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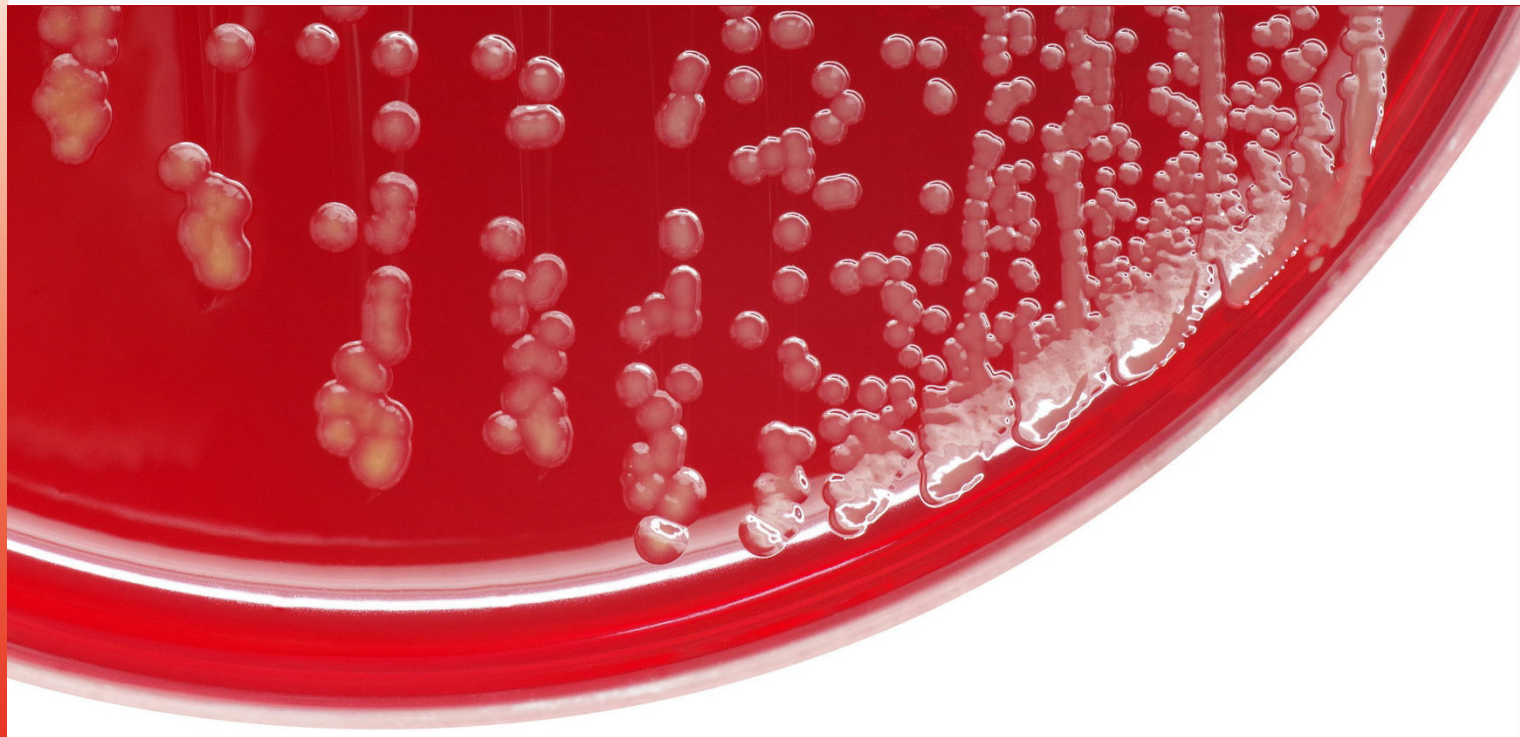

Staphylococcus aureus and its Enterotoxin: a Review

Authors

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Summary

Beside the infection in human beings and animals, *Staphylococcus aureus* can produce different enterotoxins in food. These enterotoxins can cause vomiting and diarrhea, often involving many people and causing so called staphylococcal foodborne outbreaks (SFPO). To date a lot of SFPO are of weak evidence. One reason for this could be, that currently only five, so called classical enterotoxins, out of 26 known staphylococcal enterotoxins can be analyzed using commercially available kits. A subset of the non-classical enterotoxins (s*eg, sei, sem, sen, seo and seu*) is located on the same enterotoxin gene cluster (*egc*) which is part of the *S. aureus* genomic island *v*Saβ. These enterotoxins have been described as likely being involved in SFPOs.

New tools such as whole genome sequencing and new methods for enterotoxin measurement are needed in order to be able to analyse the *S. aureus* strains and their enterotoxins and, consequently, being able to predict the enterotoxin production based on genome data. This was the main aim of this work.

In order to do so, the *egc* enterotoxins were studied at genome (*seg, sei, sem, sen, seo, and seu*), at mRNA (*seg, sei, sem, sen, seo*), and at protein level (SEG and SEI).

We demonstrate that the *in vitro* production of SEG and SEI can be predicted based on the *v*Saβ-type and the clonal complex (CC) of a strain. Furthermore, the *v*Saβ-type/CC enables the prediction of the source of an *egc*-positive SFPO strain (animal or human derived).

Enterotoxin production (SEG and SEI) under stress conditions was also tested, showing that common salt concentrations and temperature stress, mimicking conditions found in food production, seem not to affect the production of *egc* enterotoxins SEG and SEI.

With the present work a contribution can be given to a better understanding of the involvement of *egc* enterotoxins producing *S. aureus* strain in SFPOs. In addition, the results also give a first insight into the conditions and limits of *egc* enterotoxin production and a first indication in how they have the ability to be produced in food production and storage.

Zusammenfassung

Neben Infektionen bei Mensch und Tier kann *Staphylococcus aureus* in Lebensmitteln auch verschiedene Enterotoxine produzieren. Diese Enterotoxine können Erbrechen und Durchfall verursachen. Sind mehrere Personen betroffen, kommt es zu sogenannten Staphylococcal Food Poisoning Outbreaks (SFPO). Aus verschiedenen Gründen sind viele dieser SFPOs nur schwach belegt. Ein Grund dafür ist, dass von den 26 bekannten Enterotoxinen derzeit nur die fünf klassischen mit kommerziell erhältlichen Kits analysiert werden können. Eine Untergruppe der nicht-klassischen Enterotoxine (*seg, sei, sem, sen, seo und seu*) werden durch denselben enterotoxin gene cluster (egc) codiert, welcher ein Teil der *S. aureus*-Genominsel vSaβ ist. Es wird angenommen, dass diese Enterotoxine an SFPOs beteiligt sind.

Aus diesem Grund werden neue Methoden zur Voll-Genom-Sequenzierung und Enterotoxin-Messung benötigt, damit die *S. aureus*-Stämme und ihre Enterotoxine besser analysiert und darauf aufbauend die Enterotoxin-Produktion auf Basis von Genomdaten vorhergesagt werden kann. Dies ist das Hauptziel der vorliegenden Studie.

Um dieses Ziel zu erreichen, wurden die egc-Enterotoxine auf Genom-, mRNA- und Proteinebene (SEG und SEI) untersucht.

Dabei konnten wir zeigen, dass die in vitro-Produktion von SEG und SEI basierend auf dem vSaβ-Typ und dem clonal complex (CC) eines Stammes vorhergesagt werden kann. Darüber hinaus ermöglicht das Wissen um den vSaβ-Typ/CC die Vorhersage, ob ein egc-positiver SFPO-Stamm, tierischen oder menschlichen Ursprungs ist.

Die Enterotoxinproduktion (SEG und SEI) wurde unter Stressbedingungen getestet und es zeigte sich, dass eine erhöhte Salz-Konzentration (100 g/L) oder Temperatur (45 °C), die Sekretion der egc-Enterotoxine SEG und SEI nicht zu beeinflussen vermag.

Mit der vorliegenden Studie wird ein Beitrag zum besseren Verständnis der Beteiligung von egc-Enterotoxin-produzierenden *S. aureus*-Stämmen an SFPOs geleistet. Darüber hinaus ermöglichen die Ergebnisse auch einen ersten Einblick in die Bedingungen und Grenzen der egc-Enterotoxin-Produktion. Die Resultate weisen darauf hin, dass die Toxine von egc-Enterotoxin-produzierenden *S. aureus*-Stämmen in der Lebensmittelproduktion, trotz bestehender Hygienemassnahmen produziert werden können.

Résumé

Outre les infections qu'il peut causer chez l'homme et les animaux, le *Staphylococcus aureus* peut produire différentes entérotoxines dans les aliments. Ces entérotoxines peuvent provoquer des vomissements et des diarrhées, qui touchent souvent de nombreuses personnes et provoquent ce que l'on appelle des toxi-infections collectives (TIAC) à staphylocoques. À ce jour, de nombreux cas de TIAC sont peu patents, ceci pour de nombreuses raisons. L'une d'elles pourrait être qu'actuellement, seulement cinq entérotoxines, dites classiques, sur les 26 entérotoxines staphylococciques connues, peuvent être analysées à l'aide de kits disponibles dans le commerce. Une série d'entérotoxines non classiques (s*eg, sei, sem, sei, seo et seu*) est située sur le même cluster (enterotoxin gene cluster, egc) qui fait partie de l'îlot génomique vSaβ de *S. aureus*. Ces entérotoxines ont été décrites comme pouvant être impliquées dans des toxi-infections alimentaires collectives à staphylocoques.

C'est pourquoi de nouveaux outils, tels que le séquençage du génome complet (WGS) et de nouvelles méthodes de mesure des entérotoxines, sont nécessaires afin de pouvoir isoler et caractériser les souches de *S. aureus* et leurs entérotoxines et, par conséquent, de pouvoir prédire la production d'entérotoxines sur la base des données du génome. C'était le principal objectif de cette étude.

Afin d'y parvenir, les entérotoxines egc ont été étudiées au niveau du génome (*seg, sei, sem, sen, seo* et *seu*), au niveau de l'expression de l'ARNm (*seg, sei, sem, sen, seo*) et au niveau des protéines (SEG et SEI). Avec les résultats, nous démontrons que la production in vitro de SEG et de SEI peut être prédite sur la base du type de vSaβ et du clonal complex (CC) d'une souche. De plus, le type de vSaβ/CC permet de prédire la source d'une souche TIAC positive à l'egc (d'origine animale ou humaine).

La production d'entérotoxines (SEG et SEI) dans des conditions de stress a également été testée, montrant que les stress liés au NaCl et à la température, imitant les conditions rencontrées dans la production alimentaire, ne semblent pas affecter la production d'entérotoxines egc, SEG et SEI.

La présente étude peut contribuer à mieux comprendre l'implication des souches de *S. aureus* produisant les énterotoxine egc dans les toxi-infections alimentaires collectives à staphylocoques. En outre, les résultats donnent également un premier aperçu des conditions et des limites de la production d'entérotoxines egc, ainsi qu'une première indication sur leur capacité à être produites dans la fabrication et le stockage des aliments.

1 Introduction

Staphylococcus aureus is a widespread bacteria, present in different habitats like the human skin or animal ingestion. Due to contamination of raw material or/and through processing of food, the bacteria enters the food chain. Once there, under the right conditions (temperature, salt concentration, etc.), *S. aureus* can grow and often it produces enterotoxins. These enterotoxins are heat stable and whenever in a further processing step the food matrix is heated up, the bacteria will die but the enterotoxin will remain. In consequence by eating the food there is also an intake of enterotoxins. This is also the case in cheese production where the contamination happens mainly due to cow's mastitis. Before and in the first steps of cheese production *S. aureus* can grow and produce enterotoxins that will remain in cheese matrix.

Because these enterotoxins are superantigen and also have emetic activity, eating these enterotoxins causes vomiting and diarrhea. According to the European food safety authority in 2018 there were 114 foodborne outbreaks caused by *S. aureus*. Food involved seems mainly be mixed food, milk and meat products. 77 out of the 114 outbreaks are classified as weak evidence outbreaks, meaning that in these cases no causative agent could be detected in the food vehicle (EFSA, 2014). A reason for the high percentage of weak evidence outbreaks could be the fact that beside the so-called classical enterotoxins (SEA-SEE) which can be detected using commercially available kits, also non-classical enterotoxins are presumed to be involved. For these one there are no commercial kits available for their detection.

2 *Staphylococcus aureus*

2.1 Characteristics and epidemiology of *S. aureus*

In 1881 *Staphylococcus* was discovered by Sir Alexander Ogston describing staphylococcal disease and its role in sepsis (Ogston, 1882). In 1884, a German scientist (Friedrich Julius Rosenbach) was able to distinguish *Staphylococcus aureus* from *Staphylococcus albus* colonies, because of their different colours, *S. albus* was later renamed *S. epidermis* (Licitra, 2013).

S. aureus is member of the Micrococcaceae family and on microscopical examination, the organisms appear as gram-positive cocci in clusters. *S. aureus* can be distinguished from other staphylococcal species because of the gold pigmentation of colonies and positive results of coagulase, mannitol-fermentation, and deoxyribonuclease tests (Somerville and Proctor, 2009).

S. aureus is considered as an ubiquitous opportunistic and very versatile pathogen (Coelho et al., 2011; Feng et al., 2008). The microorganism is able to survive in hostile environments, colonizing the skin and mucous membranes, and causing various infections in humans and animals (Coelho et al., 2011; Malachowa and DeLeo, 2010). *S. aureus* is facultative anaerobe, and it can grow in a wide range of environmental conditions (Table 1). Doubling time in optimal laboratory condition has been described between 24 and 30 min and *in vivo* 60 min (Domingue et al., 1996).

Table 1: Factor accepting growth of *Staphylococcus aureus* (Hennekinne et al., 2012; Tatini, 1973)

	Optimum	**Range**
Temperature [°C]	37	7-48
pH	6-7	4-10
Water activity [a_w]	0.98	0.83 → 0.99*
NaCl [%]	0	0-20
Redox potential [E_h]	> +200mV	< -200 mV to > +200 mV
Atmosphere	Aerobic	Anaerobic-aerobic

Because of the adaptive capacity of *S. aureus* the species has demonstrated his success as a pathogen (Liu, 2015). It can lead to suppurative skin and soft tissue infections, which can be superficial (e.g. impetigo, furuncles), deep (e.g. abscess, osteomyelitis), systemic (bacteraemia), or foreign-body-related (Foster and Geoghegan, 2015). Further diseases caused by *S. aureus* include respiratory diseases (e.g. pneumonia) and toxin-related diseases such as food poisoning, the toxic shock syndrome, and the scalded skin syndrome (Foster and Geoghegan, 2015).

Humans are a natural reservoir of *S. aureus*. 30 to 50 % of healthy adults are colonized with this microorganism (Chen, 1986; Noble et al., 1967). Out of them 10-20 % are persistently colonized with methicillin-sensitive and methicillin-resistant isolate (Chen, 1986; Sanford et al., 1994). Persons colonized with *S. aureus* are at increased risk for subsequent infection, especially patients with diabetes, intravenous drug users and patients undergoing haemodialysis, surgical patients and patients with the acquired immunodeficiency have an increased risk of staphylococcal disease (Wenzel and Perl, 1995).

Persons that are colonized with *S. aureus* strains are at increased risk of becoming infected with these strains. Most cases of nosocomial infection are acquired through exposure to the hands of health care workers after they have been transiently colonized with staphylococci from their own reservoir or from contact with an infected patient. Outbreaks may also result from exposure to a single long-term carrier or environmental sources, but these modes of transmission are less common (Casewell and Hill, 1986; Lowy, 1998).

In dairy cows, *S. aureus* represents one of the most prevalent mastitis pathogens worldwide (Artursson et al., 2016). This microorganism is able to produce a variety of toxins and virulence factors, which contribute to the establishment of a new infection, including colonization of the mammary gland, bacterial invasion of host cells, evasion of the host immune responses, virulence, and pathogenicity (Artursson et al., 2016; Coelho et al., 2011).

2.2 *S. aureus* genome sequencing

The first genomes of S. aureus Mu50 and N315 were the first to be sequenced by (Kuroda et al., 2001). Since then a lot of genomic analysis were published about the characterisation of this species (Baba et al., 2008; Baba et al., 2002; Feng et al., 2008; Malachowa and DeLeo, 2010) (Figure 1).

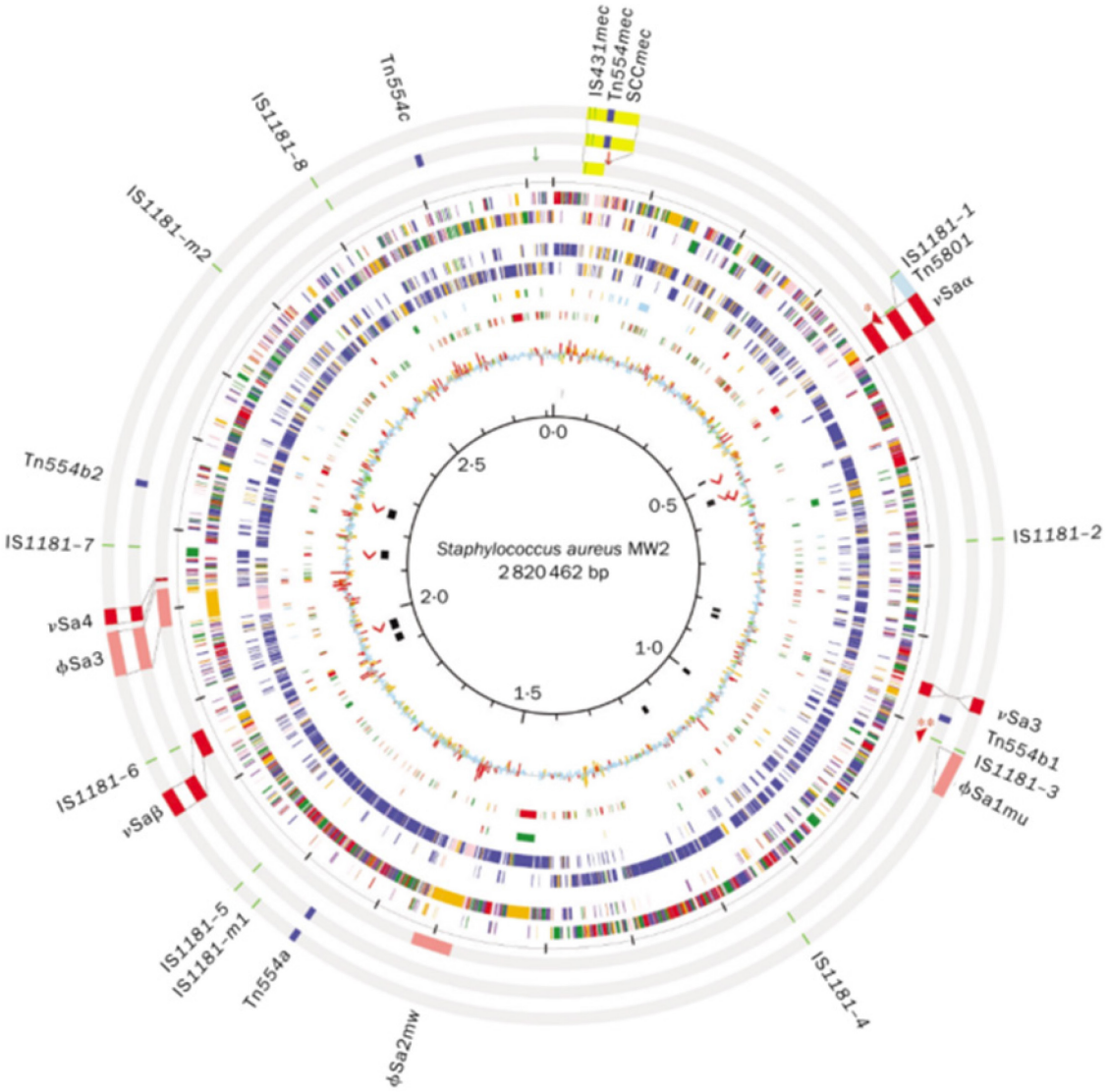

Figure 1: From the outside inward: first three circles show distribution of genomic islands (GIs) (yellow=SCCmec; red=islands; pink=prophages), transposons (light blue=Tn5801; dark blue=Tn554), and insertion sequences (green) in the chromosomes of Mu50 (first circle), N315 (second circle), and MW2 (third circle). Red arrowheads indicate the attachment sites for GI families (*) and Sa1 (**). Third circle shows location of virulence genes cna (green arrow) and seh (red arrow). Fourth circle shows every 100th open reading frame (orf). Fifth and sixth circles show orfs on plus and minus strand, respectively. Blue=cell envelope and cellular processes; green=intermediary metabolism; orange=information pathways; yellow=other functions; magenta=similar to unknown proteins; pink=no similarity. Seventh and eighth circles show taxonomic distribution of BLAST best-hit entries on the plus and minus strand, respectively. Blue=Bacillus/Clostridium group; green=firmicutes (gram-positive eubacteria); pink=viruses/insertion sequences/transposons; orange=archaea/eubacteria/eukaryota; white=no hit or ribosomal and transfer RNAs. Ninth circle shows virulence-associated orfs. Red=toxins; green=adhesins; orange=exoproteins; blue=others. Tenth circle shows orfs whose BLAST first hit entry is a human pathogen (red=streptococci; orange=Bacillus anthracis and Bacillus cereus; green=others). Eleventh circle shows GC content at the third codon (GC3) and synonymous codon-usage bias of each orf. Green=highly expressed orfs; red=putative alien orfs; orange=possible alien orfs based on GC3 skew; blue=other orfs. Size of the coloured bar=deviation of GC3 value of each orf from the average. Red arrowhead=rRNA and its orientation. Black bars=locations of tRNAs. Twelfth circle shows nucleotide position in Mb. (Baba et al., 2002)

The S. aureus genome is circular and consists of about 2.8 Mb and contains approximately 2,600 genes, and its structure is highly conserved with small variable regions (Foster and Geoghegan, 2015). Genes governing virulence and resistance to antibiotics are found on the chromosome, as well as the on extrachromosomal elements (Novick, 1990). These genes are transferred between staphylococcal strains, species, or other gram-positive bacterial species through the extrachromosomal elements (Schaberg and Zervos, 1986) (Figure 2).

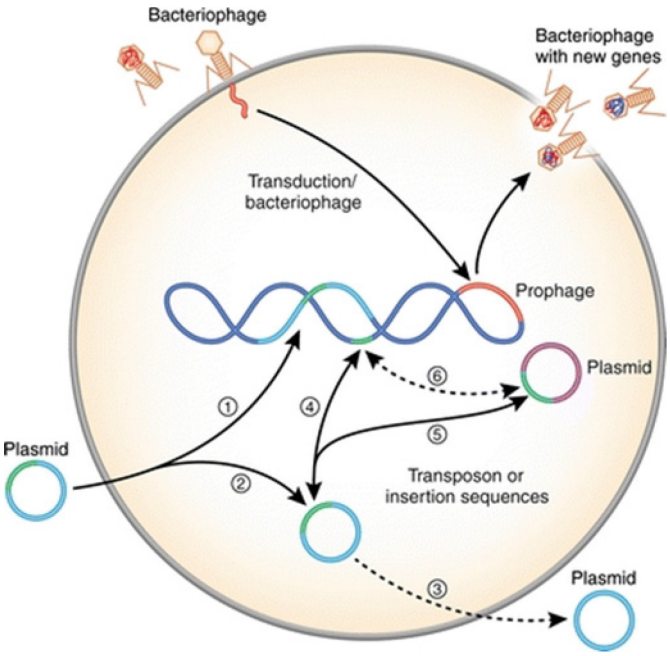

Figure 2: Acquisition of MGEs by S. aureus. 1 Incorporation of plasmids or plasmid elements into genomic DNA. 2 Plasmids can be maintained as free circular DNA. 3 Suicide plasmid. 4 Transfer of a transposon or an insertion sequence between plasmid and genomic DNA. 5 Transfer of a transposon or an insertion sequence between plasmids within the cell. 6 Transfer of a transposon or an insertion sequence from genomic DNA to another plasmid (Malachowa and DeLeo, 2010)

The core genome (genes present in all strains of a species) makes up about 75% of the total S. aureus genome, and besides housekeeping genes encoding for essential growth and survival functions, it also includes genes expressing virulence factors like surface proteins, toxins, and enzymes (Foster and Geoghegan, 2015; Malachowa and DeLeo, 2010). Variation in the core genome mostly results from single nucleotide polymorphisms (SNPs) or from differences in the length of repetitive regions (Foster and Geoghegan, 2015). S. aureus is further characterized by the presence of mobile genetic elements (MGEs), which make up about 25% of the staphylococcal genome (Malachowa and DeLeo, 2010).

2.3 The diversity of MGEs in *S. aureus* genomes

MGEs in *S. aureus* are putative and proven virulence factors with genes responsible for host adaptation and toxins (Baba et al., 2008; Baba et al., 2002; Lindsay and Holden, 2004). Following MGEs are present in *S. aureus* genome (Linear schematic of the USA300 genome (strain FPR3757) and its major MGEs (Malachowa and DeLeo, 2010).(Figure 3): Bacteriophages, Pathogenicity islands, Genomic islands, Plasmids, Transposon, insertation sequence and Staphylococcal cassette chromosome.

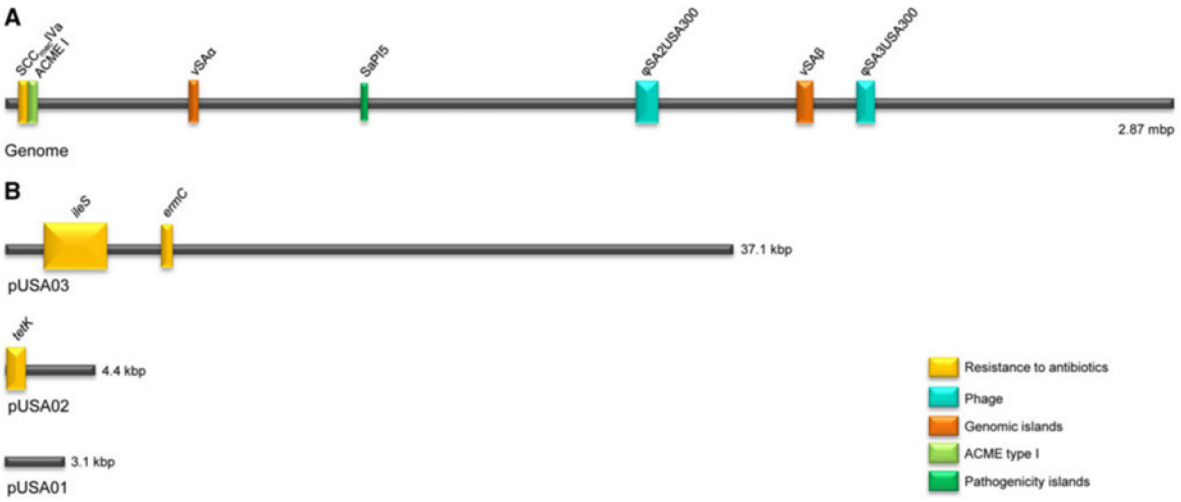

Figure 3: Linear schematic of the USA300 genome (strain FPR3757) and its major MGEs (Malachowa and DeLeo, 2010).

2.3.1 Bacteriophages and virulence

Bacteriophages (phages) or bacterial viruses seem to have an impact on staphylococcal diversity and evolution. Phages are classified into three distinctive groups: lytic, temperate, and chronic (Malachowa and DeLeo, 2010). Lytic phages are members of the Myoviridae family that has been used in phage therapy, because bacteria lyse completely during release of progeny phages. Bacteria infected with chronic phages release progeny into the extracellular environment without killing the host, which allows bacteria to grow and divide (Malachowa and DeLeo, 2010). Temperate phages, which are members of the Siphoviridae family, form the most numerous group among all *S. aureus* phages. Temperate phages have the ability to lyse bacteria after infection, but they typically form a long-term relationship with the host cell, whereby the phage DNA integrates into the staphylococcal genome as a prophage (Goerke et al., 2009; Mann, 2008). Phage can impact expression of virulence determinants by positive or negative lysogenic conversion. With a positive conversion, bacteria express prophage-encoded virulence determinants, for negative conversion there is an insertional inactivation of genes (Coleman et al., 1989; Goerke et al., 2009). Prophage-encoded virulence molecules in *S. aureus* are enterotoxins or Panton–Valentin leucocidin (Malachowa and DeLeo, 2010). Prophage can also work with other MGEs by promoting mobility of some staphylococcal pathogenicity islands or by transferring antibiotic resistance by transduction of plasmids previously incorporated into chromosomal DNA (Fitzgerald et al., 2001; Novick, 2003).

2.3.2 Pathogenicity islands

Staphylococcal pathogenicity islands (SaPIs) are MGEs of 14–17 kb in size (Malachowa and DeLeo, 2010). To date, at least 16 SaPIs have been sequenced and SaPI1 is considered as the prototype (Novick, 2003; Novick and Subedi, 2007). SaPIs form a coherent family with highly conserved core genes (Novick and Subedi, 2007; Ubeda et al., 2003). Core genes include two open reading frames encoding transcriptional regulatory proteins and a region encoding intergrase, Rep protein, and terminase. In addition to core genes, almost all SaPIs encode enterotoxins or toxic shock syndrome toxin (TSST) (Yarwood et al., 2002). SaPIbov2 is an exception to this rule, and instead it contains Bap adhesion protein, which plays a role in bovine chronic mastitis infections (Tormo et al., 2005; Ubeda et al., 2003).

2.3.3 Genomic islands

Three classes of genomic islands exist among the *S. aureus* strains whose genomes have been sequenced (Baba et al., 2002; Diep et al., 2006; Klaui et al., 2019; Lindsay and Holden, 2004). These genomic islands are named: *v*Saα, *v*Saβ, and *v*Saγ. These genomic islands are extremely stable and have highly conserved genes. The gene content is usually highly conserved but can also vary substantially between some strains. (Baba et al., 2008; Baba et al., 2002). A current notion is that genomic islands were once mobile elements acquired by horizontal gene (Waldron and Lindsay, 2006), newly studies suggest that *v*Saβ is a prophage that can still be mobilized (Klaui et al., 2019).

These genomic islands are flanked by a broken transposase gene upstream and partial restriction-modification system (RM) type I downstream. A complete RM type I comprises host specificity determinant genes *hsdR*, *hsdM*, and *hsdS*, but only *hsd*M and *hsdS* are found juxtaposed to the *S. aureus* genomic islands. Both flanking DNA segments contribute to the stability of genomic islands within the *S. aureus* chromosome (Baba et al., 2002; Ito et al., 2003; Waldron and Lindsay, 2006).

About *v*Saα it is known that they harbour a lipoprotein gene cluster (*lpl*) and staphylococcal superantigen-like genes (*ssl*) (Lina et al., 2004) and about *v*Saγ it is known for containing genes encoding b-type phenol-soluble modulins and a cluster of *ssl* genes similar to that present within *v*Saα (Gill et al., 2005).

The genomic island *v*Saβ (Figure 4) is of particular interest, as it carries two genes belonging to the type I staphylococcal restriction-modification system (*hsdM* and *hsdS*) and harbours a number of virulence-associated genes, such as a hyaluronate lyase precursor gene (*hysA*), a lantibiotic gene cluster (bacteriocins of *S. aureus* [*bsa*]), two leukocidin genes (*lukD* and *lukE*), an enterotoxin gene cluster (*egc*), and a cluster of serine protease genes (serine protease like [*spl*] genes) (Baba et al., 2008).

In older study, three different kinds of *v*Saβ were found (Baba et al., 2008). Nowadays with the new opportunities offered by whole genome sequencing a recent study (Klaui et al., 2019) identified 15 new types of *v*Saβ out of 103 clinical *S. aureus strains* (Figure 4). These new insights give a superordinate system to classify *S. aureus* strains based on genomic islands (Klaui et al., 2019).

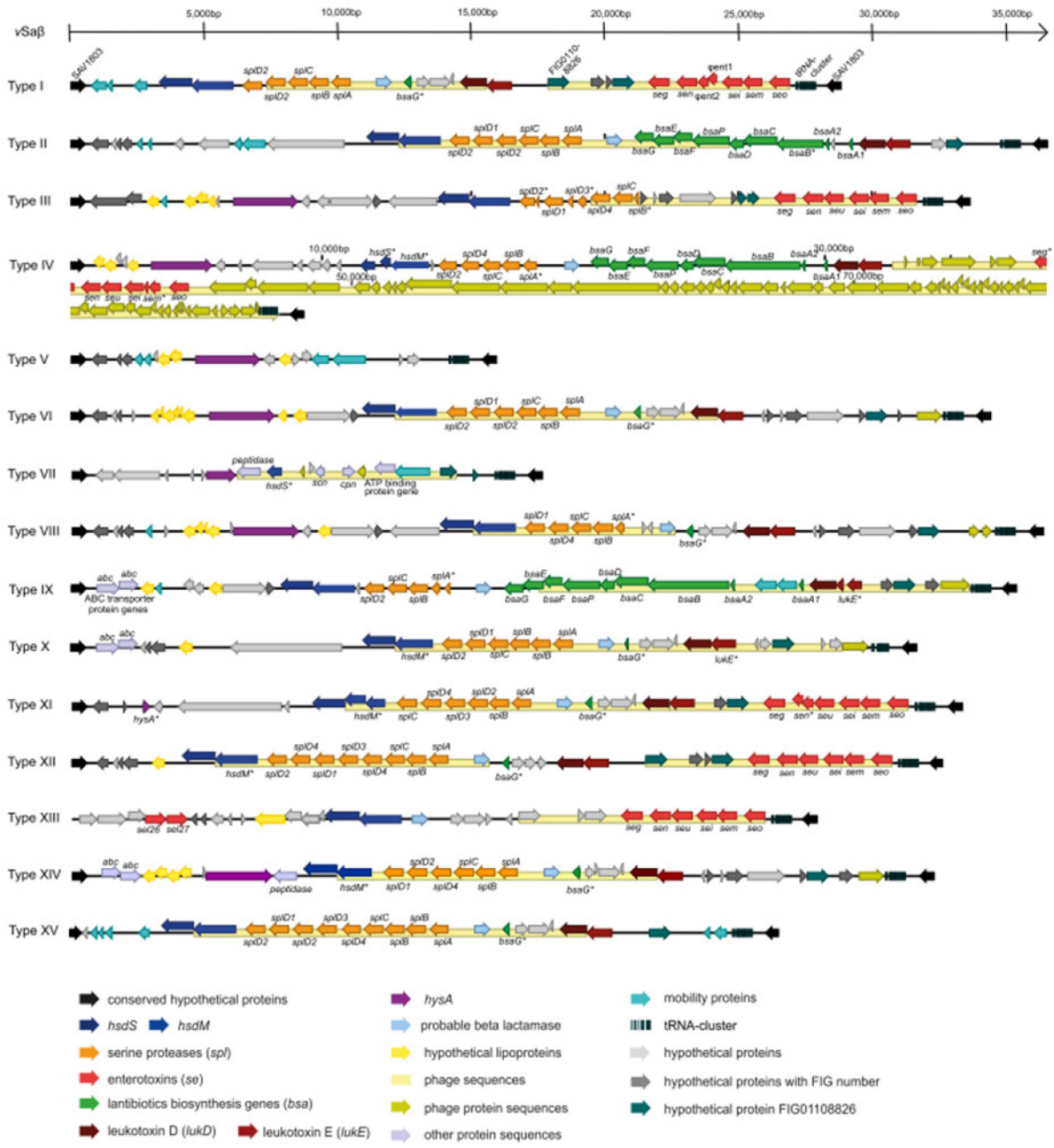

Figure 4: Representation of all Staphylococcus aureus genomic island vSaβ types I to XV, their virulence-associated genes, and other hypothetical genes located on vSaβ. For each vSaβ type, one reference strain is shown. Arrows show orientation of open reading frames. Asterisks indicate truncated or fragmented genes. Note that vSaβ IV is substantially longer due to the presence of a complete phage. All other sequences are scaled relative to each other (Klaui et al., 2019).

2.3.4 Plasmids

Plasmids are auto-replicating DNA molecules. Staphylococci typically carry one or more plasmids per cell and these plasmids have different gene content. Staphylococcal plasmids can be classified into one of the three following groups: (1) small multicopy plasmids that are cryptic or carry a single resistance determinant; (2) larger (15–30 kb) low copy (4–6/cell) plasmids, which usually carry several resistance determinants; and (3) conjugative multiresistance plasmids (Berg et al., 1998). Larger plasmids undergo theta replication (a DNA replication mechanism that resembles the Greek letter theta), whereas small plasmids usually replicate by the rolling-circle mechanism (Khan, 2005; Lindsay, 2010). As a consequence of the limited ability of *S. aureus* to acquire DNA from the environment (low natural competence) compared to bacteria such as *Vibrio cholera* or *Bacillus subtilis*, most of the intercellular transfer of staphylococcal plasmids occurs by transduction or conjugation (Morikawa et al., 2003). Upon entering the bacterial

host, staphylococcal plasmids remain as free circularized DNA or linearize and integrate into the chromosome (Malachowa and DeLeo, 2010).

Staphylococcal plasmids are known for their genes encoding antibiotic resistance as for example penicillin and more recently vancomycin, which resistance was acquired from enterococci (Weigel et al., 2003; Zhu et al., 2008).

In addition to genes encoding antibiotic resistance and molecules involved in metabolism, staphylococcal plasmids encode resistance to a variety of organic and inorganic ions, such as cadmium, mercury, arsenate, etc., which are highly toxic for living cells (Jensen and Lyon, 2009). Staphylococcal plasmids may also encode toxin genes like plasmids containing genes encoding enterotoxins. exfoliative toxin B, bacteriocin, and bacteriocin immunity (Jackson and Iandolo, 1986).

2.3.5 Staphylococcal cassette chromosome

Staphylococcal cassette chromosomes (SCCs) are relatively large fragments of DNA that always insert into the orfX gene on the S. aureus chromosome. SCC can encode antibiotic resistance and/or virulence determinants, SCCs can be classified into staphylococcal cassette chromosome mec (SCCmec) or non-SCCmec groups (Bayles and Iandolo, 1989; Malachowa and DeLeo, 2010). MRSA strains contain SCCmec encoding the methicillin resistance gene (mecA), thus conferring resistance to methicillin and all β-lactam antibiotics (Malachowa and DeLeo, 2010).

Staphylococcal cassette chromosomes can be complex and are thus not limited to encoding methicillin resistance. Non-mec SCC and wSCC (without or no functional recombinase) contain virulence or fitness/survival determinants (Malachowa and DeLeo, 2010). A non-mec SCC is the one containing the adlb gene, which is highly sensitive and specific for S. aureus GTB (a genotype often involved in mastitis), both at the analytical and the diagnostic level, enabling the very specific detection of the genotype of interest (Sartori et al., 2017).

2.3.6 Transposon and insertation sequence

Although insertion sequences (IS) can exist independently in the S. aureus genome, they often present as pairs constituting a composite transposon (Byrne et al., 1989). IS insert into various loci and may cause changes in the expression of genes in the core chromosome. Transposons and insertation sequences predominantly encode antibiotic resistance genes in S. aureus, but they can also encode for staphylococcal enterotoxin (seh) (Ren et al., 1994). The smaller transposons are usually presented in multiple copies in the staphylococcal genome, either inserted into the chromosome or into MGEs, such as SCC or plasmids. This group includes Tn554 and Tn552, which encode resistance to MLSB antibiotics and spectinomycin or penicillinase, respectively (Bagcigil et al., 2007; Ito et al., 2003).

Acquisition of MGE by horizontal gene transfer (HGT) allows S. aureus strains to have advantages for survival at a wide range of environmental conditions, including interspecies competition within particular ecological niche and antibiotic selective pressure. Although MGEs constitute only 25% of the staphylococcal genome, they encode many putative virulence factors and antibiotic determinants and thus play an important role in bacterial adaptability and survival (Lindsay and Holden, 2004; Malachowa and DeLeo, 2010).

3 Staphylococcal enterotoxins

3.1 Overview

S. aureus is a versatile pathogen that produces a wide range of exoproteins with toxicological effects on humans and animals, such as hyaluronidase, staphylokinase, nucleases, lipases, proteases, collagenases, hemolysins, exfoliative toxins, and superantigen proteins encompassing the Toxic Shock Syndrome Toxin-1 (TSST-1), Staphylococcal Enterotoxin-like proteins (SE*l*s) and staphylococcal enterotoxins (SEs). Among these exotoxins, SEs have been the most frequently associated with food-borne intoxications collectively referred to as Staphylococcal Foodborne Poisoning (SFP) (De Buyser, 2001). Staphylococcal enterotoxin was first described in 1959 (Bergdoll, 1989).

SEs and SE*l*s are of low molecular weight, single-chain basic globular proteins that specifically attack intestinal cells leading to gastroenteritis, typically evoking vomiting, diarrhoea and intestinal or gastric inflammation. They have biochemical and structural properties that make them resistant to heat, digestive proteinases, irradiation, denaturing agents, and stable to a wide pH range (Asao et al., 2003; Fung et al., 1973; Hennekinne et al., 2012).

Staphylococcal enterotoxin genes are also harboured by other Staphylococci, like: *Staphylococcus cohnii, Staphylococcus epidermidis, Staphylococcus xylosus* and *Staphylococcus haemolyticus, Staphylococcus argentus and Staphylococcus schweitzeri, Staphylococcus carnosus* and *Staphylococcus warneri* (Bautista et al., 1988; Chajecka-Wierzchowska et al., 2020; da Cunha Mde et al., 2007; Veras et al., 2008; Zell et al., 2008; Zhang et al., 2018). Recently for these coagulase negative staphylococci, their potential production of enterotoxin was studied and it was proven that they are not able to produce enterotoxins (Chajecka-Wierzchowska et al., 2020). Nevertheless the authors suggest that due to the high presence of staphylococcal enterotoxin genes coagulase in negative staphylococci should not be ignored because of their pathogenic potential (Chajecka-Wierzchowska et al., 2020).

3.2 Characteristics of the different staphylococcal enterotoxins

By now 26 different types of Staphylococcal enterotoxin have been described and the number seems constantly on the rise, due to the wider use of automated characterisation techniques at the molecular and genetic levels (Table 2). Such sophisticated, rapid and sensible techniques are expected to allow identification of new SEs/SE*l*s or reconsideration of the classification of some formerly classified SEs/SE*l*s (Benkerroum, 2018).

In 2004, the International Nomenclature Committee for Staphylococcal Superantigens (INCSS) proposed a standard nomenclature for newly discovered toxins. The INCSS naming convention is to emphasize the relevance of the food poisoning (emetic activity). To name the SE, it is required to demonstrate emetic activity via the oral route in a primate model. If an SE exhibits no emetic potential in the vomiting model or its emetic activity is not yet examined, the toxin would be named staphylococcal like enterotoxins (SE*l*) (Lina et al., 2004).

Table 2: Overview of staphylococcal enterotoxins

Toxin	Molecular weight [kDa]	Gene localisation	Reference
SEA	27.1	Prophage	(Betley and Mekalanos, 1985)
SEB	28.4	Plasmid/pathogenicity island	(Shafer and Iandolo, 1978)
SEC	27.5	Plasmid/pathogenicity island	(Bohach and Schlievert, 1987)
SED	26.9	Plasmid	(Bayles and Iandolo, 1989)
SEE	26.4	Prophage	(Couch et al., 1988)
SEG	27	Prophage *(egc)*	(Jarraud et al., 2001)
SEH	25.1	Transposom	(Ren et al., 1994)
SEI	25.1	Prophage *(egc)*	(Jarraud et al., 2001)
SE*l*J	28.2	Plasmid	(Zhang et al., 1998)

Toxin	Molecular weight [kDa]	Gene localisation	Reference
SE/K	25.4	Prophage	(Orwin et al., 2001)
SEL	24.7	Plasmid/pathogenicity island	(Fitzgerald et al., 2001)
SEM	24.8	Prophage *(egc)*	(Jarraud et al., 2001)
SEN	26.1	Prophage *(egc)*	(Jarraud et al., 2001)
SEO	26.8	Prophage *(egc)*	(Jarraud et al., 2001)
SEP	26.7	Prophage	(Omoe, Imanishi, et al., 2005)
SEQ	25.1	Prophage	(Hu et al., 2017)
SER	27	Plasmid	(Omoe et al., 2003)
SES	26.2	Plasmid	(Ono et al., 2008)
SET	22.6	Plasmid	(Ono et al., 2008)
SE/U	28.6	Prophage *(egc)*	(Thomas et al., 2006)
SE/V	23.2	Prophage *(egc)*	(Thomas et al., 2006)
SE/X	23.3	Chromosome	(Langley et al., 2017)
SE/Y	22.5	-	(Ono et al., 2015)
SE/Z	-	-	(Spoor et al., 2015)
SE/26	25.08	Prophage	(Zhang et al., 2018)
SE/27	26.9	Prophage	(Zhang et al., 2018)

In the past SEC was often divided into further subgroups like SEC_{1-3}, SEC_{bovine} ,SEC_{ovine} and $SEC_{caprine}$ (Bohach and Schlievert, 1987; Hovde et al., 1994; Marr et al., 1993). Due to the high similarity at amino acid level, this subgrouping is not anymore made and the enterotoxin is considered as one single type (SEC) (Liu, 2015). In 2020 a new enterotoxin was discovered named SE02 (Suzuki et al., 2020). By looking in detail at this enterotoxin it can be observed, that it has a great similarity at amino acids level to SEA. For this reason it was not taken into consideration in Table 2. As described in Table 2, enterotoxins can be part of different MGE, these are graphically summarized in Figure 5. A special case are also the enterotoxins located on the enterotoxin gene cluster (egc). These enterotoxins (*seg, sei, sem, sen* and *seo*) were first described in 2001 (Jarraud et al., 2001). Later on, on the same cluster also *seu* was described (Letertre, 2003). The *egc* is described to be located on the *v*Saβ, a genomic island of *S. aureus* (see 2.2) (Baba et al., 2008). According to literature, there are different variants of *egc* type and they have been named *egc1* to *egc4* (Figure 5) (Collery et al., 2009), recently also two more (*egc5*, *egc6*) were described (Chieffi et al., 2020).

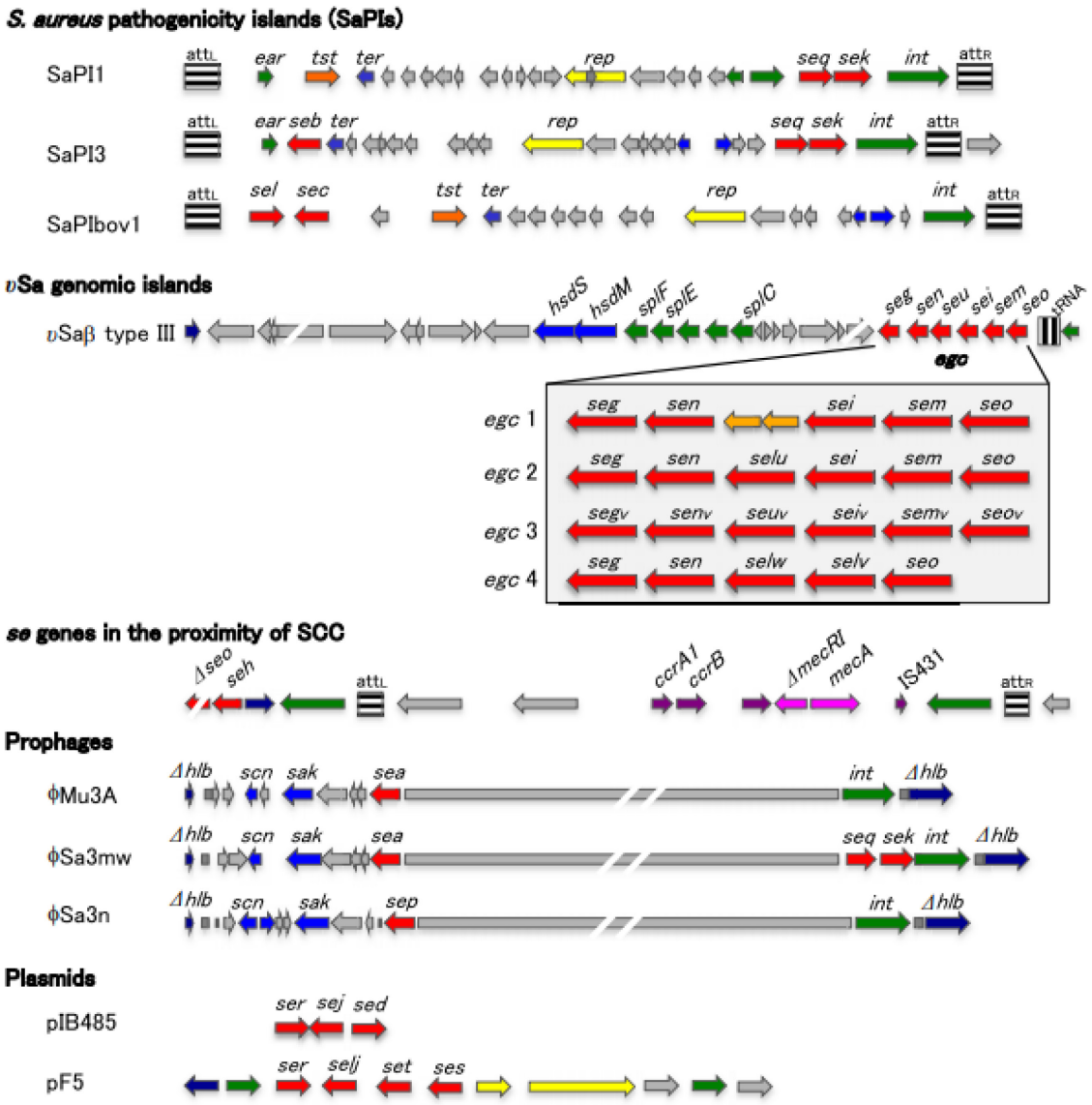

Figure 5: Staphylococcal enterotoxin (SE) and SE-like toxin genes carried by Staphylococcus aureus pathogenicity islands, υSa genomic islands, prophages, and plasmids (Hu et al., 2018).

The difference between the *egc* types is that one or more of the enterotoxin genes of the *egc* have some variants of it or are deleted. An example is *selu*: In the *egc* where *selu* is not present, two pseudogenes are present instead: *ent1* and *ent2* (*egc1*). In literature *selu* is described as enterotoxin gene that was formed by a fusion of *ent1* and *ent2* (*egc2*). *Egc3* was described as cluster having variants of each enterotoxin present (including *seu*). The last variant described is *egc4* were *sem* and *seu* are not present and instead the presence of new enterotoxins $selu_2$ or *selw* and *selv* was described. For this last *egc* type *selv* was described to be a recombination of *sem* and *sei* and $selu_2$ as a fusion of *ent1* and *ent2* (Thomas et al., 2006).

According to more recent literature, 15 different *v*Saβ types were discovered, by analysing 103 clinical *S. aureus* strains (Klaui et al., 2019). Looking at these *v*Saβ, *egc1* is described as *v*Saβ type I, *egc2* as *v*Saβ type IV, *egc3* as *v*Saβ III and *egc4* was not found in any of the analyzed genomes so no *v*Saβ type was given (Klaui et al., 2019). According to literature there are much more variants of *egc* than *egc1* to *egc6*, as there are *v*Saβ types containing single truncated or fragmented genes (*v*Saβ type IV, XI). In consequence it is questionable if *egc* 1-6 is the correct way to distinguish the different variants of the enterotoxin gene cluster. In consequence of the fact that seu_2 (or *selw*) is a variant of *seu,* in Table 2 only *seu* was considered. *Sev* seem also not to be relevant, as it was not found in the strains analyzed by Kläui et al (2019) nor in the ones analyzed by Merda et al. (2020).

3.3 Molecular structure of staphylococcal enterotoxins

Staphylococcal enterotoxins are globular, single-chain proteins with molecular weights between 19 and 29 kDa and 168-261 amino acids long (Table 2). A significant degree of similarity occurs among the primary peptide sequences of enterotoxins. Overall, 15% of the residues are entirely conserved throughout the known SEs and SE*l*s. Most of these residues are located either centrally or at the C terminus (Dinges et al., 2000). SEs can be divided into four phylogenetic groups based on their primary amino acid sequences (Thomas et al., 2006) or a phylogenetic tree can be constructed based on amino acid sequences (Figure 6).

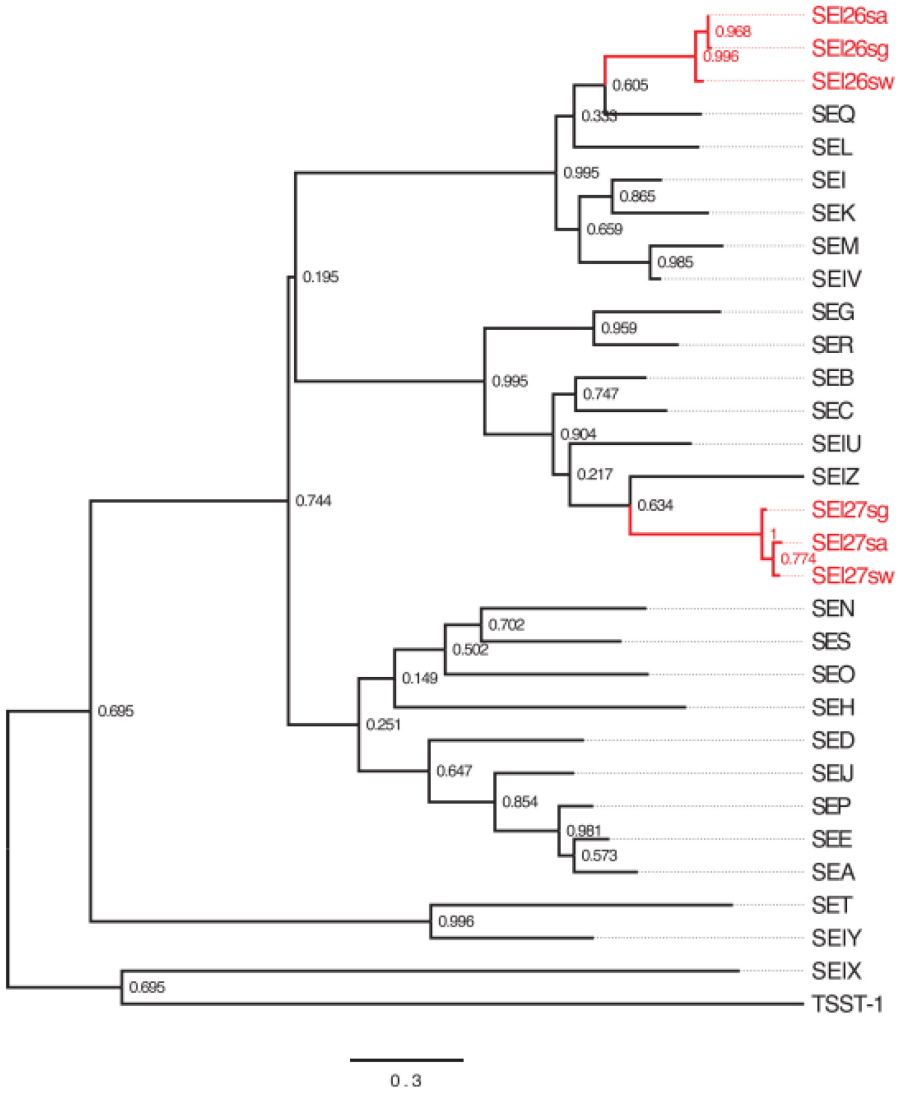

Figure 6: Maximum likelihood phylogenetic tree of staphylococcal enterotoxins, including SEI26 and SEI27. The tree was constructed based on amino acid sequences using a Poisson model in MEGA 6.06. Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points. (Zhang et al., 2018)

The three-dimensional structures of SE are conform to common protein folding what has been confirmed using crystallographic studies by (Swaminathan et al., 1995). The overall shape of SE molecules is ellipsoid, and they contain two unequal domains: A and B (Figure 7). The secondary structure is a mixture of α-helix and β-sheet components. Domain A contains both the amino and carboxyl termini, as well as a β-grasp motif. The amino-terminal residues drape over the edge of β-sheet in a loosely attached structure. The interfaces between A and B domains are marked by a set of α-helices, which form a long groove in the back side of the molecule and a shallow cavity at the top. The domain B is associated with binding to carbohydrates or nucleic acids. The internal β-barrel region is richly hydrophobic, and the external surface is covered by a number of hydrophilic residues. The characteristic SE disulphide

bond is located at the end of domain B, opposing the α-helical cap. The resulting loop structure is flexible, although this seems to vary among the SEs, depending on the length of the loop (Hu and Nakane, 2014).

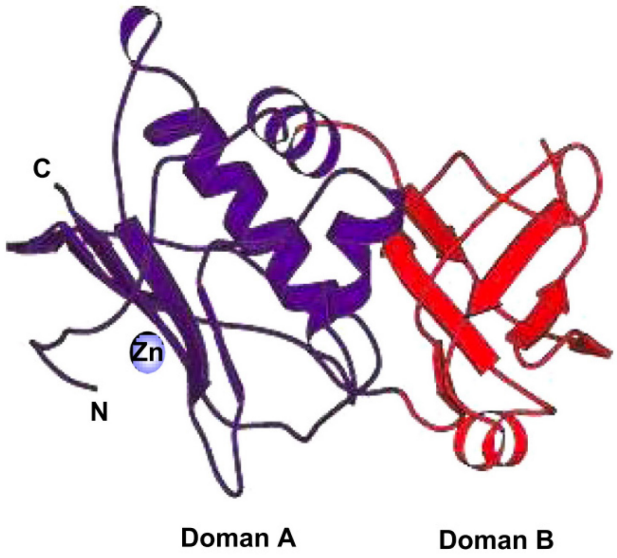

Figure 7: Three dimensional structure of SEA (Hu and Nakane, 2014).

3.4 Pathogenesis of staphylococcal enterotoxins

Staphylococcal enterotoxins have the ability to induce emetic activity beside super antigenic activity (Figure 8). These two activities are taking place in two separate domains of the protein (Dinges et al., 2000; Hovde et al., 1994). The super antigenic activity has the ability to induce excessive activation of T-cells with a subsequent massive release of cytokine, whereas the emetic activity has the ability to cause enteritis in the intestine among other symptoms (Benkerroum, 2018).

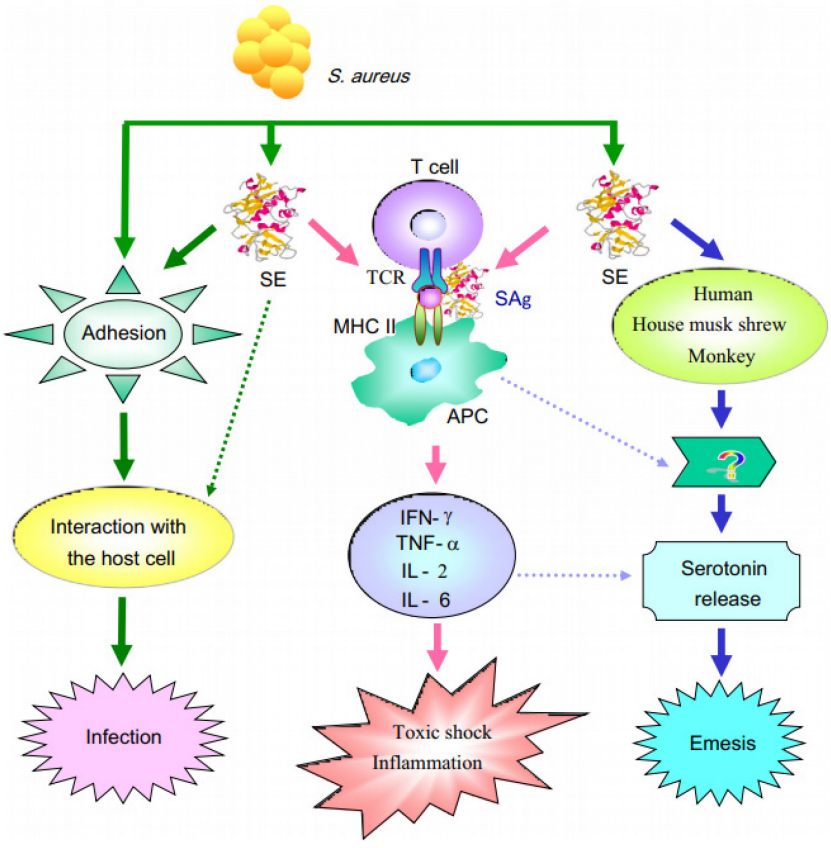

Figure 8: The biological multifunctionality of SEs in food poisoning, toxic shock and infection (Hu and Nakane, 2014)

A summary of the tested super antigenic and emetic activity of the different staphylococcal enterotoxins can be seen in Table 3. Not for all known SEs emetic activity has been tested.

Table 3: Emetic and super antigenic activity of Staphylococcal enterotoxin and Staphylococcal enterotoxin like. NE not examined, ED50 = 50% emetic dose (according to Hu et al. 2018)

SEs/SE/	Super antigenic activity	Emetic activity [µg/animal]	
		Monkey[1]	House Musk Shrew[2]
SEA	+	5 (ED_{50})	0.3
SEB	+	5 (ED_{50})	10
SEC	+	5 (ED_{50})	1000
SED	+	5 (ED_{50})	40
SEE	+	10-20 (ED_{50})	10
SEG	+	160-320	200
SEH	+	30	1000
SEI	+	300-600	1
SE/J	+	NE	NE
SE/K	+	100 (2/6)	NE
SEL	+	100 (1/6)	NE
SEM	+	100 (1/7)	NE
SEN	+	100 (1/8)	NE
SEO	+	100 (2/8)	NE
SEP	+	100 (3/6)	50
SEQ	+	100 (2/6)	NE
SER	+	<100	<1000
SES	+	<100	20
SET	+	<100	1000
SE/U	+	NE	NE
SE/V	+	NE	NE
SE/X	+	NE	NE
SE/Y	+	NE	500
SE/Z	+	NE	NE
SE/26	+	NE	NE
SE/27	+	NE	NE

[1]Oral administration

[2]Intraperitoneal administration

3.4.1 Superantigen activity

SEs and SEls are representative super antigenic toxins, which selectively activate a vast number of T cells, depending on Vβ elements in the β chain of a T-cell receptor (TCR), in direct association with the major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (APCs) (Lina et al., 2004; Uchiyama et al., 1994). They subsequently stimulate massive cytokine release and systemic shock (Figure 9).

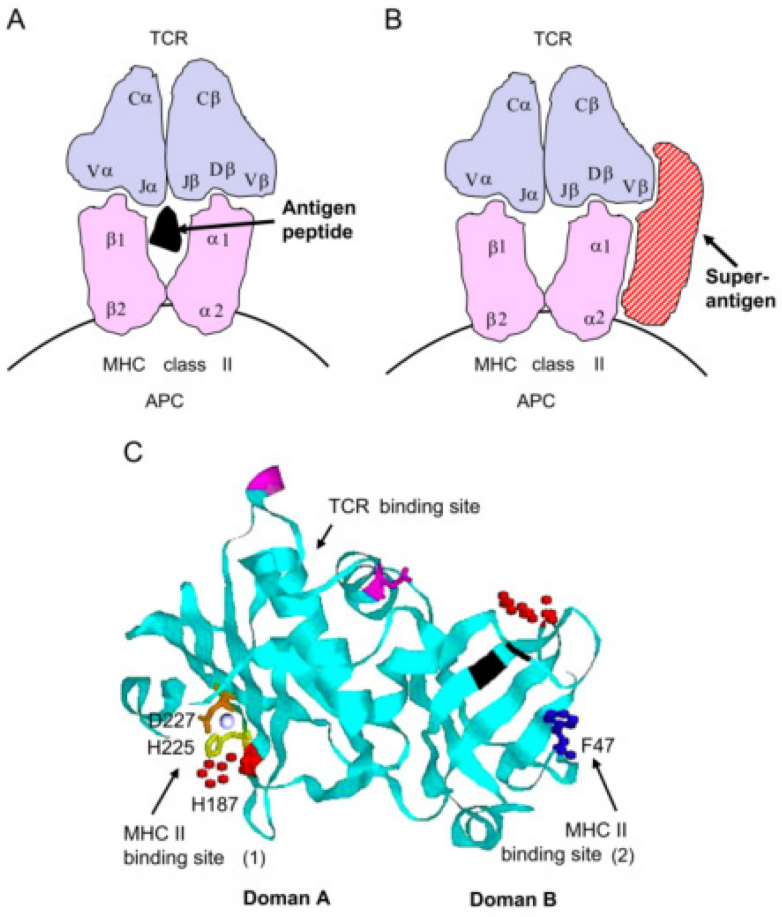

Figure 9: Diagram of normal antigen (A), superantigen binds to T cell receptor and MHC class II of APC (B), and the binding sites of SEA to these cells (C) (Hu and Nakane, 2014).

SEs have evolved in several distinct modes of interaction with MHC class II molecules. SEA contains two MHC class II binding sites (Figure 9). The zinc-dependent site is the major interaction region, and several important residues (H187, H225, and D227) were identified by mutagenesis. It is presumed that this binds MHC β chain (Schad et al., 1995). This major MHC binding site is located in domain A, near the amino terminus (Schad et al., 1995). The second (minor) binding site on SEA is F47 located in domain B, which is not zinc dependent. It may be this cooperation between the two binding sites that is responsible for the high affinity of SEA for MHC class II molecules. It could result in a trimer containing the toxin and two bound MHC class II molecules. Mutational studies were done on SEC3 (Leder et al., 1998), and showed that mutations in the TCR binding site of this toxin were capable of sharply reducing mitogenicity.

3.4.2 Emetic activity

The first well-documented report that clearly identified SEs as the cause of food poisoning outbreaks, was done by (Dack et al., 1930). They isolated a pigment-forming *Staphylococcus* present in large numbers in a Christmas cake responsible for a food poisoning incident, and sterile culture filtrate of the organism reproduced illness when ingested by human volunteers.

The target of SEs responsible for initiating the emetic reflex could be located in the abdominal viscera, where putative cellular receptors for SEs exist (Sugiyama and Hayama, 1965). Since these receptors have not yet been identified, there remains much uncertainty regarding the early events in the pathogenesis of food poisoning.

The symptoms of staphylococcal food poisoning are vomiting, abdominal cramps, nausea, sometimes followed by diarrhea after a short period of incubation (Hu et al., 2003; Le Loir et al., 2003). In contrast to these well-described clinical manifestations, the physiopathology of symptoms is only partially understood

However, little is known regarding the mechanisms by which they induce vomiting. Lack of progress in elucidating the mechanism of the emetic activity of SEs can be partially attributed to the lack of convenient and appropriate animal models. The susceptible animal species to develop human-like enterotoxigenic disease are non-human primate models, often, *Macaca mulatta* (Normann et al., 1969; Stiles, 1971). When introduced intragastrically, SEA and SEB have been shown to induce emetic responses and gastrointestinal (GI) inflammatory changes in different *Macaca* spp. (Merrill and Sprinz, 1968; Reck et al., 1988). Monkeys have been considered to be the primary animal model. However, the use of monkeys in investigating SEs is severely restricted by the high cost, the availability of these animals, and ethical considerations.

The house musk shrew, *Suncus murinus*, has been described as a small animal model for the study of emetic response to various emetic drugs (Chen et al., 1996; Okada et al., 1994). The emetic response of house musk shrew to peroral and intraperitoneal administration of SEA has been examined (Hu et al., 2001; Hu et al., 1999) (Figure 10): The emetic activity of SEA in house musk shrews was found to be dose-dependent. Multiple emetic episodes occurred during 70 to 108 min after peroral administration of SEA. Similar responses occurred during 65 to 102 min after intraperitoneal injection of SEA (Hu et al., 1999). The animals recovered clinically within 3 h.

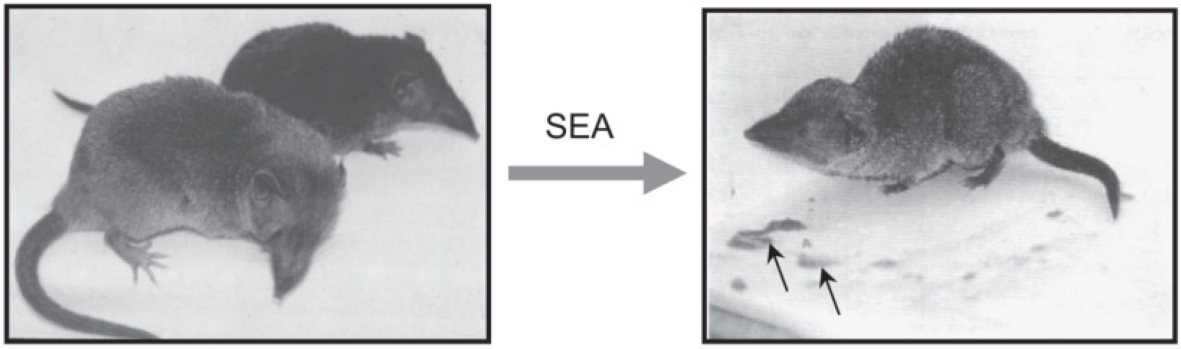

Figure 10: The emetic response of house musk shrews against SEA administration. A small emetic animal model, house musk shrews (A, arrow indicates vomit) (Hu and Nakane, 2014).

SEA passes through the mucosal epithelium in the GI lumen by an unknown mechanism and then accumulates in the submucosa (Figure 11). This translocation from the lumen to the submucosa occurs within 30–90 min, a timeframe that is consistent with the latency time of SEA-induced emesis in house musk shrew (30–120 min) (Hu et al., 2003; Hu et al., 1999). In the stomach and duodenum of house musk shrew, SEA binds to the submucosal mast cells or directly to neuron cells. The binding of SEA to an unidentified receptor expressed on the surface of these cells induces the degranulation, resulting in the release of 5-HT. At present, it is unclear what type of molecule acts as an SEA receptor on the surface of submucosal mast cells or neuron cells. Superantigens including SEA bind to MHC class II molecules expressed on surface of APCs. However, SEA and MHC class II signals were not co-localized in the GI tissues of the SEA administered animals, indicating that a receptor on mast cells is not MHC class II.

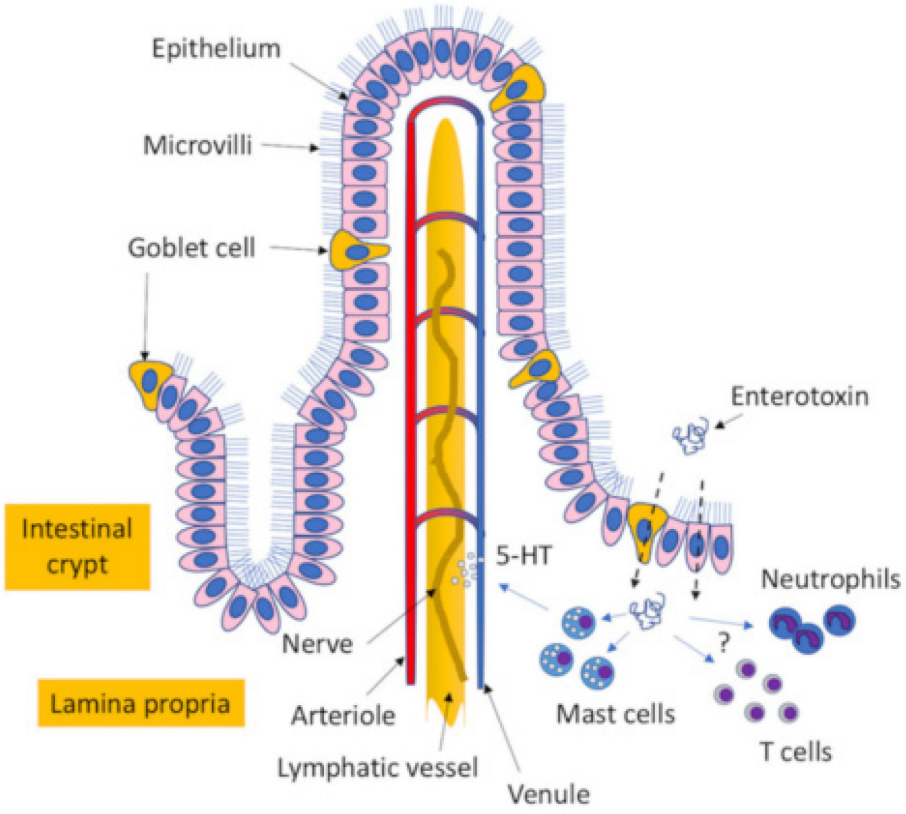

Figure 11: Proposed mechanism of enterotoxin-induced emesis. The enterotoxins transit through mucus-expelling goblet cells and epithelial cells in the intestinal epithelium to reach the lamina propria. Here, the enterotoxins can interact with mast cells to induce the release of 5-hydroxytryptamine (5-HT/serotonin precursor), which interacts with the vagus nerve to cause an emetic response. Additional cellular targets that may have possible roles in the induction of enterotoxigenic disease include different types of T cells and neutrophils (Fisher et al., 2018)

Moreover, orally administrated SEA shows tendency to bind to mast cells rather than MHC class II-positive cells in GI tract, indicating that unidentified SEA receptor on mast cells is capable of binding SEA more efficiently than MHC class II (Ono et al., 2012). Further studies on the identification and molecular cloning of the unidentified SEA receptor gene are necessary for understanding the exact molecular basis of SEA-induced emesis and elucidation of its down-stream intracellular signaling.

3.5 Prevalence of the different enterotoxins in S. aureus strains

Most of *S. aureus* genome contains one or more enterotoxin genes (Merda et al., 2020). Some of the genes seem to be more prevalent than the others and this seems to depend on the origin where the strains has been isolated (Benkerroum, 2018). A literature summary of the prevalence of the different genes harboured by different *S. aureus* strains can be seen in Table 4.

Table 4: Distribution of Staphylococcal enterotoxin types for Staphylococcus aureus strains isolated from food-poisoning cases and different foods and food-handlers 1 (Chiang et al., 2008), 2 (Merda et al., 2020), 3 (Sato'o et al., 2014), 4 (Argudin et al., 2012), 5 (Hummerjohann et al., 2014) NA = not analyzed

Description	SFPO Taiwan (147 isolates)[1]	SFPO Europe (143 isolates)[2]	SFPO Japan (42 isolates)[3]	Food and -handlers Spain (64 isolates)[4]	Cheese Switzerland (102 isolates)[5]
Se gene	**incidence [%] in *S. aureus* isolates**				
sea	29.20	34.97	70.00	38.70	21.00
seb	19.70	6.29	45.00	12.90	NA
sec	6.80	14.69	7.00	16.10	1.00
sed	2.00	11.89	5.00	22.60	44.00
see	-	2.80	-	-	NA
seg	2.00	34.97	20.00	80.70	14.00
seh	8.20	17.48	55.00	9.70	4.00
sei	29.90	37.76	20.00	8.70	16.00
seij	2.00	13.99	5.00	22.60	42.00
seik	16.30	13.99	55.00	6.50	NA
sel	6.80	13.99	3.00	9.70	NA
sem	11.60	35.66	17.00	80.70	NA
sen	10.90	37.76	17.00	80.70	NA
seo	14.30	37.06	17.00	80.70	NA
sep	27.90	11.89	10.00	9.70	1.00
seq	10.90	14.69	55.00	9.70	NA
ser	5.40	13.99	5.00	16.10	42.00
ses	NA	0.70	NA	-	NA
set	NA	0.70	NA	-	NA
seiu	14.20	25.17	NA	48.40	NA
seiv	NA	0.00	NA	NA	NA
seix	NA	12.59	NA	NA	NA
seiy	NA	89.51	NA	NA	NA
seiz	NA	9.09	NA	NA	NA
sei26	NA	0.00	NA	NA	NA
sei27	NA	0.00	NA	NA	NA

From the data of Table 4 it can be observed that not only classical enterotoxins (*sea-see*) are harboured by *S. aureus*. From the classical ones, *sea* seems to be the most prevalent one (21-70%) and *see* the less prevalent one (0-2.8%). From the non-classical ones, *sey* seems, to be the most prevalent one, as this enterotoxin is one of the newer ones, not much studies are available about it, but according to Mérda et al. 2020 it is contained in 89.51 % of the analysed strains. The second group of non-classical enterotoxins that have a high prevalence are the *egc* enterotoxins (*seg, sei, sem, sen, seo, seu*). For these enterotoxins it seems to depend a lot on the contest they were isolated from. According to the data of Table 4 their prevalence can be between 11% (SFPO isolates from Taiwan) and 80% (isolated from food and food handlers), in the study from Mérda et al. (2020) the prevalence was 37% in SFPO isolates. *Egc* seems to be especially present in association with strains isolated from humans (Jarraud et al., 2001). In this study (from France) *egc* enterotoxins were found in 57% of the strains isolated from nasal carriage and in 67% of the strains from suppurative disease. In strains isolated from animals (from Ireland) *egc* enterotoxins were present highest (86.7%) on strains isolated from chicken and lowest (10.3%) in strains isolated from goats (Smyth et al., 2005). From the non-classical enterotoxins, *seiv* wasn't found in none of the isolates analyzed.

3.6 Food borne outbreaks caused by Staphylococcal enterotoxins

The origins of staphylococcal food poisoning differ widely among countries what may be due to differences in consumption and food habits in each country. The main source of contamination are humans through contaminated food handlers via manual contact or via respiratory tract by coughing and sneezing. Often contamination occurs after heat treatment of the food. Contaminated food from animal origins, such as raw meat, sausages, raw milk, and raw milk cheese is frequently contaminated by animal carriage or infection by *S. aureus* like mastitis (Le Loir et al., 2003) (Figure 12).

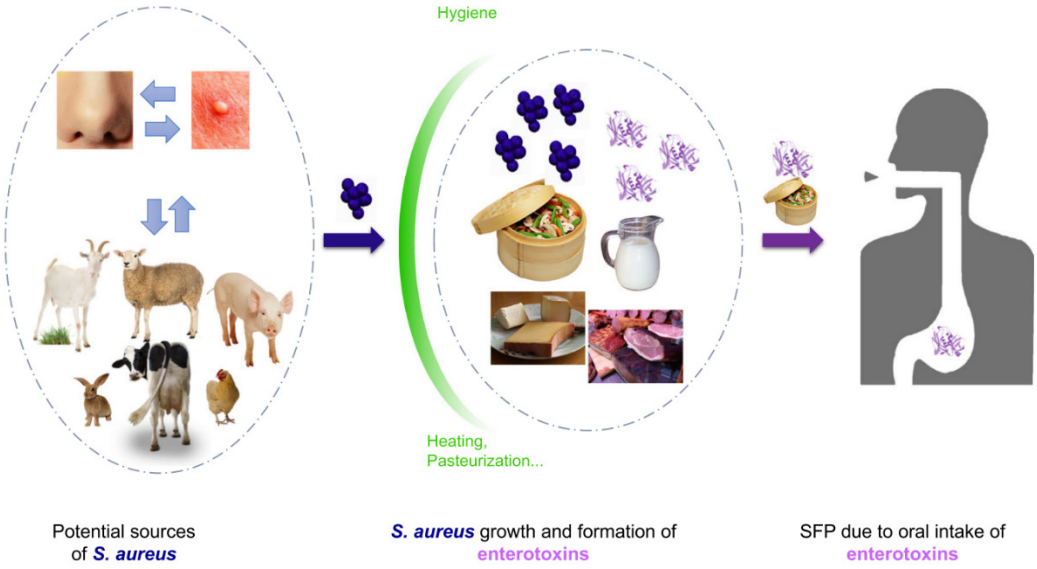

Figure 12. Overview of Staphylococcal Food Poisoning (Fetsch and Johler, 2018)

After contamination (animal or human), food is processed (e.g. heating, cooling) what can inhibit or enhance Staphylococcal enterotoxin production. Even if heating is applied after growing of *S. aureus*, enterotoxins will still remain in the food, as the enterotoxins are heat stable. The intake of SE will then be the cause of Staphylococcal food poisoning (Hennekinne, 2018).

Summarizing following series of events need to take place in order to qualify for an SFPO: (i) presence of the pathogen in raw materials or the food handler(s), (ii) contamination of the food, for example, through processing equipment or through the food preparer, (iii) inappropriate storage conditions and/or inadequate temperature control that allows for bacterial growth and enterotoxin production, and (iv) ingestion of contaminated food containing a sufficient amount of SE to trigger symptoms of the disease (Zeaki et al., 2019).

3.6.1 Overview of SFPO: EFSA Report

Outbreaks originating from bacterial toxin (other than *Clostridium botulinum*) in Europe seem to have slightly increased between 2017 and 2018 (Figure 13). Bacterial toxins considered in this case are toxins from *Bacillus*, *Staphylococcus* and *Clostridium* (other than *C. botulinum*).

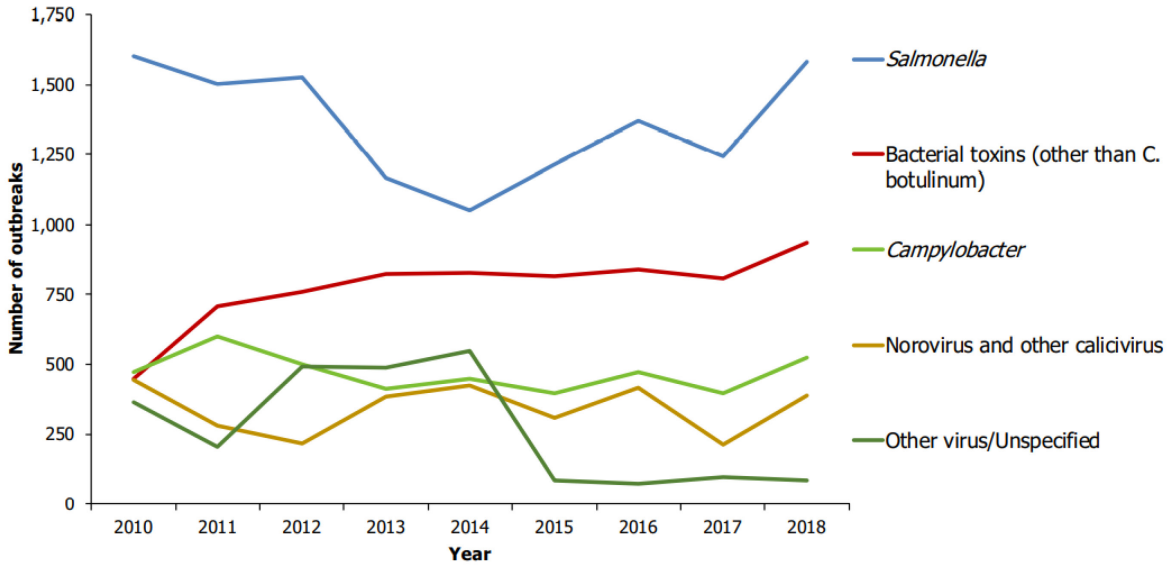

Figure 13: Number of food-borne and waterborne outbreaks in Europe, by causative agent (European Food Safety et al., 2019)

In 2018 114 outbreaks caused by Staphylococcal enterotoxin were reported, Out of them 37 were strong-evidence outbreaks and 77 weak evidence outbreaks. These outbreaks caused 1'124 human cases, out of them 167 people needed hospitalisation. The average number of people per outbreak is 9.9. In addition there are 636 weak evidence outbreaks caused by an unspecified bacterial toxin. Most of the outbreaks are reported in France due to the fact that in France SFP is a notifiable disease. South European countries in general report more SFPs than Nordic countries, probably due to inappropriate storage conditions under more critical climate conditions.

Looking in detail which kind of foods are involved in the foodborne outbreaks (Figure 14) it seems that mixed food is the most involved one, followed by "other food, meat/meat products, and milk/milk products. Buffets meals, eggs/eggs products and fish/fisheries seem to be less involved (European Food Safety et al., 2019).

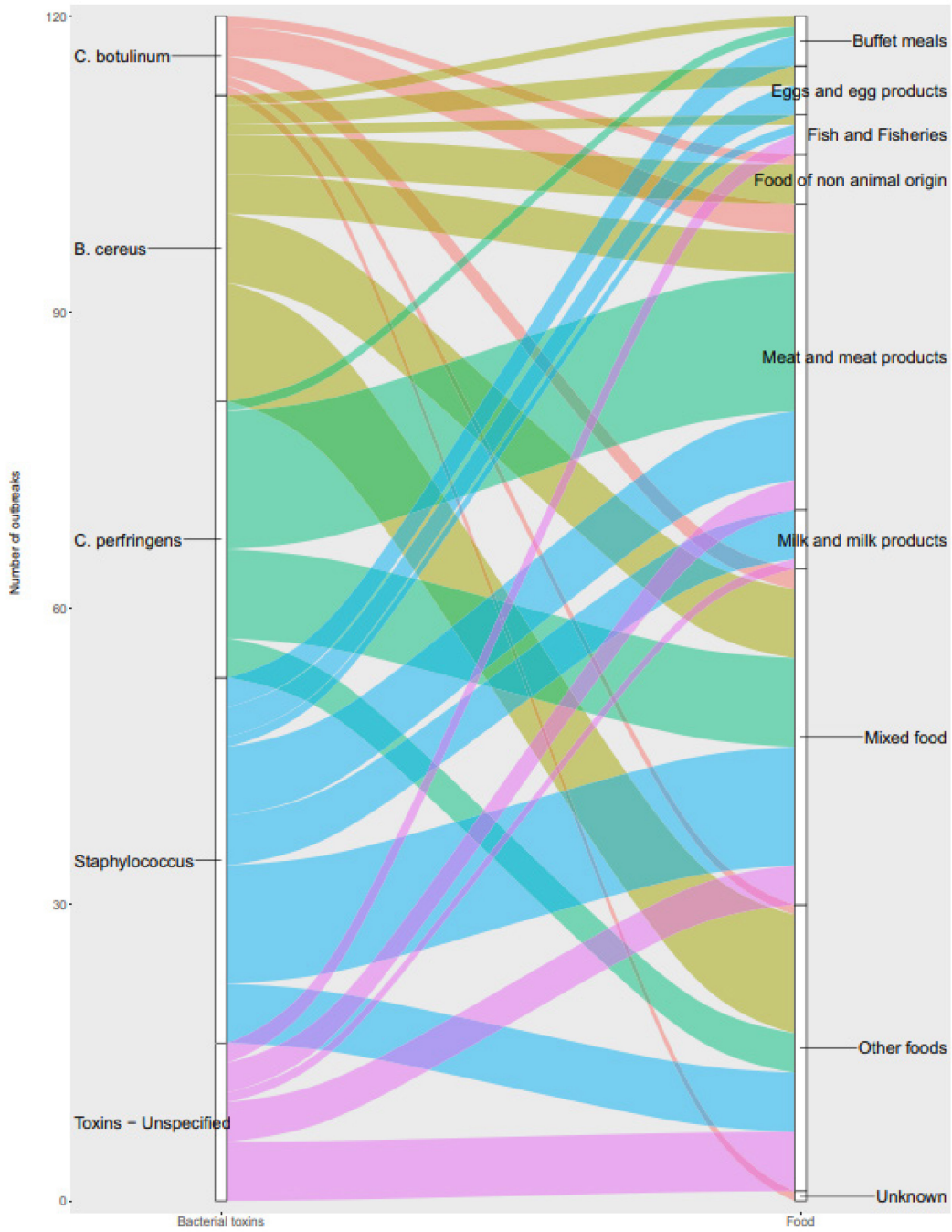

Figure 14: Sankey diagrams of the distribution of food vehicles implicated in strong-evidence foodborne and waterborne outbreaks caused by bacterial toxins (N = 120), in the EU, 2018 (European Food Safety et al., 2019)

In addition, it is remarkable that outbreaks caused by Staphylococcal enterotoxin and mixed food/meat products belong to the top-10 pathogen/food vehicle pair causing the highest number of hospitalisations. According to EFSA this number is increasing if compared to the numbers of 2010-2017. The category “mixed food” and “other foods” are both a miscellaneous group of foodstuffs including a large variety of multi-ingredients and multiorigin items. This heterogeneity makes it difficult to identify and trace back the primary food source contributing to the contamination. Mixed food was in 2018 also responsible for the highest number of illnesses and deaths among strong-evidence outbreaks at the EU level (European Food Safety et al., 2019).

3.6.2 Involvement of the different enterotoxins in foodborne outbreaks

In literature outbreaks involving classical enterotoxins (SEA-SEE) are largely described. Beside the ones described in Table 5 also older literature are describing outbreaks caused by SEA-SEE (Kerouanton et al., 2007). Especially SEA seems to have the ability to cause very large outbreak, causing more than 10'000 cases (Asao et al., 2003; Ikeda et al., 2005).

SEA-SEE are enterotoxins that can be analysed with commercially available methods like VIDAS SET2 or Ridascreen that is why this enterotoxin can easily be discovered and described (Benkerroum, 2018). Even classical enterotoxin can be detected and their respective enterotoxins gene detected too, often the strains involved are also harbouring non-classical enterotoxin genes (Table 5). Because of the fact that the detection methods (such as ELISA or other methods) for non-classical enterotoxins are not commercially available (Zhao et al., 2017a; Zhao et al., 2017b), detection of these enterotoxins remains difficult and their involvement in foodborne outbreaks cannot fully be described and confirmed.

During the last years multiple cases where described where the involvement of non-classical enterotoxin seems to be described. Among the non-classical enterotoxins SEH and the EGC enterotoxins seem to be the most involved enterotoxins (Table 5).

Table 5: Epidemiological data, coagulase positive staphylococci (CPS) concentrations, staphylococcal enterotoxin genes (se) and staphylococcal toxin (SE) detected from different staphylococcal food poisoning outbreaks reported in literature. * VIDAS set2 results (detecting SEA-SEE)

Country	Product	Cases	CPS [CFU/ml]	*Se* genes	SE confirmed	Reference
Romania	Sheep curd cheese	8	10^6 - 10^8	*sed, sej, ser*	SEA-SEE*	(Ciupescu et al., 2018)
Romania	Appetizer made with soft sheep cheese	52	10^6	*sed, sej, ser, egc*	SEA-SEE*	(Ciupescu et al., 2018)
Romania	Raw cow milk ripened salted cheese	36	10^6	*seh*	Negative*	(Ciupescu et al., 2018)
Switzerland	Tomme soft cheese	14	10^7	*sea, sed, sej, ser, egc*	SEA, SED	(Johler, Weder, et al., 2015)
Switzerland	Goat fresh cheese	5	10^6- 10^7	*egc*	Negative*	(Johler, Giannini, et al., 2015)
Switzerland	Goat semi-hard cheese	6	10^3	*egc*	Negative*	(Johler, Giannini, et al., 2015)
Japan	powdered skim milk	13420	not detected	-	SEA	(Asao et al., 2003)
Japan	Skim milk powder	10000	-	*sea, seh, egc*	SEA, SEH	(Ikeda et al., 2005)
Norway	Mashed potato with raw milk	8	10^8	*seh*	SEH	(Jorgensen et al., 2005)
Japan	Sushi, potato salad, fried barbecue/shrimp/ chicken, sandwich, raw vegetable	15	10^4-10^8	*egc*	Negative*	(Umeda et al., 2017)
Belgium	Mashed potato	28	10^2	*sea, sed, sej, ser, egc*	SEA, SED	(Denayer et al., 2017)
Belgium	Chicken, sausage, bovine meat, potato preparation	18	10^6	*sea, sec*	SEA, SEC	(Denayer et al., 2017)

Belgium	Mashed potatoes with carrots	6	10^7	*sea, egc*	SEA	(Denayer et al., 2017)
Italy	Chantilly Cream Dessert	24	10^8	*sea*	SEA	(Ercoli et al., 2017)

About SEH, two outbreaks were described where the only detected CPS was harbouring seh gene, in consequence only this enterotoxin could have been involved in the outbreak (Ciupescu et al., 2018; Jorgensen et al., 2005). In one outbreak (Mashed potato, Norway), a method was available for SEH detection and it could be detected (Jorgensen et al., 2005). For EGC enterotoxins in multiple outbreaks there is evidence of their involvement. Here again, in the two studies found (totally 3 outbreaks), CPS involved were only harbouring *egc* genes, but due to the lack of appropriate methods for enterotoxin detection, they were never analysed (Johler, Giannini, et al., 2015; Umeda et al., 2017).

3.7 Regulation of enterotoxin expression

As described in previous chapters *S. aureus* strains may harbour one or more *se/sel* genes. The harbouring of these genes does not automatically mean that they are able to express them, even under optimal conditions of growth (Carfora et al., 2015; Omoe, Hu, et al., 2005; Omoe et al., 2002). This may be due to the complexity of the genetic expression of *se/sel* genes involving an intricate network of regulatory systems acting independently or in coordination (Bronner et al., 2004).

S. aureus has a complex network of regulatory pathways to control toxin production. In consequence it responds to changes in the environment using a combination of quorum-sensing (QS) (Waters and Bassler, 2005) and other two-component systems (TCS), of which at least 16 have been discovered in *S. aureus* to date (Haag and Bagnoli, 2017), as well as many trans-acting regulatory proteins (Bronner et al., 2004). *S. aureus* relies on these systems to quickly make changes in the regulation of genes associated with important physiological features, including drug resistance, metabolism, immune evasion, and virulence. Each system can directly or indirectly control the transcription of specific sets of genes. However, the regulation of one gene may be influenced by multiple systems, leading to additional layers of regulation (Fisher et al., 2018) .

The mechanisms controlling SE production in *S. aureus* are multiple and include *se* gene promoter regions, multiple global regulators of virulence, such as the accessory gene regulator (*agr*), the staphylococcal accessory regulator (*sar*), the repressor of toxins (*rot*), the σB factor, and the two component system *S. aureus* exoprotein expression (*sae*) (Figure 15).

These regulatory mechanisms are affected in different ways by the environmental conditions in a food product, like salt content, water activity, and pH (Schelin et al., 2017). This can have a further impact on the production of the respective enterotoxins, making them difficult to control (Zeaki et al., 2019).

In addition, according to the actual knowledge, different mobile genetic elements seem to have different regulatory systems. These can be observed for SEA (prophage encoded) and SED (plasmid encoded) where the first one's regulation is linked to the life cycle of the bacteriophages and is partly regulated by the *agr* system (Zeaki et al., 2019). The consequences on enterotoxin production are that SEA is produced mostly during the exponential growth phase, at the peak of replication (Derzelle et al., 2009; Schelin et al., 2011), whereas more SED is produced during the transition from the exponential to the stationary growth phase of the microorganism (Schelin et al., 2011).

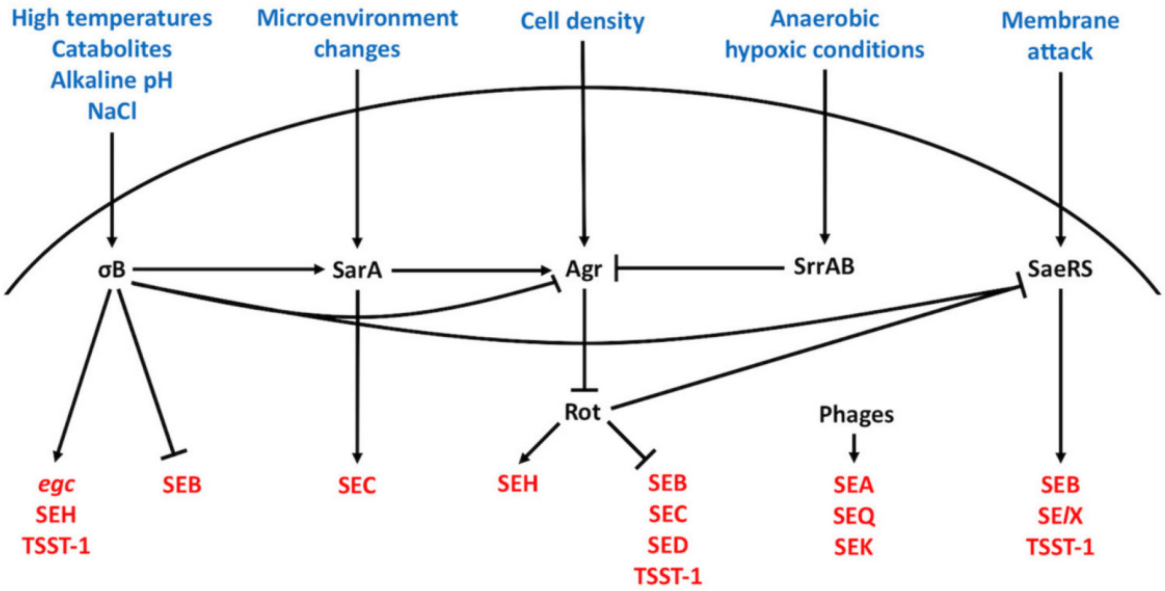

Figure 15: Regulation of staphylococcal enterotoxins expression. Harsh bacterial growth conditions, changes in the bacterial microenvironment, high cell density, hypoxia, and membrane changes direct enterotoxin expression through the alternative σ factor, SarA protein family, Agr quorum sensing system, SrrAB protein, and SaeRS two-component system, respectively. The excitatory and inhibitory action of these systems on the other regulators and enterotoxins are summarized. Arrowheads represent upregulation and bars downregulation (Fisher et al., 2018)

3.7.1 Description of the different regulation systems

The accessory gene regulator (*Agr*) is a quorum sensing system which is activated at high cell densities and is made of two transcriptional units transcribing in opposing directions: RNAII, which codes for four genes (*agrA*, *agrB*, *agrC*, and *agrD*) (Novick et al., 1995) and RNAIII, a regulatory RNA. These transcripts are controlled by the promoters P2 and P3, respectively. AgrD, which contains the sequence for the autoinducing peptide (AIP), is processed and exported out of the cell by the combined actions of the membrane-associated export protein, AgrB (Ji et al., 1997; Ji et al., 1995) and a type I signal peptidase, SpsB (Kavanaugh et al., 2007). AIP acts as the ligand for the membrane bound histidine kinase, AgrC, leading to the phosphorylation of AgrA (Ji et al., 1995). Activated AgrA binds to the P2 and P3 promoters, resulting in the perpetuation of a positive feedback loop (Koenig et al., 2004).

Expression of *agr* is affected by various trans-activating regulators, such as *Sar* family of regulatory proteins, (*SarR, SarS, SarT, SarU, SarX, SarZ, SarV, MgrA*, and Rot) (Cheung et al., 2008; Cheung and Projan, 1994; Heinrichs et al., 1996), σB (Lauderdale et al., 2009) and SrrAB (Staphylococcal respiratory response AB) (Pragman et al., 2004; Yarwood et al., 2002). Additionally, σB and Rot can affect another important two component systems called SaeRS (Kusch et al., 2011).

About σB is known to respond to high temperature, catabolites, alkaline pH, high salinity (Kullik and Giachino, 1997; Pane-Farre et al., 2006; Wu et al., 1996) on the other side *SarA* largely responds to changes in microenvironments (Cheung et al., 2004). *SrrAB* system has been shown to be particularly crucial for bacterial growth under anaerobic and hypoxic conditions (Kinkel et al., 2013; Mashruwala and Boyd, 2017; Pragman et al., 2004; Yarwood et al., 2002). *SaeRS* (*S. aureus* exoprotein expression) system responds to membrane attack by antimicrobial molecules produced by the innate host defence (Cho et al., 2015; Geiger et al., 2008; Kuroda et al., 2007; Novick, 2003). Finally *Rot*, the global gene regulator (Said-Salim et al., 2003) is known to be negatively regulated by RNAIII through an antisense mechanism (Boisset et al., 2007; Geisinger et al., 2006).

3.7.2 Regulation of the different enterotoxins

This subchapter summarizes the actual state of the art literature. However, not for all enterotoxins information on the regulation mechanisms were available.

3.7.2.1 SEA

SEA is produced the highest during the exponential growth phase, at the peak of replication (Derzelle et al., 2009; Schelin et al., 2011). The production of SEA was discovered to be closely tied to the phage's life cycle (Cao et al., 2012) and to be inducible by bacterial stress (Zeaki et al., 2015).

Sea gene encoding for SEA is located on the genome of a polymorphic family of lysogenic bacteriophages, the *Siphoviridae* family. It has been proven, through a number of studies, that SEA-producing *S. aureus* strains can be categorized into high and low SEA producers, depending on the *sea*-carrying prophage they harbour (Borst and Betley, 1994; Cao et al., 2012; Wallin-Carlquist et al., 2010). Wallin-Carlquist et al. (2010) showed that there are two *sea* variants: *sea1* and *sea2*; the high-SEA-producing strains carried *sea1*, while *sea2* was found in the low-SEA-producing strains (Figure 16**)**. The state of lysogeny, although very stable, can be disrupted by certain environmental conditions (i.e., the presence of weak acids, high NaCl concentration, UV irradiation, and DNA damage by chemical agents), and in such cases, the lytic response is initiated by the phage; a process known as prophage induction (Zeaki et al., 2019). Prophage induction has been found to increase the amount of SEA produced by some SEA-producing strains, and thus increases the probability of SFPO (Zeaki et al., 2019).

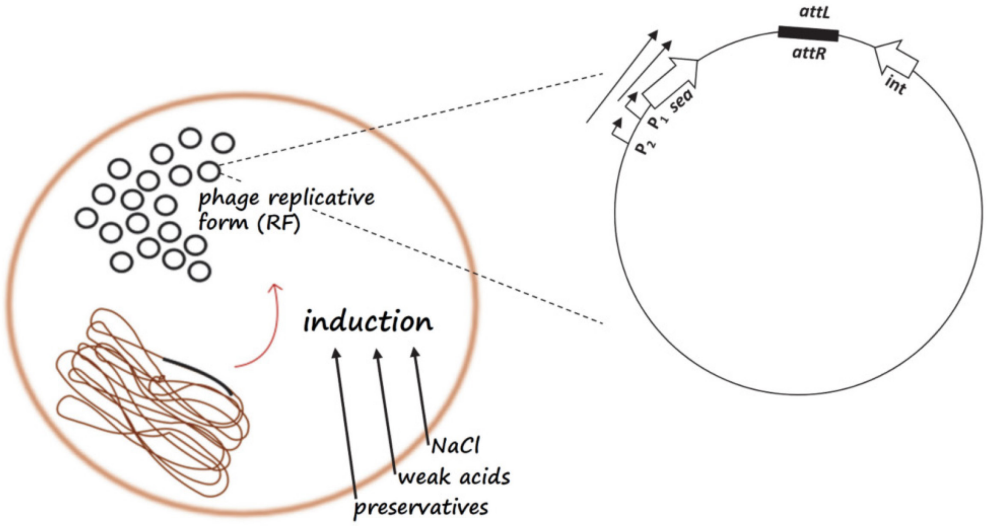

Figure 16: Schematic representation of the sea gene regulatory mechanism. Food parameters such as NaCl, weak acids, and preservatives may lead to prophage induction and replication of the circular, replicative form (RF) of the phage genome, resulting in an increase in RF copies in the cell. Prophage induction will initiate transcription from the latent promoter P2 resulting in the production of a longer sea transcript in addition to the sea transcript from the endogenous P1 promoter (Zeaki et al., 2019).

3.7.2.2 SEB, SEC, SED, SEJ

Early observations are showing that the production of SEB (Czop and Bergdoll, 1974; Derzelle et al., 2009; Gaskill and Khan, 1988), SEC (Otero et al., 1990; Regassa et al., 1991), and SED (Bayles and Iandolo, 1989) occurred between the exponential and the stationary phases of bacterial growth (Gaskill and Khan, 1988; Regassa et al., 1991; Zhang et al., 1998) suggesting that they could be regulated by *Agr*.

However, it was later shown that SEB, SEC and SED are regulated indirectly by additional factors. A summary of the factors involved in expression of these enterotoxins is illustrated in Table 6.

Table 6: S. aureus enterotoxins (SEB, SEC SED) regulated by *Agr, SarA, σB, Rot*, and *SaeRS* modified from (Zeaki et al., 2019).

Enterotoxins	*Agr*	*SarA*	*σB*	*Rot*	*SaeRS*	Reference
SEB	+/0	+	-	-	+	(Compagnone-Post et al., 1991; Kusch et al., 2011; Regassa et al., 1991; Schmidt et al., 2004; Tseng and Stewart, 2005)
SEC	+	+	+	nd	nd	(Chien et al., 1999; Regassa et al., 1991; Voyich et al., 2009)
SED	+/0	+	-	-	nd	(Sihto et al., 2016; Sihto et al., 2015; Tseng et al., 2004)

3.7.2.3 SEH

SEH seems to be produced predominantly in the late exponential phase of bacterial growth and it seems to be agr independent (Lis et al., 2012; Sakai et al., 2008). SEH was recently shown to be positively regulated by Rot, via direct binding to the seh promoter (Sato'o et al., 2015), σB (Kusch et al., 2011), several Sar homologs, and SaeR (Kusch et al., 2011; Sato'o et al., 2015).

SEIX

SaeRS appears to have a positive impact on SEX expression (Langley et al., 2017).

3.7.2.4 SEQ, SEK

SEK and SEQ, which are also found on *sea*-associated phages, can be transcriptionally induced by mitomycin C (Sumby and Waldor, 2003b).

3.7.2.5 EGC enterotoxins

The production of enterotoxins encoded in the *egc* operon (SEG, SEI, SEM, SEN, SEO, and SEIU) is highest in the earliest stages of exponential growth (Derzelle et al., 2009) and dependent on *σB* (Kusch et al., 2011).

3.8 Parameters influencing the production

Microorganisms in foods are affected by different parameters described as intrinsic factors (i.e., factors related to the food itself), extrinsic factors (i.e., factors related to the environment in which the food is stored), implicit factors (i.e., factors related to the microorganisms themselves), and finally processing factors (i.e., factors affecting the composition of the food and also the types and numbers of microorganisms that remain in the food after treatment) (Hamad, 2012). Food matrixes are a complex system where the mentioned factors interact to a great extent. The data available at present are results from laboratory experiments, where the conditions were ideal (Hennekinne, 2018). A summary of factor affecting enterotoxin production of *S. aureus* is presented in Table 7.

Table 7: Factors affecting enterotoxin production of Staphylococcus aureus (Hennekinne et al., 2012; Tatini, 1973)

	Optimum	Range
Temperature [°C]	37-45	10-45
pH	7-8	4-9.6
Water activity [a_W]	0.98	0.85 → 0.99
NaCl [%]	0	0-10
Redox potential [E_h]	> +200mV	< -100 mV to > +200 mV
Atmosphere	Aerobic (5-20% dissolved O_2)	Anaerobic-aerobic

3.8.1 Water activity (a_W)

S. aureus can grow at a wide a_w range, wider than most of the food-associated pathogens. *S. aureus* can grow at a minimum a_W of 0.83 (equivalent to about 20% NaCl) and has an optimum at an a_w of >0.99. The conditions for SE production are slightly different that the ones of growing and depend on the type of SE (Hennekinne, 2018).

SEA and SED production occurs under nearly all a_w conditions allowing growth of *S. aureus* if all other conditions are optimal. Production of SEB is overly 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 on SEB production (Ewald and Notermans, 1988; Qi and Miller, 2000). On the other side, SEE was found in media containing 10% NaCl (this concentration corresponds to a_w 0.92) (Thota et al., 1973; Troller and Stinson, 1975).

3.8.2 pH

Most staphylococcal strains grow at pH values between 4 and 10, with the optimum being 6–7. When other cultural parameters become nonoptimal, the pH range tolerated is reduced (Hennekinne, 2018). For example, the lowest pH that permitted growth and SE production by aerobically cultured *S. aureus* strains was 4.0, whereas 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). Most *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 (Bergdoll, 1989; Smith et al., 1983; Tatini, 1973).

3.8.3 Redox potential

S. aureus is a facultative anaerobic bacterium, which grows best in the presence of oxygen. Under anaerobic conditions, however, growth is much slower, and even after several days, cell numbers do not reach those attained under aerobic conditions (Hennekinne, 2018). Thus, aerated cultures produced approximately tenfold more SEB than cultures incubated in an atmosphere of 95% N_2+ 5% CO_2 (Hennekinne, 2018). 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 as 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 fewer SEA was produced than under aerobic conditions, but in both cases toxin was detected after 120 min of incubation (Belay and Rasooly, 2002).

3.8.4 Temperature

S. aureus grows between 7 and 48°C, temperature being optimal at around 37°C. The effect of temperature depends on the strain tested and on the type of the growth medium. In a 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. SE formation at 10 °C was reported by Tatini (1973) 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 (Hennekinne, 2018). S. aureus was completely inactivated in milk after application of the following temperature/time conditions: 57.2°C/80 min, 60.0°C/24 min, 62.8°C/6.8 min, 65.6°C/1.9 min, 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 aw between 0.70 and 0.80, resistance begins to decline (Troller and Stinson, 1975).

3.8.5 Nutritional factors and bacterial antagonism

Growth of *S. aureus* and SE production is also influenced by nutritional factors. *S. aureus* does not grow well in the presence of a competitive flora (Hennekinne, 2018). Its inhibition is mainly because of acidic products, lowering the pH, production of H_2O_2, or other inhibitory substances such as antibiotics, volatile compounds, or nutritional competition (Genigeorgis, 1989; Haines and Harmon, 1973). 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 (Genigeorgis, 1989; Smith et al., 1983). Starter cultures used in the production of fermented milk products such as cheese, yoghurt, buttermilk, and others can effectively prevent growth of *S. aureus* and SE formation. In the case of a failure of these cultures, however, the pathogen will not be inhibited and the product may be hazardous (Hennekinne, 2018).

3.9 Legal regulations

In food industry, food safety is ensured by preventative measures adapted to the risks linked to a specific product. These measures rely on the principles of good hygienic practices (GHP) as well as the Hazard Analysis and Critical Control Point (HACCP) system. HACCP was originally established by the Pillsbury company, NASA, and the US army to ensure the safety of food products intended for consumption in space (Bauman, 1995). Nowadays, all food business operators, except the primary producers, must implement HACCP. Good hygienic practices apply to all food business operators, including primary producers. In the European union this is regulated under Regulation EC No 852/2004 (EC, 2004) and specific microbiological criteria for foodstuffs are defined in commission regulation EC No 1441/2007 (EC, 2007) (Table 8). In Switzerland these regulations are both part of the Hygieneverordnung (EDI, 2016). In the Hygieneverordung (EDI, 2016) and in EC No 1441/2007 (EC, 2007) the criteria are divided into process hygiene criteria and food safety criteria. Process hygiene criteria indicate if the production process is performed in a good hygienic manner. These criteria define maximum cell density levels of coagulase-positive staphylococci permitted in food, and they apply during or at the end of the manufacturing process, depending on the food category. Food safety criteria define the acceptability of a foodstuff in terms of its microbiological safety, and they apply during the shelf life of a foodstuff. These criteria define that SEs must not be detected in 25 g of food in any of the sample units. Microbiological criteria for *S. aureus* counts and enterotoxin detection in foodstuffs are essential to ensure food safety. However, there are several restrictions to these criteria. Firstly, the number of *S. aureus* cells is not always a good indicator for the presence of enterotoxins since not all S. aureus strains are enterotoxigenic or express enterotoxins. In addition, even if most *S. aureus* cells were destroyed e.g. by heat treatment, the heat resistant enterotoxins might still be biologically active and could cause food poisoning. According to regulation an enterotoxin should only be analysed if values $>10^5$ CFU/g are detected, recent studied show that even in samples with lower concentrations, enterotoxins could be detected (Johler, Weder, et al., 2015; Schwendimann, Berger, et al., 2020).

Secondly, detection of enterotoxins is complex and standard detection methods are limited to classical enterotoxins (SEA–SEE). This is particularly critical since non-classical enterotoxins seem to be involved in foodborne outbreaks (see 3.6). In addition the regulation says that enterotoxins have not to be detected in 25 g, without specifying which enterotoxin.

Last but not least according to EC No 852/2004 (EC 2004) and HyV (EDI, 2016) staphylococcal tests of cheese must be conducted at the time when it is assumed that the concentration will be highest. For hard and extra-hard cheese, this is usually before the process step “scalding” starts.

Table 8: Process hygiene and food safety criteria according to commission regulation (EC) No 1441/2007 and HyV 2016

Food category	Bacteria/toxin	Sampling plan		Limits		Analytical method	Stage were criteria applies	Action in case of unsatisfactory results
		n	c	m	M			
Cheeses, milk powder and whey powder	Staphylococcal enterotoxins	5	0	Not detected in 25 g		SN EN ISO 6888-1 or 2	Products placed on the market during their shelf-life	-
Cheeses made from raw milk	Coagulase-positive staphylococci	5	2	10^4 cfu/g	10^5 cfu/g	EN/ISO 6888-2	At the time during the manufacturing process when the number of staphylococci is expected to be highest	Improvements in production hygiene and selection of raw materials. If values > 10^5 cfu/g are detected, the cheese batch has to be tested for staphylococcal enterotoxins.
Cheeses made from milk that has undergone a lower heat treatment than pasteurisation and ripened cheeses made from milk or whey that has undergone pasteurisation or a stronger heat treatment	Coagulase-positive staphylococci	5	2	100 cfu/g	1 000 cfu/g	EN/ISO 6888-1 or 2		
Unripened soft cheeses (fresh cheeses) made from milk or whey that has undergone pasteurisation or a stronger heat treatment	Coagulase-positive staphylococci	5	2	10 cfu/g	100 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	
Milk powder and whey powder	Coagulase-positive staphylococci	5	2	10 cfu/g	100 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	
Shelled and shucked products of cooked crustaceans and molluscan shellfish	Coagulase-positive staphylococci	5	2	100 cfu/g	1 000 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	Improvements in production hygiene

CPS = Coagulase positive staphylococci; SEs = Staphylococcal enterotoxins; n = number of units comprising the sample; c = maximum allowable number of sample units with values between m and M

4 Staphylococcal enterotoxin in cheese

To link the different topics mentioned in the previous chapters and to give an outlook over practical applications of the scientific results, a special sight is given to the topic of staphylococcal enterotoxins in cheese.

The production of cheese and other milk products is culturally and economically particularly important in France and Switzerland. In consequence the fact that cheese, especially fresh and soft cheese made from raw milk, are still involved in SFPO remains an important topic in these countries (Cremonesi et al., 2007; European Food Safety et al., 2019). The enterotoxins in cheese often originates from raw milk from cows with *Staphylococcus aureus* mastitis (Schmid et al., 2009). However, poor food safety practices and poor personal hygiene can also be a source of contamination (Hennekinne et al., 2012).

In the alpine region, especially in the case when the contamination with *S. aureus* happens because of cow's mastitis, two genotypes seem to be predominant: genotype B (GTB) and genotype C (GTC), representing 81% of the isolates (Fournier et al., 2008). In the case of *S. aureus* GTB, up to 87% of cows per herd showed intramammary infection, therefore, *S. aureus* GTB is a contagious mastitis pathogen, which spreads easily among cows (Fournier et al., 2008; Graber et al., 2009; van den Borne et al., 2017). In contrast, *S. aureus* GTC and most of the other genotypes were detected only in individual cows (Cremonesi et al., 2007; Fournier et al., 2008; Graber et al., 2009).

Staphylococcal genotypes are highly associated with virulence gene patterns (Cosandey et al., 2016; Fournier et al., 2008). Various enterotoxin genes, including *sea*, *sed*, *sej*, and *ser*, have been identified in *S. aureus* GTB (Cosandey et al., 2016). This genotype was also shown to persist along the food chain and its enterotoxin genes were expressed and linked to foodborne poisoning outbreaks involving raw milk cheese (Hummerjohann et al., 2014; Kummel et al., 2016). In contrast, *S. aureus* GTC was typically positive for *sec*, *seg*, *sei*, and *tst*, (Fournier et al., 2008; Graber et al., 2009) and was not linked to outbreaks traced to raw milk cheese until now (Hummerjohann et al., 2014).

In cheese production, growth of *S. aureus* can occur prior to processing, as the raw milk used in production may be stored at 8–18° C for up to 24 h before use. During cheese production, temperature, pH, competitive pressure from starter flora, and lactose starvation influence the growth of *S. aureus* and enterotoxin formation. Salt stress may also play a role in *S. aureus* growth and enterotoxin formation, although in hard and semi-hard Swiss cheeses, CPS counts start to decline during pressing or a few days after brining when the salt concentration in the cheese is still low (Bachmann, 1994). Interactions between these parameters may enhance enterotoxin production (Duquenne et al., 2016; Jakobsen et al., 2011; Walcher et al., 2014).

Specific studies were performed in the last years regarding the survival and growing of *S. aureus* as well as its enterotoxin production. Duquenne et al. (2016) reported that temperature (between 32 and 38° C) and time (15 to 45 min) were key parameters in controlling SE production during the cheese-production process. On the other side Schwendimann et al. (2020) reported the presence of enterotoxin in cheese containing 10^3 CFU/g (sample taken before salting) and after applying a scalding temperature of 56 °C. Other studies also show how bacteria like *Lactococcus lactis* (commonly used as starter culture) if added to milk may affect *S. aureus* growth and viability (Cretenet et al., 2011).

5 Methods for characterisation of S. aureus, their enterotoxin expression and protein production

5.1 Real-time PCR

The development of real-time PCR took place in 1992 (Higuchi et al., 1992). Real-time PCR is able to measure the accumulation of the amplicon instead of making an end point detection as a normal PCR does (Josefsen et al., 2012). The advantages of real-time PCR are its sensitivity and specificity but also the short duration of the analysis, the reduction of carryover contamination and the reduction of the use of toxic substances like ethidium bromide (Josefsen et al., 2012).

In real-time PCR a fluorescent reporter is introduced that binds to the PCR amplicon and reports its presence. Its presence is expressed by the fluorescence of the dyes or the probes. This fluorescence is proportional to the amount of product formed and it is monitored during the whole run (Kubista et al., 2006).

In the initial cycles the signal of the fluorescence is weak and cannot be distinguished from the background. If the target DNA is present the amount of fluorescence accumulates in subsequent reaction cycles and a signal develops that increases exponentially, until it reaches saturation (Kubista et al., 2006).

The time that is needed to reach a particular threshold fluorescence level differs depending on the amount of target sequence present at the beginning of the reaction. The number of cycles required to reach the threshold is called Ct-Value. The definition of the threshold is either chosen by the applied software, based on different methods and algorithms, or by the user (see Figure 17) (Josefsen et al., 2012; Kubista et al., 2006).

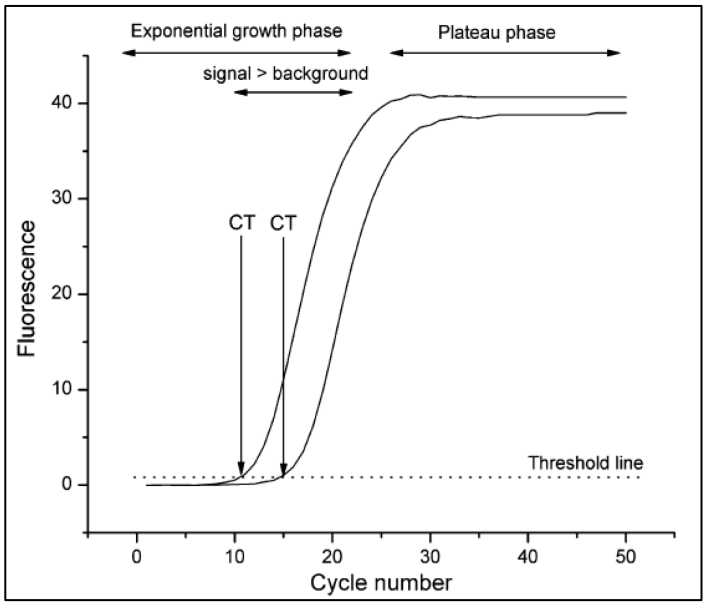

Figure 17: Summary of real-time PCR response curves. A threshold level has to be set sufficiently high to differ from the background. The number of cycles required to reach threshold, Ct- or Cp-Value are assessed (Kubista et al., 2006)

A variety of different DNA binding dyes and probes chemistry was developed for real-time PCR with the goal to provoque a change in emission of fluorescence during accumulation of the amplicon (Josefsen et al., 2012). One of the DNA binding dyes is Sybr Green. Sybr Green is an asymmetric cyanine which becomes fluorescent upon intercalating into DNA (Kubista et al., 2006). The most widely used probe chemistry is the TaqMan® probe. This is a linear target-specific probe that was designed to complement the sequence in between the forward and the reverse primers. These probes are labelled at the 5' end with a fluorescent reporter dye and at the 3' end with a quencher. When the primers are extended and the probe is encountered, part of the probe will be hydrolysed by the 5'-3' exonuclease activity of the DNA polymerase, separating the reporting dye from the quencher and allowing the emission of fluorescence (Josefsen et al., 2012).

Real-time PCR also found its application in identification and characterisation of S. aureus. Methods were developed for detection of S. aureus in milk by detecting the nuc gene (Graber et al., 2007) or for detection of the specific genotype B S. aureus (Sartori et al., 2017). For the detection of the staphylococcal enterotoxin genes also numerous studies were published (Bania et al., 2006; Boss et al., 2011; Chieffi et al., 2020)

5.2 Combined methods: PCR and sequencing

Combined methods are used in *S. aureus* analysis for subtyping the different strains. Multiple methods are available for this purpose. As these methods are not based on the detection of a single gene or genetic sequence, real-time PCR cannot be applied. These methods are often a combination of an end-point PCR and a consequent sequencing of some specific loci. In this chapter selected methods are presented.

5.2.1 MLST

Multi-locus sequence typing (MLST) is currently considered to be one of the most popular typing methods to study the molecular evolution of *S. aureus* (Deurenberg and Stobberingh, 2008; Feng et al., 2008). It is based on the sequence analysis of fragments of seven *S. aureus* housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi, and yqiL*), which are about 500 bp in length (Enright et al., 2000). The alleles resulting from the analysis of these genes build an allelic profile of the strain, which is also defined as sequence type (ST). In the pubMLST database (Jolley et al., 2018), the sequence types (STs) from MLST results can be used to allocate each strain to a clonal complex (CC). Clonal complex are suitable to study the evolutionary events within a *S. aureus* population (Deurenberg and Stobberingh, 2008).

5.2.2 Genotyping

Ribosomal spacer PCR (RS-PCR) was originally described by Jensen et al. (1993) as a rapid technique for the identification of pathogenic bacteria belonging to the genera *Listeria*, *Staphylococcus, Salmonella*, and other related species. In particular, strains of the genus *Staphylococcus* showed most significant intra- and interspecies variations in their spacer amplification products (Jensen et al., 1993). Based on this, Fournier et al. (2008) developed a rapid genotyping method for bovine *S. aureus* strains, characterized by high discriminatory power. The procedure is based on PCR amplification of the intergenic spacer region between the 16S and the 23S genes of the rRNA genetic loci, using a unified set of primers (Fournier et al., 2008; Jensen et al., 1993). The high variability in sequence and length characterizing this intergenic region enables the accurate discrimination of different subtypes of *S. aureus* (Fournier et al., 2008). After PCR amplification, amplicons are separated according to their size using a miniaturized electrophoresis system (DNA 7500 Chip, Agilent Technologies) resulting in a plot of corresponding peaks (electropherogramm), which can be translated into a pseudogel by a particular software. Results are then evaluated on a personal computer and genotypes are defined by calculating the corresponding Mahalanobis distance of informative peak sizes and comparing it to those of the reference strains (Syring et al., 2012).

5.2.3 *Spa* Typing

The spa-typing method can be used for both molecular evolution studies and the investigation of hospital outbreaks of S. aureus (Deurenberg and Stobberingh, 2008). The method relies on sequencing of the polymorphic X region of the gene encoding the protein A of S aureus (spa), which mainly consists of repeats of 24 bp in length (Deurenberg and Stobberingh, 2008). The spa gene variability can be the result of spontaneous mutations, as well as deletions or duplications occurring within the repeats (Deurenberg and Stobberingh, 2008; Harmsen et al., 2003). The Ridom StaphType software is used for data analysis and after a quality control of the constructed consensus sequence, spa-types are deduced from the order of specific repeats, to which alpha-numerical codes are assigned from the software as described by Harmsen et al. (2003).

5.3 Gene expression measurement (RT-qPCR)

For analysis of the expression of a gene, reverse transcriptase real-time PCR (RT-qPCR) is the ideal tool to investigate the transcriptome on RNA level (Schnell and Mendoza, 1997). Most gene expression studies quantify mRNAs, but research on other RNA classes such as non-coding RNAs is also evolving rapidly. The first practical real-time PCR technology, the 5’ -nuclease assay, was established in 1993 and combined the exponential PCR amplification

of a specific transcript with the monitoring of newly synthesized DNA in each performed PCR cycle. It was the most sensitive method for the detection and quantification of RNA gene expression levels, in particular for low abundant transcripts (Schnell and Mendoza, 1997).This method allowed RNA quantification from tissue samples with minor RNA concentrations, and the detection of small changes in mRNA or microRNA expression levels. While real-time RT-PCR has tremendous potential for analytical and quantitative applications, a comprehensive understanding of its underlying principles is important. The fidelity of RT-qPCR is associated with its true specificity, sensitivity, reproducibility, robustness and if performed MIQE compliant as a fully reliable quantitative method. It suffers from the problems inherent in the two single steps, the RT and the PCR, itself (Pfaffl et al., 2019).

For RT-qPCR different single steps are necessary (***Figure 18)***, these are described on the following pages.

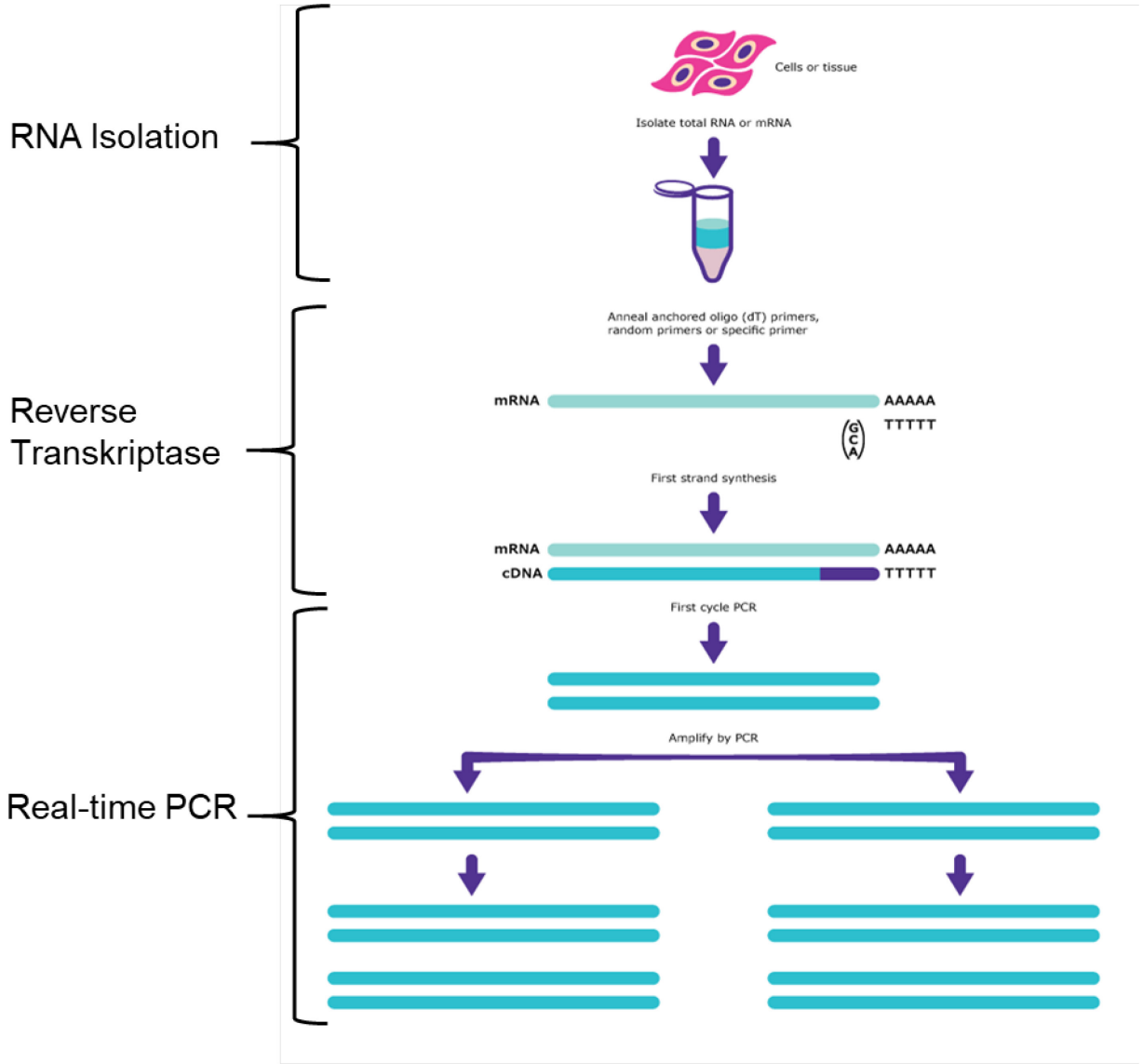

Figure 18: Summary of the processes in reverse transcriptase real time PCR (RT-qPCR). Modified according to www.sigmaaldrich.com

5.3.1 RNA extraction

RNA extraction is the first step in RT-qPCR. In this step the total RNA is extracted from the samples. In order to do so, different commercially available kits can be applied. In particular DNA rich samples an additional Dnase step needs to be performed, to eliminate DNA from the sample (Taylor et al., 2010).

The integrity of the purified RNA, after RNA extraction, is critical to all gene expression analysis and profiling techniques. For successful and reliable diagnostical use, RT-PCR needs high quality, DNA-free, and non-degraded RNA

(Becker et al., 2010). Accurate quantification and quality assessment of the starting RNA sample is particularly important for absolute quantification methods that normalize specific mRNA expression levels against total RNA ("molecules/g total RNA" or "transcript concentrations/g total RNA").

In order to check the purity of RNA according to the applied MIQE guideline (Taylor and Mrkusich, 2014) RNA integrity (RIN > 7.0) and RNA purity (A_{260}/A_{280} > 1.8) should be measured.

5.3.2 Reverse transcription

The extracted RNA is then transcribed into complementary DNA (cDNA). These steps can be combined with qPCR (one step) or performed singularly (two steps), depending on the requirements of the user (Figure 19). For the reverse transcription different types of primers can be used, again the application is depending on the user's requirements, following options are possible: Oligo(dT)s or anchored oligo(dT)s), random primers and Sequence Specific Primers (Taylor and Mrkusich, 2014).

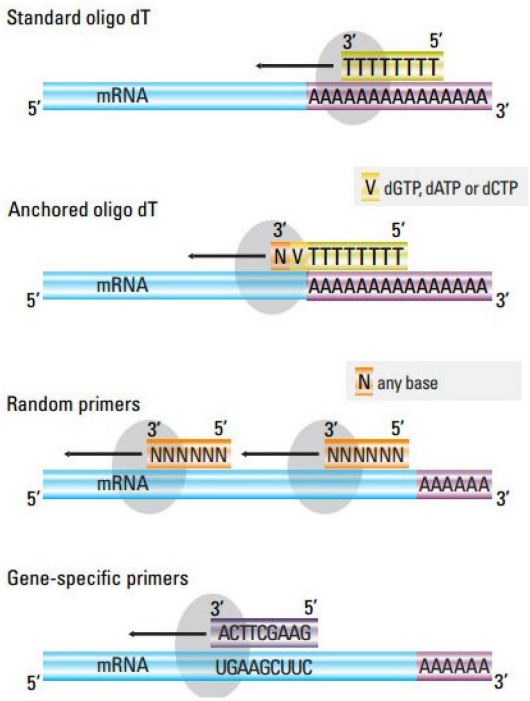

Figure 19: Four different priming methods for the reverse transcription step in two-step assays of RT-qPCR (www.thermofisher.com).

To confirm the absence of residual DNA, a minus-RT should be included in the experimental setting, as recommended by the MIQE guidelines. Additionally, it may be necessary to treat the RNA sample with commercially-available RNAse-free DNAse to get rid of any unwanted residual cellular DNA (Taylor and Mrkusich, 2014).

The RT step is the major source of variability in a real-time RT-PCR experiment, and the specific reaction conditions must be optimized for each RT enzyme. It cannot be assumed that different reaction setups have an identical cDNA synthesis efficiency. Therefore, the result can be highly variable during multiple RT reactions (Ståhlberg, Kubista, et al., 2004; Ståhlberg, Pfaffl, et al., 2004). According to literature to circumvent the high inter-assay variations in RT, target gene-unspecific primers, for example, random-hexamer, octamer, or -decamer primers should be used to synthesize the cDNA pool (Pfaffl et al., 2019).

5.3.3 Real-time PCR

Real-time PCR has already been described in chapter 5.1. For the application in RT-qPCR some specific considerations need to be considered. The amount of amplified target is directly proportional to the number of target molecules in the sample, but only during the "exponential amplifying phase" of the PCR reaction. Hence the key factor in the

quantitative ability of RT-qPCR is that it measures the product of the target gene only during this phase. Since data acquisition and analysis are performed in the same tube, this increases sample throughput, reduces carryover contamination and removes post-PCR processing as a potential source of error. In contrast, there is no direct relation of DNA input to amplified target during the plateau phase of the PCR; therefore, classical RT-PCR assays have to be stopped in late exponential and at latest in the linear phase. The exponential range of amplification has to be empirically determined for each transcript by amplifying equivalent amounts of cDNA over various PCR cycles or by amplifying dilutions of cDNA over the same number of PCR cycles (Pfaffl et al., 2019).

5.3.4 Quantification strategies

Two general quantification strategies can be performed in RT-qPCR: The levels of expressed genes may be measured by “absolute quantification” or by “relative quantification” (Figure 20). Absolute quantification relates the PCR signal to input copy number using a calibration curve, while relative quantification measures the relative change in mRNA or microRNA expression levels (Pfaffl, 2006). The reliability of an absolute real-time RT-PCR assay depends on the condition of identical amplification efficiency for both the native target and the calibration curve in RT reaction and following qPCR. Nowadays, relative quantification is more commonly performed than absolute quantification because there is no need for a calibration curve. It is based on the expression levels of a target gene versus a reference gene, usually a classical housekeeping gene.

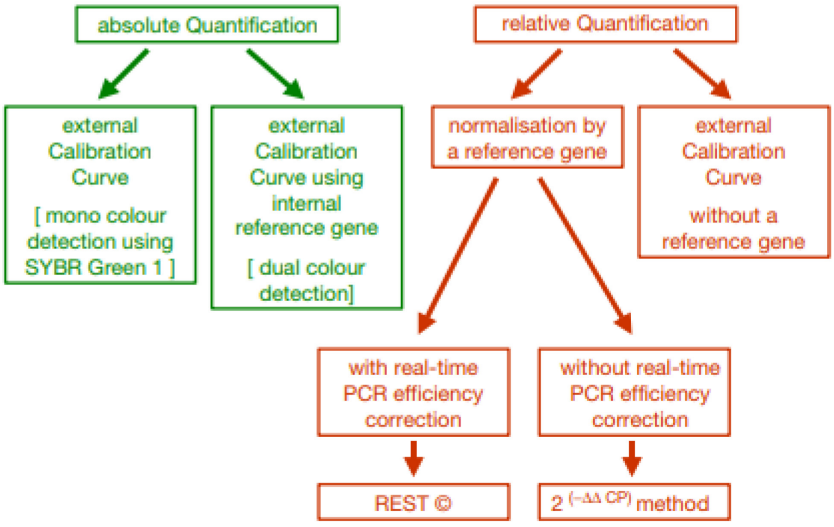

Figure 20: Absolute and relative quantification strategies in real-time RT-qPCR (Pfaffl et al., 2019).

5.3.5 Normalization of expression results

The reliability of any relative RT-PCR expression can be improved by including an invariant endogenous control in the assay to correct for sample-to-sample variations in RT-PCR efficiency and errors in sample quantification (Pfaffl et al., 2019). A biologically meaningful reporting of target mRNA copy numbers requires accurate and relevant normalization to some standard and is strongly recommended (Vandesompele et al., 2002; Vandesompele et al., 2009). But the quality of normalized quantitative expression data cannot be better than the quality of the normalizer itself. Any variation in the normalizer will obscure real changes and produce artificial changes. Real-time RT-PCR-specific errors in the quantification of RNA transcripts are easily compounded with any variation in the amount of starting material between the samples, for example, caused by sample-to-sample variation, variation in RNA integrity, differences in RT efficiency and cDNA sample loading variation (Taylor and Mrkusich, 2014). This is especially relevant when the samples have been obtained from different individuals, different tissues and different time courses and will result in the misinterpretation of the derived expression profile of the target genes. Therefore, normalization of target gene expression levels must be performed to compensate intra- and inter- RT-qPCR variations. Data normalization can be carried out against an endogenous unregulated reference gene transcript or against total cellular DNA or RNA content (molecules/g total DNA/RNA and concentrations/g total DNA/RNA). Normalization based on the amount of total cellular RNA content is increasingly used, but little is known about the total RNA content of cells or even about

the mRNA concentrations. The content per cell or per gram of tissue may vary in different tissues (*in vivo*) and cell cultures (*in vitro*), between individuals and under different experimental conditions (Pfaffl et al., 2019).

5.3.6 Application in measurement of the staphylococcal enterotoxin expression

RT qPCR has been widely applied for detection of enterotoxin gene expression. It has been used for analysing the expression of *se* under different stress parameters (Sihto et al., 2016; Sihto et al., 2015; Wallin-Carlquist et al., 2010; Zeaki et al., 2015) or by addition of mitomycin (Cao et al., 2012). These studies were mostly performed on classical enterotoxins, even they were also performed for the non-classical enterotoxins (Derzelle et al., 2009; Omoe et al., 2002; Sato'o et al., 2015)

Gene expression has also already be performed in different food matrices like pork sausages (Zeaki et al., 2014), boiled ham (Susilo et al., 2017), meat product (Sato'o et al., 2015), milk (Valihrach et al., 2014), and cheese (Duquenne et al., 2016; Duquenne et al., 2010).

5.4 Whole genome sequencing

Genome sequencing has started in the 1970-1980 when manual methods were used like Sanger and Maxam Gilbert sequencing. With these techniques whole bacteriophages and animal viral genomes were sequenced (Alberts et al., 2008). In the 90' faster and automated techniques allowed sequencing of bacteria and eukaryota. The first bacteria to be fully sequenced was *Haemophilus influenza* in 1995 (Fleischmann et al., 1995).

Microbial genome sequencing has become mainstream in the field of food microbiology due to the increasing affordability and improvements in the speed of sequencing and quality of the data. This was a consequence of the advancements in sequencing technologies collectively known as next generation sequencing (NGS). NGS encompasses both massively parallel and single-molecule sequencing which provides short and long sequencing reads, respectively. Short-read sequencing is highly accurate and produces read lengths of 100–300 bp which are then assembled into incomplete or so called, draft genomes (Jagadeesan et al., 2019). Complete genomes cannot be generated from the short reads obtained in a single sequence run due to difficulties in assembling repetitive regions and large genomic rearrangements such as insertions, deletions and inversions (Jagadeesan et al., 2019). For many applications, including comparative genomics and phylogeny, this is not an issue but where complete genomes are required and for determining complex genomic regions, longer reads are necessary. Long-read sequencing produces reads from 10 to 50 Kb in length, but this is at the cost of higher error rates (Loman and Pallen, 2015). Currently, microbial DNA sequencing can be performed on a variety of platforms Table 9.

Table 9: Summary of commonly used Whole Genome Sequencing platforms (Jagadeesan et al., 2019).

Plattform	Sequencing Technology	Read length	Output/run	Error rate	Example of use	Type of instrument and run time
Illumina	Sequencing by synthesis	Short reads 1 × 36bp – 2 × 300bp	0.3–1000Gb	Low	Variant calling	Benchtop 2–29 h
Ion Torrent	Sequencing by synthesis	Short reads 200-400bp	0.6–15Gb	Low	Variant calling	Benchtop 2–4 h
PacBio	Single molecule sequencing by synthesis	Long reads Up to 60kb	0.5–10Gb	High	De novo assembly of small bacterial genomes and large genome finishing	Large scale 0.5–4 h
Oxford Nanopore	Single molecule	Long reads up to 100kb	0.1–20Gb	High	Complete genome of isolates and metagenomics	Portable 1min-48 h

WGS of microbial pathogens has been introduced into public health surveillance relatively rapidly compared to previous methodological advancements, with reports of its use from early adopters from 2011 onward (Lienau et al., 2011; Rall et al., 2012). First it was used in retrospective analysis of foodborne outbreaks. Nowadays it becomes a powerful tool for prospective analysis and surveillance of bacterial foodborne pathogens (Allard et al., 2018; Ashton et al., 2016; Jackson et al., 2016; Moura et al., 2016). WGS is being introduced as a replacement technology, i.e. it will replace most current identification and characterization methods in the microbiology laboratory such as serotyping, virulence profiling, antimicrobial resistance determination and previous molecular typing methods. In a public health setting replacing the plethora of traditional microbiological identification and typing methods with a single efficient analytical WGS workflow makes implementation cost-effective as well as more accurate. Actionable data on public health can be collected faster than previously (Grant et al., 2018).

Analysis of WGS data by either approach is a complex process in which multiple steps are combined to produce final results, such as SNP or allele matrices and phylogenetic trees (Timme et al., 2017). The large amount of data generated in WGS brings challenges for its analysis (Deurenberg et al., 2017; Wyres et al., 2014). This has led to multiple software solutions being developed, mainly through academic endeavors, which in general require specialized knowledge and expertise to be deployed and operated. More recently commercially developed software have become available, bringing a user-friendly interface, allowing non-bioinformatics experts, with the appropriate training in both bioinformatics software and final WGS result interpretation, to conduct analyses. The commercial software may be expensive but since limited bioinformatics expertise is needed, it may nevertheless be a more cost-efficient solution for many users in food industry. Two main applications to analyze genomic data to determine the relatedness between strains, namely SNP-based and the gene by gene-based approaches (such as wgMLST and cgMLST) are available. In the SNP-based approach, sequencing reads are aligned or mapped to a known sequenced reference genome, and the nucleotide differences in both coding and non-coding regions determined (Davis et al., 2015). For each isolate, every SNP relative to the reference genome is recorded and then used to quantify the genetic relatedness between strains. CgMLST and wgMLST are an extension to traditional 7-loci multi-locus sequence typing (MLST), the genes in either a defined core genome (cgMLST) or the whole genome (wgMLST), which includes more variable accessory genes, are compared against a reference database of all known gene variants (alleles) for a particular species. CgMLST provides highly detailed phylogenetically relevant information about the genetic relatedness of a species. On the other side wgMLST provides even more discrimination than cgMLST and this can be valuable for cluster investigations to discriminate between closely related isolates. The genetic variation detected by SNP or gene-by-gene analysis can be used to infer phylogenetic relationships between bacterial isolates usually displayed in the form of a phylogenetic tree. The tree represents the calculated evolutionary model (obtained using different possible tree inference algorithms such as parsimony, maximum likelihood, and Bayesian or distance methods) of the isolates as a series of branches from the root or common ancestor. The isolates clustered together near the leaves of the tree are more closely related than other isolates elsewhere in the tree (Jagadeesan et al., 2019).

About application of whole genome sequencing in *S. aureus* can be read in chapter 2.2.

5.5 Immunoassays

Immunoassay is an analytical method that is widely applied in many fields, including pharmaceutical analysis, toxicological analysis, bioanalysis, clinical chemistry, and environmental analysis based on specific recognition between antigens and antibodies. Immunoassays are extremely sensitive and specific, rapid to operate, and can be used to detect SEs in complex samples without extensive pre-treatment. Combining antibodies as a recognition component with an appropriate transducer formed biosensor called immunosensor. In immunosensor, sensing elements play an important role in the detection process and are basic devices giving an output in the form of measurable energy that is correlated with the input quantity. According to the applied transduction patterns, these sensors can be classified into three main types: (1) optical detection techniques, (2) electrochemical detection techniques and (3) mass detection techniques (Wu et al., 2016).

Colorimetric immunoassays are belonging to the optical detection techniques and are the most common ones. They determine analytes by comparing or measuring the absorbance of a colorful substance. The transducer moiety is a key component of colorimetric immunoassays that affects performance with respect to sensitivity, specificity, response time, and the signal-to-noise ratio, due to its function of translating the detecting behavior into light absorption

ranging from 390 to 750 nm, characterized by an eye-sensitive color change. Enzyme-linked immunosorbent assay (ELISA) is a widely used colorimetric method. ELISAs are commonly performed by immobilizing artificial antigens or capturing antibodies on plastic supports. The antigen captured by the antibody support can be detected either using an enzyme-labelled antibody that is specific for the same determinant as the capture antibody or by an enzyme-labelled antibody recognizing a different epitope on the captured, multivalent antigen. Quantification of antigens is achieved by monitoring the cleavage of the chromogenic substrate (e.g., 3,31,5,51-tetramethylbenzidine (TMB)) by the enzyme (e.g., horseradish peroxidase (HRP)), which produces a blue metabolite for signal detection. These methods have many advantages, such as being easy to use, specific, and applicable for high throughput screening. SEs are routinely assayed immunologically by ELISA (Wu et al., 2016).

By looking in literature different immunological assays were developed in the last year for the non-classical enterotoxins like for SEG (Nagaraj et al., 2016), SEH (Schubert et al., 2016), SEI (Zhao et al., 2017a), SEK (Aguilar et al., 2014), SEM (Zhao et al., 2017b) and SEQ (Hu et al., 2017). Unfortunately, they are all not commercially available.

6 New literature from Agroscope about *S. aureus* enterotoxins

6.1 Growth of Staphylococcus aureus, staphylococcal enterotoxin formation, and the effect of scalding temperature during the production of Alpine cheese in a laboratory cheese-making model

6.1.1 Reference

Schwendimann, L.; Berger, T.; Graber, H.U.; Meier, S.; Hummerjohann, J.; Jakob, E. (2020). Growth of Staphylococcus aureus, staphylococcal enterotoxin formation, and the effect of scalding temperature during the production of Alpine cheese in a laboratory cheese-making model. *J Food Prot*, 83 (10): 1822–1828.

6.1.2 Abstract

To reduce the number of potential *S. aureus* contaminated cheese reaching consumers, European legislation stipulates that all cheese must be tested for coagulase-positive staphylococci (CPS) at the point in production when numbers are expected to be highest. If CPS counts exceed 10^5 CFU/mL, enterotoxin tests must be conducted. In the case the enterotoxin test shows positive results the cheese must be destroyed. Manufacturers of Swiss Alpine cheese are exempt from this legislation because enterotoxin formation in hard cheese is expected to be very unlikely, given the high scalding temperatures the cheese is exposed to during its production. Such temperatures result in inactivation of CPS in the curd. However, this assumption has not yet been scientifically demonstrated. Therefore, a laboratory-scale cheese production experiment was performed, in which the conditions corresponded with certain limitations to practical cheese-making conditions in terms of temperature and time exposure like in Gruyere or Tete de Moine Swiss type cheese. Raw milk aliquots (200 ml) were inoculated with five different strains of CPS, and scalding temperatures, ranging from 46–56° C, were applied during cheese production. The temperatures applied after pressing the curd aimed at reproducing the temperature curve in the peripheral zone of a real cheese wheel. Contrary to expectations, enterotoxin formation occurred and changed with the different scalding temperatures (52–56° C).The differences in enterotoxin formation were more associated with strain type rather than temperature. Based on these results, the mechanism of enterotoxin formation in cheese requires further study.

6.2 Staphylococcal enterotoxin gene cluster: prediction of enterotoxin (SEG and SEI) production and of the source of food poisoning based on *v*Saβ typing

6.2.1 Reference

Schwendimann, L.; Mérda, D., Berger, T.; Denayer, S.; Feraudet-Tarisse, C.; Kläui, A.; Messio, S.; Mistou, M.Y.; Nia, Y.; Hennekinne, J.A., Graber, H.U. (2021). Staphylococcal enterotoxin gene cluster: prediction of enterotoxin (SEG and SEI) production and of the source of food poisoning based on *v*Saβ typing. *Appl Env Micr.* 10.1128/AEM.02662-20.

6.2.2 Abstract

Currently only five (SEA-SEE) out of 26 known staphylococcal enterotoxins can be analyzed using commercially available kits.Six genes (*seg, sei, sem, sen, seo,* and *seu*), encoding putative and undetectable enterotoxins, are located on the enterotoxin gene cluster (*egc*) which is part of the *Staphylococcus aureus* genomic island *v*Saβ. These enterotoxins have been described as likely being involved in staphylococcal food poisoning outbreaks.

The aim of the present study was to determine if whole genome data can be used for the prediction of staphylococcal *egc* enterotoxin production, particularly enterotoxin G (SEG) and enterotoxin I (SEI). For this purpose whole genome sequences of 75 *Staphylococcus aureus (S. aureus)* strains from different origins (food poisoning outbreaks, human, and animal) were investigated applying bioinformatics methods (phylogenetic analysis using the core genome and different alignments). SEG and SEI expression was tested *in vitro* using a sandwich ELISA method.

Strains could be allocated to 14 different *v*Saβ types, each type being associated with a single clonal complex (CC). In addition the *v*Saβ type and CC were associated with the origin of the strain (human or cattle derived). The amount

of SEG and SEI produced also correlated with the *v*Saβ type and the CC of a strain. The present results show promising indications that the *in vitro* production of SEG and SEI can be predicted based on the *v*Saβ type or CC of a strain. Currently only five (SEA-SEE) out of 26 known staphylococcal enterotoxins can be analyzed using commercially available kits. Six genes (*seg, sei, sem, sen, seo,* and *seu*), encoding putative and undetectable enterotoxins, are located on the enterotoxin gene cluster (*egc*) which is part of the *Staphylococcus aureus* genomic island *v*Saβ. These enterotoxins have been described as likely being involved in staphylococcal food poisoning outbreaks.

The aim of the present study was to determine if whole genome data can be used for the prediction of staphylococcal *egc* enterotoxin production, particularly enterotoxin G (SEG) and enterotoxin I (SEI). For this purpose whole genome sequences of 75 *Staphylococcus aureus (S. aureus)* strains from different origins (food poisoning outbreaks, human, and animal) were investigated applying bioinformatics methods (phylogenetic analysis using the core genome and different alignments). SEG and SEI expression was tested *in vitro* using a sandwich ELISA method.

Strains could be allocated to 14 different *v*Saβ types, each type being associated with a single clonal complex (CC). In addition the *v*Saβ type and CC were associated with the origin of the strain (human or cattle derived). The amount of SEG and SEI produced also correlated with the *v*Saβ type and the CC of a strain. The present results show promising indications that the *in vitro* production of SEG and SEI can be predicted based on the *v*Saβ type or CC of a strain.

6.3 Behaviour of Staphylococcal *Egc* enterotoxins during bacterial growth and under Food Production-like stress conditions

6.3.1 Reference

Schwendimann, L., Berger, T., Denayer, S., Hennekinne, J.A., Ivanovic, I., Messio, S., Mistou, M.Y., Nia, Y., Graber, H.U. Behaviour of Staphylococcal Egc enterotoxins during bacterial growth and under Food Production-like stress conditions. *Unpublished manuscript.*

6.3.2 Abstract

According to the European Food Safety Authority (2019), 77 out of 114 outbreaks caused by staphylococcal enterotoxin are weak evidence outbreaks. However, only five out of over 25 enterotoxins can be analysed using commercially available kits. The presence of so-called ‘new enterotoxins’ cannot be identified. A group of these new enterotoxin genes – *seg, sei, sem, sei, seo* and *seu* – are located on the same enterotoxin gene cluster (*egc*) and might to be involved in staphylococcal food poisoning outbreaks.

The aim of the present study is to improve the understanding of the parameters and conditions under which *egc* enterotoxins are produced, which will lead to improved control of their expression during food production and storage. For this purpose, a selection of eight strains from different origins was chosen based on their genetic diversity (structure) and origin (human, animal, environment or food). The mRNA expression of *seg, sei, sem, sen* and *seo* was measured using RT-qPCR at three different points during the bacterial growing phase (start, mid-log and end-log). Based on these results, three strains were selected to study their enterotoxin expression under stress conditions: NaCl concentrations up to 100 g/L and high temperature (45°C). In addition, each sample was tested for staphylococcal enterotoxins G (SEG) and I (SEI) using an in-house sandwich ELISA method. The results showed that *egc* enterotoxins are mostly expressed in the mid-log phase of bacterial growth and switch off at the end of the log phase. Both SEG and SEI are produced at an early stage of the growing phase. Interestingly, NaCl content up to 20 g/L, a common condition of food production, did not affect the expression and production of *egc* enterotoxins. However, differences between strains were observed. The study gives insights into the production of *egc* enterotoxins under stress conditions. This information will enhance the availability of methods for controlling *egc* enterotoxins in food production and storage.

7 Discussion

The advances in whole genome sequencing and the decrease of the costs for sequencing in the past years lead to an increasing number of sequenced strains. To date sequencing data from 80'000 *S. aureus* strains can be found on NCBI. In recent years, also the number of strains sequenced coming from foodborne outbreaks are increasing (Chieffi et al., 2020; Merda et al., 2020). This data availability is an opportunity to generate new tools and methods for improving food safety. Large studies with great numbers of sequenced strains can be performed, making it easy to proof specific findings, especially if beside the genome also the isolate is available. Under these circumstances also the prediction of gene expression and protein synthesis, based on genomic data is a field that can be explored.

This is the case for *S. aureus* and its enterotoxins, as to date a lot of outbreaks are of weak evidence. Reason for that are multiple. On one side only for five out of 26 enterotoxins there are commercial detection methods available. In addition, few is known about the so called non-classical enterotoxins. For this reason new tools and methods such as WGS and new methods for enterotoxin measurements are needed in order to be able to analyse and measure the *S. aureus* strains and their enterotoxins and consequently, being able to predict the enterotoxin production based on genome data. With the present study a contribution can be given in this field since a group of the non-classical enterotoxins, the *egc* enterotoxins were studied at genome level (*seg, sei, sem, sen, seo, and seu*), at mRNA expression level (*seg, sei, sem, sen, seo*), and at protein level (SEG and SEI). By comparing the results from the different levels, models to predict SEG and SEI production based on genome data were found. They shed light on new insights into the conditions and limits of enterotoxin production under stress parameters such as NaCl and temperature.

7.1 The role of *egc* enterotoxins

Egc enterotoxins are present in about 50% of *S. aureus* strains. This percentage has been described in literature (Argudin et al., 2012; Jarraud et al., 2001; Smyth et al., 2005) and was also confirmed again in this study. Very recent studies also describe *egc* to be the most frequent virulence factor detected in MRSA and MSSA (Kwapisz et al., 2020). Beside the high prevalence, these enterotoxins are also quite particular as they are all located on a stretch forming a gene cluster. We observed that this cluster of enterotoxin genes shows no signs of recombination (unpublished data) and they seem to be on a stretch of paralogous genes. In the beginning of this study it was not clear if the gene expression of these enterotoxins is regulated together (single promoter at the start of the *egc*), or if each gene is regulated separately. With the results achieved in this study we can say that there are some signs indicating a regulation of the whole cluster, but it may be that also the promoters located just before the single enterotoxins also play a role. The whole *egc* is part of the mobile genetic element *v*Saβ, which was recently described to be a phage (Klaui et al. 2019). *V*Saβ was initially a prophage as it is still observed in *v*Saβ type IV and can still be mobilized. At the time around clone formation, the original *v*Saβ underwent multiple genetic changes resulting in the different *v*Saβ types observed today (Klaui et al. 2019).

In addition to the above-mentioned points *egc* enterotoxins express super antigenic and emetic activity. Emetic activity has been demonstrated on monkeys for SEG, SEI, SEM, SEN and SEO (Omoe et al., 2013). As emetic activity has not yet been demonstrated for SEU, this enterotoxin was not taken into consideration in our study. From a food safety point of view, literature (Johler, Giannini, et al., 2015) seems to show the involvement of these enterotoxins in foodborne outbreaks. Recent publications are also describing the super antigenic activity of these enterotoxins and they seem to have an effect particularly on human skin (Nowrouzian et al., 2017).

In the past studies of Agroscope the focus was on genotype B strains (SEA, SED, SEJ and SER) but with the aforementioned facts about the *egc* enterotoxins the focus of this study was changed to the *egc* enterotoxins, since not much is known about the expression of these enterotoxins and their involvement in SFPOs in Switzerland and France (Schwendimann et al. 2020).

7.2 *v*Saβ characterisation and subtyping of *egc* containing *S. aureus*

As soon as the WGS from the strain collection used in this study was ready, the characterisation of the *v*Saβ type was performed, as it seemed to be a good tool to subtype the strains. In addition, Klaui et al. (2019) showed its discriminatory power and the correlation between the *v*Saβ type and the clonal complex. In this study (Schwendimann et al. 2020) beside the *v*Saβ type already described in literature, we describe seven new types. As a consequence we think that, as the diversity of the clonal complex is varying, also the amount of *v*Saβ type will not be limited to 15. It is supposed that the number of *v*Saβ types will increase with additionally characterised *S. aureus* genomes and their *v*Saβ. By looking at the *egc* of the different *v*Saβ types we can see that they differ in the presence/absence of certain enterotoxins (*seu*), but sometimes also single genes are truncated or fragmented *(sen, seg, sem*). Also the single *egc* enterotoxin genes (especially *sem, seo* and *seu*) seem to have a large variability (sometimes < 90% similarity at amino acid level, Schwendimann et al. 2020), which was also described by Mérda et al. (2020). Strains having the same type of *v*Saβ have a similarity of 100% at amino acid level between them for all *egc* enterotoxins and the whole *egc*. In the past other subtyping methods for the *egc* cluster were used and described in literature (*egc* type 1-6) (Chieffi et al., 2020). With these methods different types of enterotoxins and their variants were used to define the *egc* type. Based on the new findings of this study we consider this method as too complicated (because of the different variants described) and with insufficient discriminatory power. Consequently, we propose to use *the v*Saβ typing as a tool to distinguish the different *egc* containing strains, when it considers this part of the genome as a whole and not the single enterotoxins. Using the here described method would also help to reorganise the pretty confusing *egc* nomenclature in use. Instead of using different variants like SE*l*U_1, SE*l*U_2, SE*l*W simply the type of *v*Saβ could be used. In addition, in some literature the presence of SE*l*V in the *egc* was described. This enterotoxin was not found in any of the genomes analysed in this study (Schwendimann et al. 2020) nor in the 156 genomes analysed by Mérda et al. (2020), which raises the question of the relevance of this enterotoxin. Overall I think that staphylococcal enterotoxins nomenclature and classification should be revised and newly organized since every year more studies are published describing new enterotoxins. In any case it should clearly be defined which of them are only variants of already described enterotoxins and which are completely new ones. This should be based on phylogenetic studies using the new bioinformatic tools developed in the recent years.

7.3 Prediction of enterotoxin production

After having characterized and allocated the strains to the 15 different types of *v*Saβ, the question raised if some clear differences could also be observed in enterotoxin production. At the time of the study an ELISA method for detection of SEG and SEI was available and by applying this method a subset of the strains (approx. three strains per *v*Saβ type) was analysed for their production of SEG and SEI (Schwendimann et al. 2020). We were surprised by the clear results as it could be observed and statistically demonstrated that some *v*Saβ types produced a “high” amount of SEG and SEI enterotoxins and others produced a “low” amount of them. These results and the correlation between clonal complex and *v*Saβ enabled us to create a tool to predict SEG and SEI enterotoxins based on WGS data. However, the reason for the existence of this correlation is not yet clear. We assumed that differences at the promoter level are responsible for it, depending on the *v*Saβ type. Deeper analysis of the promoter of the enterotoxins (data not included in this manuscript) showed different knowledge gaps due to the unknown TSS (transcriptional starting site) of the enterotoxins. Without knowing this site, we concluded that only presumptive promoters can be allocated and a comparison of presumptive promoters makes a comparison between *v*Saβ types difficult. A definition of TSS can only be done by performing RNA sequencing, what I assume needs to be done in future. Interestingly is also the fact that the two analysed enterotoxins (SEG and SEI) seem to follow the same expression pattern: when SEG is produced “high” also SEI is produced “high” and the same pattern for the low production level. The produced quantities of SEI are much higher than those produced of SEG. Due to this fact and due to the strong link between *v*Saβ and the clonal complex, I assume that there are some global regulation systems for the different *egc* enterotoxins in the *v*Saβ or somewhere else on the genome. They are probably correlated to the clonal evolution. Also with the approach of measuring the protein only, it was not clear if the differences in enterotoxin production are caused by differences in transcription or translation. In order to obtain more clarity in this question, the expression of the *egc* enterotoxins was measured.

7.4 Correlation between *egc* enterotoxin expression and production

Expression of *egc* enterotoxins was measured for a subset of strains using RT-qPCR (Schwendimann et al. unpublished manuscript). A method for the measurement of enterotoxin expression was developed for *seg, sei, sem, sen* and *seo*. As described in past studies (e.g. Derzelle et al. 2009), we confirmed that expression of *egc* enterotoxins seems to be highest at the mid exponential growing phase. This fact confirms that *egc* enterotoxins are non-*agr* dependent enterotoxins as otherwise they would have been expressed at a later point in the late exponential growing phase. Also at protein level SEG and SEI were surprisingly already measurable and quantifiable two hours after inoculation. For the analysis of the expression a subset of strains was chosen based on three criteria: 1) diverse in the genome, 2) different patterns of enterotoxin production (Schwendimann et al. 2020), and 3) were involved in foodborne outbreaks. Contrary to our expectations for the selected strains, no differences could be observed at the expression (mRNA) level between the strains, as the ones producing very low amounts of enterotoxin - G and I - had the same expression (mRNA) as the one producing high amounts of enterotoxins G and I. With these results I'm convinced that the *egc* enterotoxins are all expressed independently of the *v*Saβ type, but when it comes to translation from mRNA to protein a diversity in *v*Saβ/CC seems to play a role. In literature, reasons for differences in post-transcriptional steps are described to rely on translation efficiency. There are multiple causes for differences in translation efficiency such as differences in mRNA structure, differences in the Shine-Dalgarno sequence or regulation by some other RNA transcripts (Chiaruttini and Guillier, 2020; Li, 2015; Meyer, 2017). As there are some variations in the single enterotoxin genes between the different *v*Saβ I think that this could lead to some differences in RNA structure or in the Shine-Dalgarno sequence and therefore, leading to some differences in translation efficiency. Also the difference between SEG and SEI could be explained as a difference in translation efficiency. From a transcriptional point of view, as there are just minor differences between the single enterotoxins it is not yet clear if enterotoxin genes are all regulated by the same promoter, some single promoters or a combination of both.

No general conclusions on enterotoxin production can be made for enterotoxins for which a protein measurement method is not yet available like SEM, SEN and SEO: also in this case it could be that they are expressed, but maybe not translated into proteins. From literature we only know that SEM protein could be measured in strains from foodborne outbreaks (Zhao et al., 2017b).

7.5 Importance of *egc* enterotoxins for food safety

When starting this study it was not clear if *egc* enterotoxins play a relevant role in foodborne outbreaks. Only a few studies showed the possible involvement of these enterotoxins in SFPOs (Johler, Giannini, et al., 2015; Umeda et al., 2017). In this study (Schwendimann et al. 2020), the strains involved in SFPO showed their ability to produce SEG and SEI and even in "high" amounts. But it has to be noted that "high" amounts in this study, especially for SEI, are still much lower than the ones measured for SEA (Zeaki et al., 2015). Also the emetic activity is much higher (lower amounts needed) for SEA rather than for SEI (Ono et al., 2017). These facts raise the question if a single *egc* enterotoxin can be the cause for a foodborne outbreak or if it is the cumulative effect of different enterotoxins produced that causes foodborne outbreaks, because they potentially increase the severity of its single effects on human health. This is supported by the fact that all *egc* enterotoxins seem to be expressed and potentially also produced together (Schwendimann et al. unpublished manuscript). In addition and as mentioned in the previous chapter, the *egc* enterotoxins seem to be produced very fast (2 hours after inoculation), also under higher temperatures (45 °C) and high NaCl concentration (up to 100 g/L). These enterotoxins can be produced in a very wide range of conditions which are normally considered as critical for growing and enterotoxin production. Obviously there is a big impact for the production of cheese. Finally and not to underestimate is the fact that *egc* seems to be present in approx. 50% of the strains. These findings support the fact that *egc* enterotoxins may play a role in foodborne outbreaks. At the moment an *egc* enterotoxin caused SFPO would fall into the category of weak evidence outbreak, as no laboratory would be able to measure *egc* enterotoxins from the strains nor in the food. The inability of measuring these and other staphylococcal enterotoxins is controversy to the fact that according to the Swiss and European law Staphylococcal enterotoxins should not be present in foods. A very disappointing situation that should be solved by further research and invention.

7.6 Ideas for outbreak investigation with suspected involvement of *egc* enterotoxins

Considering the fact that *egc* enterotoxins seem to be more involved in SFPO than expected also new strategies and tools should be developed to determine *egc* enterotoxins caused outbreaks. As already mentioned in the previous chapter, in my opinion the whole *egc* should be considered as a unity from an analytical and epidemiological point of view. This perspective would simplify the analytical procedure and the full investigation of *egc* enterotoxin caused outbreaks (Figure 21). Linking together all new insights of this study leads to a suggestion on how an *egc* caused outbreaks could better be clarified in the future.

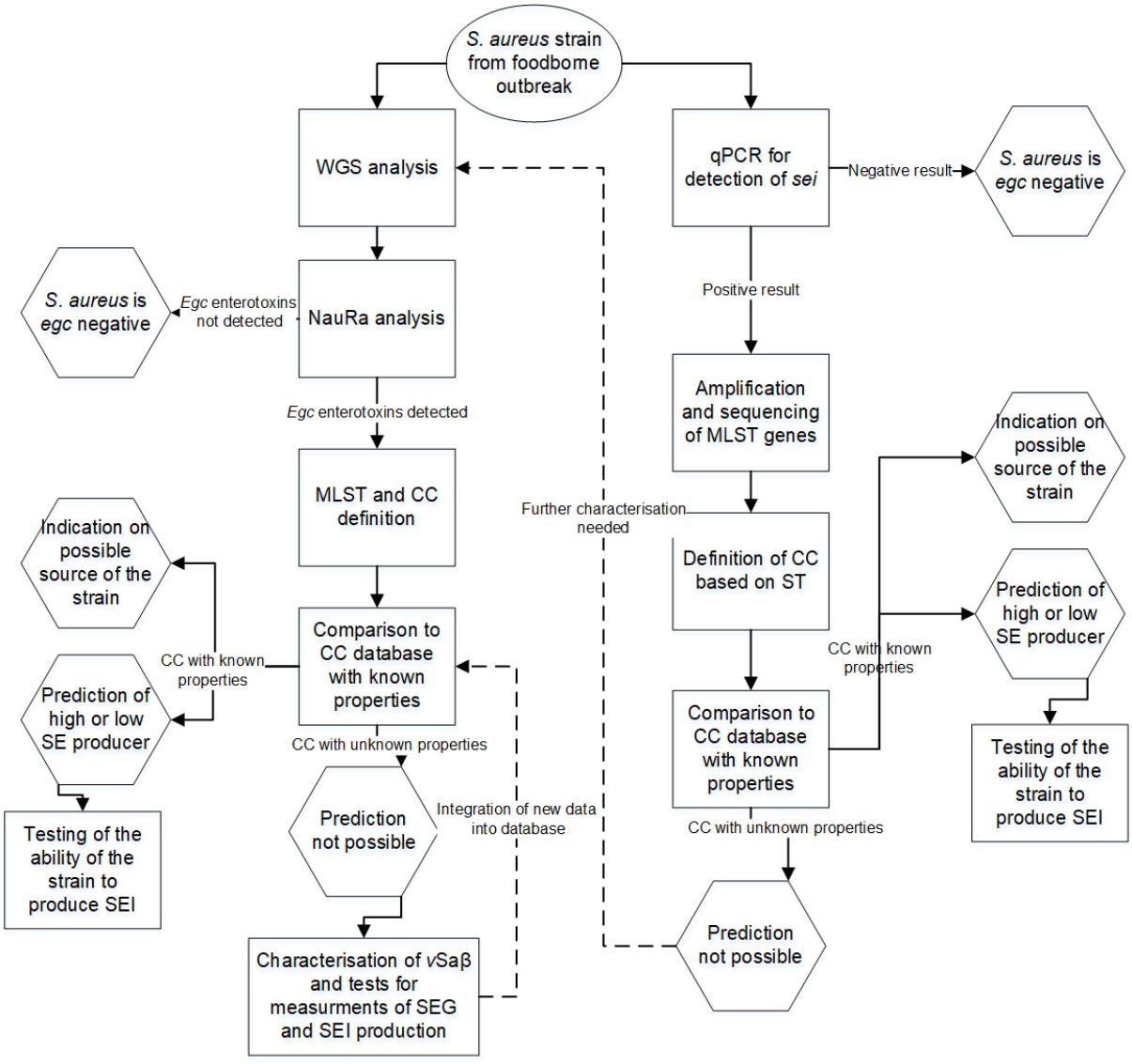

Figure 21: Characterisation, prediction and source tracking of an egc containing S. aureus strain potentially involved in a foodborne outbreak

First step is taking an isolated *S. aureus* from the presumptive food matrix involved in a foodborne outbreak. Then it has to be decided if the strain is going to be directly sequenced or a series of qPCRs is going to be performed. Taking the first option has a lot of advantages as all genome information will be available sooner. On the other side the sequencing of the strain will cost more time and money.

With the sequencing data, the NAuRA tool developed by Mérda et al. (2020) can be used for identifying all enterotoxins present in the genome. Additionally, also the definition of the CC should be performed (as described in Schwendimann et al. unpublished manuscript). When knowing the CC of an *egc* positive strain, it can be compared to the ones already described in literature. The results of our study shows (Schwendimann et al. 2020) that only a

small number of such CCs are involved in SFPOs in Europe, because most of the strains were allocated to only five different kinds of *v*Saβ type/CCs (Schwendimann et al. 2020). Consequently, there is a high probability that information on the CC found is already known. With this comparison to known CCs additional information will be available (Schwendimann et al. 2020): 1) It is possible to know if the strain is a “high” or “low” enterotoxin producer. If it appears that e.g. the strain is a CC705 (having *v*Saβ IV and being a low producer) it is clear that in this case *egc* enterotoxins would not have been involved in the outbreak. In the case of a CC45 the contrary could be assumed. 2) A CC can give an indication of the possible source of the involved *S. aureus* strain. Again, assuming it is CC705, even when SEG and SEI enterotoxins are not involved, with the new insights we can assume that the source of this strain is cattle. On the other hand, a strain being CC45 is most probably from human origin. By knowing this information, the possible source of contamination can more easily be found and eliminated.

If the results of the sequencing show a CC where neither enterotoxin production studies nor *v*Saβ characterization studies have been performed, no information on prediction or source can be given. In this case these SEG and SEI production studies should be performed with the isolated *S. aureus* strain and *v*Saβ should be characterized. Applying these methods the data base will continuously be enlarged and the cases of unknown CC will become rare. In addition, if in the future methods for the detection of SEM, SEN and SEO are available, these enterotoxins show maybe the same pattern and CC dependency as SEG and SEI. For a strong evidence outbreak proof, the enterotoxins need to be detected in the food matrix and also evidence that the isolated strain is able to produce these enterotoxins. With the new information of this study, in future it might be necessary to only analyze one enterotoxin (e.g. SEI) instead of all of them (SEG, SEI, SEM, SEN, SEO).

If the *S. aureus* strain is not directly going to be sequenced, qPCR for the detection of enterotoxins needs to be performed. In such a situation, I suggest to only use one qPCR for the detection of *sei*. Here again the new insights of *egc* should be used: As we know that *sei* is always present on each *v*Saβ type, not being truncated and having a low variability (Schwendimann et al. 2020, Klaui et al. 2019), this enterotoxin gives an indication of the presence or absence of *egc*. If *egc* is present, CC should be identified by PCR and sequencing of the seven housekeeping genes. Afterwards the procedure is the same as described in the case of sequencing of the strain.

8 Conclusions and outlook

The main goal of this study was to find a model that can predict staphylococcal *egc* enterotoxin production based on WGS data. This goal has been reached and as a side effect some more insights on *egc* enterotoxins and *v*Saβ have been found.

The correlation between clonal complex and *v*Saβ type, together with the fact that enterotoxin G and I production is dependent on the *v*Saβ type and can statistically be allocated to “high” and “low” production, made it possible to predict SEG and SEI production based on whole genome data. In addition, the new insights also show that based on the CC indication the possible source of contamination can be determined. This new knowledge can be used in SFPOs cases in order to evaluate the involvement of a specific strain in an outbreak.

Beside the new insights, I nevertheless think the prediction model needs to be further extended due to the diversity of clonal complexes and by genotypical and phenotypical characterisation of the not already described CCs. This should be done by analysis of the *v*Saβ and the test for the production of enterotoxins G and I. In addition, once a quantitative method for detection of enterotoxin SEM, SEN and SEO is available the strains of the different *v*Saβ types should be analysed for the production of these enterotoxins. If the results also give the same insights as the one for SEG and SEI, the model could be extended to all *egc* enterotoxins.

In the beginning the reason for the differences in the correlation of *v*Saβ and enterotoxin production were assumed to be in the diversity of the promoters. Due to the absence of information about the TSS, promoter studies could not be performed as planned. In consequence, I think that RNAseq would be needed to be able to define the TSS. In addition, with this method it could also be confirmed if *egc* enterotoxins are all expressed together or singularly.

From the point of view of food safety also some new insights into production of SEG and SEI under different parameters were gained with this study. Under the stress parameters used here, expression (mRNA) was measured for all *egc* enterotoxins. Surprisingly, SEG and SEI production of these enterotoxins could already be measured two hours after inoculation, with an incubation temperature of 45°C or NaCl concentrations up to 100 g/l. These results show that SEG and SEI can also be produced under harsh conditions. Therefore, it should be further observed how they are produced in a food matrix. Especially with foods where a lot of human handling is required (e.g. artisanal cheese making and similar), challenge tests should be performed in order to find out if these enterotoxins have the ability to be produced under real life conditions. Nevertheless, statements at the protein level cannot be made for SEM, SEN and SEO, as we have no knowledge if translation from mRNA to protein happened for these enterotoxins.

The enterotoxin gene cluster is composed of five enterotoxin genes for which emetic activity is demonstrated. In my opinion this cluster should be considered as one and in the future, when more knowledge will be available about the single enterotoxins and their behaviour, maybe only one representative enterotoxin out of the five can be used for demonstrating the implication of an *egc* positive *S. aureus* strain in a SFPO. Before reaching this level, a detection method should be made available for all five enterotoxins in order to be able to study the complete toxin production pattern and to see if the total amount of enterotoxin produced correlates with the high implication of *egc* enterotoxins in SFPOs.

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