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Staphylococcal Food Poisoning and Novel Perspectives in Food Safety

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<http://dx.doi.org/10.5772/62177>

Abstract

Ingestion of food is the major way for human beings to obtain nutrient substances for basic living, and therefore, the quality and safety of food is a major concern. Foodborne illness includes any illness resulting from the consumption of contaminated food that contains pathogenic bacteria, viruses, or chemical or natural toxins. Consequently, food safety is considered to be a globally expanding issue and thus a leading topic in public health, no longer limited to foodborne illnesses but extended to nearly every safety issue regarding “farm to table” food approaches. Bacterial foodborne infections occur by ingestion of food contaminated with growth of pathogenic bacteria, toxin production, and continuous bacterial growth in intestines. In the past decade, a large number of cases or reports have been available on food containing unhealthy, harmful, or toxic substances (other than food poisoning outbreaks) worldwide. Foodborne microorganisms, primarily associated with pathogenic bacteria and toxic substances produced in food, have presented major challenges for food safety. As a global foodborne pathogen, *Staphylococcus aureus* is typically capable of causing a large number of infections, including food poisoning. Thus, this chapter aims to review several factors contributing to the rise of staphylococci as a growing concern for the food safety industry, including the growth of *S. aureus*, production and regulation of staphylococcal enterotoxins, the viable putative but nonculturable (VPNC) state, and antimicrobial resistance of *Staphylococcus* caused by the indiscriminate use of antibiotics in both clinical and veterinary settings.

Keywords: *Staphylococcus aureus*, Enterotoxins, Food poisoning, Biofilm

1. Introduction

Foodborne illness, also known as foodborne infection, foodborne diseases, or food poisoning, is defined as any illness resulting from the consumption of contaminated food that contains pathogenic bacteria, viruses, or chemical or natural toxins (such as poisonous mushrooms) [1,

2]. Remaining as the leading concern in public health and food safety globally, the annual occurrence of foodborne illness has been reported to be 47.8 million foodborne cases (with 128,000 hospitalizations and 3,000 deaths) in the United States, 750,000 cases (with 113,000 hospitalizations and 460 deaths) in France, and 5.4 million cases (with 18,000 hospitalizations and 120 deaths) in Australia [3–5]. Foodborne infections and diseases are caused by a large variety of pathogens that contaminate food and related products, accounting for the major source of all foodborne illnesses, with an estimate of 14 million illnesses, 60,000 hospitalizations, and 1,800 deaths per year [2]. Therefore, bacterial foodborne infections occur when ingested food is contaminated with pathogenic bacteria, toxins produced (even if the growth of host bacteria is terminated), and continuous bacterial growth in intestines (for setting up an infection that causes illness).

1.1. Staphylococci

As a group of Gram-positive, facultative aerobic, frequently unencapsulated, osmotolerant microorganisms, staphylococci are carried, mostly transiently, by approximately 50% of healthy adults on the skin and anterior nares and widespread in untreated water, raw milk, and sewage. Staphylococci are responsible for various tissue infections and a multitude of diseases [6–9]. Up to date, more than 30 distinct types of staphylococci strains have been verified to be infectious for humans, and the associated illnesses and diseases range from mild to severe, from no treatment required to even potentially fatal [6, 7, 10]. Most of these infections are caused by *Staphylococcus aureus*, which has been regarded as a leading human and zoonotic pathogen implicated in both clinical medicine and food safety and typically is capable of causing a large number of infections, including skin infections and sometimes pneumonia, endocarditis, osteomyelitis, gastroenteritis, scalded skin syndrome, and toxic shock syndrome (TSS) [6]. Aside from this bacterium, coagulase-negative staphylococci (CoNS) are regarded as a frequent cause of nosocomial infection and bacteremia, especially in patients with indwelling medical devices [12, 13]. CoNS have also become the most frequently isolated pathogens in intravascular catheter-related infections (CRI), accounting for an estimated 28% of all nosocomial bloodstream infections [14].

1.2. Staphylococcal Food Poisoning (SFP)

Staphylococcal food poisoning (SFP) is a noncontagious gastrointestinal illness caused by the ingestion of food contaminated with preformed staphylococcal enterotoxins (SEs), with low fatality and symptoms commonly including nausea, retching, vomiting, stomach cramps, exhaustion, and diarrhea [1, 15]. The onset of SFP symptoms commonly occurs between 0.5 and 6 h, and the illness typically lasts for 1 day (up to 3 days), with rapid recovery [1]. Serving as one of the most economically important foodborne diseases, SFP is currently a major issue for global public health programs worldwide [16, 17]. In the United States, *Staphylococcus* has been among the top 5 pathogens responsible for foodborne illnesses. From 1983 to 1997, the annual number of SFP cases had been estimated to be 185,000, with 1,750 hospitalizations and 2 deaths, totaling a cost of 1.5 billion dollars [1, 2]. Based on the recent surveillance in 2011, SFP was found to be account for 241,148 foodborne illnesses, 1,064 hospitalizations, and 6

deaths per year [1, 18]. In Europe, SFP ranks as the fourth most common causative agent of foodborne illness, with 926 SFP outbreaks reported in 15 European Union (EU) countries from 1993 to 1998 [2, 19]. In Japan, according to the Ministry of Health and Welfare of Japan, for a period of 20 years (1980–1999), a total of 2,525 outbreaks of SFP were reported, which involved 59,964 persons, resulting in 3 deaths [17]. In addition, an extensive SFP outbreak occurred in Japan in 2000, with a large number of patients (13,420 cases) ingested with dairy products contaminated with SEs [20]. In China, for a majority of regions, *S. aureus* was recovered from more than 15% of food samples, and in occasional outbreak cases, the identification rate approached 90% [2, 8, 21]. However, due to the lack of comprehensive surveillance and investigation and the prevalence and occurrence of SFP that varied considerably among different regions and areas in China, this discrepancy may be explained by different local eating habits and food product usage. In addition, staphylococcal strain-dependent differences may also contribute to the variation.

Food commonly involved in staphylococcal intoxication include protein food (even salty) such as ham, raw or processed meat, puddings, pastries tuna, chicken, sandwich fillings, cream fillings, potato and meat salads, custards, raw milk, milk products (especially unpasteurized milk), cheese products, and creamed potatoes [22, 23]. In China, raw meat, milk and dairy products, frozen products, and cooked food have been found as major food types contaminated by *S. aureus*, taking up 38%, 20%, 16%, and 14%, respectively [2, 8, 21]. In Europe, meat and related products have been the common food vehicles for SFP. In Japan, SFP frequently occurs in rice balls and Japanese-style desserts [24]. Food made by hand requiring no further cooking or handled frequently during preparation are major targets for *Staphylococcus* contamination and at highest risk for the production of bacterial toxins, which eventually cause SFP. Although *S. aureus* contamination can be readily avoided by heat treatment of food, its ability to grow in a wide range of temperatures (7–48.5°C), pH [4.2–9.3] and sodium chloride concentrations (up to 15% NaCl) facilitates the contamination and transmission of this organism to various types of food [16, 17, 25, 26].

2. Staphylococcal Enterotoxins (SEs)

Some *S. aureus* strains are capable of producing SEs, and several CoNS species have also been reported to produce SEs, including *Staphylococcus epidermis* and *Staphylococcus haemolyticus*, among others [27–29]. Classified into members of the pyrogenic toxin superantigen family [30], SEs are a series of extracellular single-chain proteins primarily produced in food or culture media and secreted by some *Staphylococcus* strains, and the causative agent of SFP, which after ingestion may cause intoxication exhibited by vomiting (emetic action on the abdominal viscera) and diarrhea (epithelium of the intestinal tract and inhibition of water absorption in intestine), even collapse in severe cases [15]. In most SFP cases, a single enterotoxigenic staphylococcal strain isolated from the contaminated food is suspected to be the responsible strain [31]. However, from a number of SFP outbreaks with symptoms similar to gastrointestinal syndromes mediated by SEs, only nonenterotoxigenic staphylococci have been isolated, which may be explained by the outgrown enterotoxigenic staphylococci by the nonentero-

toxigenic ones. Containing low α -helix and high β -pleated sheet content, SEs have similar and flexible structure, with a low molecular weight between 24,000 and 30,000 Da [32]. One of the characteristic features of SEs is their heat stability (withstanding heating to 121°C for 10 min), as SEs are commonly produced ranging from 10°C to 50°C, with the optimum at 30°C to 40°C [24, 26, 33–35]. Consequently, when *Staphylococcus* strains grow in food under appropriate conditions and produce SEs undetectable by taste or smell, SEs remain active even when bacteria itself have been eliminated by heating. As concluded, effective ways include the prevention of food contamination from staphylococci, staphylococci organisms from growing, and SEs from being produced under either heating or freezing. SEs are also highly hydrophilic, with pH ranges from 4.8 to 9.0 (optimum between 5.3 and 7.0) and water activity (*aw*) ranges from 0.87 to 0.99 (optimum at 0.90) [24, 36, 37]. Being well studied and documented, SEs are found to possess a number of biological properties, including superantigenicity (induction of T-cell mitogenicity and human interferon), emetic activity, and pyrogenicity [38]. In addition to gastrointestinal symptoms in SFP as aforementioned, SEs have also been implicated in other diseases such as atopic eczema [39–41], rheumatoid arthritis [41–43], and urticaria [41, 44]. With binding of functional SEs (not presursor) to both the α -helical regions of the major histocompatibility complex (MHC) class II molecules outside the peptide binding groove of the antigen-presenting cells (APC) and the variable region ($V\beta$) on the T-cell receptor (TCR), a bridge between T cells and APC is formed, leading to nonspecific activation and proliferation of a large number of T cells [41, 45, 46] resulting in robust inflammatory cytokine release.

As classified according to the distinct immunological entities, until recently, a total of 25 types of SEs (A–V and X, with 3 subtypes for C) have been identified. The finding of SEs has been in accordance with the development of identification methodologies. In early studies, animal testing experiments had been commonly used for the observation of SE activity, requiring monkey, feline, or cavy models [47–49]. After feeding with contaminated food, animals exhibited abnormal behavior or gross morphology changes, and the SEs contained in food were determined by the number of vomiting events, the time until the first vomiting event, and behavioral changes [47]. These types of animal feeding experiments had been commonly used for the characterization of emetic activities of SEs, thus determining the roles for emetic activities of SEs during SFP. However, such methodologies were also significantly limited by low sensitivity and specificity, poor reproducibility, high expense and laboratory operation, diversity in animals, and incapability of quantification and accurate identification [47]. Therefore, the identification of individual single type of SEs was reported by the availability of serological assays. Based on the specific reaction between antigen and antibody, identification of SEs via specific antibody [50] was first reported in the 1930s. Ouchterlony double immunodiffusion, also known as agar gel immunodiffusion, had become applicable in 1948 [51], and the first serological study on distinct SEs was conducted in 1958 [52]. From 1960s to 1970s, a number of SEs with emetic activities were identified and reported by the employment of serological methodologies including Ouchterlony double immunodiffusion, radial immunodiffusion, and enzyme-linked immunosorbent assays (ELISA). With the development of molecular biotechnology such as PCR and even genome sequencing in the 1990s and 2000s, a large number of newly identified SEs (G–V and X) as well as variants (for instance, 17 distinct variants of *sel-x*) had been discovered on enterotoxin gene cluster (*egc*), staphylococcal

pathogenicity islands (SaPIs), mobile genetic elements (MGEs), and even the bacterial genome (*sel-p* on genome of N315).

2.1. Classical SEs

Although investigation on the observation and detection of SEs from animal feeding experiments could be dated back from 1930s, the immunological characteristics were not clarified until a number of serological studies had been conducted in the 1950s and 1960s. These studies revealed 6 types (A, B, C₁, C₂, D, and E) of SEs that were characterized and further referred to as classic SEs (including C₃ reported in 1984). Antigenicity of SEs was validated when the antisera prepared from rabbits infected with SEs was demonstrated to protect cats [52], and later from a further serological study, the origins of two heat-resistant types of SEs had been verified [52, 53]. These extracellular immunologically distinct SEs responsible for the clinical manifestation of SFP were first referred to as type F (food poisoning) and type E (mostly produced by strains of “enteritis” origin) and later designated as A and B for a better sequential numbering system, with only type A commonly associated with food poisoning (A-1963). Sharing a similar basic three-dimensional structure, the 5 serological groups of SEs (A–E) exhibit nucleotide sequence identity of 50% to 85%, with types A, D, and E categorized into one group (52–83% amino acid identity) and types B and Cs falling into a separate, more closely related group (62–64% amino acid identity) [53, 54]. As origins and sources, types B and C are important causes of nonmenstrual-associated TSS, and types A and D are common causes of SFP with types B and C to a lesser extent [49].

2.1.1. *Staphylococcal Enterotoxin A* (*sea* or *SEA*)

SEA was first identified in 1959 from *S. aureus* strain FRI-196E [52, 55] and then named as type A [56], which has been considered to be the most commonly detected SE associated with food poisoning, with its minimal toxic dose for humans ranging from 20 to 100 ng [24]. Also, SEA has been verified to be responsible for a number of SFP outbreaks, including an extensive outbreak caused by ingestion of dairy products in Osaka, Japan, in 2000 [24, 56, 57]. After identification by serological methodology, the production of SEA in different media or condition had become a major concern. The maximal level of SEA production was found to occur during exponential phase. In semisolid BHI agar (pH 5.3), the production of SEA was acquired, and trace amounts of SEA and SEB were obtained with cellophane sac culture [58, 59]. As food samples were concerned, SEA production was detected in a number of meat samples (raw beef and pork, cooked beef and pork, and canned ham). Better growth of *S. aureus* and production of SEA were detected in cooked meat than raw meat despite no significant difference obtained, and such diversity may be explained by the bacterial competition between anaerobic and aerobic conditions (with the latter preferable for *S. aureus*) [50, 58, 59]. In milk, SEA production was also found to be associated with staphylococcal growth [60]. In fermented sausage, SEA production had been detected aerobically at pH 5.1 (with an inoculum of 4×10^7 cells/g sausage) but not anaerobically at pH 5.7 [61]. For co-growth food microorganisms, inhibition was observed to be more common than stimulation, including inhibition of both staphylococcal growth and SE formation with no apparent

effect on growth [62]. However, such influence of other microorganisms on *Staphylococcus* was affected markedly by environmental conditions, and discrepancy on inhibition had been also noticed between plate test and meat slurries [58, 59]. Despite acquisition of appropriate staphylococcal growth in both pure culture and in the presence of other food microorganisms, production of SEA was only formed in pure culture [58, 59]. Although SEB was reported to be produced in much larger quantities and more diverse among strains than SEA, SEA had been implicated in a larger number of food poisoning cases [63]. Resembling a primary metabolite (with SEB as a secondary metabolite), SEA is secreted by the bacterium during the exponential phase of growth, with various of factors affecting its production, including salt concentration (NaCl, NaNO₂, and NaNO₃ showing no influence), surfactants (increase in SEA secretion), pH (optimal ranging from 6.5 to 7.0), and antimicrobial agents (inhibition by chloramphenicol or 2,4-dinitrophenol, with streptomycin or penicillin G exhibiting no influence), which may explain the higher frequency and incidence of SEA in food poisoning [63–66]. In addition, temperature and inoculum size play important roles in SEA production. As temperature was concerned, SEA production was detected under a broad range of temperatures from 10°C to 50°C (in BHI broth) but not at low temperatures (such as 8°C or 10°C as reported) [24]. SEA was detected in the exponential phase from 15°C to 37°C, and its production increased with the elevation of temperature. Also, SEA was detected in the stationary or death phase at 10°C despite acquisition of the lowest SEA concentration at this temperature. Similar to SEE, SEA contains 2 MHC-II binding sites (Zn²⁺ dependent) and thus possesses strong superantigenicity for T-cell activation [67, 68]. Carried by a polymorphic family of lysogenic phages [69, 70], the gene encoding SEA has a length of 771 bp, and its translational product is SEA precursor of 257 amino acids. With 24-residue N-terminal hydrophobic leader sequence further processed, the mature form of SEA was composed of 233 amino acids [38, 53, 68–74]. Unlike other classic SEs (*seb*, *sec*, and *sed*), expression of *sea* had been found to be independent of *agr* regulation [54, 75, 76].

2.1.2. *Staphylococcal Enterotoxin B (seb and SEB)*

Being the first identified (from *S. aureus* strain FRI-243) and the most studied SE, SEB was initially named as type E and subsequently designated as type B. As the most potent SE and requiring much lower quantities for toxic effect than synthetic chemicals, SEB are capable of causing multiorgan system failure and death at low concentration. As an exotoxin secreted by *S. aureus*, production of SEB had been reported from diverse clonal complexes, including CC8 (the most common), CC20, and CC59 [32, 77–79]. As a superantigen capable of cross-linking APCs and T cells to form a ternary complex between MHC-II and TCR at specific V β chain, SEB had been well studied as a causative agent for food poisoning, TSS, atopic dermatitis (common colonization of *S. aureus* and frequent occurrence of SEB-specific antibody from patients with AD), and respiratory diseases (asthma and nasal polyps) [32, 77, 80, 81]. As a well-characterized protein, SEB had been found to be extremely stable (retaining its activity even in acidic environment), water soluble, heat stable (among the most heat-stable proteins, with intact protein under 78°C to 80°C for 30 min), broadly pH tolerant [4–10], and resistant to proteolytic digestion (such as pepsin, trypsin, and papain) [47, 82, 83]. Nevertheless, SEB formation and production were influenced by a number of factors, including inhibition of SEB

formation in BHI broth by medium filtrates (such as K_2HPO_4 , KCl, $CoCl_2$, NaF, acriflavine, phenethyl alcohol, streptomycin sulfate, chloramphenicol, spermine phosphate, spermidine phosphate, and Tween-80) [82], decrease of SEB production by either temperature depletion (without affecting staphylococcal growth) or curing salt concentration elevation (more rapid reduction of SEB production than staphylococcal growth) [84], catabolite repression [85], and minerals (double SEB production was obtained when magnesium and potassium are under appropriate concentration) [34, 86, 87]. Generally, maximal SEB production occurs in postexponential growth. From early studies in the 1950s and 1960s, SEB was considered to be irrelevant to food poisoning [50, 88]. Located in either chromosome (strain FRI-243, FRI-277, or S6) or plasmid (strain DU-4916), *seb* is 705 bp in length, and the mature SEB consists of 239 amino acid residues sharing nucleotide and amino acid sequence homology with *sec*₁ and streptococcal pyrogenic exotoxin A [89–91]. Regulated by the staphylococcal two-component system, accessory gene regulatory (*agr*), the region between 59 and 93 nucleotides upstream of the transcription ignition site was found to be essential for transcription and expression [92–94]. *seb* had been commonly found in toxin-mediated foodborne and clinical *S. aureus* strains, and recently, *seb* (by PCR and Western blotting) was detected from 5% of 300 clinical *Staphylococcus* strains [95].

2.1.3. *Staphylococcal Enterotoxin C (sec and SEC)*

According to the new numbering system agreed on the American Society for Microbiology (ASM) meeting in 1962, the first verification of SEC was then reported in 1965, with its toxicity and specificity also confirmed [96]. In this study, the enterotoxins from *S. aureus* strains FRI-137 and FRI-361 were both discovered to react with a specific antibody; thus, strain FRI-137 (ATCC 19095) was selected as the prototype of SEC [96]. However, 2 years later, enterotoxins from strain FRI-137 and FRI-361 were purified as distinct enterotoxins [96, 98] and consequently labeled as SEC₁ (strain FRI-137) and SEC₂ (strain FRI-361). In 1984, the third enterotoxin C (SEC₃) was discovered from a *S. aureus* strain FRI-913 from prawn in England, which were serologically and chemically similar to SEC₁ and SEC₂ but identical by isoelectric focusing, radioimmunoassay (RIA), and N-terminal analysis [99, 100]. Despite cross-reactivity with same antibody, each of the 3 SEC had antibodies that reacted with minor determinants [99]. Located on chromosome (SaPIs), *sec* is composed of 801 bp and encodes a precursor protein of 267 amino acids, with the mature toxin of 239 amino acids [101, 102]. Aside from 3 types of classic SECs, additional *sec* variants (such as *sec*-bovine from SaPI_{bov}) possessing >95% deduced amino acid homology among them had been also reported [103–107]. As SEC₁, SEC₂, and SEC₃ are all emetic enterotoxins with equal toxicity to that of SEA and SEB in both oral administration and intravenously [96, 99], SEC have been responsible for numerous SFP outbreaks (mostly caused by milk) [108]. Maximal production of SEC occurs during postexponential growth. SEC-positive strains of *S. aureus* are commonly associated with bovine, ovine, and caprine dairy products [109]. Yet interestingly, SEC expression has been noted to be reduced in cheeses [110]. From a recent study, milk environment was found to dramatically change the expression profiles of enterotoxin genes despite no influence on staphylococcal growth. In particular, SEC production was substantially reduced in milk compared to the

laboratory medium on the protein level, which may be explained by the down-regulation of the *agr* system [111].

2.1.4. *Staphylococcal Enterotoxin D (sed and SED)*

In 1967, SED was first reported from *S. aureus* strain FRI-293 (which also produced SEC; thus, strain FRI-494 was selected as the prototype strain, also known as ATCC 23235) and its emetic activity in cats, and specific neutralization of biological activity by antisera had been verified [112]. The production of SED alone and in combination with SEA was considered to play a key role in food poisoning (ranking second in frequency after SEA) and recognized as one of the most commonly recovered enterotoxins in SFP outbreaks [112, 113]. Encoding a toxin of 228 amino acids, *sed* is located on a 27.6-kb penicillinase plasmid pIB485 [114]. SED was found to be partially activated by *agr* via RNA III-mediated reduction of Rot (repressor of toxin) during postexponential growth phase, as independent formation from *agr* was found under high concentration. As a consequence of *agr* regulation via quorum sensing, during growth in BHI broth, a modest postexponential induction ratio (<10-fold) was obtained as *sed* reached maximal production during transition from exponential to stationary phase of growth [113, 115]. With the existence of a consensus -10 sequence, a less conserved -35 sequence, and a TG dinucleotide motif, the presence of 52-bp sequence (from -34 to +18) and transcription from +1 to +18 were important for promoter function and *agr* regulation [116]. Aside from regulation by the *agr* system, NaCl stress was capable of decreasing *sed* expression, although no significant effect was further verified. However, regulation under NaCl stress may be highly strain specific variable [117]. As food samples were concerned, in cheese manufacturing (with starter culture including 10^3 CFU/ml of milk), *sed* expression was not induced even when inoculated at 10^6 CFU/ml (equal to 10^8 CFU/g of cheese), presenting a low level of expression and a prolonged pattern that was similar to SEA [113, 118]. In different ham products, when *S. aureus* was inoculated for optimal growth in cultivation broth for 7 days, continuous *sed* expression was observed throughout the entire incubation period for both boiled and smoked ham [115]. However, much less production of *sed* (9 times less) was detected in the latter. For Serrano ham, SED was only detected after 5 days of incubation (*sed* expression still too low to determine), similar to which a second increase had been obtained for boiled and smoked ham after the same time span of incubation [115].

2.1.5. *Staphylococcal Enterotoxin E (see and SEE)*

In 1971, SEE was reported from a food poisoning *S. aureus* strain FRI-326, which produced distinct SEE having no immunoreactivity with specific antibodies to other SEs [119]. Its toxicity (in rhesus monkeys), specificity, and neutralization with specific antibody were also validated [119]. Located on the phage, *see* is composed of 771 bp and encodes a precursor with a molecular weight of 29,358 Da, which was further processed to a mature extracellular form with a molecular weight of 26,425 Da [120]. Containing a single polypeptide chain, SEE consists of 259 amino acid residues (no free sulfhydryl groups found), with serine and threonine as the NH_2 - and COH-terminal amino acids, respectively [121]. Under extreme acidic (pH 2) and basic (pH 12) conditions as well as heating, the toxicity (emetic activity)

and antigenicity (serological activity) were found to be destroyed, which is likely due to conformational change [121].

2.2. Staphylococcal enterotoxin-like toxins

Before the 1990s, a total of 7 types of classic SEs (*sea*, *seb*, *sec₁*, *sec₂*, *sec₃*, *sed*, and *see*) had been known as causative agents of SFP in humans due to emetic activity. However, starting from the discovery of *seh* in 1994 (aside from discovery of *sef* on 1981), a large variety of novel SE or SE-related toxins (as well as variants) had been reported (G–V and X) based on genetic homology with classical SEs. In 2004, the International Nomenclature Committee for Staphylococcal Superantigens has proposed that only staphylococcal superantigens inducing emesis after oral administration in a primate model should be designated as SEs, whereas other related toxins lacking either emetic properties in a primate model or verification of emetic activity should be otherwise designated as staphylococcal enterotoxin-like toxin type X [122, 123].

2.2.1. Staphylococcal Enterotoxin-Like Toxin Type F (*sel-f* and SEI-F)

In 1981, Bergdoll et al. had noticed an enterotoxin-like protein recovered from 93.8% [61/65] *S. aureus* strains sampled from patients with TSS, representing the first evidence of *sel-f* [124]. With its purification and preparation of specific antibody, *sel-f* was also recovered from 11.5% [3/26] of laboratory *S. aureus* strains, compared with only 4.6% [4/87] from other sources, which suggested the association between SEI-F and TSS [124]. However, from an investigation on the spread of a TSS strain, a temporal association of antibodies to SEI-F with cessation of recurrences of TSS was found, indicating that its production may not either reach clinically significant levels during infection or is insufficient to cause TSS [125]. Generally, studies and reports on SEI-F have been rarely available.

2.2.2. Staphylococcal Enterotoxin-Like Toxin Type G (*sel-g* and SEI-G)

In 1998, SEI-G and SEI-I (from *S. aureus* strains FRI-572 and FRI-445, respectively) had been identified and characterized, including verification on emesis (eliciting emetic response in rhesus monkeys) and superantigenicity (proliferation of T cells) [126]. *sel-g* consists of 777 nucleotides and encodes a precursor protein of 258 amino acids, which has typical bacterial signal sequences and is then cleaved to form mature toxin with 233 amino acids and with a molecular weight of 27,043 Da [127, 128]. SEI-G showed higher homology to SpeA, SEB, SEC, and SSA (38–42% amino acid identity) and exhibited similar epitopes with SEC₁ [126].

2.2.3. Staphylococcal Enterotoxin-Like Toxin Type H (*sel-h* and SEI-H)

In 1994, the first discovery of *sel-h* from *S. aureus* strain D4508 was reported, with its nucleotide and amino acid sequences identified [129]. One year later, SEI-H was identified and purified from *S. aureus* strain FRI-569, which elicited an emetic response in monkeys and was found to be antigenically distinct from other existent SEs [51]. SEI-H shares about 35% amino acid identity with SEA, SED, and SEE [130]. As a superantigen homologous to SEA subfamily, SEI-H displays unique MHC-II binding properties. As a potent T-cell mitogen, SEI-H was capable

of activating large amounts of T cells by cross-linking APC and T cells via V α domain (V α 10, TRAV27) of TCR (with no TCR V β -specific expansion) by direct interaction between SEI-H and TCR V α domain [131, 132]. With emetic activity, *sel-h* was commonly detected alone or together with *sea* [133] and responsible for a number of SFP outbreaks. In 1996, an outbreak was caused by cheese and *S. aureus* strains isolated from cheese were found to produce SEI-H [133]. From the SFP outbreak caused by reconstituted milk in Japan, SEI-H was also detected along with SEA. In a survey on 146 *S. aureus* strains isolated from humans, cows, and bovine in Japan, 7 and 4 strains were found to harbor *sea*⁺*sel-h*⁺ and *sel-h* alone, respectively [57, 133]. In December 2003, a suspected SFP outbreak involving 8 persons (3 adults and 5 children) with symptoms of vomiting, stomach cramps, and diarrhea shortly after lunch was caused by contaminated mashed potato, and *S. aureus* strains contained in raw bovine milk for preparation of mashed potato were found to produce sufficient SEI-H for food poisoning [134]. SEI-H production was influenced by a variety of factors, including aeration and pH conditions. Higher production level of SEI-H was acquired under aerobic incubation or pH controlled at 7.0, with decrease in SEI-H production under anaerobic condition or slight change of pH (such as 6.5 or 7.5) [135].

2.2.4. Staphylococcal Enterotoxin-Like Toxin Type I (*sel-i* and SEI-I)

As aforementioned, SEI-I were identified together with SEI-G in 1998 [126]. Unlike SEI-G, SEI-I was more similar to SEA, SED, and SEE (26–28% amino acid identity). *sel-i* consists of 729 nucleotides and encodes a precursor protein of 242 amino acids, which contains typical bacterial signal sequences and is further cleaved to form mature SEI-I of predicted 218 amino acids with a molecular weight of 24,928 Da [127, 128]. Although separated by DNA related to other SEs, linkage of *sel-g* and *sel-i* was discovered, and this enterotoxin gene cluster was designated as *egc*, with *sel-g* located 2002 bp downstream of *sel-i* [127]. In southern France, carriage of *sel-g*⁺*sel-i*⁺ and *sec*⁺*sel-g*⁺*sel-i*⁺ was detected from 41.9% and 24.5% of 155 *S. aureus* strains isolated from various food samples [128]. In Taiwan, 14.5% [8/55] *S. aureus* strains of human origin and 9.4% [13/139] strains isolated from frozen food, Chinese sausage, and meal boxes were found to harbor *sel-g*, *sel-h*, and/or *sel-i*, suggesting a minor role that such SEs play in SFP outbreaks [136]. However, a discrepancy between the presence of *sel-g* and *sel-i* and the production of enough quantities of SEG and SEI was also noticed [128]. In 2004, 10.1% [11/109] wild *Staphylococcus* spp. strains were found to contain SEs and *egc*, and the *egc* from strain AB-8802 present variants of *sel-g* and *sel-i* (*sel-gv* and *sel-iv*) [137].

2.2.5. Staphylococcal Enterotoxin-Like Toxin Type J (*sel-j* and SEI-J)

In 1998, *sel-j* was first found to be located on the plasmid pIB485 encoding *sed*, which was separated from *sed* by 895 bp of intergenic region containing a perfect inverted repeat (with each arm of the repeat having a length of 21 bp) [138]. Most of *sel-j* was detected on *sed*-encoding plasmid, suggesting the coexistence of these 2 SEs and their relative contribution to the food poisoning symptomology [138]. With transcription in opposite directions, both *sel-j* and *sed* were capable of expression in *S. aureus* strains, with *sed* only under the transcrip-

tional control of *agr* [138]. Containing 269 amino acid residues, sequence of SEI-J showed substantial similarity to the SE family of *sea*, *sed*, and *see*.

2.2.6. Staphylococcal Enterotoxin-Like Toxin Type K (*sel-k* and SEI-K)

Despite observation of the *sel-k* gene on SaPI1 (in 1998) and *egc* from *S. aureus* strains A900322 (in 2001), the first designation of *sel-k* from *S. aureus* TSS isolates MN NJ was reported in 2001, with its identification on SaPI3 together with *seb* [139, 140]. Possessing biochemical and biological properties similar to classic SEs, including superantigenicity ($V\beta$ -specific T-cell activation), pyrogenicity, emesis, and lethality in primates, SEI-K was secreted by clinical *S. aureus* strains, with a molecular weight of 26,000 Da and a pI between 7.0 and 7.5 [48, 140]. An increase in the secretion of SEI-K was obtained when coexpressed with SEB (K-2014). However, regardless of the variation in SEI-K secretion amount *in vitro*, similar levels of SEI-K accumulation were found *in vivo* [141]. SEI-K was commonly detected in clinical isolates (more than half) and almost all USA300 strains. In addition, a genetic variation of *sel-k* was discovered, with 6 variants found among 20 clinical isolates [141].

2.2.7. Staphylococcal Enterotoxin-Like Toxin Type L (*sel-l* and SEI-L)

First noticed on *egc* from *S. aureus* strain A900322 [142], *sel-l* was identified on pathogenicity island SaPIbov (15,891 bp) from a bovine mastitis *S. aureus* isolate RF122 (*sel-l*) in 2001, with a molecular weight of 26,000 Da and an isoelectric point of 8.5 [105]. Lacking emetic activity, SEI-L was found to exhibit a number of biological properties similar to other SEs, including superantigenicity, pyrogenicity, enhancement of endotoxin shock, and lethality in rabbits when administered via subcutaneous mini-osmotic pumps, but the protein lacked emetic activity [105].

2.2.8. Staphylococcal Enterotoxin-Like Toxin Type M (*sel-m* and SEI-M)

In 2001, *sel-m* was reported to be located on the *egc* (enterotoxigenic gene cluster) together with *sel-g*, *sel-l*, *sel-k*, and *sel-i*, and SEI-M was found to exhibit superantigenicity activity with specific $V\beta$ pattern [142]. However, the emetic activity has not been elucidated yet. Most clinical *S. aureus* strains harboring *egc* were found to carry such SEs regardless of the diseases, suggesting the potential derivation of SEs and the putative cluster of SE genes from *egc*.

2.2.9. Staphylococcal Enterotoxin-Like Toxin Type N (*sel-n* and SEI-N)

From the *egc* reported in 2001, *sel-n* was also found to be located between *sel-i* and *sel-g* [142]. A study was conducted on the cloning and expression of *sel-m* and *sel-n* from *S. aureus* strain FRI-1230, demonstrating that SEI-M and SEI-N were capable of stimulating T cells and inhibiting K562-ADM and B16 cells with an equivalent level to that of SEC₂ [143]. Although superantigenicity had been verified, the emetic activity of SEI-N is still unclear [144].

2.2.10. *Staphylococcal Enterotoxin-Like Toxin Type O (sel-o and SEI-O)*

In 2001, *sel-o* was identified from the *egc* cluster, on which other 4 SEs and 2 pseudogenes were also located, including *sel-i*, *sel-j*, *sel-m*, *sel-n*, *Ψent1*, and *Ψent2* [142]. However, the biological and biochemical properties of *sel-o* remains unclear despite validation of its superantigenicity [144].

2.2.11. *Staphylococcal Enterotoxin-Like Toxin Type P (sel-p and SEI-P)*

In 2001, *sel-p* (previously called *sep*) was first discovered from the genome of MRSA N315, and its biological properties were fully characterized in 2005 (with *sel-p* from *S. aureus* strain Sagal isolated from an SE unidentified food poisoning outbreak in Japan), including superantigenicity (induction of a substantial proliferative response and production of cytokines) and emetic activity (at relatively high dose as 50–150 µg/animal) [123, 145]. According to this study, SEI-P was detected in 60% of the 30 *sel-p*-positive *S. aureus* isolates, and all 10 strains harboring *seb* and *sel-p* produced SEB but not SEI-P, suggesting that inactivation of the *sel-p* locus associates with a particular SE genetic constitution [123]. Most recently, colonization with *sel-p*-positive MRSA increased the risk of bacteremia, which indicated *sel-p* as a predictive virulence factor for invasive disease [146].

2.2.12. *Staphylococcal Enterotoxin-Like Toxin Type Q (sel-q and SEI-Q)*

In 2002, a member of the new subfamily (group V), *sel-q* (from *S. aureus* strain MN NJ) was identified and located directly 5 of *sel-k*, with a molecular weight of 26,000 Da and isoelectric point between 7.5 and 8.0 [147]. Despite a lack of emetic activity (incapability in neither lethality in rabbits nor emetic activity in monkeys), *sel-q* had been found to possess superantigenicity, pyrogenicity, and ability to enhance endotoxin shock.

2.2.13. *Staphylococcal Enterotoxin-Like Toxin Type R (sel-r and SEI-R)*

In 2003, *sel-r* was recovered and identified from 4 *S. aureus* strains (Fukuoka 5, Fukuoka 6, Fukuoka 7, and Fukuoka 8) isolated from patients with nausea, vomiting, and diarrhea from a food poisoning outbreak occurred at a lunch-box shop in Fukuoka prefecture of Japan in September 1997 [148]. Located on 2 types of plasmid, pBI485 (and pBI485-like plasmids, encoding *sed* and *sel-j* as well) and pK0311 (pF5, pF6, and pF7), *sel-r* was found to most closely related to *sel-g* [148]. Investigation on the biological properties of SEI-R revealed its superantigenicity (T-cell stimulation activity via MHC-II) and emetic activity (induction of a reaction in animals within 5 h at 100 µg/kg) [148–150]. SEI-R production was also verified in seropositive *S. aureus* strains [148, 149]. A survey was conducted on the SEI-R production from 24 *sed*-positive *S. aureus* isolates, and *sel-r* expression was detectable from 22 isolates despite carriage of variant *sed* gene for seven strains lacking SED production [151].

2.2.14. *Staphylococcal Enterotoxin-Like Toxin Type S (sel-s and SEI-S)*

Two novel SE-like genes, *sel-s* and *sel-t*, had been reported on the plasmid pF5, where *sel-j* and *sel-r* were located. SEI-S (rSES) was characterized for biological properties, including super-

antigenicity (specific stimulation of human T cells via MHC-II APC) and emetic activity (induction of emetic reactions in monkeys) [150].

2.2.15. *Staphylococcal Enterotoxin-Like Toxin Type T (sel-t and SEI-T)*

As aforementioned, a first identification of *sel-t* was reported on plasmid pF5 harbored by *S. aureus* strain Fukuoka 5 from SFP. Similar to SEI-S, SEI-T was found to exhibit both superantigenicity and emetic activity (induction of a delayed reaction after 24 h or 5 days postadministration). Data from the emetic study on SEs involved in the SFP outbreak in Fukuoka in 1997 combined with emesis studies in house musk shrews (similar as in the monkeys) suggest that SEI-R and SEI-S were validated to be the causative toxins of vomiting [150].

2.2.16. *Staphylococcal Enterotoxin-Like Toxin Type U (sel-u and SEI-U)*

From sequencing of 24 *S. aureus* strains harboring *egc*, *sel-u* was identified on 4 of the tested strains [152]. SEI-U was found to result from sequence divergence in the Ψ_{ent1} and Ψ_{ent2} pseudogenes, as *sel-u* was located between *sel-iv* and *sel-n* in *egc* of strain 382F (AY158703) with replacement of the Ψ_{ent1} and Ψ_{ent2} between *sel-iv* and *sel-n* in *egc* of strain Mu50 (AP003363) [144, 152]. A variant *sel-u*, designated as *sel-u2*, was recovered from an atypical *egc* locus and generated by a limited deletion in the pseudogenes Ψ_{ent1} and Ψ_{ent2} , which contained superantigenicity for activation of T-cell families V β -13.2 and V β -14 [144].

2.2.17. *Staphylococcal Enterotoxin-Like Toxin Type V (sel-v and SEI-V)*

In a broad surveillance on *egc* from 666 clinical *S. aureus* isolates, 63% [421/666] strains were positive for *egc* locus [144]. The archetypal *egc* harboring 5 SEs and 2 pseudogenes was found in 409 strains, and a novel SE-like toxin, designated as *sel-v*, was discovered from an atypical *egc* locus from *S. aureus* strain A900624 [144]. SEI-V was generated by recombination between *sel-m* and *sel-i*, and its superantigenicity for activation of T-cell families V β -6, V β -18 and V β -21 has also been validated.

2.2.18. *Staphylococcal Enterotoxin-Like Toxin Type X (sel-x and SEI-X)*

In 2011, *sel-x* was discovered from the core genome of 95% of phylogenetically diverse *S. aureus* strains with human and animal origins, including 17 distinct allelic variants (*sel-x1* to *sel-x14*, *sel-xov*, *sel-xbov1*, and *sel-xbov2*). Acquisition of *sel-x* includes the horizontal transfer by a *S. aureus* progenitor, allelic diversification by point mutation, and assortative recombination, which explains the high genetic diversity of *sel-x*. With a unique predicted structure, SEI-X was well characterized by biological activities, including superantigenicity (activation of V β -specific T cells), pyrogenicity, and endotoxin enhancement. It is also noteworthy that SEI-X produced by strain USA300 (CA-MRSA) had been found to be responsible for the lethality in a rabbit model, which suggested a novel virulence determinant of CA-MRSA disease pathogenesis.

2.3. Pathogenicity Islands (PAIs) and *S. aureus* PIs (SaPIs)

2.3.1. PAIs

In 1986, before the first report of PAIs by Hacker et al. in Werner Goebel's group of Germany in 1994, two large segments had been found to be capable of deletion and thus enable the host bacterial to produce hemolysin and loss of *P. fimbriae* [139, 153, 154]. Considered to be foreign DNA segments integrated into the bacterial genome, such segments existed within pathogenic isolates (cause of virulence) but not on highly genetically similar nonpathogenic strains [153]. As a subclass of genomic islands, PAIs are defined as a group of gene clusters encoding bacterial virulence on a large DNA segment (ranging from 20 to 100 kb) located on the bacterial chromosome [139, 154–156]. PAIs are acquired by microorganisms via horizontal gene transfer via transduction, conjugation, and transformation. Acquisition of PAIs may rapidly and radically alter the genome of a bacterium, consequently strengthening or reducing its fitness within the host [154, 157]. Pathogens are capable of harboring one or more PAIs associated with one or more virulence genes. PAIs are capable of encoding genetic products, including secretory proteins (such as type III secretion system), cell surface proteins (such as erythrocytolysin, fimbriae, and heme binding factors), signal transduction systems, and regulation systems [139, 155–157].

As distinct DNA regions are present in the genome of pathogenic bacteria and absent in nonpathogenic strains (despite same or close species), typical PAIs are composed of mobility genes (such as integrases) commonly located at the beginning of the island and close to the tRNA locus or the respective attachment site. A number of virulence genes (V1–V4) are frequently interspersed with other mobility elements including insertion sequence (IS) elements (Isc, complete insertion element) or remnants of IS elements (ISd, defective insertion element) [153, 155, 156]. Commonly flanked by direct repeats (DR) and IS elements, PAIs are often genetically unstable and comprise some potential mobile components, such as IS elements, integrase, transposase, and plasmid replication initiation sites. As DNA sequences ranging from 16 to 20 bp (with maximum of 130 bp) with sequence repetition, DR plays a critical role in insertion and deletion (as recognition sites), such as integration of bacteriophages. Although combination of IS elements may be capable of mediating transfer of large DNA fragments, insertion mediated by IS also leads to inactivation of genes as well [153, 154, 156]. Consequently, PAIs are capable of deletion with distinct frequencies and loss of virulence traits encoded by PAIs are reported to occur at higher frequency than that encoded by mutation. PAIs are commonly inserted in the backbone genome of the host strain, typically located to specific sites such as tRNA loci or adjacent to tRNA genes, or sites associated with plasmid and phage integration, due to highly conserved genes encoding tRNAs among various bacterial species [153, 154].

PAIs differ from host chromosomes in GC content and codon usage, which may account for the discovery of novel PAIs and maintenance of the divergent nucleotide composition from the horizontally acquired DNA. Based on significant differences with respect to bacterial virulence, GC content, and codon usage, a hypothesis was proposed that such characteristics

may be bestowed from DNA segments on the plasmid and phage; thus, acquisition of PAIs and the emergence of new pathogenic organisms can be correlated [153, 156].

2.3.2. SaPIs

As mobile pathogenicity islands with length ranging from 14 to 17 kb and carriage of genes for superantigen toxins and other virulence factors, SaPIs have been responsible for the TSS and other superantigen-related diseases, especially SE-like toxins. Located in specific loci of the chromosome and induced by bacteriophages, SaPIs are capable of incorporating small infective phage-like particles via a program of excision-replication-packaging. Containing most of the staphylococcal toxins and virulence factors, SaPIs facilitate the horizontal acquisition of MGEs and, thus, play an important role in the evolution of *Staphylococcus* [139, 154, 155].

2.3.2.1. SaPI families

Aside from SCCmec (introduction in detail as below), a large number of toxin genes have been found in SaPIs, including SE-like toxins and TSST. Several types of SaPIs have been identified. SaPI1 was found to be inserted in an *attC* site close to the *tyrB* gene and flanked by the region of *tst* gene, with a length of 15,233 bp [139]. The characteristic features of SaPI1 include mobility and instability, whereas SaPI2 was identified as a second locus. Transduction between SaPI1 and SaPI2 by via helper phage was demonstrated, and stable integration of these 2 SaPIs without phages had also been verified [139, 155, 156]. Inserted at the 3' end of the GMP synthase gene, SaPIbov was identified in a bovine isolate of *S. aureus* by PAI related to SaPI1, with a length of 15,891 bp and carriage of *sec*, *sel-l*, and *tst*. SaPI3 was identified to contain *sel-k* and *sel-q* [157]. SaPIs with similar structure between SaPI3 and SaPI1 had also been reported.

2.3.2.2. vSa families

Up to date, 7 conservative PAI types had been discovered in *S. aureus*, namely, vSa1 (including SaPI1 and SaPI3), vSa2 (SaPIbov), vSa3, vSa4 (including SaPI2), vSa α , vSa β , and vSa γ [139, 155–157]. vSa1 to vSa4 were found to contain integrase genes as putative elements of genetic mobility. Derivation of *int* and *att* sites from phage genome was pointed out, as both were found in SaPIs. On the contrary, vSa α and vSa β harbor transposase genes, which may be derived from transposons. Comprising SaPI1 and SaPI3, the vSa1 locus of CA-MRSA also carries a large number of genes encoding enterotoxins and TSST. Similarly, the loci of both vSa2 from CA-MRSA and SaPIbov were found to contain enterotoxins and TSST. Capable of high-frequency deletion and formation of an episomal circular DNA, vSa3 was identified in CA-MRSA MW2 and Mu50, and one type of vSa3 harbors novel allelic forms of *sec* and *sel-l*. With a lower frequency of excision than that of vSa3, the vSa4 family contains several allelic forms of a genomic island, and type I vSa4 carries *sec* and *sel-l* [157]. Despite the presence of both vSa α and vSa β in all sequenced *S. aureus* genomes, the size and number of ORFs in vSa α , as well as the size and gene composition of vSa β , were found to be highly variable, with neither SaPIs spontaneously excised from the chromosome [145]. The composition of vSa α includes 11 allelic forms of *set* genes (encoding exotoxins), *lukDE* genes (encoding leukotoxins), and lipoprotein gene clusters. However, all varieties of vSa β contain a gene cluster for serine

proteins and superantigen genes absent in CA-MRSA but present in HA-MRSA strains. Adjacent to short DRs, the locus *etd* PAI contains exfoliative toxins *etd* and *edin-B* (encoding exfoliative toxins), IS element, and restriction/modification system [158].

2.3.2.3. Development and evolution of pathogenicity

The mechanisms of horizontal gene transfer in prokaryotic cells include transduction, conjugation, and transformation, among which phage transduction has been the primary transmission drivers of genes among different species and thus plays an important role in the formation of PAI [159]. The formation of PAIs may include 5 stages [160] as follows: (i) acquisition of virulence gene via horizontal gene transfer regulated by an operon and derived from “gene pool” of varied environment; (ii) integration of foreign genes (commonly derived from various complex genes of different donors) into the bacterial chromosome or plasmid via site-specific recombination or other mechanisms, following similar rules to complete integration and shaping obvious structure of genetic island; (iii) evolvement of MGEs into PAI via restructure, gene elimination, and acquisition of other genetic materials, during which the gene components associated with mobility may be inactivation or lost, such as origin of plasmid replication, self-transmissible plasmid *tra* and phage *int*; (iv) induced expression of foreign genes under temperate environment; and (v) acquisition or elimination of genetic information constantly via a series of recombination, insertion or elimination, by which PAI may retrieve MGE and obtain the ability of excision and transfer the whole PAI from chromosome to another recipient bacterium.

Evolution of the bacterial genome may significantly influence its pathogenicity, mainly including point mutations, recombination, and horizontal gene transfer. Despite slow evolution due to relatively low frequency of point mutation, the horizontal gene transfer of large genetic segments (such as PAIs and SaPIs) undoubtedly speeds up the exchange of bacterial genes (as “quantum leap” in short time), leading to the consequent appearance and spread of various novel mutations or variants [161, 162].

3. Novel perspectives of *Staphylococcus* associated with food safety

3.1. Antimicrobial resistance mediated by MGEs

Antibiotics, as compounds or substances that kill or inhibit the growth of microorganisms, have been regarded as one of the greatest contributions to medicine and humanity and used to treat a wide range of infectious diseases caused by bacteria for both animals and human beings [5, 165]. Abuse of existing antibiotics contributes to the spread of antibiotic resistance and poses a predicament for the treatment of several bacterial infections, including therapy for individuals with food poisoning. In China, as one of the currently worst areas for antibiotics abuse, the annual prescription of antibiotics, including both clinical and veterinary treatment, is approaching 140 grams per person and has been roughly estimated to be 10 times higher per capita than that in United Kingdom [6, 7, 163, 164]. From a retrospective study conducted on 1,739 *Staphylococcus* isolates from a hospital in Guangzhou, China, from 2001 to 2010,

antimicrobial resistance of tested drugs (exclusively for teicoplanin and vancomycin) was commonly observed among the isolates examined, with high resistance rates for β -lactamases (94.0% and 73.7% for penicillin and oxacillin, respectively) and resistance percentages for cefoxitin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, trimethoprim-sulfamethoxazole, and tetracycline ranging from 83.9% to 19.4% [165]. As a consequence, antibiotic resistance in microorganisms still remains one of the core concerns in global public health, with methicillin-resistant staphylococci (MRS) strains representing one important group, commonly considered as “super bugs” [7]. Since their first discovery in 1961, MRS (including MRSA and MRCNS) have become among the most prevalent pathogens causing nosocomial infections throughout the world [13, 16, 17, 19, 166]. With the first report of an MRSA-mediated gastrointestinal illness outbreak [174], MRS strains have been considered a major contributor to both health-care-associated and foodborne illnesses. MRS had been identified from contamination of various food samples, such as milk, pork, chicken, veal, beef, turkey, and lamb meat [168–170] as well as in food production animals, such as cattle, chickens, pigs, and cows and are closely connected with the newly discovered MRSA designated as livestock-associated MRSA (LA-MRSA) [171–173]. Carriage of MRS strains in a wide variety of food and food production livestock may not be limited to only food hazard but also poses a significant occupational risk for the industrial staff, such as handlers, asymptomatic carriers, and uncolonized individuals. MRS strains show resistance to nearly all β -lactam antibiotics and commonly multiple other drugs due to the *mecA* and other resistance genes carried by an MGE designated as staphylococcal cassette chromosome *mec* (SCC*mec*). Additionally, the role of integrons as a mobile genetic mechanism in horizontal transfer of antibiotic resistance has also been well established [174–177].

3.1.1. SCC*mec*

As one of the major foodborne infectious pathogens, *S. aureus* (in particular, MRSA) has been considered to be a potential “super bug,” posing a challenge to hospital infection control and a threat to global food safety. Due to the carriage of the *mecA* gene encoding a novel specific penicillin-binding protein (PBP2a), which exhibits a decreased binding affinity to antibiotics, MRSA presents resistance to virtually all β -lactam antibiotics [178]. Evolution from methicillin-susceptible *S. aureus* (MSSA) to MRSA occurred with the acquisition of a genomic island, the staphylococcal cassette chromosome (SCC*mec*). Harboring *mecA* and a large number of functional and regulatory genes, SCC*mec* is an MGE present in *Staphylococcus* species. With accurate excision and integration mediated by site-specific recombinase genes *ccrA* and *ccrB*, SCC*mec* is capable of integration into the bacterial chromosome, leading to the rapid spread of antimicrobial resistance (to β -lactam or other antibiotics) among staphylococcal strains.

MRSA was found shortly after the common use of methicillin, which was first licensed to treat penicillin-resistant *S. aureus* infections in Britain in 1959 [179], resulting in outbreaks of MRSA occurring worldwide. In the 1980s, an extraordinary large chromosomal DNA segment greater than 30 kb carrying *mec* was found to contain no allelic equivalence in MSSA strains by direct chromosome analysis of MRSA strains; this region was designated as *mec* DNA [180–183]. In 1987, the sequence of *mecA* gene cloned from a Japanese MRSA strain was determined [184,

185]. In consideration of the threat caused by this pathogen, the characteristics of MRSA were studied and SCC mec was found to be an MGE in MRSA. Additionally, the types of SCC mec were found to be genetically diverse. In 1999, the cloning and determination of the structure of the entire *mec* DNA sequence from a Japanese *S. aureus* strain N315 (first isolated in 1982) was reported [186]. Based on the structure, it was reported that *mec* DNA was a novel genomic element designated as staphylococcal cassette chromosome *mec* (SCC mec) driven by two site-specific recombinase genes referred to as cassette chromosome recombinases A (*ccrA*) and cassette chromosome recombinases B (*ccrB*) [178]. This was the first time that SCC mec was defined, and *ccrA* and *ccrB* were proposed as a novel set of recombinases, defining a new family of staphylococcal genomic elements. After the discovery of SCC mec , various types of SCC mec were continuously found by scientists around the world. In 2001, the identification of two additional types of SCC mec was isolated in other countries of the world and designated as type II SCC mec (found in N315) [187]. The two additional types of SCC mec were type I found in NCTC10442, which is the first MRSA isolate in England in 1961, and type III found in 85/2082 isolated in New Zealand in 1985. In 2002, a novel type of SCC mec designated as type IV was identified from CA-MRSA strains [188]. According to the foundation of type IV SCC mec , a new type of SCC mec designated as VI, which was originally mistaken as type IV SCC mec , was explored [189, 190]. In 2004, type V SCC mec was found in the chromosome of a CA-MRSA strain (WIS [WBG8318]) isolated in Australia [191]. Since 2008, novel types of SCC mec were found globally. Types VII and VIII SCC mec were identified in MRSA strains JCSC6082 (a Swedish isolate) [192] and C10682 (a Canadian isolate) [193], respectively. Novel types of SCC mec designated as types IX and X were identified in MRSA strains JCSC6943 and JCSC6945, respectively [194]. The latest type of SCC mec to be classified was designated as type XI; it was discovered in the MRSA strain LGA251 genome with a divergent *mecA* homologue (*mecALGA251*) [195].

3.1.1.1. Structure and types

As a major member of the SCC family and a carrier for gene exchange in staphylococci strains, SCC mec is located near the replication origin of the *Staphylococcus* chromosome and inserted at *attB* site located at the 3' end of a novel ORF with unclear function (*orfX*). Typical SCC mec comprise 3 basic genetic elements: (i) *Ccr* complex, composed of two site-specific recombinase genes (*ccrA* and *ccrB*), and surrounding ORFs. Via site-specific recombination mediated by *ccrA* and *ccrB*, multiple antibiotic resistance and heavy metal resistance genes are capable of insertion into SCC mec . SCC mec is further integrated to the staphylococcal chromosome by accurate excision and integration, leading to adaption of the bacterial host to different environments and pressure of antibiotic selection. According to the different types of *ccrA* and *ccrB*, *Ccr* complex was classified into 8 allotypes: type 1 for *ccrA1* and *ccrB1*, type 2 for *ccrA2* and *ccrB2*, type 3 for *ccrA3* and *ccrB3*, type 4 for *ccrA4* and *ccrB4*, type 5 for *ccrC1*, type 6 for *ccrA5* and *ccrB3*, type 7 for *ccrA1* and *ccrB6*, and type 8 for *ccrA1* and *ccrB3*. (ii) *Mec* complex harboring *mecA* and related regulatory genes. Based on the regulatory genes located upstream and downstream of *mecA* and the difference of insertion sequences, *mec* complex was classified to five classes: class A carried the integrated *mecI-mecR1-mecA-IS431* structure, class B carried devoid the *IS1272-ΔmecR1-mecA-IS431* structure that contains integration insertion sequence,

class C carried the *IS431-mecA-ΔmecR1-IS431* structure that contains two copies of insertion sequence *IS431*, class D carried the *IS431-mecA-ΔmecR* structure, and class E carried the *blaZ-mecALGA251-mecR1LGA251-mecILGA251* structure. Class C *Mec* complex was divided into two different class designations: class C1 (two *IS431s* were arranged in the same direction) and class C2 (two *IS431s* were arranged in the opposite direction) by the inserted direction of *IS431*. (iii) A junkyard region (J region) is located between *Ccr* complex and *Mec* complex. According to its location in *SCCmec*, J region was classified into J1 (also known as L-C region), J2 (also known as C-M region), and J3 (also known as M-R region) region, located at the upstream of *ccr* gene complex and downstream of *Ccr* complex and the upstream of *Mec* complex and downstream of *Mec* complex, respectively.

Up to date, *SCCmec* elements are classified into types I to XI based on the nature of *Ccr* and *Mec* complex and are further classified into different subtypes in accordance with diverse J region. Types and subtypes of *SCCmec* are described in detail as follows: (i) Type I *SCCmec* was first discovered and had a long history dating back to the 2000s. The characteristic MRSA strain carrying type I *SCCmec* was identified, designated as NCTC10442, which was recovered in the United Kingdom in 1961, representing the first MRSA strain [187]. Type I *SCCmec*, carrying class B *Mec* complex and type 1 *Ccr* complex, carried a *pls* regulator in J1 region. A subtype within type I *SCCmec* was designated as IA, containing a plasmid pUB110 located in J3 region [202]. According to the nomenclature proposed in 2006 [271], type I *SCCmec* was designated as 1B.1.1 and its subtype IA was designated as 1B.1.2. (ii) The characteristic MRSA strain carrying type II *SCCmec* had been identified and designated as N315, which was first isolated in 1982 and discovered in 1999 [178, 186, 196, 197]. Type II *SCCmec* harbored class A *Mec* complex and type 2 *Ccr* complex. In J3 region, an integrated copy of staphylococcal plasmid pUB110 was found and a *kdp* regulator was found in J1 region. A number of subtypes were designated as IIA, IIB, IIC, IID, IIE, and IIb and a variant in type II *SCCmec* in consideration with the difference of J1 and J3 regions. According to the nomenclature reported in 2006 [196], type II *SCCmec* was named 2A.1.1 and type IIb was designated as 2A.2. IIA, IIB, IIC, IID, and IIE were designated as 2A.3.1, 2A.3.2, 2A.3.3, 2A.3.4, and 2A.3.5, respectively. The variant of type II was designated as 2A.1.2. (iii) A Zelanian isolate designated as 85/2082 first isolated in 1985 was found carrying type III *SCCmec*, which was first discovered in 2001 together with type I *SCCmec* [196] and was known as the representative MRSA strain of type III *SCCmec* until now. Type III *SCCmec* carries class A *Mec* complex, type 3 *Ccr* complex, and an integrated copy of plasmid pT181 encoding tetracycline and mercury resistance in J3 region. Regarding the difference within the J3 region, there were several subtypes in type III *SCCmec* designated as IIIA and IIIB and two variants designated as IIIC and IIID. According to the 2006 nomenclature [196], type III *SCCmec* was designated as 3A.1.1, IIIA was designated as 3A.1.2, and IIIB was designated as 3A.1.3. (iv) The two commonly characteristic MRSA strains carrying type IV *SCCmec*, which was first discovered in 2002, were designated as CA05 (JCSC1986) and 8/6-3p (JCSC1978) [188]. Type IV *SCCmec* was found to have a unique combination of class B *Mec* complex and type 2 *Ccr* complex, and transposon Tn4001 was found in J3 region of type IV *SCCmec*. Diversity in subtypes of type IV *SCCmec* was obtained, including IVa, IVb, IVc, IVd, IVE, IVF, IVA, IVg, IVh, IVi, IVj, and IV1. Based on the 2006 nomenclature [196], IVa, IVb, IVc, and IVd were designated as 2B.1.1, 2B.2.1, 2B.3.1, and 2B.4, respectively. IVE was named 2B.

3.3, IVF was designated as 2B.2.2, and IVA was designated as 2B.N.2. IVg, IVh, IVi, IVj, and IVk were designated as 2B.5.1, 2B.6.1, 2B.7.1, and 2B.8.1, and IV1 was designated as 2B.new.1. (v) The CA-MRSA strain WIS (JCSC3624) isolated in Australia was the characteristic MRSA strain carrying type V *SCCmec* [191], which carried class C2 *Mec* complex and type 5 *Ccr* complex. No subtype had been found so far within the group of type V *SCCmec*. According to the nomenclature proposed in 2006 [196], type V *SCCmec* was designated as 5C.1. (vi) In 2001, type VI *SCCmec* was first identified from a pediatric MRSA clone named HDE288, which was first reported in 1992 [189, 190]. Type VI *SCCmec* carries a class B *Mec* complex and type 4 *Ccr* complex. Until now, no subtype of type VI *SCCmec* has been found, which was designated as 4B according to the 2006 nomenclature [196]. (vii) CA-MRSA strain designated as JCSC6082 (p5747/2002) was isolated in 2002 [198] and identified to carry a type VII *SCCmec* in 2008 [192]. Type VII *SCCmec* carries a class C1 *Mec* complex that was different from class C2 *Mec* complex carried by type V *SCCmec* and type 5 *Ccr* complex. There was no subtype of type VII *SCCmec* reported. According to the nomenclature put forward in 2006 [196], type VII *SCCmec* was designated as 5C1. (viii) Type VIII *SCCmec* was first identified from a Canadian MRSA strain designated as C10682 isolated in 2003 [193]. It harbored a novel combination of class A *Mec* complex and type 4 *Ccr* complex. No subtype of type VIII *SCCmec* has been found currently. According to the nomenclature proposed in 2006 [196], type VIII *SCCmec* was designated as 4A. (ix) Type IX *SCCmec* was first reported to be identified in a MRSA strain designated as JCSC6943 isolated from a Thailand participant [194]. It was found carrying class C2 *Mec* complex and type 1 *Ccr* complex. No subtype of type IX *SCCmec* was found so far. According to the nomenclature put forward in 2006 [196], type IX *SCCmec* was designated as 1C2. (x) Together with type IX *SCCmec*, type X *SCCmec* carried class C1 *Mec* complex and novel type 7 *Ccr* complex was identified in a Canadian MRSA strain designated as JCSC6945 [194]. So far, no subtype of type X *SCCmec* has been found. According to the 2006 nomenclature [196], type X *SCCmec* was designated as 7C1. (xi) Type XI *SCCmec* isolated from MRSA strain LGA254 in southwest England of 2007 was a novel type different from other *SCCmec* with carriage of distinct class E *Mec* complex and type 8 *Ccr* complex. No subtype was found in type XI *SCCmec*, and according to the 2006 nomenclature, it was designated as 8E [195, 196, 199].

A thorough understanding of the molecular epidemiology and evolution of MRSA may aid in the further identification, control, prevention, and therapy of *Staphylococcus*-mediated human diseases, necessitating *SCCmec* typing as an essential tool for discrimination of different types and subtypes. Currently, there are several *SCCmec* typing methods available for the global evolutionary study of MRSA, with multiplex PCR as the major and widely used methodology. Multiplex PCR was first developed in 1988 and put in use to distinguish different types and subtypes of *SCCmec* in 2002 [198, 200]. In various multiplex PCR strategies, specificity of primer design has been the major concern determining the application of *SCCmec* typing. The multiplex PCR assay described in 2006 [201] was applicable for unique and specific typing of types and subtypes I, II, III, IVa, IVb, IVc, IVd, and V, respectively. After years of validation, this multiplex PCR strategy had been demonstrated to be a rapid, simple, and feasible method for *SCCmec* typing and serves as a useful tool for further prevention and control of *Staphylococcus*-mediated infections by clinicians and epidemiologists. However, with emergence of

novel SCCmec (11 types and various subtypes to date), inclusive and novel SCCmec typing methodologies are desperately required.

3.1.1.2. Prevalence and occurrence

As different types and subtypes of SCCmec have been verified to influence the multidrug resistance and the antimicrobial MIC of β -lactam, a thorough understanding of the prevalence of SCCmec may aid in the further identification, control, prevention, and therapy of *Staphylococcus*-mediated human diseases. Consequently, surveillance of SCCmec has been performed globally in past decades. As the first identified type, type I SCCmec was nonpredominant in the 1970s, which was reported in a limited number of areas, including Brazil, Iran, Japan, Philippines, Spain, Switzerland, and the United States [202–210]. Type II SCCmec had been commonly found in Japan, Korea (occasionally in China), and the United States [206, 207, 211–215] and occasionally detected in Algeria, Brazil, China, Iran, Turkey, and Thailand [205, 216–221]. Type III SCCmec has been most frequently found among HA-MRSA and remains the predominant type in many countries or areas including Asia (China, Hong Kong, Iran, Malaysia, Singapore, Taiwan, and Thailand), Europe (Poland, Portugal, and Turkey), and South America (Brazil) [205, 208–214, 217, 220, 222–231]. Types IV and V have been implicated as CA-MRSA-associated SCCmec. A large number of variants (subtypes) have been reported within type IV, which is also the predominant type in Algeria, Brazil, Denmark, Korea, New Zealand, Portugal, Philippines, Sweden, Switzerland, Spain, and the United States [202–204, 209, 219, 221, 231–238]. Other types of SCCmec are rarely detected and reported [166]. According to our preliminary studies, from 2001 to 2006 in Guangzhou, analysis of the distribution of SCCmec type in 262 *Staphylococcus* strains demonstrated that the classic nosocomial SCCmec type (I–III) dominated among the tested strains, and none of the tested strain carried type IV or V. For MRSA strains, 3 and 198 strains belonged to SCCmec types II and III, respectively, with 8 strains untypeable. For MRCNS strains, 9, 24, and 12 strains were classified as SCCmec types I, II, and III respectively, with 8 strains untypeable. From a retrospective study conducted on 1,739 *Staphylococcus* isolates from a local hospital in Guangzhou from 2001 to 2010, SCCmec typing was performed on 263 randomly selected MRSA strains. Type III SCCmec was most frequently observed with an identification rate of 94.7% [249/263], with type II detected in 4 isolates (one individual isolate in 2001, 2002, 2005, and 2008, respectively) and 10 untypeable MRSA strains were recorded [165]. However, diversity in SCCmec types had been obtained from SCCmec surveillance of MRSA from another medical setting in Guangzhou from 2009 to 2012, as types I, II, III, IIIA, IV, V, and VI SCCmec carriage were found to be 17.6%, 56.8%, 6.2%, 10.7%, 4.1%, and 2.1%, respectively.

3.1.2. Other resistance determinants in *Staphylococcus*

Aside from SCCmec, the role of integrons as a mobile genetic mechanism in the horizontal transfer of antimicrobial genes or determinants among microorganisms has been recently well characterized, established, and documented, which may contribute to the broad distribution and spread of antibiotic resistance and ultimate emergence and unleashing of “super bugs” [174–177]. A complete and functional integron platform comprises three elements: (i) the

integrase gene (*intI*) encoding an integrase, (ii) a proximal primary recombination site *attI*, and (iii) a promoter gene (Pc) functionally demonstrated for all integrons [240]. Several classes of integrons have been identified and distinguished by differences and divergence in the *intI* sequences, and integron classes 1 to 3 are so-called multiresistant integron (RIs) with a capability of acquiring identical gene cassettes [173]. Class 4 integron is considered to be a distinct type of integron and termed super integron (SI), which was found on the small chromosome of *Vibrio cholerae* and known to be an integral component of various γ -proteobacterial genomes [17, 241, 242]. As a direct result of the linkage to Tn402-like transposons and associated with Tn3 transposon family (Tn21 or Tn1696), the class 1 integron platform has been the most ubiquitous among microbes and remains the focus of numerous studies, with a large variety of clinical Gram-negative organisms and a few Gram-positive bacteria reported to harbor this integron class [243–245]. The first observation of class 1 integron within *Staphylococcus* spp. was reported in 2004, with species including *Staphylococcus lentus*, *Staphylococcus nepalensis*, and *Staphylococcus xylosus* [246]. In Guangzhou, class 1 integrons were commonly found in MRSA strains (31.6%, 83/263) during 2001 to 2010, with decreasing identification rates observed [6, 7, 165, 166, 247]. From 2001 to 2004 in Guangzhou, the detection rate of class 1 integron for MRSA and MRCNS was 51.7% [46/89] and 56.6% [30/53], respectively [6, 13, 165, 247, 248]. From 2007 to 2010, class 1 integron was found in MRS isolates based on a series of studies of systematic integron investigation in hundreds of staphylococci strains from 2001 to 2006 [165, 247, 248]. Nevertheless, only 38.3% [46/120] of MRSA isolates carried class 1 integron. Undoubtedly, the commonly detected integron-based antimicrobial resistance mechanisms have contributed to the evolution of the resistance of MRSA and may further lead to dissemination of new waves of “super bugs.” Class 2 integron has an organization similar to that of class 1 but is associated with the Tn7 transposon family [174, 249]. Class 3 integron contains a comparable structure to that of class 2 integron and up to date has only been found in a limited number of microorganisms, including *Pseudomonas*, *Alcaligenes*, *Serratia marcescens*, and *Klebsiella pneumoniae* [249–252]. Class 4 integron harbors hundreds of gene cassettes encoding adaptations that extend beyond antibiotic resistance and pathogenicity [253]. The remaining classes of integrons may also contain antibiotic resistance gene cassettes, but knowledge of their worldwide prevalence remains limited [240, 254]. As a genetic element existing in 9% of bacteria and representatives from a broad range of phyla and environments, integrons play a core role in antibiotic resistance among clinical organisms and contribute to the evolution and adaption of bacteria.

3.1.3. Mobility and evolution of MGEs in staphylococci

As a commonly found MGE with an antibiotic resistance gene (*mecA*) and site-specific recombinase genes (*ccrA* and *ccrB*), *SCCmec* has been classified into 11 types, various subtypes, and variants and plays a core role in antibiotic resistance, molecular epidemiology, and evolution of staphylococci. Through recognition of recombination sites (*attB*, *attSCC*, *attI1*, *attC*, secondary sites, etc.) and via this site-specific recombination event, MGEs are capable of capturing foreign genes. The mobility of MGEs is defined as being associated with mobile DNA elements (transposons or plasmids) and antibiotic resistance genes in addition to having a small array size and substantial heterogeneity in recombination sites [187, 229] From

Southern hybridization analysis in preliminary studies, 58 staphylococci isolates were found to harbor one copy of class 1 integron on the chromosomal instead of plasmid DNA compared with their frequent location on plasmids for facilitation of conjugative-mediated transfer [13]. As natural capture systems and assembly platforms, MGEs in *Staphylococcus* (SCCmec or integrons system) allow bacteria to incorporate foreign genes and convert them to functional proteins by ensuring the correct expression. Despite affinity for self-transposition, integron systems are commonly associated with the transposons and conjugative plasmids serving as vehicles for the intra- and interspecies transmission of genetic material as well as gene cassettes capable of mobilizing to other integrons or to secondary sites in the bacterial genome [255]. This event has been regarded as a key mechanism in the dissemination and spread of resistance genes responsible for the swift spread of resistance genes and the rapid evolution of resistance to a wide range of unrelated antibiotics among diverse bacteria [251, 256]. Any ORF existing in the environmental “gene pool” is conceivably capable of being structured into the bacterial genome through the recombination platforms, and MGEs consequently have the potentially limitless capacity to exchange and stockpile functional genes, which enables rapid adaptation to selective pressure and may ultimately endow additional fitness and advantage to the bacterial host. In addition, a vast number of MGEs (such as conjugative plasmids, transposons, insertion sequences, and even entire chromosome) and the captured genes comprise the vast reservoirs of integrons and lead to the longstanding concept of a single massive “gene pool” that is available and temporally shared among bacteria [73]. The common observation of MGEs in microorganisms from the general environment and its enormous sequence diversity detected from such microbes, as well as various products unrelated to antibiotic resistance, strongly suggests that MGEs are ancient genomic structural elements and have played a general role in evolution and adaptation for a considerable period of time [43].

As a genomic island (G island) and MGE demarcated by a pair of DRs and inverted repeats, SCCmec has a set of site-specific recombinase genes (*ccrA* and *ccrB*) required for its movement and is inserted at the 3' end of *orfX* and located adjacent to the replication origin [220]. In the chromosome of staphylococci, SCCmec may have evolved from a primordial mobile element SCC, into which the *mec* complex was inserted. However, the function of the putative SCCmec may not be limited as the conveyer of antimicrobial resistance (mediated by *mec* complex) alone, and this MGE may serve as a vehicle for the exchange of useful genes for the better survival for staphylococci in various environments. In addition, SCCmec is a general genetic information exchange system of staphylococci with *ccrA* and *ccrB* involved in the recombination events (integration and excision), which plays a significant role in the evolution of *Staphylococcus*. MGEs serve as the reservoir for various genes and possess the function of interspecies genetic exchange. It is interesting to speculate whether multiple MGEs carried by staphylococci would speed up the rate of gene exchange or genome evolution, although these hypotheses require further investigation. From previous surveys, the influence of carriage of multiple MGEs on antimicrobial resistance had been investigated in MRSA. The presence of multiple MGEs was found to be strongly correlated with antimicrobial resistance, including erythromycin, gentamicin, tetracycline, and trimethoprim-sulfamethoxazole, which further limits the therapeutic options for deep-seated *Staphylococcus* infection and diseases. For treatment of complicated *Staphylococcus* infections, gentamicin is commonly prescribed by

many clinicians in combination with vancomycin due to enhanced efficacy based on synergistic antibacterial activity [257]. For penicillin-allergic patients, erythromycin has been frequently used. As the first choice for suspected CA-MRSA cutaneous infections, trimethoprim-sulfamethoxazole has also been commonly used in combination with rifampin for MRSA in carriers despite the high recurrence (up to 50%) and frequent emergent resistance of this organism.

Up to date, the most known functional genes carried by MGES are found to encode resistance to the oldest groups of antibiotics (such as tetracycline, streptomycin, and spectinomycin) that have been discontinued in clinical settings for decades but still available in veterinary practice. Although the indiscriminate use of these older antibiotics is no longer occurring in the clinical setting, their use in veterinary medicine may contribute to a novel and significant concern in food safety. Abuse of antibiotics leads to the emergence of antibiotic resistance and poses a predicament for the future treatment of bacterial infection, with MGEs undoubtedly facilitating the rapid spread and dissemination of a vast number of resistance genes among microorganisms.

3.1.4. *Livestock-Associated MRSA (LA-MRSA)*

As a common pathogen for both clinical medicine and food safety, MRSA was first reported as hospital associated before the 1990s and thus designated as HA-MRSA. Since the 1990s, CA-MRSA strains have increasingly been reported among groups of patients with no apparent connection to hospitals. It is noteworthy that a large number of such CA-MRSA-infected patients or carriers were pediatric associated. Aside from HA-MRSA and CA-MRSA, LA-MRSA has been recently documented and is known to be more persistent in food products from swine and cattle [258], which is also responsible for pneumonia, endocarditis, and necrotizing fasciitis by LA-MRSA carriers [259]. Nowadays, LA-MRSA acts as an increasing risk for public health and a challenge to livestock farming and related food products. LA-MRSA was mostly found among animals (particularly pigs) and humans with frequent contact to livestock farming or livestock food products [260–262]. After the first isolation of MRSA from livestock (cows with mastitis) [263], a extremely limited number of reports were focused on LA-MRSA. However, after an initial LA-MRSA case occurred in humans, described in 2005 [262], LA-MRSA have been the focus of numerous recent studies. In 2007, a transmission of MRSA (ST1, spa-type t127) between cows and humans was reported, verifying the transmission between animals and humans [264]. Afterwards, different types of LA-MRSA have been continuously discovered globally, and the prevalence and occurrence of LA-MRSA vary significantly in different areas. In Europe and America, the majority of LA-MRSA strains belong to sequence type (ST) 398, whereas ST9 is frequently discovered in Asia [265–268]. Both livestock and humans are potential carriers of LA-MRSA, and individuals working in animal clinics and livestock production environments with direct contact or exposed to MRSA-positive animals or ingestion of the MRSA-positive livestock food products have an increased risk of becoming MRSA carriers [269, 270]. A high risk of animal to human transmission of ST398 was found to result from direct association between animal and/or human MRSA

carriages in the farm setting [261, 269, 271–273] despite much lower occurrence of transmission between humans by LA-MRSA and that of HA-MRSA [274–276].

As a clone of typical LA-MRSA, ST398-LA-MRSA has been responsible for serious infections and outbreaks worldwide [277, 278]. Containing various spa-types, ST398-LA-MRSA strains are mostly found to carry type IV or V SCC mec , which are nontypeable by standard PFGE using *SmaI* digestion due to protection from digestion by the presence of a restriction/methylation system [271, 279, 280]. According to the virulent properties of ST398 strains, most animal-associated ST398-LA-MRSA strains lack the major virulence factors in staphylococci, such as Panton-Valentine leukocidin (PVL), TSS toxin 1, and exfoliative toxins [281]. However, various resistance genes commonly present in staphylococci of human and animal origins are also recovered in ST398-LA-MRSA strains, including the β -lactamase gene cluster *blaZ-blaI-blaR*, the tetracycline resistance genes *tetM* and *tetK*, the macrolide-lincosamide-streptogramin B (MLSB) resistance genes *ermA*, *ermB*, and *ermC*, the lincosamide resistance gene *lnuA*, and arrays *aacA-aphD* or *aadD* for resistance to gentamicin-tobramycin-kanamycin or kanamycin-neomycin, respectively [281–285]. In addition, novel resistance genes were also discovered in ST398-LA-MRSA strains, such as *dfrK* (trimethoprim resistance), ABC transporter genes *vgaC* and *vgaE* (pleuromutilin-lincosamide-eptogramin A resistance), and *apmA* (apramycin resistance) [286].

Emergence, spread, and dissemination of ST398-LA-MRSA from animals, as well as its transmission between humans and animals, strongly suggest that the antimicrobial resistance caused by veterinary antibiotic abuse poses a hazard to both humans and animals regarding food safety challenges associated with animal origins.

3.2. Viable Putative but Nonculturable (VPNC)

In nature, bacteria exist in various states such as normal growth state, dead state, dormant state, and VPNC state, which was first reported in 1986 [287]. Differing significantly from the “starvation survival” state, VPNC state [previously known as viable but nonculturable (VBNC)] is a specific state under which bacteria remain alive but fail to form a colony on routine bacteriological media that normally support their growth. Consequently, routine bacteriological detection methodology fails to detect the VPNC bacteria. However, given the right conditions, bacteria in the VPNC state remain active and can “resuscitate” to the normal state. Hence, the VPNC food spoilage or pathogenic bacteria are considered to be a stealth source of contamination, posing a significant concern for traditional surveillance and control methodologies of foodborne pathogens.

3.2.1. Induction and resuscitation

Entering into the VPNC state is considered to be a survival mechanism for nonsporulation bacteria under a number of harsh environmental conditions, which is described in detail as follows: [1] Nutrient starvation [288]. Without essential nutrients, bacterial growth and metabolism may be terminated and thus enter the death-like status. Nutrient starvation, such as the absence of carbon source or nitrogen source, which is an extreme condition for the

growth of bacteria, can induce the VPNC state. [2] Extreme temperature [289]. The appropriate temperature for typical bacterial growth ranges from 20°C to 37°C, and termination of growth usually occurs under extremely high or low temperature. The temperature of 4°C or -20°C, at which bacteria stop growth and metabolism, is frequently used for induction of VPNC state. The combination of nutrient starvation and low temperature has also been widely applied as an induction condition. [3] pH value [290]. Most microorganisms grow in neutral and slightly acidic or alkaline pH conditions. Strong acidity or alkalinity may lead to bacterial death-like states, which has been occasionally used to induce the VPNC state. [4] Salinity [291]. As an extreme condition for bacterial growth, high salinity has been found to enable the entering of VPNC state. [5] Osmotic stress [291]. Extremely high osmotic stress was reported to be applied for VPNC state induction. [6] Oxygen availability [292, 293]. In an aerobic environment, anaerobic bacteria would enter into the VPNC state and the absence of oxygen would induce the VPNC state of aerobic bacteria and vice versa. [7] Existence of heavy metals [294, 295]. [8] Common food preservatives (cryopreservation, vacuum preservation, etc.). Currently, numerous bacteria are reported to have the ability to enter into VPNC state, such as *Salmonella* spp. [287, 296, 297], *Enterococcus* spp. [298–300], *Vibrio* spp. [301–309], *Campylobacter* spp. [310], *Pseudomonas* spp. [307, 311–313], *Shigella* spp. [314, 315], *Lactobacillus* spp. [316, 318], *Escherichia coli* [313, 316], and *Staphylococcus* spp. [318–324]. Furthermore, it has been well established and documented that bacteria in VPNC state can resuscitate and regain culturability when provided with appropriate conditions [308, 325, 326]. A variety of processes, including elevation of temperature gradually or directly [308], heat shock treating [325], adding nutrients [326], and adding organic matter (Tween-20, Tween-80, catalase, sodium pyruvate, etc.) were found to be applicable for resuscitation from the VPNC to normal state. The resuscitated bacteria are comparatively similar to their exponential-phase bacterial counterparts.

Currently, only 2 species of *Staphylococcus*, *S. aureus*, and *S. epidermidis*, were capable of entry into VPNC state [318–324]. In 2009, formation of VPNC *S. aureus* by radiation was reported for the first time, representing the first evidence of *Staphylococcus* cells entering the VPNC state [318]. One year later, induction of VPNC state by starvation of the *Staphylococcus* cells at low temperature (4°C) was also obtained [319]. Resuscitation of *S. aureus* strain under VPNC state was induced by temperature upshift (from 4°C to 22°C) or rich medium supplemented with sodium pyruvate [319, 320]. The prevention of resuscitation was observed by deficiencies in catalase or superoxide dismutase, indicating the relation of VPNC formation of *S. aureus* to oxidative stress [319, 320], constituting the initial studies on the mechanism of the formation and resuscitation of *S. aureus* in VPNC state. In addition, *S. aureus* cells in biofilm were found to enter into a VPNC state under antibiotic pressure (vancomycin or quinupristin/dalfopristin) [320, 321], suggesting that central venous catheter (CVC) or medical implant-associated biofilms may be potential reservoirs for *S. aureus* and *S. epidermidis* in the VPNC state [323]. Thus, both biofilm formation and VPNC induction may augment clinical challenges associated with antibacterial treatment options. *S. epidermidis* biofilms were reported to enter into the VPNC state when grown in excess glucose presumably due to accumulation of acidic compounds as the degradation products of glucose metabolism. This process was counteracted by high extracellular levels of calcium and magnesium added to the culture medium allowing

modulation of the proportions of VPNC bacteria within *S. epidermidis* biofilms [324]. Although the induction and resuscitation of *Staphylococcus* cells in VPNC state has been verified, relatively little is known with respect to inducing and resuscitating condition, necessitating further investigation into this fascinating bacterial survival strategy.

3.2.2. Characteristics and mechanisms

Remaining metabolically or physiologically active, bacteria in VPNC state maintain cell integrity but exhibit dwarfing, which contribute to protect against a wide variety of stressors. The maintenance of metabolic activity and continuous gene expression under VPNC state [327, 328] indicates that potentially ingested bacteria may still be capable of causing foodborne illnesses. Such microorganisms also possess the capacity to regain culturability *in vivo* [329], exhibiting high ATP level, membrane potential [298], and retained plasmids, presenting higher autolytic capability than exponentially growing cells. The outer membrane protein profile also alters with entry into VPNC state [330]. Due to the diversity of VPNC bacteria, various characteristics among different species of microorganisms are being discovered worldwide. Regarding the mechanism of the VPNC state, the up- or down-regulation of genes and proteins associated with VPNC status compared to the exponential phase and the resuscitated status is considered to be potential factors for entering and exiting of VPNC state. However, it is currently unclear as to which genes are essential for these processes.

As for the pathogenicity of *Staphylococcus* cells under VPNC state, the viable cell numbers and gene expression had been found to remain constant in VPNC state by examination of epifluorescence microscopy, flow cytometry, and reverse transcription-PCR (RT-PCR) [320, 321]. This finding implied that *S. aureus* cells are likely still pathogenic in VPNC state and thus pose a significant concern on its threat to food safety.

3.2.3. Detection and identification

VPNC pathogenic bacteria are considered to be a threat to public health and food safety due to incapability of detection by the “gold standard” methodology for identification of food-associated microorganisms. Hence, the development, evaluation, validation, and further application of rapid and accurate detection methodology for VPNC bacteria are considered to be the leading concerns for the surveillance of bacterial cells in VPNC state as well as further understanding of the mechanisms on their survival and persistence in the extreme environment. The conventional detection method for VPNC bacteria was the combination of acridine orange direct count (AODC) (for total bacterial cell number counting), bright-field microscopy with nalidixic acid (for metabolically active cell number counting), and plate counting (for determination of culturability). The occurrence of entry of bacterial cells into VPNC state was validated and confirmed when colony counts were totally depleted on culture plates (with no observed colonies), which was designated as nonculturable, whereas the total bacterial and metabolically active cells still remained countable. Despite the limited application of nalidixic acid on Gram-negative microorganisms, the novel LIVE/DEAD Bacterial Viability Kit with requirement on differential fluorescence was employed for the detection of both Gram-positive and Gram-negative bacteria [331]. In consideration of the carcinogenesis and expense of

fluorescence substances, the development of molecular assays, such as random amplified polymorphism DNA and RT-PCR [328], was recently applied to identify bacterial cells in the VPNC state. As *Staphylococcus* species were concerned, an immunosensing system using impedance spectroscopy measurements was recently developed and applied for rapid verification and quantification of *S. aureus* cells in the VPNC state [322], with high sensitivity and specificity obtained.

In conclusion, foodborne pathogens, especially *S. aureus* strains, which contain various virulence genes, are capable of forming VPNC state and resuscitating into active and pathogenic state under specific conditions, posing a significant threat to food safety. The “farm to table” process includes food ingredients, processing, transportation, and storage, which involves a large variety of conditions. A number of such conditions (such as low temperature during refrigeration) may be sufficient for entry into the VPNC state, complicating the use of routine diagnostics by resulting in high “false-negative” rates of pathogen detection. However, once resuscitation occurs under proper conditions, foodborne pathogens remain active and virulent, which thus are highly likely to cause food poisoning outbreaks.

4. Concluding remarks

Ingestion of food is the major (although, not only) way for human beings to obtain nutrient substances for basic living; therefore, the quality and safety of food have recently become a major concern. Considered to be an expanding global problem and leading topic in public health, food safety is no longer limited to foodborne illnesses but has been extended to all safety issues associated with “farm to table” food approaches. In the past decade, a large number of worldwide cases or reports have been available regarding food containing unhealthily, harmful, or toxic substances (other than food poisoning outbreaks). Foodborne microorganisms, previously limited to pathogenic bacteria and toxic substances produced in food, have played a critical role in food safety. However, now due to diversity in the genus and species of microbes, variety of mechanisms on the regulation of growth and survival, and complexity of ecosystem involving polymicrobial interaction and environmental factors, a number of novel microbial issues associated with food safety have been recently acknowledged. Microorganisms may very well be capable of surviving the journey from farm to table via various evasion mechanisms at various food processing stages, including source (antimicrobial resistance caused by the use of drugs in veterinary medicine or livestock feed), processing (formation of biofilm and further survival of bacterial elimination), storage (formation of VPNC state and “false-negative” detection), and even after cooking (production of heat-stable toxins that remain active despite elimination of host bacteria). The contributions of aforementioned and novel evasion mechanisms with respect to food safety undoubtedly require further investigation *in vitro* and *in vivo* for improved diagnostic and decontamination procedures.

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