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Chapter

# Extracellular Vesicles and Their Role in *Staphylococcus aureus* Resistance and Virulence

*Brenda Silva Rosa da Luz, Vasco Azevedo, Yves Le-loir and Eric Guedon*

## Abstract

*Staphylococcus aureus* is a pathogen of great importance to clinical and veterinary medicine. Recently, there has been a growing interest in *S. aureus* extracellular vesicles (EVs) in the pathogenesis of this bacterium. Released by living cells into the extracellular milieu, EVs are membranous structures carrying macromolecules such as proteins, nucleic acids, and metabolites. These structures play several physiological roles and are, among others, considered a mechanism of intercellular communication within *S. aureus* populations but also in *trans* kingdom interactions. *S. aureus* EVs were shown to transport important bacterial survival and virulence factors, such as β-lactamases, toxins, and proteins associated with bacterial adherence to host cells, and to trigger the production of cytokines and promote tissue inflammation. In this chapter, we will review the main studies regarding *S. aureus* EVs, including their composition and roles in host-pathogen interactions, and the possible applications of EVs for vaccines and therapy development against staphylococcal infections.

**Keywords:** EV, membrane vesicles, composition, bacterial survival, cargo delivery, immunomodulation, host-pathogen interactions, immunization, vaccine, therapy

## 1. Introduction

### 1.1 EVs characteristics

The release of extracellular vesicles (EVs) is a long-known phenomenon widely reported, mainly in eukaryotes [1–4]. Archaea and Bacteria also release EVs, making their occurrence an evolutionally conserved feature among all three kingdoms [5]. They can be referred as membrane vesicles, microvesicles, ectosomes, exosomes, apoptotic bodies, outer membrane vesicles (OMVs), and others, depending on their origin and characteristics [5, 6]. The study of these particles is of great interest, as they are considered a mechanism of cell-free intercellular communication and *trans* kingdom interactions [7]. They are composed of a lipid bilayer and range from 20 to 1000 nm. They carry several bioactive molecules, such as proteins, lipids, metabolites, and nucleic acids, and were shown to modulate the metabolism and physiology of local or distant target cells [8]. Recently, the study of bacterial EVs has gained attention since they can affect pathogen-host interactions and contribute to bacterial pathogenesis.

### 1.2 History of bacterial EVs

The first study regarding bacterial EVs dates back to 1966, when lipid-like structures purified from culture supernatants of *Escherichia coli* were observed under electron microscopy [9]. In Gram-negative bacteria, vesiculation occurs from the budding out of the outer membrane (OM) that captures components present in the periplasm. This process forms nanoparticles called outer membrane vesicles (OMVs), which are released in the extracellular milieu [10]. Gram-positive bacteria lack an outer membrane and have a thicker peptidoglycan (PGN) cell wall, which was regarded as a barrier to EV release. This might explain why the first observations of EV release in Gram-positive bacteria were reported much later, in 2009,

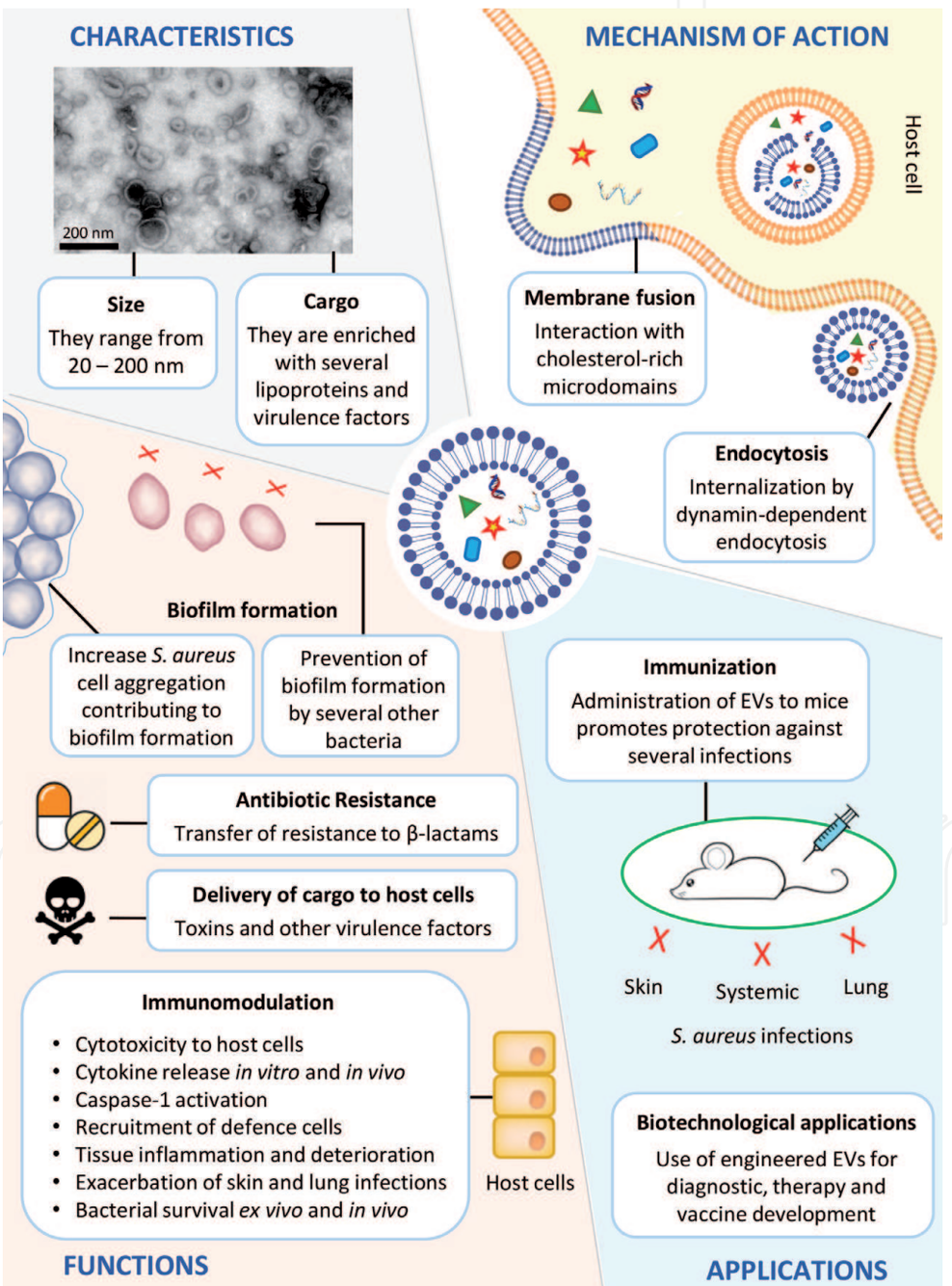


**Figure 1.**
*General features of* S. aureus *EVs.*

when Lee and collaborators demonstrated the production of EVs by *Staphylococcus aureus* [11]. Ever since, other studies confirmed EVs release by other Gram-positive bacteria belonging to various genera such as *Bacillus sp*, *Bifidobacterium sp*, *Cutibacterium sp*, *Clostridium sp*, *Enterococcus sp*, *Lactobacillus sp*, *Mycobacterium sp*, *Propionibacterium sp*, and *Streptococcus sp*, among others [12–22].

### 1.3 *S. aureus* and its derived EVs

*S. aureus* is a bacterium that asymptomatically colonizes the nasal track of 20–80% of the human population without causing disease [23]. *S. aureus* is also a major opportunistic pathogen in humans, being a common cause of nosocomial infections [24]. It is a causative agent of life-threatening diseases such as sepsis, endocarditis, pneumonia, and minor infections in soft tissues [25]. *S. aureus* is also an important pathogen in veterinary medicine. It is one of the main etiological agents of mastitis, an inflammation of the mammary gland that affects dairy herds and causes vast economic losses worldwide [26]. The type and severity of infections depend on strain-specific virulence factors, mostly expressed from accessory genetic elements [27]. Secreted and surface-exposed *S. aureus* virulence factors are responsible for weakening the host immune response, immune evasion, damage to host tissues, and infection onset [28].

One emerging field of great interest is the involvement of EVs in the infections caused by *S. aureus*. Recent studies have shown that *S. aureus* EVs carry important bacterial survival and virulence factors, such as β-lactamases, superantigens, toxins, coagulases, and proteins associated with bacterial adherence to host cells [11, 29–34]. In some cases, they trigger production of cytokines and promote tissue inflammation [35–38]. As EVs are also regarded as potential vehicles for biotechnological and clinical applications, such as the development of vaccines [39–42], their study is an attractive area in microbiology and the future development of new strategies against bacterial infections. Here, we will address the main studies regarding *S. aureus* EVs, their biogenesis, composition, and roles in bacterial resistance, virulence, host-pathogen interactions, and the possible applications of EVs for diagnostic, therapy, and vaccine development against diseases caused by this bacterium (see **Figure 1**).

## 2. Biogenesis of bacterial EVs

Several models have been proposed to elucidate how bacteria release EVs. Since the study of Gram-negative bacteria OMVs dates to the '60s, this phenomenon is better established and documented. Several hypotheses are proposed to explain EVs production, which include one or a combination of many processes [43]. It has been proposed that the accumulation of molecules in the periplasm space alters turgor pressure, promoting OMV release [44, 45]. In another model, alterations in lipid structure and topology could lead to modifications in the membrane curvature, resulting in vesicle bubbling from the outer membrane [46]. On the contrary, EVs biogenesis is still poorly understood in Gram-positive bacteria [47] due to the recent discovery of EV release by these microorganisms [11]. Notably, efforts have been made to better understand how EVs can get through the thick PGN layer present in the Gram-positive bacteria's cell wall structure.

In *S. aureus*, phenol-soluble modulins (PSMs) were shown to be associated with EVs release. These small proteins have surfactant-like properties and are

considered crucial staphylococcal virulence factors since they can play various biological roles [48–50]. The staphylococcal PSMs were reported to have cytolytic and membrane-damaging activities, be proinflammatory, participate in biofilm formation, and be responsible for mobilizing lipoproteins from the staphylococcal cytoplasmic membrane, and the export of cytoplasmic proteins [51–55]. Since *S. aureus* EVs are generally enriched for both lipoproteins and cytoplasmic proteins, some studies investigated the role of PSMs in EV biogenesis. Wang et al. showed that deletion of *psmα* genes in *S. aureus* strain JE2 resulted in a significant decrease in size and number of EVs recovered from the culture supernatant [40]. Similarly, another study with strain USA300 revealed striking differences in EV production between the wild-type and a Δ*psmα3* mutant [56], supporting a conserved process in *S. aureus* species. It was shown that PSMα3 promotes EVs release by an increase in membrane fluidity, and that bacterial turgor under hypotonic osmotic conditions could be an important driving force for EV release in *S. aureus* [56]. Likewise, lipoproteins can also play a role in EV biogenesis since their absence resulted in an increase in membrane fluidity of *S. aureus*, as well as alterations in the protein content, the yield, and the size of EVs [57].

In addition to the importance of PSMs and lipoproteins in staphylococcal EV biogenesis, it was demonstrated that penicillin-binding proteins (PBPs) and autolysins also influence *S. aureus* EV release in acting likely on cell wall porosity to allow EVs to cross the cell wall. PBPs are involved in PGN cross-linking, a crucial EV release factor [40]. The autolysins Atl and Sle1 are PGN hydrolases that play an important role in cell division, modifying, therefore, cell wall integrity. Accordingly, a *pbp4* mutant, which was shown to significantly reduce PGN cross-linking [58], presents an increased EV production, whereas isogenic mutants for both Atl and Sle1 showed a significant decrease in EV size and release, consistent with their roles in peptidoglycan metabolism [40]. In another Gram-positive bacteria, *B. subtilis*, Toyofuku et al. evidenced that prophage-encoded endolysins create holes in the PGN, allowing, therefore, the protruding of biological components to form EVs that are released in the extracellular environment [59].

## 3. *S. aureus* vesicle cargo composition

### 3.1 *S. aureus* vesicle protein cargo

Different molecules may be incorporated into EVs during their biogenesis: nucleic acids, proteins, lipids, and metabolites [5, 8, 60, 61]. Most studies on *S. aureus* EV cargo composition, however, focused mainly on their proteome. The first study characterizing the proteome of *S. aureus* EVs identified with high confidence 90 proteins, distributed in cytoplasmic (56.7%), membrane (16.7%), and extracellular (23.3%) locations [11]. They included N-acetylmuramoyl-L-alanine amidase, which could have a predatory role in competing with other bacteria, transporters (SecD/SecF), and proteins related to antibiotic resistance, such as penicillin-binding proteins PBP1, PBP2 and PBP3, and β-lactamase [11]. They also found that *S. aureus* EVs comprise key virulence factors, such as superantigens (SSaA1 and SSaA2), toxins that disrupt host cell wall (α- and δ-hemolysins), coagulase factors, and immunomodulatory proteins, such as staphylococcal protein A (Spa), and immunoglobulin-binding protein (Sbi). Since then, several studies characterized the EV protein content of other *S. aureus* strains, revealing from 90 to 617 identified proteins, including numerous virulence factors (**Table 1**).

| Strain | No. of proteins | Function | Ref. |
|---|---|---|---|
| 01ST93 | | Non-cytotoxic to host cells (Hep-2) | [31] |
| 03ST17 | 143 | Non-cytotoxic to host cells (Hep-2, HaCaT) | [31, 38] |
| | | Cytotoxic to host cells (HaCaT) | [62] |
| | | Immunomodulation *in vitro* and *in vivo* (e.g., ↑ IL-1β, IL-6, IL-8, TNF-α, and MCP-1) | [38, 62] |
| | | Mast cell recruitment and exacerbation of skin inflammation | |
| 06ST1048 | | Cytotoxic to host cells (Hep-2) | [29, 31] |
| | 143 | Delivery of Spa protein through EVs (Hep-2) | [29] |
| 8325–4 | | Induction of the MAPK pathway (THP-1 and MLE-12) | [63] |
| | | Cytotoxicity to host cells (HeLa) | [64] |
| | | Hemolytic activity | [63, 64] |
| 8325-4Δ*hla* | | Low cytotoxic to host cells (HeLa) | [64] |
| | | Weaker induction of MAPK pathway (THP-1 and MLE-12) | [63] |
| ATCC 14458 | 90 | ND | [11] |
| | | Cytotoxic to host cells (HaCaT) | [30] |
| | | Immunomodulation *in vivo* (↑ IL-1β and IL-6, ↓ TNF-α) | |
| | | Immunomodulation *in vitro* and *in vivo* (e.g., ↑ IL-6, INF-γ, MIP-1α, eotaxin) | [37] |
| | | Immunomodulation *in vitro* and *in vivo* (e.g., ↑ IL-6, TNF-α, IL-12, INF-γ) | [35] |
| | | Induce skin inflammation in mice | [30, 37] |
| | | Promote lung inflammation in mice | [35] |
| | | Protective against lung infections | [42] |
| | | Transfer of resistance to β-lactams | [32] |
| ATCC 25923 | | Cytotoxic to host cells (HaCaT) | [65] |
| | | Immunomodulation *in vitro* (↑ IL-1β, IL-6, TNF-α, IL-8, and MCP-1) | |
| | | Prevention of biofilm formation by other bacteria | [66] |
| ATCC 6538 | | Non-cytotoxic to host cells (HDMECs) | [36] |
| | | Induce recruitment of monocytes (THP-1) | |
| | | Immunomodulation *in vitro* (e.g., ↑ E-selectin, ICAM1 and VCAM1, IL-6) | |
| BWMR22 | | Exogenous EVs from vancomycin treated culture promote *S. aureus* aggregation | [67] |
| CI1449 | | Exogenous EVs confer bacterial resistance to whole blood killing | [68] |
| JE2 | 180 | Cytotoxic to host cells (human leukocytes, THP-1 cells, human macrophages MΦ) | [40, 57] |
| | | Immunomodulation *in vitro* (↑ IL-1β, IL-18, and caspase-1 activation) | |

| Strain | No. of proteins | Function | Ref. |
|---|---|---|---|
| JE2 Δ*arg*, Δ*sae*, Δ*lukAB*, Δ*lukSF-PV*, Δ*hla* | | Decreased cytotoxicity and immunomodulation (THP-1 cells) | [57] |
| JE2-Δ*agr*-Δ*spa* | 212 | Non-cytotoxic to host cells (human leukocytes, A549, HL60, and rabbit erythrocytes) | [40] |
| | | Non-protective against lethal sepsis | |
| JE2Δ*agr*Δ*spa* pHla$_{H35L}$-LukE | | Non-cytotoxic to host cells (human leukocytes, A549, HL60, and rabbit erythrocytes) | [40] |
| | | Protective against lethal sepsis | |
| JE2Δ*lgt* | 198 | Decreased cytotoxicity to host cells (human macrophages) | [57] |
| | | Defective in the induction of IL-1β, IL-18, and IL-6, and caspase-1 activation *in vitro* | |
| M060 | 153 | Cytotoxic to host cells (Hep-2, COS-7 and HaCaT) | [31, 65] |
| | 153 | Immunomodulation *in vitro* (↑ IL-1β, IL-6, TNF-α, IL-8, and MCP-1) | [65] |
| MSSA476 | LB[1]: 131 BHI[2]: 617 | Exogenous EVs promotes bacterial survival *ex vivo* and *in vivo* (human whole blood and neutrophils) | [69] |
| MW2 | 168 | ND | [34] |
| N305* | 222 | Non-cytotoxic to host cells (PS and MAC-T) | [33] |
| | | Immunomodulation *in vitro* and *in vivo* (e.g., ↑ IL-8, IL-1β, TNF-α, DEFβ1, MIP-2, BAFF) | |
| | | Induction of neutrophil recruitment *in vivo* | |
| | | ND | [34] |
| Newman | | Immunomodulation *in vitro* (↑ IFN-β mRNA) | [70] |
| O11* | 164 | ND | [34] |
| O46* | 171 | ND | [34] |
| RF122* | 160 | ND | [34] |
| RN4220 | 92 | ND | [41] |
| RN4220 Δagr | 119 | Engineered EVs protect mice against viral infections | [41] |
| ST692 | [3]: 137 [4]: 156 | Transfer of resistance to β-lactams | [71] |
| USA300 | | Immunomodulation *in vivo* (↑ IGM, total IgG, IgG1, IgG2a, and IgG2b) | [56] |
| | | Protective against systemic and skin infections | [69] |

*Note: Production of EVs was also demonstrated for S. aureus strains ATCC 35556 [72], ATCC 700699 [29], NRS135 [68], NRS77$_{phage}$ [68], RN4220$_{phage}$ [68], RN6390 [32], and TSST-1 103D [29], however, proteomic or functional characterization were not performed.* *Animal isolates; ND, not determined.*
[1]*Luria-Bertani Medium.*
[2]*Brain Heart Infusion Medium.*
[3]*Optimal condition.*
[4]*Sub-inhibitory concentration of ampicillin.*

**Table 1.**
S. aureus-*EVs characterization and functions.*

As shown in **Table 1**, *S. aureus* EVs comprise several proteins. The numbers of proteins vary from one study to another because of the proteomic approaches used and the growth conditions. Sometimes EV proteome comprises up to 24% of the whole bacterial predicted proteome. It is expected that different methods of protein detection may give divergent results, and, indeed, some studies have evidenced such variations. Lee et al. identified 41 and 84 proteins with In-gel and In-solution digestion methods, respectively, with only 35 proteins shared by both sets of proteins identified [11]. In another study, Askarian et al. demonstrated that 43 and 286 proteins are exclusively identified when using either In-solution and Lipid-Based Protein Immobilization (LPI) methods, respectively [69]. These results highlight the impact of detection methods for EVs characterization. Therefore, comparison of EVs produced by different *S. aureus* strains should be done carefully, like other comparative proteomic analysis.

In this regard, a recent study characterized and compared the proteome of EVs derived from several *S. aureus* strains using the same experimental approach [34]. This work was carried out on EVs produced by five *S. aureus* strains of diverse host origins (human, bovine, and ovine). A total of 253 proteins were identified (from 160 to 218 EV proteins according to the strain), 119 of which were common to EVs derived from all strains. This conserved EV proteome included several proteins related to nutrient uptake, antibiotic resistance, virulence, and pathogenesis, reinforcing the importance of EV cargo for bacterial survival and staphylococcal infections [34]. Numerous of these core EV proteins are also present within EVs produced by phylogenetically distant species supporting the existence of specific and conserved rules for protein loading into EVs that remain to be uncovered [34].

### 3.2 Selective protein cargo sorting into EVs

Since EVs bud out of the cytoplasmic membrane, it is natural that their composition mainly reflects the physiological state of the producing cells, as it has been shown by several studies characterizing the EV cargo [73, 74]. However, several studies showed strong evidence that protein cargo sorting is a selective regulated process in both Gram-negative and Gram-positive bacteria [8, 34, 75, 76]. As mentioned before, OMV biogenesis involves the capture of components associated with the periplasm and the OM. Interestingly, OMVs derived from *Serratia marcescens* lack proteins abundant in the OM and, in contrast, can be enriched with proteins that are absent in this compartment [77]. As another example, *Porphyromonas gingivalis* OMVs also exclude proteins abundant in the OM and are enriched with several virulence factors [78]. Regarding Gram-positive bacteria, studies demonstrated that the non-pathogenic *B. subtilis* secretes EVs enriched with lipoproteins and siderophore-binding proteins, which are essential to survival [13]. *Mycobacterium bovis* and *Mycobacterium tuberculosis* were also shown to be enriched with several lipoproteins, some of which can modulate the host response in a TLR2-dependent fashion, contributing to mycobacterial virulence [21].

Several studies demonstrated that *S. aureus* EV cargo comprises secreted, cell wall-anchored, membrane, and cytoplasmic proteins. The latter are their most abundant component [11, 33, 34, 69]. This feature is interesting since it is the unique known pathway of a Gram-positive bacteria to secrete cytoplasmic proteins, which lack any export signals. Moreover, compared to whole-cell proteome, *S. aureus* EVs were also enriched with virulence-factors, extracellular proteins, and lipoproteins [11, 34]. For instance, Lee *et al*. demonstrated that Sbi is highly enriched in *S. aureus* EVs and is localized at the vesicle surface, enhancing its ability

to bind to host cells [11]. Furthermore, secreted virulence factors such as coagulases, β-lactamase, and hemolysins were also enriched [11]. Finally, comparative proteomics revealed that lipoproteins of five *S. aureus* clinical and animal isolates accounted for approximately 20% of the EV content, while they corresponded to only 2.5% of the whole predicted proteome [34]. These data show that some protein populations are enriched in *S. aureus* EVs, and they reinforce the hypothesis that the selection of protein cargo occurs through a dynamic mechanism common to the strains of *S. aureus* species. To date, the molecular mechanisms that drive the recruitment of proteins into EVs remain unclear. Nevertheless, it was proposed that abundance, charge, and subcellular location of proteins could influence their availability and packing into *S. aureus* EVs [34].

### 3.3 *S. aureus* vesicle cargo: other components

As mentioned earlier, data regarding the characterization of the other components of staphylococcal EVs apart from proteins are scarce. Although some studies demonstrated that lipids, carbohydrates, or nucleic acids are also associated with *S. aureus* EVs, they did not perform an extensive characterization of these components. Schlatterer et al. used a fluorescent membrane dye (FM4–64) to quantify lipids present in the membrane of *S. aureus*-derived EVs and demonstrated that lipid release is also dependent on PSMs [56]. In another study, the Fourier Transform InfraRed spectroscopy (FTIR) approach showed that administration of the antibiotic vancomycin induced chemical changes on *S. aureus* EVs, including the reduction of carbohydrate yield in comparison to untreated cells [67]. Regarding nucleic acids, in the study by Andreoni et al., quantification with PicoGreen dsDNA kit revealed the association of DNA molecules to *S. aureus* EVs [68]. Finally, Rodriguez and Kuehn recently demonstrated that *S. aureus* Newman strain secrets EVs containing DNAs of ~500 base-pair long and RNAs with sizes of <300 nucleotides in length [70]. However, further investigations are necessary to better characterize the nucleic acid content of *S. aureus* EVs.

## 4. *S. aureus*-EVs functions

First considered "trash bags" to remove unwanted molecules from cells, nowadays, it is well-established that EVs play essential roles for bacterial fitness. Several described biological functions of OMVs and EVs include offensive and defensive mechanisms, such as quorum sensing, competition, delivery of toxins, resistance to antibiotics, horizontal DNA transfer, and transfer of regulatory RNAs (sRNAs), which can hijack the host immune response altering host-pathogen interactions. *S aureus* EVs were shown to participate in several metabolic and infectious processes, exhibiting several functions (**Table 1**).

### 4.1 *S. aureus*-EVs in cell toxicity

Studies demonstrated that *S. aureus* EVs can be cytotoxic and can induce cell death by delivering their toxin content. For example, δ-hemolysin (*hld*) and the exfoliative toxin A (ETA) were shown to be delivered to HEp-2 cells, inducing cytotoxicity [31]. Moreover, exposition of human macrophages THP-1 to *S. aureus* JE2 EVs during 24 h also occasioned significant cellular cytotoxicity, a result that was sharply decreased when EVs were isolated from mutant lacking several pore-forming toxins (PFTs) [57]. In another study, Thay et al. showed

that *S. aureus* EVs contributed to HeLa cell cytotoxicity and erythrocyte lysis in a dose-dependent manner [64]. These results were tightly associated with biologically active α-hemolysin within EVs since their cytolytic and cytotoxic effects were significantly attenuated when EVs were isolated from an isogenic *hla* mutant [64]. Furthermore, *in vivo* experiments conducted by Hong et al. revealed that only *S. aureus* EVs could disrupt the skin barrier and cause dermal inflammation, which was not observed in the presence of purified α-hemolysin or EVs from strains that lack this protein [30]. More interestingly, they showed that EV-associated α-hemolysin was more cytotoxic than the purified toxin itself, and while the first induced necrosis, soluble α-hemolysin induced apoptotic cell death [30]. Together, these findings highlight the critical role of EVs in host cell death during staphylococcal toxicity.

### 4.2 *S. aureus*-EVs in antibiotic resistance and biofilm formation

Besides delivering toxins to host cells, *S. aureus* EVs were shown to play an important role in antibiotic resistance. Lee et al. demonstrated that biologically active BlaZ, a β-lactamase protein, is present inside *S. aureus* EVs [32]. EVs containing BlaZ were able to confer a transient resistance against ampicillin to susceptible surrounding Gram-negative and Gram-positive bacteria, including different strains of *E. coli*, *Salmonella enterica* serovar Enteritidis, *Staphylococcus epidermidis,* and *S. aureus* [32]. In a more recent report by Kim et al., the protective effect of EVs derived from the methicillin-resistant *S. aureus* (MRSA) strain ST692 grown in the presence of ampicillin was evaluated. Accordingly, ST692 EVs were shown to protect susceptible ATCC29213 strain against six different β-lactam antibiotics in a dose-dependent manner [71]. In another study, the addition of exogenous EVs purified from the culture supernatant of strain BWMR22 grown in the presence of a sub-inhibitory concentration of vancomycin was able to increase *S. aureus* adhesion and cell aggregation, contributing to biofilm formation [67]. Finally, it was shown that application of *S. aureus* EVs to polystyrene surfaces reduces biofilm formation by several other pathogenic bacteria, including *Acinetobacter baumannii*, *Enterococcus faecium*, and *Klebsiella pneumonia* [66]. This can be explained by the ability of *S. aureus* EVs to increase the hydrophilicity of surfaces, a key parameter for the initiation of biofilm formation [66]. This conversion of surface properties confers a vital competitive advantage that could explain the prevalence of *S. aureus* as a nosocomial pathogen.

### 4.3 *S. aureus*-EVs in immunomodulation

Various studies also demonstrated the role of *S. aureus* EVs on immunomodulation and their contribution to the induction or exacerbation of pulmonary and skin inflammations. Detection of *S. aureus* EVs in house dust led Kim et al. to investigate their role in lung infection models. Repeated airway exposure of mice to these particles resulted in a local increase in cytokine production and neutrophilic pulmonary inflammation [35]. Regarding cutaneous infections, it was shown that *S. aureus* EVs induce atopic dermatitis (AD) inflammation by enhancing cutaneous production of various cytokines, which promote infiltration of the dermis by mast cells and eosinophils, and consequently the increase in epidermal thickening in mice [30, 37]. In addition to that, *S. aureus* EVs were also shown to exacerbate inflammation in an AD mouse model [38]. Topical application of *S. aureus* EVs resulted in severe eczematous dermatitis, skin thickening, and a massive infiltration by inflammatory and mast cells [38]. These symptoms were not observed when

animals were treated with lysed EVs [38]. Finally, an *in vitro* study showed that human dermal microvascular endothelial cells exposed to *S. aureus* EVs produce cell adhesion molecules, such as E-selectin, ICAM1, and VCAM1, which efficiently promote endothelial cell activation and monocyte recruitment, contributing, therefore, to the infiltration of immune cells [36].

Wang et al. demonstrated that EVs derived from the S. aureus JE2 strain could activate TLR2 signaling of NLRP3 inflammasomes in human macrophages through K+ efflux and apoptosis-associated speck-like protein (ASC) recruitment [57]. ASC is a key adaptor complex required for caspase-1 activation, which leads to the release of the mature forms of IL-1β and IL-18 cytokines. They also investigated whether EVs derived from a mutant for the *agr* quorum-sensing system and the SaeRS two-component system could affect inflammasome activation since they control the release of several PFTs, such as hemolysins and leukocidins. Indeed, the Δ*arg*Δ*saeRS* EVs packed a minimum amount of PFTs, leading to the absence of caspase-1 activation and a consequent decrease in the release of IL-1β and IL-18 by human macrophages [57]. Similarly, a mutation in a gene involved in lipidation and maturation of lipoproteins (Δ*lgt*) also decreased the levels of Hla and of the leukocidin LukS-PV present inside EVs, and, consequently, their ability to induce caspase-1 activation and cytokine release [57].

A recent study conducted by Rodriguez et al. demonstrated that nucleic acid associated with *S. aureus* EVs is immunomodulatory [70]. They identified DNA and RNA populations associated with EVs derived from Newman strain and provided evidence that these nucleic acids are delivered into host endosomal compartments [70]. *In vitro* experiments showed that murine macrophages exposed to EVs presented a strong IFN-β mRNA expression after 3 hours of stimulation [70]. Pretreatment of macrophages with inhibitors of endosomal acidification strongly reduced IFN-ß mRNA expression after EV stimulation, suggesting that EVs' processing depends on the acidic endosomal environment to release their immunomodulatory cargo and promote TLR signaling [70]. These results were corroborated when the exposition of TLR3−/−, TLR7−/−, and TLR9−/− mouse macrophages to EVs reflected in a substantial decrease in IFN-ß mRNA expression [70].

As described above, most studies regarding *S. aureus* EVs have focused mainly on clinical human isolates, and to date, there is only one report describing the biological functions of EVs derived from a *S. aureus* animal strain. Tartaglia et al. demonstrated that EVs derived from the bovine mastitis strain Newbould 305 carry several virulence factors and induce cytokine production in a bovine mammary epithelial cell *in vitro* without altering their viability [33]. Additionally, they showed that the intraductal inoculation of EVs in the mouse mammary gland promotes inflammation, tissue deterioration, and cytokine and chemokine production in murine mammary glands [33]. Altogether, these data indicate that staphylococcal EVs can interact with and modulate host cells' immune response, suggesting that EVs can play an important role in staphylococcal pathogenesis.

## 5. *S. aureus*-EVs delivery to host cells

### 5.1 *S. aureus*-EVs integrity and cell toxicity

Secretion of molecules and virulence factors is an essential component of *S. aureus* pathogenesis, including toxins, adhesins, and invasins. Molecules such as proteins or nucleic acids released in the surrounding medium may be rapidly degraded by the proteases or nucleases secreted in the extracellular milieu. The bilayered EVs thus appear as protective vehicles for efficient delivery of components

in a concentrated manner. Gurung et al. were the first to evidence that Spa delivery via EVs was responsible for host cell death only when EVs were intact, establishing *S. aureus* EVs as effective delivery vehicles to target cells [29].

Other studies confirmed this role of EVs. For instance, disrupted EVs produced by *S. aureus* ATCC 25923 strain were shown to be four times less cytotoxic than intact EVs [65]. Again, whole and lysed EVs derived from strain 03ST17 were both cytotoxic and proinflammatory, however, these properties were more intense when EVs were intact [38, 62]. Nevertheless, in some cases, EV integrity does not influence their cytotoxic properties, as it is the case of *S. aureus* M060 EVs, that in both intact and disrupted states had the same cytotoxicity levels towards HaCaT cells [65]. These results highlight that EVs' integrity is essential and can lead to different outcomes depending on the mode of action of the effector molecules and the mechanism of EV cargo delivery.

### 5.2 *S. aureus*-EVs internalization into host cells

As important as the transport of cargo by EVs is how they transfer their cargo to recipient cells. They can act extracellularly through ligand-receptor interactions or intracellularly after their internalization into target cells and cargo release [79]. In the latter case, EVs' internalization may occur through several pathways, which all subsequently lead to an intracellular release of their cargo. These pathways include membrane fusion, phagocytosis, macropinocytosis, and lipid-raft-, caveolin- or clathrin-mediated endocytosis [80].

Studies showed that *S. aureus* EVs could interact with host cells via cholesterol-rich membrane microdomain. The cholesterol-sequestering agent Filipin III prevents EV membrane fusion and cargo delivery into host cells [29, 64]. Another study demonstrated that of all pretreatments of human macrophages with different inhibitors for clathrin-, lipid raft-, actin-, and dynamin-dependent endocytosis, only dynasore inhibited the entry of EVs into host cells, suggesting that EV uptake is mediated by dynamin-mediated endocytosis [57]. This finding is supported by a recent report by Rodriguez et al., where macrophages exposed to *S. aureus* Newman EVs had a substantial decrease in IFN-β mRNA expression when cells were also pretreated with dynasore [70]. They also provided visual evidence through molecule labeling and confocal microscopy that EV-associated RNAs are efficiently delivered into macrophages [70]. Both membrane fusion and endocytosis depend on the integrity of EVs. This may explain why intact EVs usually present higher cytotoxicity since they allow direct delivery of concentrated components into host cells, enhancing, therefore, cell damage and immunomodulation. Although these recent findings highlight the role of cholesterol-rich domains and dynamin in *S. aureus* EV uptake, one cannot exclude that staphylococcal EVs exploit diverse entry routes for their cargo delivery host cells.

## 6. *S. aureus*-EVs environmental modulation

### 6.1 Impact of growth conditions in *S. aureus*-EV release

Besides intrinsic bacterial factors, several external factors were also shown to modify EV production. In *S. aureus*, exposure to the antibiotic penicillin significantly increased EV number, size, and protein yield compared to untreated bacterial cultures. In contrast, treatment with the antibiotic erythromycin did not affect EVs release [40]. This can be explained by the nature of each antibiotic action with penicillin affecting cell wall biosynthesis, whereas erythromycin is active on protein

translation. Likewise, in another study, *S. aureus* had a significant increase in EVs release after exposure to the β-lactam antibiotics flucloxacillin and ceftaroline due to their ability to weaken the PGN wall [68]. Again, addition of the β-lactam ampicillin increased *S. aureus* EV production in a dose-dependent manner, which corresponded to a 22.4-fold increase at 64 μg/mL concentration [71]. The PGN present in the bacterial cell wall of most bacteria has a rigid structure formed of highly cross-linked polymers composed of polysaccharide chains and short peptides [81]. β-Lactams have been shown to decrease PGN cross-linking by serving as a substrate that irreversible binds and inactivates a transpeptidase involved in cell wall biosynthesis. As a result, it increases cell wall permeability due to the presence of a looser PGN matrix structure, allowing vesicles to cross the cell wall with less resistance, generating, therefore, particles in higher numbers and sizes. The correlation between vesicle release and PGN cross-linking has also been reported for Gram-negative bacteria [10, 82].

### 6.2 Impact of growth conditions in *S. aureus*-EV cargo composition

Culture conditions also alter EV content since bacteria modulate gene expression and protein secretion to cope with environmental changes. Indeed, comparative proteomic analysis revealed that 131 and 617 proteins were identified in EVs derived from *S. aureus* strain MSSA476 grown in Luria-Bertani (LB) and Brain-Heart Infusion (BHI) broth, respectively, with 109 proteins identified in both conditions [69]. Moreover, EVs derived from LB cultures were two-fold larger than those derived from BHI cultures, even though the latter presented higher protein diversity, which may also explain their significantly higher cytotoxicity towards neutrophils following brief exposure compared to LB-derived EVs [69]. In another study, proteomics identified 156 and 137 proteins in EVs derived from cultures in the presence and absence of a sub-inhibitory concentration of ampicillin, respectively, while only 67 proteins were shared by both conditions [71]. Another example of changes in EVs content was observed in the chemical composition of *S. aureus* EVs following treatment with vancomycin at 1 mg/ml. Compared to EVs produced by untreated bacteria, EVs prepared from vancomycin-treated cultures presented an increase in the ratio of protein relative to carbohydrates [67].

Additionally, EV content can also be impacted by a combination of several factors. For instance, Andreoni et al. evidenced that EVs produced by lysogenic strains had a significantly higher amount of DNA than those of the cured strains when a DNA-damaging SOS antibiotic was used, while the DNA content was unchanged in EVs purified from cultures treated with β-lactam [68]. This can be explained by the prophage-induced cell lysis caused by SOS-response triggering components, leading to an increase of DNA inside EVs, which does not occur with β-lactams since they target bacterial cell wall biosynthesis. These findings evidence that both intrinsic and external factors impact EV release and content, but much research is necessary to better elucidate EV biogenesis and cargo selection in *S. aureus* as well as in other bacteria.

## 7. *S. aureus*-EVs and host cells specificity

### 7.1 *S. aureus*-EVs strain specificity

Cytotoxicity and immunomodulation of EVs towards host cells vary according to the *S. aureus* strain and host cell line studied since both can have specific

characteristics. As virulence factors vary from an *S. aureus* strain to another, so does the cargo of EVs. This affects cytotoxicity and host cell response to EVs contact. It was demonstrated that the presence of α-hemolysin in EVs is directly related to host cell death, and EVs from α-hemolysin-negative strains have very low or no cytotoxic effect on different cell types [30, 64]. Similarly, EVs from M060 *S. aureus* strain containing exfoliative toxin A (ETA) were highly cytotoxic towards HEp-2 cells, contrary to EVs purified from three other *S. aureus* isolates that lacked the ETA protein [31]. Furthermore, it was also demonstrated that EVs from *S. aureus* ATCC 25923 induces a stronger immune response in HaCaT cells than that of M060 EVs at the same concentrations [65]. These data show that EVs from different *S. aureus* strains indeed have different effects on host cells.

### 7.2 Host cell lines specificity

On the other hand, the cell lines used *in vitro* also have different responses reflecting differences in host cells-EVs interactions, which result in variable cytotoxicity, and immunomodulation levels. EVs derived from *S. aureus* subsp. *aureus* Rosenbach MSSA476 induced extensive cell death in human neutrophils and THP-1 cells, while it had very low cytotoxicity in HaCaT at the same concentrations [69]. In another study, *S. aureus* JE2 EVs were showed to be less cytotoxic to airway epithelial cells (A549) than to erythrocytes and neutrophil-like HL60 cells [40]. As another example, after exposure to ATCC 14458 *S. aureus* EVs, alveolar macrophages produced TNF-α and IL-6, while A549 cells produced only IL-6 [35]. Together, these findings show that EVs' role in host cell toxicity and immune response is strongly affected by the variations in EV cargo, which itself vary from an *S. aureus* strains to another, and to variations in molecular and physiological characteristics of the host cell types.

## 8. Applications of bacterial EVs

### 8.1 Use of EVs as a vaccine platform

As reviewed above, EVs interact with host cells leading to cytotoxicity, immunomodulation, tissue disruption, and other effects that mimic those caused by living bacteria during infection. These characteristics make EVs interesting vectors for delivering antigens and other components, some of which may have adjuvant properties. These features make EVs good candidates for vaccine development. Several studies have shown that EVs can induce adaptive immunity and confer protection against infections caused by both Gram-negative and Gram-positive pathogenic bacteria [83–85]. For instance, mice immunized with 1 μg of *E. coli* derived OMVs resulted in 100% protection against a lethal dose challenge, while the survival rate was only 20% in the untreated group [86]. In another study, intraperitoneal administration of *Streptococcus pneumoniae* BAA-255 EVs protected mice against the EV-producing cells and the pathogenic KCCM-41569 strain, demonstrating EVs' ability in eliciting a cross-protection against different strains [14].

### 8.2 Use of EVs against *S. aureus* infections

Regarding *S. aureus*, several studies have already reported the use of its derived EVs for immunization, revealing its potential in vaccine design. In 2015, Choi *et al*.

demonstrated that exposition of bone marrow-derived dendritic cells to ATCC 14458 EVs during 24 h enhanced the expression of co-stimulatory molecules CD80 and CD86 and of proinflammatory mediators such as TNF-α, IL-6, and IL-12, suggesting the induction of adaptive immunity [42]. As expected, intramuscular administration with three doses of >5 μg of ATCC 14458 EVs resulted in 100% protection against challenge with a lethal dose of bacteria in a mouse pneumonia model, with a reduction of bacterial colonization, pneumonia, and production of cytokines [42]. They revealed that immunization is mediated mainly by CD4+ T cell response, and transfection of these cells from EVs-immunized mice to naïve mice results in 70% protection after a lethal-dose challenge of *S. aureus*. Finally, they demonstrated that ATCC 14458 EV immunization provides long-term protective immunity and that it is a safe method since the administration of EV doses 10-fold higher were not cytotoxic to mice [42].

In another study, Askarian et al. demonstrated that intraperitoneal vaccination with USA300-derived EVs promoted a high production of antibodies, in addition to the protection of mice against subcutaneous and systemic *S. aureus* infections [69]. Another example of *S. aureus* EVs' application as a vaccine was shown by Wang et al. EVs were purified from the JE2 Δ*agr*Δ*spa* strain containing a plasmid coding for non-toxic Hla and LukE toxins under control of the *spa* promoter, whose activity is enhanced in the absence of the *arg* quorum sensing system [40]. They demonstrated that recombinant non-toxic Hla and LukE are immunogenic, and engineered EVs carrying these detoxified cytolysins protected mice against lethal sepsis infection [40]. Remarkably, reports on OMVs used as vaccine platforms against *S. aureus* infections were also explored. Irene et al. used OMVs derived from *E. coli* to incorporate five *S. aureus* antigens, Hla, SpA, FhuD2, Csa1, and LukE. They were successfully integrated into *E. coli* OMVs, corresponding from 5–20% of the total protein content [87]. The engineered OMVs conferred significant protection against sepsis, kidney, and skin *S. aureus* experimental infections in mice [87].

### 8.3 Use *S. aureus*-EVs against other infections

Interestingly, Yuan et al. used EVs derived from the *S. aureus* RN4220-Δ*agr* strain to produce particles with a reduced content of virulence factors and a decreased toxicity to generate a safe platform against viral infections [41]. Major components of *S. aureus* EVs were fused to tag sequences able to incorporate viral antigens, generating PdhB-FLAG and Eno-FLAG proteins associated with envelope E domain III, the primary protective domain for prevention of dengue virus (DENV) [41]. These heterologous viral antigens were successfully integrated into EVs, which induced antibodies against four DENV serotypes and protected mice against lethal challenge with DENV-2 [41].

## 9. Conclusions

As addressed here, EVs transport various types of biomolecules that have been reportedly associated with bacterial survival and host-pathogen interactions. *S. aureus* is, to date, one of the best-documented bacteria in this field. Yet, several research questions remain to be elucidated. First, EVs biogenesis is still poorly understood in Gram-positive bacteria even though recent studies showed *S. aureus*-EVs biogenesis can be affected by a range of intrinsic and external factors, such as PSMs, autolysins, and environmental conditions, such as antibiotics.

Moreover, several studies evidenced that selective cargo sorting exist. Since the EV cargo determines their biological functions, clarifying which components are selected, and how, is of crucial value to understand their role in pathogenesis, and to their use as delivery systems. Second, most studies on *S. aureus* EVs have focused on proteomes. As well as proteins, nucleic acid cargo could play essential roles in *S. aureus* survival and pathogenesis. They could be associated to horizontal gene transfer for antibiotic resistance, and regulation of host cell expression by small regulatory RNAs. Therefore, more research is necessary in this field. Third, the physiological role of *S. aureus* EVs remains elusive. Staphylococcal EV cargo was shown to induce host cell toxicity, and skin and pulmonary inflammations, however, to the best of our knowledge, the exact contribution of EVs during infection remains unclear. The study of EV-free *S. aureus* strains in the infection context could reveal valuable clues to their real contribution to pathogenesis. Nevertheless, to the present, this phenomenon is unknown. Finally, their ability to induce a host immune response has arisen interest in using EVs as vehicles for vaccination. Several studies reported that administration of *S. aureus* EVs induce protection against systemic, pulmonary, and cutaneous infections. Although being a recent field of study, these promising data sheds light onto the possible application of engineered EVs to prevent diseases caused by this important human pathogen.

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## Conflict of interest

The authors declare no conflict of interest.

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