinogenic by enterotoxins *seg* and *sei*. Genes encoding the SE-like proteins (*sel*), namely, *selm*, *seln*, *selo* and *selv*, were also identified in this group. Grispoli et al. (2019b) analysed *S. aureus* isolates from 12 cattle dairy farms in central Italy. The isolates presented a prevalence ranging from 47% for *see* to 6% for *seb* and *sec*. Khemiri et al. (2019) reported the presence of *S. aureus* in 47 out of 150 bovine milk samples and in 9 out of 100 ovine milk samples from nine small family farms distributed in five regions in Tunisia. Sixteen enterotoxin-encoding genes were detected, with a prevalence ranging from 47% for *sed* to 2% for *ser* and *seip*.

Less data is available about enterotoxins producing *S. aureus* isolated from non-milk samples. Zhou et al. (2017) reported that of 74 nasal swabs of goats collected from 10 herds in the Chongqing municipality (China), 43% were positive for *S. aureus*. Enterotoxin genes were detected in the majority (53%) of the strains, and *sej* gene was found in 22% of them, followed by *seb*, *sec* and *see* in 19% isolates each, *sei* (9%) and *seg* (3%). In a study published by (Zhang et al. 2018), 130 *S. aureus* isolates from samples

associated with pork production were tested for the presence of 18 SEs genes. Ninety-five percent of isolates from different stages of pork meat production harboured one or more SEs genes forming 37 different enterotoxin gene profiles. *Seb* was present in 60% of the isolates, the highest among the genes tested. The genes *sed*, *sej*, *seo*, *sep*, *ser* and *seu* were not found. The five classical SEs genes had lower prevalence than the *egc* gene cluster. Velasco et al. (2018) analysed *S. aureus* isolated from the pork chain supply in Chile. Samples from living animals were taken from the nostrils and the skin. The overall prevalence of *S. aureus* was 34%. A higher prevalence was detected on carcasses (57%), in pigs sampled at farms (41%) than in pigs sampled at slaughterhouses (23%) and in non-packaged retail meat (43%), than packaged retail meat (5%). The presence of SEA, SEB, SEC and SED was determined by agglutination test. Only one strain isolated from a meat sample was positive for SEB.

The wide variety of data that can be observed in different studies reflects the differences in the *S. aureus* populations around the world. These differences must be taken into account during the development of SFP risk assessment models because the production of SEs can vary significantly.

### Prevalence and detection of SEs in food matrices

The most commonly reported food categories involved in SFP outbreaks in the EU are mixed food (29.7%), meat and meat products (20.8%), cheese and dairy products (14.4%), bakery products (8.4%) and fish and fish products (6.5%; Nia et al. 2016). Even though EC Regulation 2073/2005 defines a criterion for SEs only for cheese and dairy products, this data highlights the involvement of other food categories, especially mixed food and meat products. The official method in the EU to detect SE types SEA to SEE in all categories of food is the European Screening Method (ESM) developed by the European Union Reference Laboratory for Coagulase Positive Staphylococci (EURL CPS; Ostyn et al. 2020). ESM is based on an extraction step with dialysis concentration, followed by qualitative detection using either of two validated commercial assays: immunoenzymatic Vidas® SET2 (bioMérieux, Marcy l'étoile, France) and/or RIDASCREEN® SET Total (R biopharma, Darmstadt, Germany; Hennekinne et al. 2007; Ostyn et al. 2011). The EURL CPS annually organises Inter-Laboratory Proficiency Testing Trials (ILPT) to evaluate the competence of the European Countries' National Reference

Laboratories to analyse SE content in food matrices. Eight food matrices were used for ILPT over the period 2013–2015, including cheese, freeze-dried cheese, tuna, mackerel, roasted chicken, ready-to-eat food, milk and pastry. Food samples were spiked with four SE types (SEA, SEC, SED and SEE) at various concentrations. The analysis of results obtained by participants for a total of 155 blank and 620 contaminated samples indicated sensitivity (>98%) and specificity (100%) of ESM (Nia et al. 2016). These immunological detection methods of the five classic SEs have demonstrated that SEA is the major contributor (almost 80%) to SFP outbreaks, followed by SED, SEB, SEC and SEE (Hu and Nakane 2014; Denayer et al. 2017; Grispoldi et al. 2019b).

There are no immunological detection kits available in commerce for newer SEs, even though a wide variety of diagnostic methods have been developed in order to understand their potential role in SFP. To date, molecular analysis to detect SE encoding genes remains the most common method used: in the last two decades, many studies have reported the presence of non-classic, enterotoxin encoding genes (in particular *egc*-encoded genes) in SFP outbreaks around the world (Blaiotta et al. 2004; Grumann et al. 2008; Yan et al. 2012; Viçosa et al. 2013; Chao et al. 2015; Johler et al. 2015; Cheng et al. 2016; Song et al. 2016; Shen et al. 2017; Umeda et al. 2017). In addition, an increasing number of immunological-assays has been presented in literature for SEG (Nagaraj et al. 2016), SEH (Su and Wong 1996), SEI (Zhao et al. 2016), SEK (Aguilar et al. 2014), SEM (Zhao et al. 2017) and SEQ (Hu et al. 2017). All this data indicates that multiple SEs are most likely present and can contribute to each SFP outbreak. Some authors have suggested that the expansion of the existing multiple assays would be the most efficient strategy to detect all SEs. However, some concerns regarding the high risk of cross-reaction have been raised and vigorous testing is required to develop this idea (Liang et al. 2015; Adhikari et al. 2016; Nia et al. 2016).

### Predictive microbiology and risk assessment models

Given the wide variety of SEs and food-related factors that can influence SE production, it is difficult to estimate human exposure to the hazard and evaluate the safety of a food production process. Predictive, microbiological models, if validated in the food under examination, are precious tools to estimate the growth and production of enterotoxins by *S. aureus* (Castillejo-

Rodriguez et al. 2002; Ross and McMeekin 2003). In literature, kinetic models of *S. aureus* response to food-related factors can be found. These models predict growth and lag time on the basis of various input values, e.g. temperature, aerobic/anaerobic conditions, water activity, pH and nitrite concentration. They can be built by using free software, including the growth model in the ComBase modelling toolbox and the growth and survival models in the US FDA pathogen modelling programme (PMP; McMeekin et al. 2006). In addition, many growth and inactivation curve studies are available online. This data can be useful to design food processes to prevent the growth and production of SEs (Obeso et al. 2010; Grispoldi et al. 2019c). The main challenges in the development of predictive models are that food contamination is often incidental with a difference of just a few cells, together with a highly variable strain level. Some studies have pointed out highly variable behaviour of *S. aureus* in foods at low levels of contamination and at single cell level. These differences were masked when studies were performed at population level (Vora et al. 2003; Lindqvist 2006). This emphasises the usefulness of probabilistic approaches to predictive modelling, using distributions of parameters instead of single, fixed values. On the other hand, there is a lack of predictive models of the kinetics of different SE production and expression of enterotoxin genes in foods. Most of the models that can be found in literature are fitted to the formula by Baranyi and Roberts (1994), which developed a dynamic approach for the prediction of bacterial growth in food, and to the modified equation of Gompertz (Gibson et al. 1987). A study by Fujikawa and Morozumi (2006) described an extended logistic model shown by the following equation:

$$dN/dt = rN\{1 - (N/N_{\max})m\}\{1 - (N_{\min}/N)n\}$$

where  $N$  is the bacterial population (CFU/g) at time  $t$  (h),  $r$  is the rate constant of growth (1/h),  $N_{\max}$  is the maximum population (CFU/g) and  $N_{\min}$  the initial populations (CFU/g). Parameters  $m$  and  $n$  (>0) are related to the curvatures of the deceleration and lag phase of growth, respectively. This model was shown to describe microbial growth with performance very similar to the Baranyi model. The model validation was performed with root mean square error (RMSE). They described a linear increase of SEA production in sterile milk between 15 °C and 32 °C with the following equation:

$$p = 0.0376xt - 0.559$$

where  $p$  is the amount of SEA (ng/ml h) and  $t$  is the temperature (°C). Higher levels of SEA were produced

above 32 °C, although correlation was no longer present. The same author (Fujikawa 2021) applied the previously described equation to predict the detection time of SE A formed in hydrated batter mix. The results of this study suggested that the kinetic model could well predict the SEA production from the temperature history.

A study by (Soejima et al. 2007) developed the following equation to describe the correlation between the toxin production (SEA) and the cell count in milk:

$$\text{Tox} = 0.9300751 \times C - 6.662092$$

where Tox is the toxin production (log ng/ml) and C is the number of cells (log cfu/ml).

Castillejo-Rodriguez et al. (2002) analysed the growth of *S. aureus* in commercially available vacuum-packaged cooked ham, turkey breast meat and chicken breast meat at different temperatures (2.3 to 17.7 °C). Growth rates observed were compared with those predicted on the basis of various growth models found in literature by using the mean square error (MSE), the bias factor ( $B_f$ ) and the accuracy factor ( $A_f$ ). Their results indicate that only two models (Dengremont and Membrè model) accurately reflected *S. aureus* behaviour while the others overestimated the growth.

Mansur et al. (2016) developed a predictive model for *S. aureus* growth in raw pork, ham and sausage at isothermal conditions of different temperatures (10–40 °C). Data obtained were fitted to the Baranyi and Roberts model (Baranyi and Roberts 1994) and the Ratkowsky's square root model (Ratkowsky et al. 1983) was then used to describe the *S. aureus* maximum growth rate ( $\mu_{\max}$ , log CFU/h) as a function of temperature with the following equation:

$$\sqrt{\mu_{\max}} = c \times (T - T_0)$$

where  $c$  is the regression coefficient,  $T$  is the temperature (°C) and  $T_0$  the theoretical minimum temperature for bacterial growth. The model was then compared with the ComBase predictor. Results showed that this model seems suitable for the prediction.

Gunvig et al. (2018) developed a dynamic mathematical model to predict formation of SE during heating and fermentation of meat products. A total of 78 experiments were carried out in a meat model system, covering a range of different temperatures (10–40 °C), pH (4.6–6.0), water phase salt (WPS; 2.2–5.6%) and sodium nitrate concentrations (0–150 ppm). The system was inoculated with a cocktail consistent of three different SEs producing strains at a concentration of approximately  $10^3$  CFU/g. The SEs production and growth models were validated on separate data sets

including both static and dynamic conditions. According to authors, the model is dependable and the prediction errors are comparable to laboratory reproducibility. The models are available at [www.dmri-predict.dk](http://www.dmri-predict.dk).

Hazard identification in risk assessments varies from *S. aureus* in general to enterotoxigenic strains and/or enterotoxin. Poor personal hygiene, incorrect food handling practices and inadequate refrigeration of foods are identified as the main factors contributing to SFP. Although it is well known that temperatures below 7 °C and 10 °C can reduce the risk of *S. aureus* growth and production of enterotoxin, respectively, many foods are stored at higher temperatures for sensory reasons, and many processes include steps under growth permissive conditions (Schelin et al. 2011).

The hazard characterisation for a toxigenic micro-organism should focus on dose-response relations and threshold models (Buchanan et al. 2000; Schelin et al. 2011). Due to lack of knowledge, this step for *S. aureus* is often based on food poisoning symptoms and cell counts in foods used as a proxy for potentially hazardous doses of enterotoxins. For example, (Lindqvist et al. 2002) expressed the threshold level as the number of *S. aureus* cells between 5 and 8 log CFU per g. On the basis of outbreak data, threshold levels of 20 ng (Kim et al. 2009), 94 ng (Heidinger et al. 2009) and 100 ng (Soejima et al. 2007) per serving were used.

Exposure assessment based on measurements of SEs in food has been reported. However, it relies basically on data of *S. aureus* prevalence, initial level of contamination, growth and inactivation due to cooking or processing the food (Schelin et al. 2011). On the other hand, consumer handling and consumption or the exposure of susceptible groups of population have rarely been described.

The main limitation of the risk characterisation step is linked to the complexity of the dynamics that occur between bacterial growth, gene expression and enterotoxin production in foods. As also expressed in this manuscript, the potential uncoupling between cell count and the level of enterotoxin produced should be taken in account.

## Conclusion

SFP is a major public health concern worldwide. Consumers' increasing demand for ready-to-eat foods, together with the high stability and resistance of SEs to the majority of the conditions that inactivate the vegetative form of *S. aureus* and its wide distribution

in animals and humans make SFP a serious threat. The traditional approach is to consider SE production correlated with bacterial growth (more cells equal more toxin), and the colony count is the parameter considered to determine whether or not a food product is safe for human consumption. However, explanations have been given of the way in which different parameters found in foods can influence *S. aureus* survivability, growth and enterotoxin production and the extent to which the behaviour of these bacteria in actual food environments can differ from what can be observed in liquid cultures. In the light of these discoveries, the importance of developing new risk assessment models for *S. aureus* based on real foods in order to improve consumer safety and food product quality is clear.

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## Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

## Author contributions

LG: Writing – original draft; AA: Writing- Reviewing and Editing; BC-G: Conceptualisation, Writing- Reviewing and Editing, Supervision, Project administration, Funding acquisition.

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