

Review

# Methicillin-resistant food-related *Staphylococcus aureus*: a review of current knowledge and biofilm formation for future studies and applications

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## Abstract

There is increasing concern about the public health impact of methicillin-resistant *Staphylococcus aureus*. Food and animal are vectors of transmission, but the contribution of a contaminated environment is not well characterized. With regard to this, staphylococcal biofilms serve as a virulence factor, allowing MRSA strains to adhere to surfaces and other materials used in the food industry.

Methicillin resistance and biofilm-forming capacity may contribute to the success of *S. aureus* as a human pathogen in both health care and community settings and the food production chain. This review summarizes current knowledge about the significance of food- and animal-derived MRSA strains and provides data on attachment and biofilm formation of MRSA. In addition, the impact of quorum sensing on MRSA gene expression and biofilm formation is examined.

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**Keywords:** *Staphylococcus aureus*; MRSA; Biofilm; Quorum sensing; Virulence

## 1. An overview

Highly virulent strains of staphylococci are emerging which are resistant to many antimicrobial molecules, such as methicillin-resistant *Staphylococcus aureus* (MRSA). Currently, MRSA is the most commonly identified antibiotic-resistant pathogen in many parts of the world [1]. While long recognized as a nosocomial infection [2], the epidemiology of MRSA has changed in recent years with the emergence of community-acquired MRSA [3]. At present, new evidence suggests that domestic animals, including food animals, are capable of serving as reservoirs and shedders of MRSA and that transmission between host species may be possible [4]. The emergence of MRSA in food-producing animals has provoked great concern about the presence of MRSA in

associated foodstuff. MRSA strains have been isolated from foods, posing a threat concerning their possible dissemination through the food production chain [5,6]. Both methicillin-sensitive *S. aureus* (MSSA) and MRSA have an inherent ability to form biofilms on various surfaces [7,8]. Staphylococcal biofilms serve as a virulence factor allowing the MRSA strains to adhere to surfaces including implanted devices and other materials used in the food sector. Scott et al. [8] reported that MRSA strains were found on various household surfaces.

## 2. Methicillin-resistant *S. aureus* (MRSA)

MRSA strains were first reported in humans in the 1960s and subsequently, emerged as important nosocomial pathogens with multiple healthcare-associated (HA-MRSA) clones being internationally disseminated. Until the 1980s, MRSA infections were restricted to hospitals and were primarily associated with immunocompromised individuals. In the late 1980s and early 1990s, MRSA emerged as an important agent

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of community-acquired (CA-MRSA) infections, first in the Oceania region and later throughout the world [9,10].

CA-MRSA differ from the HA-MRSA as they show a more virulent phenotypic profile. They frequently produce the Panton-Valentine leukocidin, a toxin often associated with severe skin infections. These isolates have been reported to affect individuals without classical risk factors for MRSA infections [11,12]. In addition, nosocomial infections can now be caused by CA-MRSA lineages, making it difficult to distinguish between CA- and HA-MRSA isolates [11].

In 2010, it was estimated that MRSA caused illness in more than 150,000 persons annually in healthcare facilities in the EU [13]. Recent statistics indicate that invasive HA-MRSA infections have declined, but CA-MRSA infections are still increasing [14]. MRSA isolates display a remarkable clonal structure and pandemic clones are associated with few specific lineages [15]. Currently, MRSA is distributed worldwide and constitutes a major concern in human health because of its complex epidemiology and its ability to acquire novel antibiotic resistance mechanisms [16].

Different genetic techniques are currently used for classification of *S. aureus* strains, including pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and DNA sequencing of the X region of the protein A gene (spa typing). Consequently, a single *S. aureus* isolate can have more than one valid name, depending on the test used for typing. Examples of strain names are USA100, CMRSA1 or EMRSA1, based on PFGE typing, ST followed by a number (ST398) based on MLST typing, or “t” followed by a number (t011) in spa typing. MRSA are also grouped, by MLST, into clonal complexes (CC398), which contain genetically related ST types. Isolates may be identified with a combination of tests for a more complete description. The most common MRSA multilocus sequence typing (MLST) clonal complexes (CC) worldwide are CC1, CC5, CC8, CC22, CC30 and CC45 [15]. More recently, distinct lineages of MRSA have been identified from livestock, known as livestock-associated MRSA (LA-MRSA), highlighting the adaptation of the species to diverse ecological niches. Worldwide, in fact, various clones of MRSA have been reported in domestic pets, livestock, wild birds and other animals [17]. These findings revealed that companion, livestock and wildlife animals can play a major role as MRSA reservoirs. These strains (LA-MRSA) show a different profile compared to HA- and CA-MRSA. LA-MRSA has been identified as being less aggressive, not encoding many of the toxins often associated with *S. aureus* [18]. Many of the LA-MRSA strains belong to lineage multilocus sequence type 398 (ST398) and they show a broader host range compared to most other *S. aureus* lineages [10]. It has been postulated, that LA-MRSA originated as methicillin-sensitive *S. aureus* in humans and were transferred to pigs, where they acquired methicillin and tetracycline resistance via the uptake of mobile genetic elements and then transferred back to humans [19].

Methicillin resistance is, in staphylococci, conferred by the carriage of staphylococcal cassette chromosome *mec* (SCC*mec*) [20]. SCC*mec* is a mobile genetic element that

includes the *mec* gene complex, composed of the *mecA* gene encoding penicillin-binding protein 2a (PBP2a), which shows low affinity for  $\beta$ -lactam antibiotics such as penicillin and methicillin, the two regulation genes *mecR1* (encoding the signal transducer protein MecR1) and *mecI* (encoding the repressor protein MecI) and the associated insertion sequences [21,22]. SCC*mec* also carries unique site-specific recombinases designated as cassette chromosome recombinases (*ccr*), responsible for mobility of the elements [22,23]. In addition to the *ccr* and *mec* genes, three other regions (J regions) are included in SCC*mec* typing [24]. J regions typically contain pseudogenes and truncated copies of transposons and insertion sequences and were originally designated junkyard regions. Currently, they are commonly referred to as joining regions, since they encode important functions such as resistance to additional antibiotics and to heavy metals [25].

Structural organization and the genetic content of SCC*mec* are highly diverse and the chromosomal cassettes are classified by a hierarchical system into: (i) types based on a combination of the type of *ccr* gene complex and the class of the *mec* gene complex and (ii) subtypes defined by structural differences in J-1, -2 and -3 regions [22,23]. Despite differences in size (from ~21 kb to 67 kb) and gene content, all SCC*mec* elements share common features. First of all, all SCC*mec* carry the *mecA* gene in a *mec* gene complex and the *ccr* gene(s) (*ccrAB* and/or *ccrC*) in a *ccr* gene complex. Integration of SCC*mec* at a specific site, the attB integration site sequence (ISS) present at the 3' end of the *orfX* gene in the staphylococcal chromosome, which serves as a target for *ccr*-mediated recombination, is another shared characteristic, as is the presence of flanking direct repeat (DR) sequences containing the ISS [25].

The *ccr* gene(s) and surrounding open reading frames (ORFs) compose the *ccr* complex. To date, three phylogenetically different *ccr* genes (*ccrA*, *ccrB* and *ccrC*, with nucleotide identity of <50%) have been found in *S. aureus* strains. The *ccrA* and *ccrB* genes were classified into four allotypes, based on sequence variations between them (DNA sequence identities are between 50% and 84%) [26]. The *ccr* gene complexes reported thus far can be divided into two groups, one carrying two adjacent *ccr* genes (*ccrA* and *ccrB*) and the other carrying the *ccrC*, and five types were identified: type 1 (carrying *ccrA1B1*), type 2 (carrying *ccrA2B2*), type 3 (carrying *ccrA3B3*), type 4 (carrying *ccrA4B4*) and type 5 (carrying *ccrC*) [27].

The *mec* gene complex is classified into five classes (A to E). The prototype of the *mec* gene complex is class A *mec*, which contains *mecA*, the complete *mecR1* and *mecI* regulatory genes upstream of *mecA*, and the hypervariable region (HVR) and insertion sequence IS431 downstream of *mecA*. The other four classes contain truncated *mecR1* genes resulting from insertion of IS1272 or IS431 and/or differences in spatial arrangement of the elements composing the complex. Several variants of the major classes have also been described [25].

Subtypes of SCC*mec* within the same *mec-ccr* complex are defined by structural differences in J regions. J1 is the region between the right chromosome junction and the *ccr* complex,

J2 is between the *ccr* gene complex and the *mec* gene complex and J3 is between the *mec* complex and the left chromosomal junction [26].

Some SCC*mec* cassettes include other resistance genes besides the *mecA* gene, which encode resistance to other antibiotics and/or heavy metals. Such genes are part of integrated copies of plasmids or transposons [28]. Recently, a divergent *mecA* homologue, termed *mecC* (formerly *mecALGA251*), was identified in *S. aureus* isolates from dairy cattle in United Kingdom [29]. *mecC* shows 70% nucleotide identity to *mecA* and is located in a novel SCC*mec* element designated SCC*mec* type XI. Routine culture and susceptibility testing will identify *S. aureus* isolates containing *mecC*; however, molecular confirmatory methods will not identify them as MRSA [29]. *S. aureus* containing *mecC* has been found in a range of multi-locus sequence type lineages isolated from humans and other animal species [17,29,30].

To date, 11 major SCC*mec* variations have been described (I to XI), but types I–V are the most common. SCC*mec* types I–III are relatively large and are typically found in strains associated with healthcare facilities (HA-MRSA). SCC*mec* types IV and V are smaller and are usually found in MRSA associated with community-acquired infections (CA-MRSA) and often encode virulence factors such as the Panton Valentin leukocidin (PVL). In addition, the SCC*mec* contains additional insertional sequences that allow incorporation of additional antimicrobial resistance markers [31]. These insertional sequences explain why many methicillin-resistant staphylococci are resistant to non- $\beta$ -lactam antimicrobials, which act through mechanisms other than interference with bacterial cell wall synthesis such as macrolides, fluoroquinolones [32].

SCC*mec* can spread horizontally between staphylococcal populations [32–34]. Molecular analyses of numerous MRSA strains indicate that resistance genes have been transferred to various methicillin-susceptible *S. aureus* strains (MSSA) on multiple occasions [35]. Methicillin resistance transfer between *S. aureus* strains in the laboratory is also possible at low frequencies by transduction [36,37]. A restricted number of *S. aureus* lineages are able to acquire SCC*mec*, with multilocus sequence type clonal complexes (CCs) CC1, CC5, CC8, CC22, CC30 and CC45 predominating among MRSA isolates [35,38,39]. Multiple independent transfers of resistance in different countries are required for possible generation of resistance lineages from originally susceptible strains. The presence of restriction-modification systems that block horizontal gene transfer (HGT) into *S. aureus* in a lineage-specific manner [40], and the sequence variations around the attB site that impede the integration of SCC*mec* into the chromosome of some strains [41], are likely responsible for the apparent ability of only a limited number of lineages to acquire SCC*mec*.

Moreover, MRSA may also resist vancomycin [42]. With regard to this, many MRSA strains, particularly HA-MRSA strains, are also resistant to other classes of antibiotics [43]. In any case, according to the Clinical and Laboratory Standard Institute (CLSI), MRSA is defined as *S. aureus* having the

*mecA* gene or CLSI breakpoints, according to broth-based methods, to oxacillin and methicillin are 2 mg/L and 4 mg/L, respectively. In addition, the mechanisms of methicillin resistance in MRSA are diverse [44]. The earliest published report of MRSA in farm animals described the detection of MRSA, in 1972, in dairy cows with mastitis in Belgium [45]. A new MRSA strain, ST398, was detected in 2003 in swine and swine farmers in the Netherlands [46]. Following its detection in animals and in-contact humans such as pigs and people professionally exposed to pig farming [47,48]. CC398 LA-MRSA is now recognized as an important cause of zoonotic infections in several countries throughout Europe, Asia and the USA, and has recently been identified in a pig in Northern Ireland [32,49]. However, case control studies in humans identified working with live pigs and cattle as a risk factor for testing positive for MRSA CC398 [50,51]. Later studies revealed the presence of that lineage in other food-producing animals. Other studies on the prevalence of this clone in food-production animals showed its presence in pigs in several countries in Europe, Canada, the US and Asia [32]. The clone is also widely spread in veal calves in the Netherlands [52]. In poultry, an observation on the presence but not the prevalence of MRSA in Belgium was published [53]. In dairy cattle, systematic monitoring is lacking in Europe, but initial reports suggest a low prevalence [54]. CC398 has also been found in companion animals and horses, but the primary reservoir is in food production animals [53]. Moreover, a different swine-associated MRSA strain, CC9, is circulating among pigs and pig farmers in China [55]. It appears, from genome analysis of numerous human and animal strains belonging to the CC398, that LA-MRSA ST398 was derived from a human MSSA ST398 strain that was transferred to pigs [19].

### 3. MRSA as a foodborne pathogen

While long recognized as a nosocomial infection [2], the epidemiology of MRSA has changed in recent years. The emergence of MRSA in food-producing animals, in fact, has provoked great concern in the presence of MRSA in associated foodstuff [56]. The prevalence of MRSA in food greatly varies depending on the animal's origin and country. Many reports have identified the presence of MRSA in different retailed meat products [57]. In general, foods from which MRSA were isolated included raw meat (including pork, beef, lamb, chicken, turkey and on one occasion rabbit), dairy products (milk and cheese) and, in one instance, pancakes. Based on literature data available, it seems that, while pork had the highest contamination rate in the USA and Canada [58–60], in the Netherlands and Denmark, the highest prevalence was found in poultry [16]. In some cases, the MRSA isolates were identified as HA- or CA-MRSA, indicating that food handlers were likely to be the source of the bacteria [58–61]. In other surveys, LA-MRSA strains were the primary isolates, indicating an animal source of contamination [4,62]. In surveys of raw meat conducted in the European Union, MRSA rates of up to 37.2% have been reported [62], with CC398 LA-MRSA

predominating. Of further concern, rates in excess of 60% have been observed in turkey meat [32]. In details, in surveys carried out between 1999 and 2006 in Korea, Hungary, France, Japan, Italy and Jordan, the frequency of MRSA isolation was low when different types of food were tested, with results ranging from 0 to 1.2% [63–71]. A similar percentage was found in a study carried out in Spain (1.3%) in food samples of animal origin [72]. However, higher rates of contamination with MRSA were observed in Pakistan (10.4%) in raw milk [73], and in two studies in the Netherlands (2.5% and 11.9%) and in the United States (5%) in raw meat at retail [4,58,74]. In particular, Normanno et al. [70] isolated MRSA strains from bovine milk and some cheese varieties in Italy. Several studies carried out typing of the MRSA isolates recovered from food samples. Two studies performed in the Netherlands identified CC398 in one isolate recovered from raw food of pork origin [74] and in 85% of MRSA food isolates of different origins (chicken, turkey, pork, beef, veal, lamb and fowl) [4]. In Spain, MRSA CC398 was obtained in a pork meat sample [72]. In addition, specific MRSA strain CC398 has been linked to different foods animals and humans, and a new MRSA form, livestock-associated (LA-MRSA) has arisen. LA-MRSA has been isolated from both human and animal infections, as well as from bovine mastitis cases [57]. Other sequence types (ST5 and ST125) associated with human infections have been detected in other studies [67,68,72]. When biotyping was carried out, human biotypes were identified in two studies [65,66] and animal biotypes in one study [70]. When SCCmec characterization was carried out with MRSA isolates of food origin, SCCmec III and IV (including a new subtype SCCmec IVg) were identified in isolates recovered from cows' milk and chicken [66–68]. Foodborne illness causing severe symptoms due to MRSA has been documented, illustrating the potential impact of this pathogen on human health [5]. In conclusion, it is difficult to compare the risks of different food types, given the wide variety of methodologies used and the lack of quantitative data on MRSA levels in most studies. It has been shown that MRSA can frequently be detected in raw meat in low numbers. However, foodborne MRSA infections have been formally demonstrated on several occasions [75,76], and food as a transmission route of successful LA and CA-MRSA lineages should not be neglected. On the basis of limited data currently available, the question of whether occurrence of MRSA in food is a cause for concern with regard to human health cannot be answered [77]. Moreover, MRSA strains commonly carry enterotoxin genes [75]. If foods are cooked properly, MRSA cells are killed. However, under conditions of temperature abuse, MRSA cells can grow in foods, produce heat-stable enterotoxins and cause foodborne intoxication. In individuals whose normal biota has been depleted by antibiotic treatment, MRSA cells on-ready-to eat foods including processed meats and cheeses can cause staphylococcal enterocolitis. However, MRSA present in foods can potentially cause colonization or infection from handling or consumption of contaminated food. Consumers should be educated concerning the risk of MRSA transmission from raw

foods and/or the environment. Such contamination can be avoided by adhering strictly as a transmission route of successful LA and CA-MRSA lineages to rules of hygiene.

#### 4. Food-initiated MRSA outbreaks

Three separate outbreaks of MRSA in a special-care infant unit have been described [78]. A milk bank worker was identified as a carrier of the MRSA isolate, and it is postulated that infant milk served as a vehicle. To date, only two reported outbreaks have been associated with MRSA-contaminated food [75,76]. One case of community-acquired food-borne illness caused by an enterotoxin C-producing MRSA and involving three persons occurred in Tennessee in 2000 [75]. Moreover, a case of MRSA infection following consumption of MRSA-contaminated food was described in a Dutch hospital [76]. This outbreak involved 27 patients and 14 health-care workers. MRSA was detected in the food and throat of a dietary worker who prepared food for patients. Five of the 21 patients who developed clinical disease died. MRSA colonization, or MRSA infection by contact with MRSA-contaminated food, has not been described. However, MRSA swabbed on pork loins was transferred at low levels to knives and cutting boards, indicating the potential for cross-contamination and exposure in persons working with pork [79]. In addition, concerning food-handlers, a study involving carriage of *S. aureus* by 200 food handlers in Botswana identified 115 of them as *S. aureus*-positive and 33 strains were identified as MRSA [80]. Studies among food handlers in Europe revealed a different situation. In Spain, Argudin et al. [81] did not find MRSA among 14 food handlers sampled. A study conducted among professional meat handlers in the Netherlands in 2008 [82] revealed that all samples taken from 95 employees working in the cold meat-processing industry and in institutional kitchens were negative for MRSA. The authors concluded that the risk for professionals of colonization by MRSA from handling raw meat was low, and that the general population was at an even lower risk of being colonized/infected through meat handling [82]. It is noteworthy that MRSA can be transmitted between humans and animals during close contact. Persons with occupational exposure to animals carrying MRSA are more likely to carry related animal MRSA strains than the general population. Colonization with livestock-associated MRSA, in fact, has been reported frequently in people who work with these animals [50,83]. Transmission of MRSA was reported between Hungarian cows with subclinical mastitis and an agricultural worker who was throat-swab-positive [84]. In 2004, a Dutch pig farmer's wife developed MRSA mastitis and pleural effusion. Subsequent screening found her husband and daughter to be MRSA carriers. Six months later, with the baby and parents still colonized, wider sampling revealed that 3 co-workers and 10 pigs from the closest holding were carriers of PFGE non-typeable MRSA, all of which were identical [85]. Agricultural workers and their families in contact with pigs and (to a far lesser extent) cattle have a high likelihood of MRSA

colonization, with up to 23% of Dutch pig farmers being nasal carriers of MRSA [47]. Based on data available, the risk from contact with contaminated food appears to be small, much lower than that following contact with live animals or humans. In conclusion, the most frequently reported instances of foodborne MRSA occurred through contamination by infected food handlers rather than food itself.

## 5. *S. aureus* attachment and biofilm formation

*S. aureus* is a well known pathogen living in a wide variety of environments [86]. It also has the inherent ability to form biofilms on biotic and abiotic surfaces [7]. In addition, biofilm production is recognized as an important virulence factor for bacteria of the genus *Staphylococcus* [87–89]. Biofilm formation is, in fact, important for survival of staphylococci in the food industry, and studies have already shown the adhesion capacity of food-related staphylococcal strains. On the other hand, since *S. aureus* has emerged as one of the most important pathogens causing infections with indwelling medical devices, and while this type of device-associated infection is mainly characterized by the pathogenic capacity to colonize the surfaces of such equipment [90], *S. aureus* biofilm formation has been studied in depth in medicine.

The *S. aureus* biofilm mode of growth is tightly regulated by complex genetic factors. However, the mechanisms and/or processes of biofilm formation in *S. aureus* are poorly understood, and studies on the expression profiles of genes involved in biofilm mechanism are still limited.

*S. aureus* biofilm on food-contact surfaces poses a serious risk of food contamination [91]. It has been frequently found in surfaces of food processing plants and is responsible for outbreaks related to consumption of fresh and processed foods worldwide [92–96]. Both methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) can form biofilm on different surfaces. Environmental contamination with MRSA has been reported [97,98]. On pig farms, MRSA have been found in various samples such as dust, and possibly even in air. In abattoirs that slaughter CC398 carrier pigs, MRSA was detected in a number of areas [99]. The environment of abattoirs and food production units could be contaminated with MRSA [100]. Scott et al. [8] reported that MRSA was isolated from 9 out of 35 homes, and they were found on various household surfaces such as sponge/cloth, drain and worktop, etc. *S. aureus* can produce a multilayered biofilm embedded in a glycocalyx or slime layer, with heterogeneous protein expression throughout [101]. The process of biofilm formation involves diverse quorum sensing mechanisms. *S. aureus* can develop at least two types of biofilm: ica-dependent (i.e. promoted by the *ica* operon) and ica-independent biofilm matrixes (Fig. 1).

Polysaccharides and, in particular, polysaccharide intercellular adhesin (PIA), are the primary determinants of the accumulation phase of staphylococcal biofilm formation [102,103]. PIA consists of linear  $\beta$ -1,6-linked glucosaminylglycans.

### 5.1. PIA-dependent biofilm formation

PIA is produced in vitro from UDP-N-acetylglucosamine via products of the intercellular adhesion (*ica*) locus [104]. The genes and products of the *ica* locus [*icaR* (regulatory) and *icaADBC* (biosynthetic) genes] have been demonstrated to be necessary for biofilm formation and virulence, and are upregulated in response to anaerobic growth, such as conditions seen in the biofilm environment [105].

The staphylococcal respiratory response regulator SrrAB is responsible for PIA induction under anaerobic environments via binding of a 100 bp DNA sequence upstream of the *icaADBC* operon [106]. Other environmental factors can also play a role in regulation of *ica* [107]. In addition, PIA expression is repressed by TcaR, a transcriptional regulator of the teicoplanin-associated locus; however, deletion of the *tcaR* gene had no effect on PIA synthesis [108]. Strong negative regulation is conferred by IcaR through binding of the *ica* cluster promoter; deletion of the *icaR* gene results in enhanced *ica* cluster gene expression [108,109]. However, the protein regulator of biofilm formation, Rbf, expresses transcription of IcaR, albeit indirectly, leading to augmented *ica* gene expression, PIA production and biofilm formation [110]. In addition, Spx, a global regulator of stress response genes, was shown to have a negative regulatory impact on biofilm formation, seemingly by modulating IcaR [111].

### 5.2. PIA-independent biofilm formation

Despite the importance of the *ica* gene locus in biofilm development, biofilms can occur in an ica-independent fashion. The *arlRS* two-component system was shown to repress biofilm development and, when deleted, led to enhanced attachment and PIA production [112]. However, biofilm synthesis was unaffected by additional deletion of the *icaADBC* operon, suggesting that, in this double deletion mutant, PIA was not essential for biofilm development [112]. In addition, Fitzpatrick et al. [107] showed that biofilm formation in MRSA strain BH1CC was unaffected by *ica* locus deletion. However, other mutant strains lost the ability to form biofilm. Interestingly, when *S. aureus* *icaADBC* operon-deletion mutants are categorized by methicillin susceptibility, MRSA strains are capable of biofilm development, whereas MSSA strains are impaired in biofilm formation [113]. These data propose that biofilm formation in an ica-independent manner is strain-specific. In an *ica*-deletion mutant *S. aureus* strain, protein A (SpA) production was found to be essential for biofilm formation [114]. Fibronectin binding proteins (FnBPs) can also arbitrate biofilm formation through an essential role by the major autolysin (Atl) and *sigB* regulation [115]. In addition, biofilm-associated protein (Bap) and Bap-related proteins of *S. aureus* can confer biofilm development independently of PIA production through cell-to-cell aggregation [116].

Another important component of staphylococcal biofilm is extracellular DNA (eDNA). Rice et al. [117] discovered that a mutation of *cidA*, a gene encoding an effector of murein

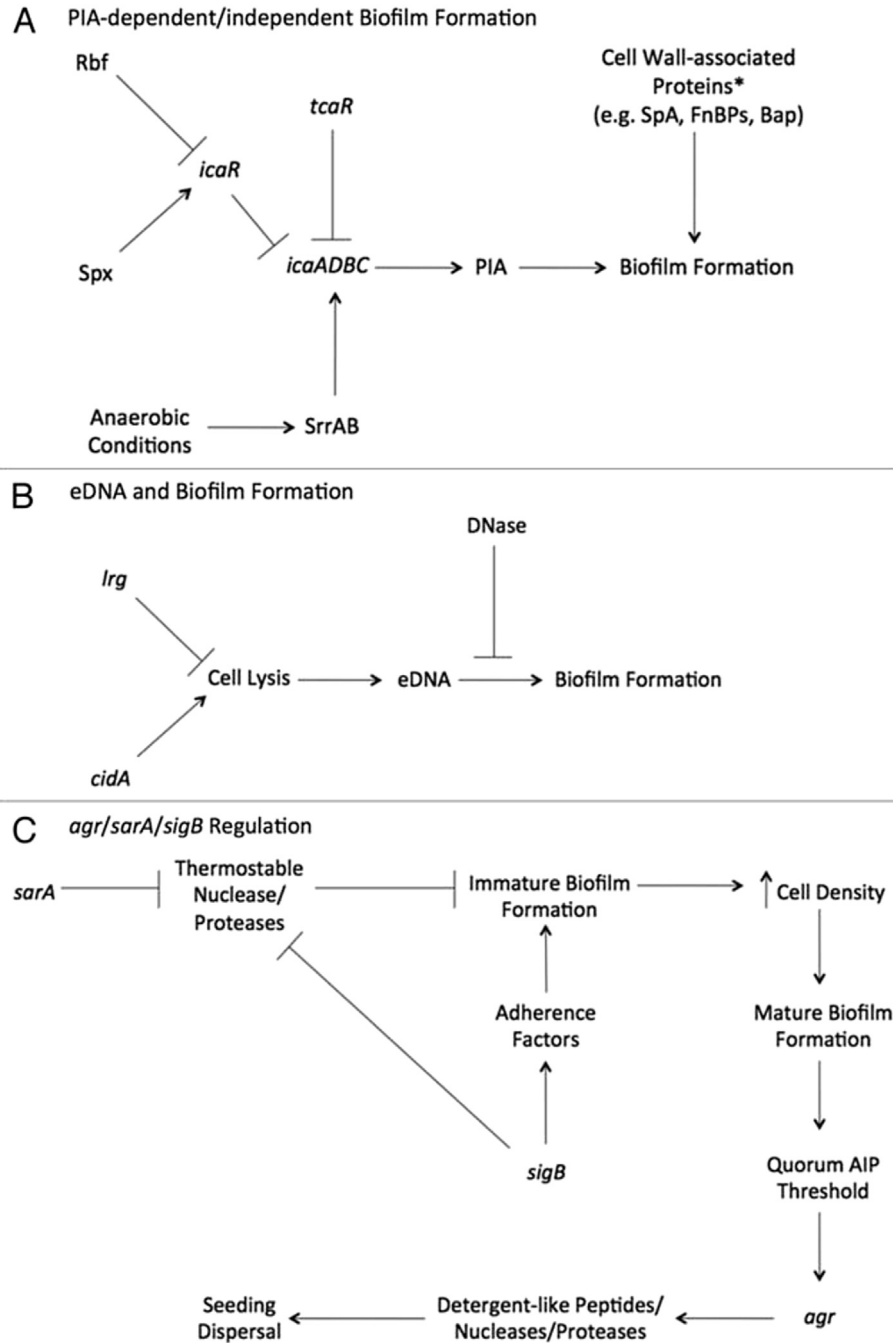


Fig. 1. Flow chart of regulatory factors involved in *S. aureus* biofilm formation [101]. (A) PIA-dependent biofilm formation Expression of *icaADBC* gene cluster can be suppressed by production of *tcaR* and *icaR*, resulting in downregulation of PIA and biofilm formation. In the case of the *icaR* gene, expression can be up- or down-regulated by proteins Spx and Rbf, respectively. Consequently, Spx induction of *icaR* expression results in downregulation of *icaADBC* expression, PIA production and biofilm formation. Conversely, Rbf inhibits *icaR* expression, leading to upregulation of *icaADBC* expression, PIA production and biofilm formation. Additionally, anaerobic conditions induce production of SrrAB, causing expression of the *icaADBC* gene cluster, PIA production and biofilm formation. PIA-independent biofilm formation can be mediated through cell wall-associated protein cell-to-cell (SpA, FnBPs and Bap) adhesion (MRSA-specific). *icaADBC*, intercellular adhesion biosynthetic genes; PIA, polysaccharide intercellular antigen; *tcaR*, transcriptional regulator of the teicoplanin-associated locus; *icaR*, intercellular adhesion regulatory gene; Spx, global regulator of stress response genes; Rbf, protein regulator of biofilm formation; SrrAB, staphylococcal respiratory response regulator; SpA, *S. aureus* protein A; FnBPs, fibronectin-binding proteins; Bap, biofilm-associated protein. (B) eDNA and biofilm formation. eDNA leads to enhanced biofilm formation. DNA release is arbitrated through cell lysis and controlled by *lrg* and *cidA* gene expression. Upregulation of the *lrg* gene results in inhibition of cellular lysis, DNA release and biofilm formation. Conversely, *cidA* gene expression enhances cellular lysis, DNA release and biofilm formation. eDNA, extracellular DNA; *lrg*, regulator of murein hydrolase and cell death; *cidA*, regulator of murein hydrolase and cell death. (C) *agr/sarA/sigB* regulation. Expression of the *sarA* gene results in downregulation of proteases and the thermostable nuclease, allowing for development of an immature biofilm. Expression of *sigB* similarly downregulates protease production, but additionally promotes expression of adherence factors that aid in initial biofilm formation. The immature biofilm increases in cell density until a mature biofilm develops. At this stage, the density of AIPs throughout the bacterial community reaches a quorum sensing threshold and induces expression of the *agr* gene. Induction of *agr* results in upregulation of detergent-like peptide, protease and thermostable nuclease expression, leading to release of bacterial cells from the mature biofilm, termed seeding dispersal. *sarA*, staphylococcal accessory regulator; *sigB*, *sigmaB*; AIP, auto-inducing peptides; *agr*, accessory gene regulator.

hydrolase activity and regulating cell death, led to less adherent biofilm that contained lower levels of genomic DNA. The *cidA* gene has been shown to be a holin homolog involved in cell lysis, and these data suggest that this cell lysis activity is necessary for DNA release and biofilm development. Cell lysis and subsequent genomic DNA release must also occur early in cell attachment for proper biofilm formation, as shown by Mann et al. [118]. The *lrg* gene, another regulator of murein hydrolase and cell death, counteracts the effects of *cidA* DNA release, as revealed by enhanced biofilm attachment and matrix-associated eDNA in *lrgAB* mutants. Other globally upregulated genes in *S. aureus* biofilms included autolysin [119,120]. Control of cell lysis and eDNA release is another branch in the complex regulation of biofilm formation by *S. aureus*.

The major global regulators, staphylococcal accessory regulator (*sarA*) and accessory gene regulator (*agr*), have also been implicated in biofilm formation. A two-component regulatory gene locus encoded by *arlRS*, a member of the OmpR-PhoB family of response regulators, is regulated by *agr* and *sarA* loci [121]. When upregulated, the product of *arlS* prevents biofilm formation and may mediate attachment to polymer surfaces by affecting peptidoglycan hydrolase activity. Transcripts of *sarA* are upregulated in biofilms when compared with planktonic cultures [122].

In addition, the capacity to form biofilm is reduced in *sarA* mutants [123]. Biofilm formation can be recovered in *sarA* mutants by concurrently mutating the gene encoding the *S. aureus* thermostable nuclease, *nuc* or addition of protease inhibitors, as *nuc* and extracellular proteases were found to be transcriptionally upregulated in *sarA* mutants [124]. By inhibiting *nuc* expression and protease activity, *sarA* expression may prevent degradation of extracellular DNA and protein, important biofilm structural components. Although *sarA* affects and is affected by *agr*, the effect of *agr* in vitro mutants on biofilm development is minimal and has been shown to be an *agr*-independent pathway [122,124,125]. Despite this, there is evidence that the *agr* locus is associated with biofilm development. The *agr* quorum sensing system has been shown to downregulate genes of cell-wall-associated adherence factors [126]. This would lead to lesser adherence and thus, indirectly, decreased initial biofilm formation. Repression of *agr* has been shown to be necessary for biofilm formation [127]. Additional levels of control are accomplished through the *sigB* operon product  $\sigma^B$  in *S. aureus* [128]. Factors necessary for the early stages of biofilm formation, including clumping factor, fibronectin binding protein A (FnbpA) and coagulase, are all upregulated by  $\sigma^B$  [129].

In addition, factors that correlate with seeding dispersal and a planktonic phenotype are all negatively controlled [130]. Rachid et al. [131] revealed that a *sigB*-deficient *S. aureus* could not form biofilm. In contrast, Valle et al. [122] found that a *sigB* deletion mutant still effectively developed biofilm. These conflicting reports suggest that the *sigB* operon may act in a strain-specific manner in controlling biofilm formation.

Ica-independent biofilms appear to be the most important bacterial films produced by MRSA isolates. Although the

exact composition of these biofilms and the mechanisms involved in biofilm regulation are poorly defined, treatment with proteases and protein-denaturing substances suggests that proteins are major components of *S. aureus*, including MRSA biofilm [132].

Fibronectin-binding proteins (FnBPs) were shown to play an important role in ica-independent biofilm formation by human MRSA isolates [113]. In addition, the major autolysin (Alt) plays a role in the early stages of ica-independent biofilm formation by clinical MRSA strains [115]. More recently, two studies suggested that penicillin-binding protein 2a (PBP2a) is also an important factor in biofilm accumulation [133].

Currently, data available on the prevalence of MRSA in the environment of food production premises are scarce. Recently, Bardiau et al. [134] reported that biofilm formation ability was present in all MRSA isolates analyzed from bovine mastitis in Belgium. In any case, few studies have investigated the abilities of MRSA strains to form biofilms, and most of these studies addressed clinical aspects related to biofilm formation by MRSA strains [135–138]. Mirani et al. [7] reported the ability of MRSA from food to form biofilm on Congo red agar plates and glass slides. Quantitative and qualitative analysis showed that 37/64 (57.8%) of MRSA isolates were biofilm producers, and 27/64 (42.1%) showed no sign of biofilms either on Congo-red agar plates or glass slides. The interesting finding of this study was the relation of SCCmec type IV, *agr* type II and biofilm formation. All biofilm-positive isolates, in fact, belong to SCCmec type IV, and most (91.8%) carry *agr* type II. Biofilm-negative isolates, on the other hand, belong to SCCmec type V. Kwon et al. [139] also supported this finding and reported that MRSA strains with SCCmec type IV have a higher probability of biofilm formation compared with other types of SCCmec. Cafiso and co-authors [140] recently reported that strong biofilm-producing strains belong to *agr*-type II; these isolates possess a defective *agr* system and show early transcription of *icaA*. This suggests that SCCmec type IV and *agr* type II make a good combination for biofilm formation in food-borne MRSA isolates.

In conclusion, understanding the processes of biofilm formation by *S. aureus* can facilitate the study of the ability of wild strains to adhere to and form biofilms when they are exposed to conditions simulating the environments found in health care settings and food processing plants, enabling a better definition of control strategies.

## 6. Approaches to studying biofilm formation and virulence (i.e. –omics technologies) by MRSA

Quantifying biofilm that develop from microorganisms is the main problem in studies related to biofilm formation. The most frequently applied technique for quantification of biofilm cells is crystal violet staining [141–147]. Other proposed methods include the determination of biofilm by counting colony-forming units (CFUs) after detachment of biofilm cells by the bead vortexing method [148–151]; a semi-quantitative method for MRSA biofilm estimation is the PBP2a latex agglutination test [145,146,152]. However, information

provided by these methods is limited to estimation of biofilm cells, with the capacity for biofilm formation depending on the microorganism, growth conditions or the presence of an antibiofilm agent. On the other hand, abundant techniques are currently available for studying and understanding bacterial physiology. Indeed, several -omics techniques developed during the last decades are applied in different studies for understanding biofilm formation and the virulence of different pathogens. In general, these methods enable a rapid and reliable monitor of pathogen physiology, providing a clear view of microbial behavior in a particular environment. Staphylococcal biofilm have been studied by several researchers; in a recent review, intra- and interspecies interactions within staphylococcal biofilm were well documented [153]. However, to our knowledge, limited works related to biofilm have focused on MRSA. These limited studies are listed below; observations obtained in most of them regarding gene expression, biofilm formation, virulence and quorum sensing will be analyzed in the future.

In the study of MRSA biofilm, the *S. aureus* surface protein (SasC) involved in cell aggregation and biofilm accumulation was characterized by SDS-PAGE and western blotting and detected by immunofluorescence microscopy [154]. Similarly, the contribution of the *ica* operon to biofilm development in *S. aureus* clinical isolates was investigated by reverse transcriptase PCR (RT-PCR) [107]. Western and northern blotting were also performed recently for detection of PBP2a and determining the effect of oxacillic acid and mupirocin on *mecA* gene transcription in MRSA [155]. In another study, a combination of proteomic (2-DE) and genomic (qPCR, microarrays) analysis was applied to investigate the effect of manuka honey on proteomes and genomes of MRSA [156]. Similarly, Qin et al. [144] used a combination of transcriptomics, i.e. RNA seq, RT-real-time PCR and scanning electron microscopy (SEM), to study MRSA biofilm inhibition by ursolic acid and resveratrol. The same experimental procedure was followed to study biofilm inhibition by ursolic acid and resveratrol of methicillin-susceptible *S. aureus* (MSSA) [157]. The dynamics of biofilm formation and interactions between MRSA and *Candida albicans* was studied by the application of SEM, along with a method for quantification of extracellular hydrolytic enzymes [147]. In another study, atomic force microscopy (AEM) was recently applied to investigate the binding strength and cell surface localization of staphylococcal adhesion (SdrG) [158]. On the other hand, Chen et al. [159], proposed a label-free near-infrared surface-enhanced Raman scattering (NIR-SERS) method for discrimination of pathogenic bacteria, and it was suggested that some differences could be observed between two strains of MRSA isolated from clinical samples.

From the above research works and applied techniques, it seems that abundant tools are available and may be applied for understanding and/or controlling MRSA biofilm, extrapolating important results associated with infections caused by this type of staphylococcal biofilm. In addition, the application to a food or food-related processing environment represents a promising research topic in food safety and security.

## 7. Impact of quorum sensing on MRSA gene expression and biofilm formation

Around 50 or more accessory genes (known as virulons) involved in the pathogenesis of *S. aureus* encode proteins that enable the organism to evade host defenses, to adhere to cells and the tissue matrix, to spread within the host and to degrade cells and tissues, for both nutrition and protection [160]. Establishment of infection implies that bacteria will orchestrate expression of a group of molecules that determine pathogenicity, collectively known as virulence factors.

Among bacterial global regulatory systems, cell–cell communication and quorum-sensing (QS) systems have gained broad attention in the scientific community, while the agr (accessory gene regulator) is the most important and well studied quorum sensing system in *Staphylococcus* species. It should be noted that it has been difficult to define the entire virulon one gene at a time, because with the exception of the toxins, staphylococcal disease is dependent on the combined action of many exoproteins [160]. However, the agr global regulator seems to control expression of exoprotein genes. Molecules known to be implicated in the agr QS system are RNA II, RNA III and AIP [160–162]. RNA II constitutes the Agr-sensing mechanism, transcribed by genes *agrA*, *agrC*, *agrD* and *agrB* (P2 operon). RNA III (the P3 transcript), which is the intracellular effector of the agr regulon [163], acts reciprocally, upregulating transcription of most extracellular protein genes and downregulating that of many surface protein genes [163]. On the other hand, AIP (an autoinducer protein), is generated from its precursor ArgD and secreted out of the cell through the action of the AgrB membrane protein. Four different classes of AIPs have been identified. AIP from one *S. aureus* strain is capable of activating the agr regulon in itself and inhibits agr activation of other strains [164,165]. It seems that the agr system of *S. aureus* centers around AIPs that interact with cognate AgrC sensor kinases of the same group to regulate exotoxin production and biofilm dispersal [166]. In another study, AP4-24H11, a developed monoclonal antibody (MAbs), was able to alter production of various *S. aureus* exoproteins, including known virulence factors, and to increase biofilm formation in vitro, while also limiting *S. aureus* pathogenicity in vivo [167].

The major autolysin of *S. aureus*, Atl, is the peptidoglycan hydrolase thought to play an important role in cell wall turnover, cell division and cell separation, in the lysis of bacteria induced by the  $\beta$ -lactam antibiotics and biofilm formation [168]. However, another autolysin/adhesion (Aaa), involved in cell-wall metabolism and in some adhesins, has been previously described [169]. Biswas and co-authors [168] observed that staphylococci are able to cope with the loss of Atl by upregulation of aaa expression. It is also noteworthy that Aaa upregulation is not sufficient to prevent cell aggregation or tetrad formation, or to bring back the capacity for biofilm formation. It appears that upregulation of aaa is a regime for the extreme emergencies; it allows survival, but does not cure the severe consequences of an atl mutation. Another important component of *S. aureus* is extracellular genomic DNA

(eDNA), which is released from bacteria by cell lysis and may implicate an additional role for the major *S. aureus* autolysin Atl in biofilm development apart from its function in initial attachment [117]. At low density, bacteria produce protein factors such as the MSCGRAMMS and other adhesions that promote attachment and biofilm accumulation [162]. In addition, ribosomal protein L2 (RAP) is an alternative agr activator believed to act early in growth. On the other hand, downregulation of genes encoding surface proteins early in growth and upregulation of those encoding secreted proteins upregulated post-exponentially are correlated with the population density sensing agr two-component system, which is activated in mid-exponential phase [161,170]. It is also worth noting that the role of the luxS system in *S. aureus* biofilm formation and pathogenicity could not be detected, while its role in metabolism was suggested [171].

Infections due to staphylococci are nearly always monospecific [172], although it is well known that many biofilms are polymicrobial and QS molecules are used for interspecies cross-talk. In a recent review, these intra- and interspecies interactions within biofilms of *S. aureus* are elucidated [153]. A synergistic interaction of *S. aureus* and *Pseudomonas aeruginosa* was observed in CF lungs, as *S. aureus* seems to become, or to selectively grow as, small colony variants (SCVs) which also display reduced susceptibility to other antimicrobial agents and increased ability to form biofilms [173–176]. This induction is mediated by secretion of 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) by *P. aeruginosa* [173], which activates alternative sigma factor B in *S. aureus* [177]. Sigma factor B altered expression of several virulence factors, including those that regulate the ability to adhere to, invade and persist within host cells and facilitate emergence of the SCV phenotype [177]. It had been also suggested that formation of *S. aureus* SCVs is a survival strategy to withstand competition by *P. aeruginosa* [168]. Thus, it seems that *S. aureus* and *P. aeruginosa* do not always mediate synergistic interactions and, in some cases, when co-cultured, *P. aeruginosa* can outcompete *S. aureus* [168]. In brief, *P. aeruginosa* produces various QS-controlled respiratory toxins such as pyocyanin and hydrogen cyanide, which kill *S. aureus*; however, the presence of yayurea A and B (QS inhibitors isolated from *Staphylococcus delphini*) protects *S. aureus* in co-cultivation with *P. aeruginosa* [178,179]. On the other hand, Sandiford and Upton [180] reported that epidermicin NI01 produced by *S. epidermidis* strain 224 is effective against *S. aureus* and biofilm-forming *Staphylococcus epidermidis*. Similarly, in vitro studies have shown that endopeptidase lysostaphin derived from *Staphylococcus simulans* is capable of killing *S. aureus* cells [181] and treating biofilms on in welling catheters in mice [182]. Similarly, *Actinobacillus actinomycetemcomitans* produces dispersin B, a glycoside hydrolase that degrades PNAG with a very high degree of specificity at its  $\beta(1,6)$ -linkage, making it highly effective against staphylococcal biofilms [183]. Furthermore, farnesol, a QS molecule produced from *Candida*, was found to be able to inhibit *S. epidermidis* biofilm formation in vitro [184]. In the same study, a significant reduction in biofilm and

cell viability in a mouse model of catheter-related infection and demonstrated synergy in commonly used antibiotics was observed. On the other hand, *C. albicans*, MSSA and MRSA were found to co-exist in biofilm without antagonistic effects and with an apparent synergistic effect, as the presence of *C. albicans* increased staphylococcal biofilm due to the higher available amount of glucose on the *Candida* matrix [147].

Different approaches have been proposed for blocking bacterial signaling via quorum sensing, i.e. inhibition of the agr (accessory gene regulator) system, controlled expression of PSMs or creating interference using signaling molecules from different microorganisms [183]. However, in the same work, it was suggested that the strain and growth conditions as well as the type of infection (biofilm or planktonic) should not be underestimated, as inhibition may vary because of these factors. A recent transcriptomic and proteomic study of MRSA treated with manuka honey demonstrated reduced expression of virulence markers by downregulation of global regulators such as agr, sae and sarV [156]. In the same work, they concluded that the observed differential expression of proteins and genes caused by manuka honey provided valuable insight into mechanisms by which growth and pathogenicity in MRSA were inhibited. In a previous study, researchers noted that the use of ursolic acid may inhibit biofilm formation, but the amount of  $\delta$ -hemolysin may be increased [144]. In the same study, it was suggested that combined use of ursolic acid and QS inhibitors may overcome this phenomenon. However, the differing response of MRSA and MSSA strains to resveratrol and ursolic acid treatment had been previously reported [144,157], suggesting a different response of closed strains to the same agent. Fitzpatrick et al. [107] also concluded that regulatory pathways controlling the biofilm phenotype in reference strains may be different from those used by clinical isolates, possibly driven by genetic exchange and exposure to different and complex environments. Similarly, resistance to beta-lactam antibiotics varies according to strain variation, as the majority of MRSA strains produce a unique heterogeneous phenotype in which a subpopulation of extremely high and low beta-lactam MIC values co-exist [185]. It has been reported that the delayed antimicrobial action of factors like ampicillin may result in the emergence of increased resistance due to prolonged and repeated exposure of MRSA to the antimicrobial agent [145,186]. It is worth noting that ampicillin treatment was also found to increase the amount of PBP2a (penicillin binding protein 2a), a protein that confers resistance to beta-lactam antibiotics [145]. However, reduction of PBP2a levels in MRSA biofilm was observed in the presence of fraction F-10 from the plant *Duabanga grandiflora* and the 9EA-FC-B fraction from *Acalypta wilkesiana*, possibly attributed to physicochemicals detected, i.e. alkaloids, tannins, etc. [145,146].

In a recent publication, pathogen protection against the action of disinfectants in multispecies biofilms was reviewed [187]. Dual-species biofilm consisting of *S. aureus* and *Escherichia coli* was more resistant to essential oils extracted from citronella and lemon compared to monospecies biofilm [188]. Similarly, *Bacillus subtilis* biofilms protected *S. aureus*

from the action of peracetic acid [189]. Moreover, the efficiency of chlorhexidine digluconate (CHX) and commonly used mouth rinses was particularly lessened, when confronted with polyspecies biofilms, by *Streptococcus mutans*, *S. aureus* and *P. aeruginosa* on titanium discs compared to mono-species [190].

## 8. Conclusions and future research requirements

The most important issue with pathogens like MRSA is the control of biofilms by inhibition of biofilm formation or removal of established biofilm. The biofilm is a sessile microbial community of cells embedded in a matrix of extracellular polymeric substances and exhibiting an altered phenotype with respect to bacterial physiology, metabolism and gene transcription. Thus, prior to application of an antimicrobial agent to control biofilm formation, its effect on antibiotic resistance, virulence, etc. must be studied in depth. In every study related to biofilm formation, the effect of the compound used for inhibition of biofilm formation or removal of established biofilm on virulence factors must be taken into account. Moreover, the different response of specific strains to the same or different treatments should not be underestimated. The presence of other species and their effect on MRSA biofilm formation or resistance to disinfectants should be considered. The application of nanofilms on surfaces that will eliminate biofilm formation could be of interest. However, their effect on virulence and antibiotic resistance needs to be further studied.

A significant amount of data has been produced in recent years to monitor human pathogens in both health care and in the food production chain. However, the presence of MRSA in associated foodstuffs was underestimated in the past, apart from MRSA inherent ability to form biofilm on various surfaces and materials used in the food sector. With this aim, the present review focused on the prevalence of MRSA in various foods and the ability of strains to adhere to surfaces. In addition, the impact of virulence and QS on MRSA gene expression and biofilm formation, and/or vice-versa, as well as the most recent tools used to study these phenomena, have been reviewed. In conclusion current approaches to the study of biofilm formation via genomics and metagenomics will help to elucidate the structure of biofilm ecosystems associated with food environments. These approaches will hopefully be complemented by transcriptomic and metatranscriptomic analyses of pathogens in order to further contribute to advances in our knowledge of quorum sensing and pathogen virulence.

## Conflict of interest

The authors have declared no conflict of interest.

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