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Brenda Silva Rosa da Luz

CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* EXTRACELLULAR VESICLES AND THE STUDY OF THEIR IMPACT ON THE HOST IMMUNE RESPONSE

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“Somewhere, something incredible is waiting to be known.”

Carl Sagan

Resumo

As vesículas extracelulares bacterianas (VEs) são nanopartículas esféricas, circundadas por uma bicamada lipídica, produzidas pela maioria das células e secretadas no meio extracelular. Elas desempenham um papel essencial na comunicação entre as células (intra e interespécies) e na vetorização das propriedades biológicas das células, devido à sua capacidade de transportar moléculas de uma célula produtora para uma célula receptora. *Staphylococcus aureus*, uma bactéria de grande importância médica e veterinária, secreta VEs envolvidass em interações com o hospedeiro. Até o momento, a maioria dos trabalhos em VEs de *S. aureus* se concentrou em isolados clínicos e na caracterização de seu conteúdo de proteína. Aqui, nós fornecemos um perfil transcriptômico e proteômico completo da cepa clínica HG003 de *S. aureus* e suas VEs derivadas de diferentes condições ambientais. Note que esta é a primeira caracterização extensa do conteúdo de RNA de VEs de *S. aureus* na literatura. Em média, 78.0% dos transcritos anotados no genoma HG003 foram identificados nas VEs, compreendendo todas as categorias de RNA, incluindo pequenos RNAs regulatórios (RNAs). No entanto, apenas ~ 5% foram identificados como altamente cobertos por leituras (≥ 90% de cobertura), indicando que a maioria dos RNAs nas VEs estão fragmentados. O conteúdo de proteínas das VEs também inclui vários elementos importantes, como fatores de virulência, reguladores transcricionais e enzimas de várias vias metabólicas. Embora a composição das VEs seja impactada pelo estado fisiológico das células produtoras, observou-se que tanto a natureza quanto a abundância de proteínas e RNAs diferiram entre VEs e células produtoras, sugerindo o empacotamento seletivo do conteúdo das VEs. Em termos de suas funções biológicas, a adição de VEs supplementares de HG003 melhorou o crescimento de bactérias cultivadas em meios pobres em nutrientes. Este é o primeiro estudo que mostra o impacto positivo de VEs de *S. aureus* no crescimento bacteriano. No contexto das interações hospedeiro-patógeno, apesar da ausência de citotoxicidade das VEs para diferentes linhagens celulares humanas, elas induziram a expressão de diversas citocinas pró-inflamatórias. No entanto, a resposta celular induzida pelas VEs difere daquela induzida por bactérias vivas, sugerindo que VEs exercem funções específicas. Resultados semelhantes foram obtidos a partir da análise comparativa do transcriptoma de uma linhagem de células epiteliais de glândula mamária bovina na presença de VEs derivadas do isolado bovino N305 de *S. aureus*. Esses resultados mostram, na escala do genoma, a extensão da atividade das VEs de *S. aureus* nas células hospedeiras. Nosso estudo revelou novos elementos das VEs de *S. aureus*, seus potenciais papéis funcionais, e fornece novos conhecimentos sobre o papel das VEs intra-espécies, nas interações hospedeiro-patógeno e na patogênese estafilocócica.

Palavras-chave: vesículas de membrana; vancomicina; RNA-seq; proteômica; fatores de virulência; interações hospedeiro-patógeno.

Résumé

Les vésicules extracellulaires bactériennes (VEs) sont des nanoparticules sphériques, entourées d'une bicouche lipidique, produites par la plupart des cellules et sécrétées dans le milieu environnant. Elles jouent un rôle essentiel dans la communication entre les cellules (intra et inter-espèce) et dans la vectorisation des propriétés biologiques des cellules, de par leur capacité à transporter des molécules d'une cellule productrice à une cellule receveuse. *Staphylococcus aureus*, bactérie d'une grande importance médicale et vétérinaire, secrète des VEs impliqués dans les interactions avec l'hôte. A ce jours, la plupart des travaux sur les EVs de *S. aureus* ont concerné des isolats cliniques et la caractérisation de leur contenu en protéine. Ici, nous fournissons un profil transcriptomique et protéomique complet de la souche clinique HG003 de *S. aureus* et de ses VEs à partir de différentes conditions environnementales. Remarquablement, il s'agit de la première caractérisation approfondie du contenu en ARN des VEs de *S. aureus* dans la literature. En moyenne, 78,0 % des transcrits annotés dans le génome de HG003 ont été identifiés dans les VEs, qui contiennent toutes les catégories d'ARN, y compris les petits ARN régulateurs (ARN). Seulement 5 % de ces ARN pourraient se trouver sous une forme intacte, la grande majorité des ARN identifiés étant fragmentée. Le contenu en protéines des VEs est tout aussi divers avec, entre autres, des facteurs de virulence, des régulateurs transcriptionnels et des enzymes de voies métaboliques variées. Bien que la composition des VEs soit affectée par l'état physiologique des cellules productrices, des différences de composition en protéines et ARN entre les VEs et les cellules productrices ont été détectées, suggérant des processus d'empaquetage sélectif de leur contenu en protéines et ARN. Au niveau de leurs fonctions biologiques, nous avons montré que l'ajout de VEs de HG003 à des cultures bactériennes de *S. aureus* améliorait leur croissance en conditions carencées. Dans le cadre des interactions hôte-pathogène, les VEs de HG003 induisent l'expression de plusieurs cytokines pro-inflammatoires dans différentes lignées cellulaires humaines, sans pour autant être cytotoxiques. Cependant, la réponse cellulaire induite par les VEs diffère de celle induite par la bactérie vivante. Ce résultat suggère que les VEs exercent des fonctions différentes de celles des bactéries. Des résultats similaires ont été obtenus à partir de l'analyse comparée du transcriptome d'une lignée de cellules épithéliales de la glande mammaire bovine en présence de VEs d'un isolat bovin de *S. aureus* et de la bactérie. Ces résultats originaux montrent, à l'échelle du génome, l'étendue de l'activité des VEs de *S. aureus* sur les cellules de l'hôte. Notre étude a découvert de nouveaux éléments des VEs de *S. aureus*, leurs rôles potentiellement fonctionnels et fourni de nouvelles connaissances sur le rôle des VEs de *S. aureus* intra-espèce, dans les interactions hôte-pathogène et dans la pathogenèse staphylococcique.

Mots-clés : Vésicule membranaire; vancomycine; ARN-seq; protéomique; facteurs de virulence; interactions hôte-pathogène.

Abstract

Bacterial extracellular vesicles (EVs) are spherical nanoparticles, surrounded by a lipid bilayer, produced by most cells and secreted into the surrounding environment. They play an essential role in communication between cells (intra and inter-species) and in the vectorization of the biological properties of cells, due to their ability to transport molecules from a producer cell to a recipient cell. *Staphylococcus aureus*, a bacterium of great medical and veterinary importance, secretes EVs involved in interactions with the host. To date, most work on *S. aureus* EVs has focused on clinical isolates and the characterization of their protein content. Here, we provide a complete transcriptomic and proteomic profile of the *S. aureus* clinical HG003 strain and its derived EVs under different environmental conditions. Remarkably, this is the first in-depth characterization of the RNA content of *S. aureus* EVs in the literature. On average, 78.0% of the annotated transcripts in HG003 genome were identified in EVs, comprising all RNA categories, including small regulatory RNAs (RNAs). Only ~5% of these RNAs were highly covered by reads (≥90% coverage) indicating that most EV RNAs were fragmented. The protein content of EVs also comprised virulence factors, transcriptional regulators and enzymes of various metabolic pathways. Although the composition of EVs is affected by the physiological state of producer cells, differences in protein and RNA composition and abundance between EVs and producer cells have been detected, suggesting processes of selective packaging of their content. In terms of their biological functions, we have shown that the addition of HG003 EVs to bacterial cultures of *S. aureus* improved their growth in restringent conditions. This is the first study showing the positive impact of *S. aureus* EVs on bacterial growth. In the context of host-pathogen interactions, despite of the absence of HG003 EVs cytotoxicity towards different human cell lines, they induced the expression of several pro-inflammatory cytokines. However, the cellular response induced by EVs differs from that induced by live bacteria, suggesting that EVs exert specific functions. Similar results were obtained from the comparative analysis of the transcriptome of a bovine mammary gland epithelial cell line in the presence of EVs derived from the bovine isolate N305 of *S. aureus*. These results show, at the scale of the genome, the extent of the activity of *S. aureus* EVs on host cells. Our study uncovered new elements of *S. aureus* EVs, their potentially functional roles, and provided new insights into the role of *S. aureus* EVs intraspecies, in host-pathogen interactions and in staphylococcal pathogenesis.

Keywords: Membrane vesicle; vancomycin; RNA-seq; proteomics; virulence factors; host-pathogen interactions.

List of abbreviations

ASC	Apoptosis-associated speck-like protein
BCAA	Branched-chain amino acids
BHI	Brain-heart Infusion
CA-SA	Community-associated Staphylococcus aureus
CCL	Chemokine (C-C motif) ligand
CHIPs	Chemotaxis inhibitory protein of Staphylococcus aureus
Clf	Clumping factors
CNA	Collagen adhesin
Coa	Coagulase
CP	Capsule polyssaccharide
CXCL	Chemokine (C-X-C motif) ligand
Ecb	Extracellular complement binding protein
ECM	Host extracellular matrix
ETs	Exfoliative toxins
EVs	Extracellular vesicles
FISH	Fluorescence in-situ hybridization
FLIPr	Formyl peptide receptor-like-1 inhibitory protein
FnBP	Fibronectin binding protein
FPR	Formyl peptide receptor
FTIR	Fourier transform infra-red spectroscopy
GTP	Guanosine-5'-triphosphate
HA-SA	Health-care associated Staphylococcus aureus
HK	Histidine kinase
IFNγ	Interferon gamma
IgG	Immunoglobulin
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-17a	Interleukin 17a
IL-1β	Interleukin 1β
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-5	Interleukin 5
IL-8	Interleukin 8
KEGG	Kyoto Encyclope of Genes and Genomes
LB	Luria Broth
LC-ESI MS/MS	Liquid chromatography coupled to electrospray ionization tandem mass spectrometry
LPI	Lipid-based protein immobilization
MCP	Monocyte chemoattractant protein

MGEs	Mobile genetic elements
MIP	Macrophage inflammatory protein
MLST	Multi-locus sequencing typing
MPO	Myeloperoxidase
MRSA	Methicillin-resistant Staphylococcus aureus
MSCRAMMS	Microbial surface components recognizing adhesive matrix molecules
NCBI	National Center for Biotechnology Information
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor
NOD1	Nucleotide-binding oligomerization domain-containing protein 1
NTA	Nano Tracking Analysis
OM	Outer membrane
OMVs	Outer membrane vesicles
PCR	Polymerase chain reaction
PFT	Pore-forming toxins
PGN	Peptidoglycan
PIA	Polyssaccharide intercellular adhesin
PIs	Pathogenic islands
PSMs	Phenol-soluble modulins
PVL	Panton-valentine leukocidin
RNAP	RNA polymerase
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
RR	Response regulator
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
Sak	Staphylokinase
Sbi	Immunoglobulin-binding protein
SCFAs	Short-chain fatty acids
SCIN	Staphylococcal complement inhibiter
SCV	Smal colony variants
SEs	Staphylococcus enterotoxins
SFP	Staphylococcal food poisoning
Spa	Staphylococcys protein A
SRD	Staphylococcal regulatory RNA database
SspA	Serine protease V8
SSSS	Staphylococcal scalded skin syndrome
TCSs	Two-component regulatory systems
TEM	Transmission electron microscopy
TLR2	Toll-like receptor 2
TLR3	Toll-like receptor 3
TLR4	Toll-like receptor 4
TLR7	Toll-like receptor 7
TLR9	Toll-like receptor 9

TLR10	Toll-like receptor 10
TNF-α	Tumor necrosis factor α
TSST-1	Toxic shock syndrome toxin-1
UCCC	C-rich sequence motif
VISA	Vancomycin-intermediate Staphylococcus aureus
VRSA	Vancomycin-resistante Staphylococcus aureus
vWbp	von Willebrand factor binding protein
WTA	Wall teichoic acid

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Presentation

1. International collaboration

This thesis' project was conducted under a joint supervision agreement (cotutelle) between France and Brazil. It was developed in the frame of the International associated laboratory (LIA) BactInflam, which is the result of a 20-year collaboration between the Laboratory of Cellular and Molecular Genetics (LGCM) in Brazil, and UMR1253 STLO (Science and Technology of Milk and Eggs), in France.

LGCM is coordinated by Prof. Dr. Vasco Azevedo and is associated with the Postgraduation Program in Genetics at the Federal University of Minas Gerais (Universidade Federal de Minas Gerais, UFMG). Dr. Yves Le-Loir directs the Science et Technologie du Lait et de l'Oeuf (STLO) research unit. It is associated with the French National Research Institute for Agriculture, Food and Environment (INRAe) and the French School of Agriculture, Food, Horticultural and Landscape Sciences (Agrocampus Ouest).

This Ph.D. project was supervised by Dr. Vasco Azevedo, Dr. Yves Le-Loir and Dr. Éric Guédon (also from STLO).

2. Funding

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3. Thesis' context

Staphylococcus aureus is a non-spore-forming, anaerobic Gram-positive bacterium among the main opportunistic pathogens in humans and a common cause of nosocomial infections. It is responsible for causing life-threatening diseases such as sepsis, endocarditis, pneumonia, and minor soft tissue infections. In addition, *S. aureus* is also the principal causing agent of mastitis, an inflammation of the mammary gland that affects dairy herds, causing significant impacts on veterinary medicine and food sector production. Therefore, this bacterium is a global concern in medical and veterinary medicine, especially with the increasing appearance of antibiotic-resistant strains.

In 2009, extracellular vesicles (EVs) released by Gram-positive bacteria were first characterized in *S. aureus*, and since then, several reports have shown the release of EVs by other Gram-positive bacteria. EVs are spherical nanoparticles released by all living cells that carry biocomponents, such as proteins, lipids, metabolites, and nucleic acids, that participate in signaling and communication between cells. They contribute to critical physiological and pathological processes, such as competition, nutrient acquisition, antibiotic resistance, and immunomodulation. There is, therefore, a growing interest in the role played by bacterial extracellular vesicles (EVs) in health and disease.

S. aureus is a bacterium of global impact, and the study of EVs is an attractive area for the future development of new strategies to fight bacterial infections. Indeed, recent studies have shown that essential virulence factors are associated with *S. aureus* EVs and play important roles in biofilm formation, antibiotic resistance, and immunomodulation. However, since it is a recent research area, knowledge is still lacking in this field. This Ph.D. work broadens the characteristics of EVs released by *S. aureus* and their role in host-pathogen interactions.

4. Thesis' organization

The thesis has been divided as follows:

Chapter 1 comprises a literature review addressing the theoretical background of this work, providing information about *S. aureus* characteristics and pathogenesis and the current knowledge of the main features and functions of *S. aureus*-derived EVs.

Chapter 2 provides this work's research context, hypothesis, and objectives.

Chapters 3 and **4** introduce two original research articles describing the *S. aureus* EV content, which notably includes the first extensive characterization of RNA cargo in this bacterium.

Chapters 5 and **6** comprise additional results addressing the role of these EVs on host-pathogen interactions and immunomodulation, also comprehending the first RNA-seq data of eukaryotic cells exposed to *S. aureus* EVs.

Chapter 7 presents a general discussion and the perspectives of the work developed during this thesis.

Thesis outputs present a list of the products derived from this thesis, including research articles and scientific communications in events and congresses.

Résumé étendu present an extended abstract written in French.

References contain the list of scientific studies cited in the thesis.

Annexes present supplementary information.

Chapter 1. Introduction

Staphylococcus aureus

1.1 General characteristic of *Staphylococcus aureus*

1.1.1 Definition, morphology, and biochemical characteristics

Staphylococcus aureus (*S. aureus*) is the most important bacterium from the genus *Staphylococcus* spp due to its impact on animal and human health worldwide. The *Staphylococci* were first isolated from a clinical case in 1880 by the Scottish surgeon Alexander Ogston. The bacterium genera name describes the phenotype observed microscopically after Gram staining, being *staphyle* for its grape-like structural arrangement, and *kokkos* for its cocci-shape (Fig. 1.1) (LICITRA, 2013; ZHANG et al., 2013). In 1884, a golden-colored strain was named *Staphylococcus aureus* ('aureus' = golden in Latin) by the German physician Friedrich Julius Rosenbach (ROSENBACH, 1884).

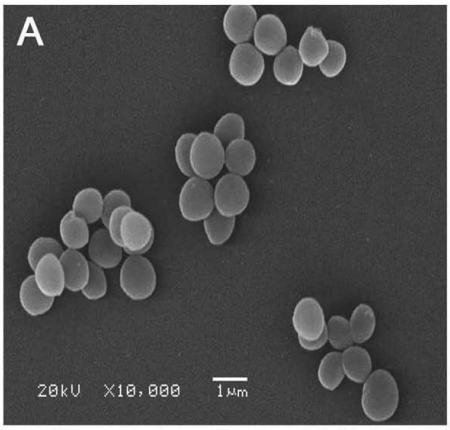

Figure 1.1. Scanning electron microscopy of *S. aureus* (from Zhang et al., 2013).

S. aureus is a non-motile and non-spore forming Gram-positive bacterium. It is an aerobe and facultative anaerobe organism ranging from 0.5 to 1 µm that grows in temperatures ranging from 18-40 ºC (FOSTER, 1996). Most strains present yellow-golden color when grown in rich nutrient media, resulting from the expression of staphyloxanthin, a carotenoid involved in *S. aureus* resistance to reactive oxygen species (ROS) (CLAUDITZ et al., 2006; MARSHALL; WILMOTH, 1981; XUE et al., 2019).

When grown in blood solid media, *S. aureus* hemolytic activity is evidenced by blood cells lysis caused by one of the four types of hemolysins (alpha (α), beta (β), gamma (γ), and delta (δ)) (DIVYAKOLU et al., 2019). *S. aureus* is generally described as catalase and coagulase positive, characteristics widely used for its identification (KATEETE et al., 2010). However, exceptions for these biochemical properties were reported (AKINEDEN et al., 2011; BERTRAND; HUGUENIN; TALON, 2002; ÖVER; TÜÇ; SÖYLETIR, 2000).

1.1.2 Genome

The *S. aureus* average genome ranges from 2.8 to 2.9 Mb and encodes around 2.800 genes (BOSI et al., 2016; LINDSAY; HOLDEN, 2004). Of these, approximately 56% correspond to the core genome shared by all *S. aureus* strains (BOSI et al., 2016). Most of the *S. aureus* core genome comprises housekeeping genes involved in transcription, translation, and metabolic processes (BOSI et al., 2016; LINDSAY; HOLDEN, 2004). In addition to housekeeping genes, the core genome also holds virulence factors involved in capsule polysaccharide synthesis, production of cytotoxins, iron acquisition, and extracellular adhesins (BOSI et al., 2016). On the other hand, *S. aureus* accessory and unique genomes comprise genes related to bacterial survival, defense mechanisms, and resistance to antibiotics, which are mainly found in mobile genetic elements (MGEs) (e.g., plasmids, transposons, pathogenic islands (PIs)) (BOSI et al., 2016; LINDSAY; HOLDEN, 2004). These MGEs provide high genetic plasticity to *S. aureus*, reflecting its intraspecies diversity and a broad spectrum of exploited ecological niches (LINDSAY; HOLDEN, 2004; ZIEBANDT et al., 2010).

1.1.3 Epidemiology

S. aureus is a versatile bacterium of global distribution that can colonize a wide spectrum of hosts, including several mammals, reptiles, and birds (CUNY et al., 2010; MATUSZEWSKA et al., 2020). Despite this vast range of colonized hosts, molecular analyses suggest that its primary ancestor host was humans (WEINERT et al., 2012). Multi-Locus Sequence Typing (MLST) is a powerful tool widely used to study *S. aureus* population structure and epidemiology. Indeed, MLST data provides valuable information on the global distribution of clonal complexes and their association with specific hosts (PETON; LE LOIR, 2014; RICHARDSON et al., 2018a).

Regarding clinical isolates, parameters such as molecular characteristics, antibiotic susceptibility, and origin of infection have long been used to distinguish healthcare-associated *S. aureus* (HA-SA) from community-associated *S. aureus* (CA-SA) strains (UHLEMANN et al., 2014). Nowadays, these definitions have been blurred by the growing appearance and persistence of CA-SA in nosocomial settings, which started in the late 1990s (DAVID et al., 2008; OTTER; FRENCH, 2012). In contrast, it appears that HA-SA strains are restricted to healthcare. Rudkin and collaborators (2012) suggested that higher expression of the *mecA* gene in HA-SA strains interferes with the bacteria cell wall and the *arg* quorum sensing system (RUDKIN et al., 2012). It results in decreased toxin expression and virulence, which, in consequence, prevents their dissemination out of nosocomial settings (RUDKIN et al., 2012).

In veterinary medicine, *S. aureus* is a causative agent of various infections that affects economically significant livestock animals, including poultry, cattle, small ruminants, pigs, and rabbits (FITZGERALD, 2012; HAAG; ROSS FITZGERALD; PENADÉS, 2019). The rapid spread of *S. aureus* to other species over the years probably results from the common closeness of farmers to livestock animals, which may have contributed to the jump of *S. aureus* from humans to these animals (SHEPHEARD et al., 2013; SPOOR et al., 2013). Genomic studies suggested that these cross-infections may have occurred several times (Fig. 1.2), and some *S. aureus* strains lack host tropism (RICHARDSON et al., 2018a). In contrast, some *S. aureus* strains seem to be host specific, such as ruminant-infecting strains (PETON; LE LOIR, 2014; SAKWINSKA et al., 2011; SMYTH et al., 2009). These differences in host specificity are often associated with MGEs, which carry elements that contribute to bacterial adaptation and survival in particular niches (BEN ZAKOUR et al., 2008; MATUSZEWSKA et al., 2020; SUNG; LLOYD; LINDSAY, 2008).

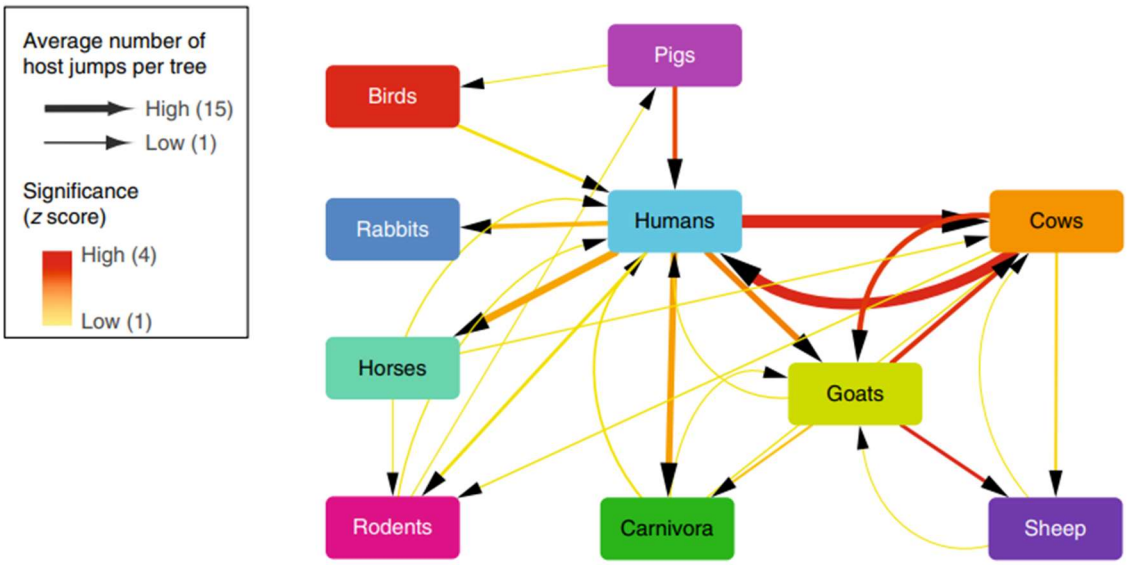

Figure 1.2. Quantification of the number of host-switching events in *S. aureus*. Network-based on the BEAST Markov Jumps model (from Richardson et al., 2018).

1.2 *Staphylococcus aureus* niches

1.2.1 Commensal bacterium

The anterior nares of healthy adults are the primary *S. aureus* colonization site and persistently colonized individuals comprise approximately 20% of the population (KLUYTMANS; VAN BELKUM; VERBRUGH, 1997; SIVARAMAN; VENKATARAMAN; COLE, 2009; WERTHEIM et al., 2005a; WILLIAMS, 1963). Nasal carries can be divided into at least three types: persistent, intermittent, and non-persistent (SAKR et al., 2018; VAN BELKUM et al., 2009b). Despite being found mainly in the human nasal tract, *S. aureus* secondary niches also include skin, mucous membranes, and gastrointestinal and vaginal tracts (GUINAN et al., 1982; MUENKS et al., 2016; RIMLAND; ROBERSON, 1986; WERTHEIM et al., 2005b; WILLIAMS, 1963). Accordingly, there is a correlation between the bacterial load in the nasal nares and the likelihood of *S. aureus* colonization of other body sites, suggesting that the nares may be the primary source of inoculation (MERMEL et al., 2011).

S. aureus colonization occurs mainly via skin-to-skin contact and can take place at the early stages of life, being a portion of newborns colonized right at delivery (BOURGEOIS-NICOLAOS et al., 2010; PEACOCK et al., 2003). Nonetheless, *S. aureus* carriage is complex, and rates vary in different groups, being higher in children, white people, and men (ARMSTRONG-ESTHER; SMITH, 1976; ERIKSEN et al., 1995; HERWALDT et al., 2004; NOBLE; VALKENBURG; WOLTERS, 1967; WILLIAMS, 1963). On the other hand, artificial colonization attempts revealed that approximately 75% of non-carries return to their original state, suggesting that they are inherently resistant to *S. aureus* colonization (NOUWEN et al., 2004). However, the mechanisms behind this resistance are yet to be fully understood.

1.2.2 Clinical medicine

S. aureus is also an opportunistic human pathogen. Although *S. aureus* carriage by healthy adults is common, it is an occurrence of global clinical concern since it is associated with a higher risk of staphylococcal infections, which results in high treatment costs, morbidity, and mortality in healthcare settings (BODE et al., 2010; EIFF et al., 2001; NOUWEN et al., 2006). For instance, it was demonstrated that *S. aureus* nasal carries presented three times more risk of developing bacteremia, while bacteremia-related mortality was 46% higher in non-carries, suggesting that partial immunity may play an essential role in these outcomes (WERTHEIM et al., 2004). Predisposing factors that can result in the progression from harmless colonization to the disease include prolonged hospitalization, invasive procedures, and immunocompromising conditions (SAKR et al., 2018; VAN BELKUM et al., 2009a; WERTHEIM et al., 2005a).

Figure 1.3 below shows the clinical manifestations caused by *S. aureus*, ranging from non-lethal to life-threatening diseases (SALGADO-PABÓN; SCHLIEVERT, 2014; SIVARAMAN; VENKATARAMAN; COLE, 2009). They comprise several chronic and acute infections, including bones and joints, meningitis, pneumonia, bacteremia, and skin and soft tissue syndromes,being the last the most frequent (LOWY, 1998; SUNDERKÖTTER; BECKER, 2015). *S. aureus* is also responsible for several toxin-induced syndromes, such as the staphylococcal scalded skin syndrome (SSSS), enterotoxins staphylococcal food poisoning (SFP), and the toxic shock syndrome toxin-1 (TSST-1) (FISHER; OTTO; CHEUNG, 2018; MISHRA; YADAV; MISHRA, 2016; SILVERSIDES; LAPPIN; FERGUSON, 2010).

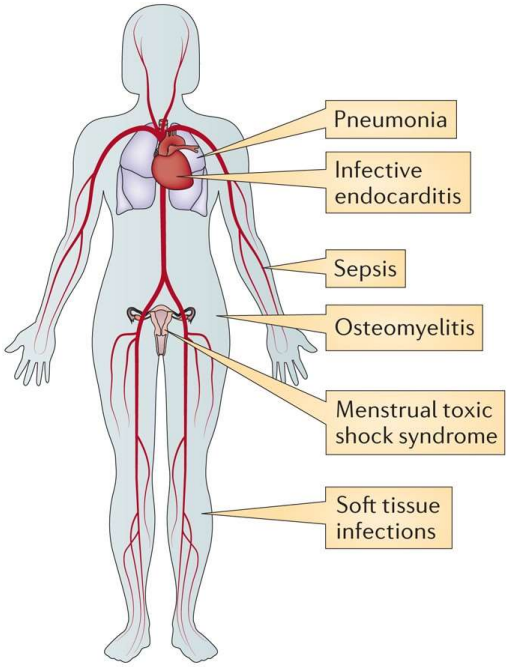

Figure 1.3. Human infections are caused by *S. aureus* (from Salgado-Pabón and Schlievert, 2014).

1.2.3 Veterinary medicine

In veterinary medicine, *S. aureus* has been reported to cause tissue lesions in pigs (MEEMKEN et al., 2010; SUN et al., 2015), skin abscesses in horses (DEVRIESE; NZUAMBE; GODARD, 1985), skin diseases and mastitis in rabbits (VIANA et al., 2007), and several manifestations in poultry. The last presents sepsis and infections on bones, joints, liver, lungs, and skin, the most common the foot pad dermatitis, also known as “bumblefoot” (MCNAMEE; SMYTH, 2000; PETON; LE LOIR, 2014).

Mastitis, an inflammation of the mammary gland, is among the most important diseases caused by *S. aureus* in veterinary medicine. This highly occurring disease that generally affects cows, sheep, and goats, represents a significant economic concern to the dairy industry worldwide (ABRIL et al., 2020). It modifies the quantity and quality of milk infected animals produce, resulting in significant economic losses. Moreover, altered milk may also have severe consequences for human health due to stable toxins capable of promoting food poisoning (ARGUDÍN; MENDOZA; RODICIO, 2010; LE LOIR; BARON; GAUTIER, 2003).

1.3 *Staphylococcus aureus* molecular basis of virulence and pathogenesis

As discussed earlier, *S. aureus* is present in several ecological niches. It can colonize the host without causing disease, but as an opportunistic pathogen, *S. aureus* can lead to chronic and recurrent infections in humans and animals. Following lesions on the epithelial protective layer, caused by minor scratches or even the action of α-toxin compromising the cytoskeleton, *S. aureus* may break through this barrier and develop invasive infections (POPOV et al., 2015; STRYJEWSKI; CHAMBERS, 2008). After overcoming the epithelial barrier, *S. aureus* infection success relies on potent mechanisms to evade the host defenses. At last, *S. aureus* invades organs and tissues, where it persists through the formation of small colony variants (SCV), biofilm, or abscesses.

This bacterium produces several virulence factors that contribute to bacterial adhesion, invasion, evasion, inflammation, survival, and persistence within the host, including surface proteins, exoenzymes, cytotoxins, and superantigens (CHEUNG; BAE; OTTO, 2021; TONG et al., 2015). Moreover, the expression of these virulence factors is finely controlled by complex regulatory systems that ensure *S. aureus* adaptation to diverse environmental conditions. Figure 1.4 summarizes the main *S. aureus* virulence factors, which will be further discussed in the context of pathogenesis.

1.3.1 Main virulence factors

1.3.1.1 Surface proteins

S. aureus surface elements are mainly related to hosting cell adhesion, invasion, and evasion (FOSTER et al., 2014; HEILMANN, 2011). The major class of *S. aureus* surface proteins belongs to the family of Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs). Other cell wall elements include the three-helical bundle and the NEAT motif protein families (FOSTER, 2019a).

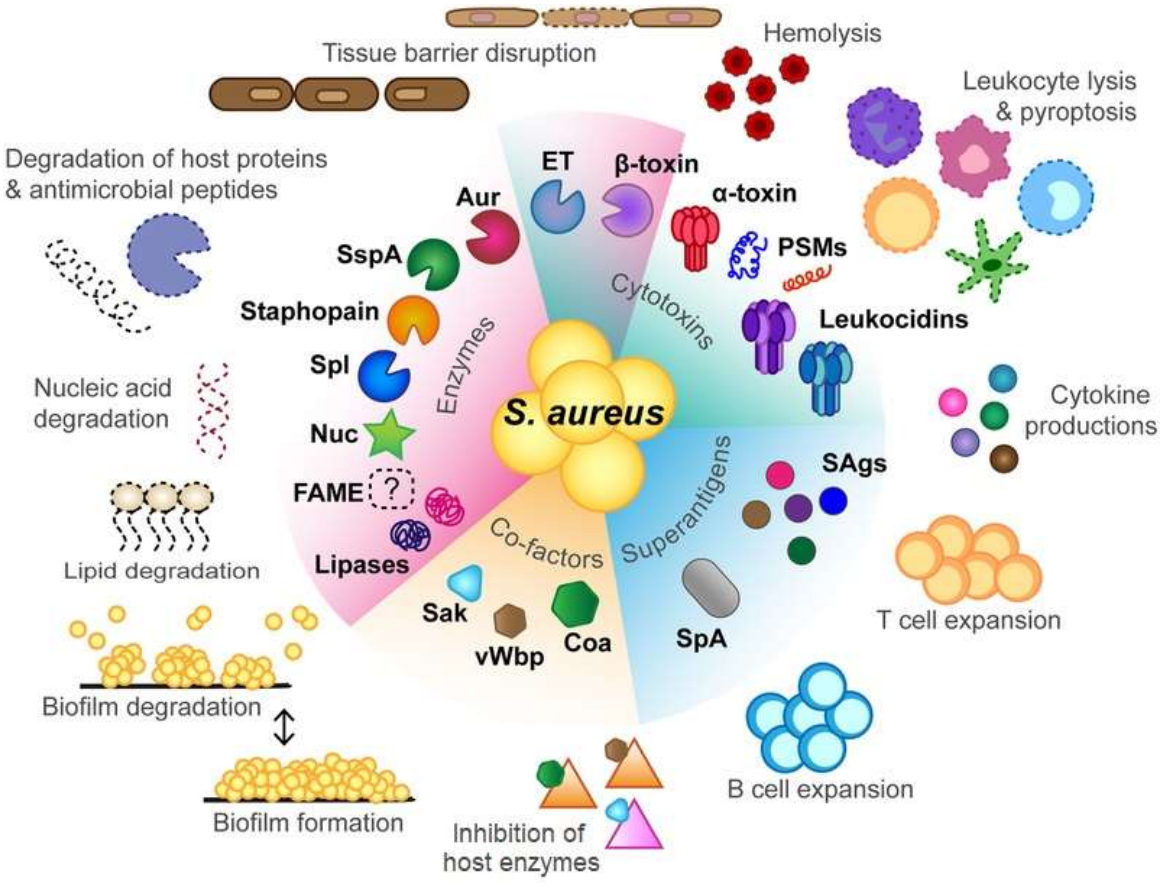

Figure 1.4. *S. aureus* major virulence-factors. Superantigens promote T and B cell expansions leading to massive cytokine production. Cytotoxins also stimulate cytokine production, promoting cytotoxicity, cytolysis, hemolysis, and tissue barrier disruption. Other enzymes, such as nucleases and proteases, can degrade host proteins or biofilms, contributing to bacterial dissemination. Finally, cofactors such as coagulase (Coa) can bind and inactivate host enzymes, mediating clot formation and coagulation. Altogether, this vast repertoire of *S. aureus* enzymes and toxins contribute to bacterial immune evasion and survival, playing an essential role in bacterial virulence and pathogenesis (adapted from Tam and Torres, 2019).

1.3.1.1.1 MSCRAMM family

S. aureus adherence to the host extracellular matrix (ECM) is essential for initiating infection. The MSCRAMM family englobes proteins containing at least two tandem repeats of IgG-like folded domains at the N-terminal A region, which enable ligand binding and bacteria adhesion to components of ECM, such as collagen,

fibrinogen, and fibronectin (FOSTER, 2019b). MSCRAMMs can contribute to the invasion of both phagocytic and non-phagocytic cells (FOSTER, 2019a) and are involved in biofilm formation of both the host and the surface of medical devices, contributing to bacterial persistence and pathogenesis (ARCHER et al., 2011; DONLAN, 2001; IDREES et al., 2021; REFFUVEILLE et al., 2017).

Collagen-binding protein

Since collagen is highly abundant in vertebrates, the interaction of bacterial proteins with this protein can greatly benefit pathogenesis, allowing bacterial adherence, invasion, and persistence in several bacterial infections (ARORA; GORDON; HOOK, 2021). Indeed, the collagen adhesin (Cna) was shown to be a critical *S. aureus* virulence factor, and its binding efficiency to host collagen is associated with several diseases, such as arthritis, osteomyelitis, endocarditis, and mastitis (ELASRI et al., 2002; HIENZ et al., 1996; MADANI; GARAKANI; MOFRAD, 2017; MAMO; FROMAN; MULLER, 2000; PATTI et al., 1994; ZONG et al., 2005). The significance of this staphylococcal protein can also be explained by its protagonism in *S. aureus* evasion from the host immune system. The binding of Cna to the collagenous domain of C1q obstructs C1r-C1q interactions, resulting in C1 complex inactivation and inhibition of the classical complement activation pathway (KANG et al., 2013).

Fibrinogen-binding proteins

Other important *S. aureus* surface proteins belonging to the MSCRAMM family are the fibronectin-binding proteins A and B (FnBPA and FnBPBB). FnBPs interacted with the fibronectin receptor integrin α5β1 host cell and contributed to the adherence and bacterial invasions of several cell types, such as endothelial cells, osteoblasts, fibroblasts, and keratinocytes (EDWARDS et al., 2011; PALMQVIST et al., 2005; PEACOCK et al., 1999; SINHA et al., 1999). Moreover, FnBPs capture serum plasminogen and stimulate its conversion into the serine protease plasmin, facilitating bacterial spreading in infected tissues (FOSTER, 2019b; PEETERMANS et al., 2014). FnBPs also capture antimicrobial histones to prevent their access to the bacterial cell surface and increase *S. aureus* resistance to the activity of neutrophil extracellular traps (NETs) (FOSTER, 2019b; PIETROCOLA et al., 2019). Overall, FnBPs were associated with systemic infections,

kidney abscess formation, and septic arthritis in murine models, highlighting their importance in *S. aureus* infections (PALMQVIST et al., 2005; SHINJI et al., 2011).

Clumping factors

The clumping factors A and B (ClfA, CflB) are involved in *S. aureus* adhesion and plasma agglutination (O'BRIEN et al., 2002a). ClfA binds to the γ chain of host fibrinogen, promoting bacterial clumping and platelet aggregation (HAWIGER et al., 1982; MCDEVITT et al., 1997; PEACOCK et al., 2002). More importantly, CflA coating of fibrinogen to the *S. aureus* cell surface prevents opsonization and interferes with recognition by host phagocytes, presenting an anti-phagocytic effect (HIGGINS et al., 2006). Indeed, some studies demonstrated that prevention of agglutination driven by interactions between host fibrinogen and ClfA of *S. aureus* reduces the success of staphylococcal septicemia in humans (FLICK et al., 2013; MCADOW et al., 2011).

CflA also contributed to *S. aureus* immune evasion through a complement-mediated mechanism. CflA binds to the complement regulator factor I (fI), which increases the cleavage and inactivation of the potent opsonin C3b, resulting in diminished *S. aureus* phagocytosis (CUNNION; HAIR; BUESCHER, 2004; CUNNION; BUESCHER; HAIR, 2005; HAIR et al., 2008, 2010). Because of all these functions, CflA is required for full bacterial virulence and usually is present in all *S. aureus* strains since mutants for this protein present decreased infection ability (JOSEFSSON et al., 2001; KWIECINSKI; JIN; JOSEFSSON, 2014).

CflB protein binds to the α chain of host fibrinogen, in addition to cytokeratin 10 and loricrin, two proteins found abundantly in squamous epithelial cells (FOSTER, 2019b; FOSTER; HÖÖK, 1998; WALSH et al., 2004; XIANG et al., 2012). Accordingly, it was demonstrated that ClfB plays a vital role in nasal colonization of humans and mouse by promoting adhesion to the squamous epithelium of the nasal tract (MULCAHY et al., 2012; O'BRIEN et al., 2002b; SCHAFFER et al., 2006; WERTHEIM et al., 2008). The binding ability of ClfB to squamous epithelial cells could indicate its role in the colonization of other body niches, which remains to be elucidated.

Serine-aspartase repeat proteins

Another important group of proteins belonging to the MSCRAMM family is part of the *S. aureus sdr* locus, encoding the serine–aspartate repeat proteins SdrC, SdrD, and SdrE, together with the bone sialoprotein-binding protein (Bbp) (JOSEFSSON et al., 1998). It was demonstrated that, similar to CflB, SdrC and SdrD proteins also contribute to the *S. aureus* adherence to squamous nasal epithelial cells and could be involved in nasal colonization (CORRIGAN; MIAJLOVIC; FOSTER, 2009). Interestingly, SdrC was also shown to bind to the neuronal protein β-neurexin. However, the physio-pathological implications of this interaction remain unknown (BARBU et al., 2010). Similar to CflA, SdrE promotes evasion of the host immune response by a complement-mediated mechanism, directly affecting bacterial resistance to host cell killing (SHARP et al., 2012). Finally, the Bbp protein, a ligand of bone sialoprotein that plays a significant role in osteomyelitis, was also shown to bind the Aα chain of fibrinogen (Fg), resulting in the inhibition of thrombin-induced blood coagulation in humans (TUNG et al., 2000; VAZQUEZ et al., 2011).

1.3.1.1.2 Three-helical bundle family

Proteins in this family class contain three-helix bundles arranged in folding structural domains. The Protein A (Spa) of *S. aureus* is a multifunctional protein that contains five homologous immunoglobulins (IgG) binding modules at the N-terminus, each of which comprises single folded three helical bundles (CEDERGREN et al., 1993; DEISENHOFER, 1981; MAZIGI et al., 2019) that can bind to various host ligands. This diverse binding capacity makes Spa an important *S. aureus* virulence factor, which can inhibit complement activation, opsonization, and phagocytic killing, in addition to promoting the superclonal expansion and apoptotic collapse of B cells through superantigen activity (CRUZ et al., 2021; FALUGI et al., 2013; GOODYEAR; SILVERMAN, 2003, 2004).

The immune evasion Sbi protein also belongs to this family class and contains four IgG-binding domains, two of which are homologous to those of Spa protein (BURMAN et al., 2008). The Sbi can be found anchored to the bacterial cell wall and extracellularly as a secreted protein and gives *S. aureus* the ability to avoid opsonophagocytosis and neutrophil killing (SMITH et al., 2011, 2012). It was shown that

both forms play a role in immune evasion by interaction with IgG, whereas only the secreted form inhibits complement activation by binding C3 (SMITH et al., 2011, 2012).

1.3.1.1.3 NEAT motif family

This group of proteins presents the near iron transporter, also known as the NEAT motif family. The NEAT domain promotes the capture of heme from hemoglobin, contributing to bacterial survival in the host environment, where iron is limited (ELLIS-GUARDIOLA; MAHONEY; CLUBB, 2021) (Fig. 1.5). *S. aureus* carries nine iron-regulated surface (Isd) proteins, which represents the predominant system for iron acquisition in this bacterium (GRIGG et al., 2010). The proteins IsdA, IsdB, and IsdH are anchored in the cell-wall by sortase A activity and serve as heme receptors. On the other hand, the IsdC heme receptor is embebbed in the cell wall by sortase B, which, together with the IsdDEF complex, is involved in the pumping of heme across the membrane to the cytoplasm (EBERLE et al., 2009; MARESSO; SCHNEEWIND, 2006; MAZMANIAN et al., 2017; VILLAREAL et al., 2008). Once inside the cells, iron is released from heme by the proteins IsdI and IsdG (SKAAR; GASPAR; SCHNEEWIND, 2004).

Interestingly, the cell-wall anchored Isd proteins exert other vital functions besides iron acquisition. It was demonstrated that IsdA, a protein expressed under iron-limited conditions, binds a broad spectrum of host elements, including fibrinogen, fibronectin, cytokeratin 10, involucrin, and loricrin (CLARKE et al., 2007, 2009; CLARKE; FOSTER, 2008; CLARKE; WILTSHIRE; FOSTER, 2004). This multi-binding capacity of IsdA is involved in *S. aureus* nasal carriage and bacterial resistance against bactericidal lipids and antimicrobial peptides, such as lactoferrin (CLARKE et al., 2006, 2007; CLARKE; FOSTER, 2008).

The IsdB protein interacts with other elements such as host ECM fibronectin to promote *S. aureus* invasion of non-phagocytic cells (PIETROCOLA et al., 2020; ZAPOTOCZNA et al., 2013). Moreover, the IsdB protein's direct interaction with the platelet integrin GPIIb/IIIa results in platelet aggregation and activation (MIAJLOVIC et al., 2010). Finally, the IsdH protein was shown to play a role in *S. aureus* evasion from neutrophil killing by accelerating the degradation of the opsonin C3b through a yet unknown mechanism (VISAI et al., 2009).

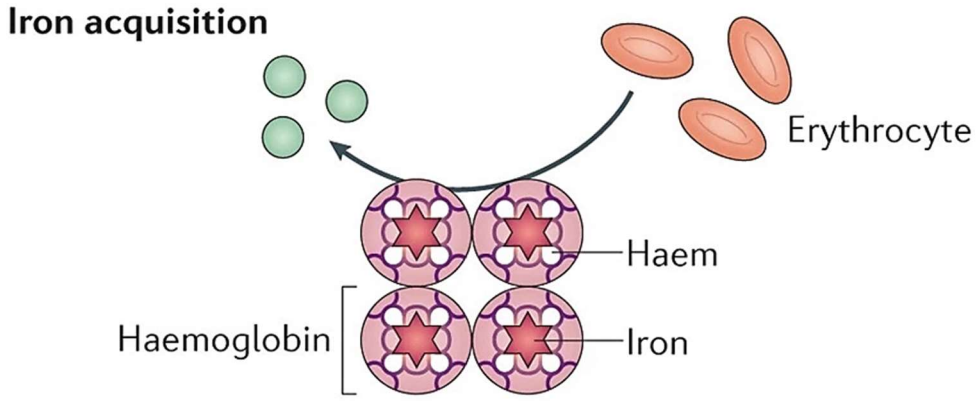

Figure 1.5. *S. aureus* iron acquisition. The surface protein Isd binds host hemoglobin, extracts and transports heme across the membrane to the cytoplasm, where iron is released (adapted from Foster et al., 2014).

1.3.1.2 Exoenzymes

In addition to the extensive repertoire of surface elements, *S. aureus* also secretes several proteins and enzymes that affect immune evasion. For example, the *S. aureus* formyl peptide receptor-like-1 inhibitory protein (FLIPr) prevents neutrophil chemotaxis (STEMERDING et al., 2013). This is also the case of the chemotaxis inhibitory protein of *S. aureus* (CHIPS) that presents high binding affinity to C5a and neutrophil formyl peptide receptor (FPR), leading to defective neutrophil activation and detection of bacterial peptides (DE HAAS et al., 2004; POSTMA et al., 2004; ROOIJAKKERS et al., 2006). The staphylococcal complement inhibitor (SCIN) prevents complement cascade activation by interfering with C3b deposition and forming C5a, resulting in decreased phagocytic killing (ROOIJAKKERS et al., 2005, 2006).

S. aureus also secretes clotting factors involved in the prevention of bacterial clearance. Coagulase (Coa) and von Willebrand factor binding protein (vWbp) trigger similar modifications in the coagulation cascade by activating prothrombin and converting fibrinogen to fibrin, resulting in the clotting of plasma and blood (BJERKETORP et al., 2002; MCADOW; MISSIAKAS; SCHNEEWIND, 2012). Another important exoenzyme is the Staphylokinase (Sak), a bacteriophage encoded protein that interacts with host plasminogen and activates the high proteolytic enzyme plasmin, leading to tissue damage and bacterial invasiveness (JIN et al., 2004; PEETERMANS et al., 2014).

S. aureus also secretes several proteases. The cysteine protease Staphopain A cleaves the host chemokine receptor CXCR2 preventing the ligand binding of potent chemoattractants important for neutrophil recruitment (LAARMAN et al., 2012). The serine protease V8 (SspA) is able to disminish bacteria's adhesion due to degradation of cell surface fibronectin-binding proteins (FREY; CHAPUT; SHAW, 2021; MCGAVIN et al., 1997). Finally, the metalloprotease aureolysin (Aur) prevents opsonization by interferring with complement activation and protects bacterial killing through the cleavage of the host antimicrobial peptide LL-37 (KUBICA et al., 2008; LAARMAN et al., 2011; SIEPRAWSKA-LUPA et al., 2004).

1.3.1.3 Exotoxins

After the establishment of colonization, *S. aureus* expresses a plethora of exotoxins that contribute to spreading and pathogenesis, including pore-forming toxins (PFTs), exfoliative toxins, and superantigens. These components often share similar functions and target the host defense cells, such as macrophages, neutrophils, and lymphocytes (THAMMAVONGSA et al., 2015).

1.3.1.3.1 Pore-forming toxins (PFTs)

Receptor-dependent PFTs

The α-toxin is one of the most critical *S. aureus* virulence factors, exerting several functions, such as cell lysis, disruption of the epithelial barrier, cell signaling, autophagy, immunomodulation, and others (BERUBE; WARDENBURG, 2013; BHAKDI; TRANUM-JENSEN, 1991; BONIFACIUS et al., 2020; CRAVEN et al., 2009; EIFFLER et al., 2016; POPOV et al., 2015). α-toxin cytolytic activity results from forming heptameric pores on the membrane of several cell types (Fig. 1.6A), including epithelial cells, endothelial cells, neutrophils, macrophages, and monocytes (BERUBE; WARDENBURG, 2013; BHAKDI; TRANUM-JENSEN, 1991). Moreover, α-toxin also compromises the structure of the epithelial barrier by triggering the cleavage of cell adherent junctions, which leads to the disruption of the protective layer, bacterial dissemination, and pathogenesis (INOSHIMA et al., 2011; POPOV et al., 2015).

S. aureus also expresses seven leukocidins, a complex of bi-component proteins composed of 'F' and 'S' subunits, being the last responsible for specific binding to host cellular receptors (Fig 1.6B) (ALONZO; TORRES, 2014; MILES; MOVILEANU; BAYLEY, 2002). All *S. aureus* strains produce at least three leukocidins, LukAB/GH and the γ-hemolysins AB and CB (HlgAB, HlgCB), while other strains can also secret LukSF-PV (or Panton-Valentine leukocidin, PVL) and LukED, all of which are associated to human infections (ALONZO; TORRES, 2014). On the other hand, LukMF' and LukPQ leukocidins have been linked to animal infections (KOOP et al., 2017; VRIELING et al., 2016; YAMADA et al., 2005).

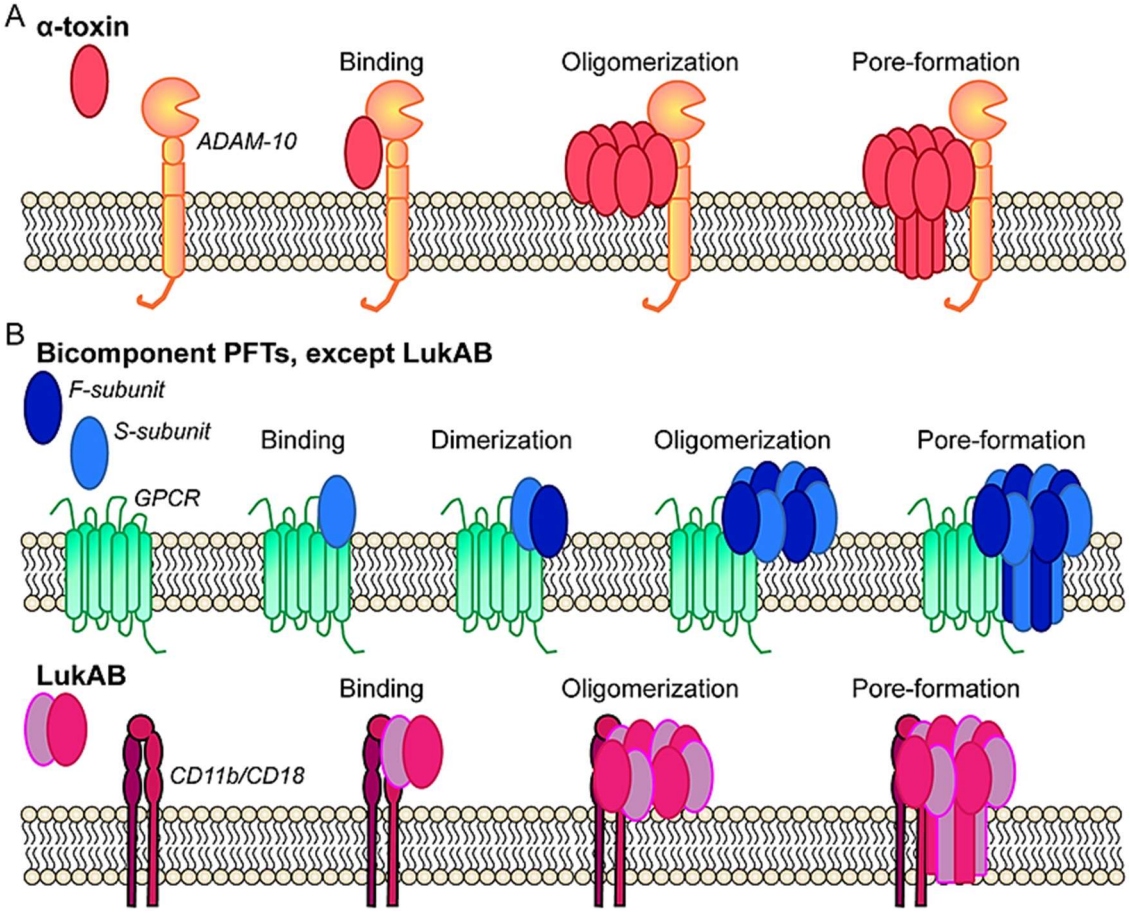

Figure 1.6. Action of *S. aureus* pore-forming toxins (PFTs). A: α-toxin monomer binds to the host receptor (ADAM-10) to form a heptameric β-barrel pore on the host cell membrane. B: The bicomponent PFTs monomers (except the dimer LukAB) bind to host cell surface receptors GPCRs (except for LukAB, which binds to CD11b), which dimerize and oligomerize to form an octameric β-barrel pore, thus disrupting the target cell membrane (from Thammavongsa et al., 2015).

As represented in Figure 1.6B, the ‘S’-subunit recognizes the host receptor and recruits the ‘F’ subunit to form dimers that subsequently oligomerize, leading to β-barrel pore-formation (ALONZO; TORRES, 2014; YAMASHITA et al., 2011). One exception is the LukAB leukocidin, which already binds to the host cell in a heterodimer form (Fig 1.6B) (DUMONT et al., 2014).

These bi-component toxins are important *S. aureus* virulence factors since they target several host cells, including monocytes, macrophages, neutrophils, and leukocytes (TAM; TORRES, 2019). The formation of pores on the host cell affects membrane permeability, leading to membrane disintegration, inflammasome activation, production of pro-inflammatory cytokines, and host cell death (HOLZINGER et al., 2012; MELEHANI et al., 2015; PERRET et al., 2012; SPAAN et al., 2014; STAALI; MONTEIL; COLIN, 1998; YANAI et al., 2014).

Receptor-independent PFTs

S. aureus phenol-soluble modulins (PSMs) are a family of toxic peptides involved in several steps of bacterial pathogenesis. The *psm*α operon encodes PSMα1– PSMα4 peptides that are ~ 20–25 amino acids long, while PSMβ1 and PSMβ2 (~ 44 amino acids) are encoded by *psm*β (WANG et al., 2007). Finally, the δ-toxin is encoded by *hld*, a gene within RNAIII, the effector molecule of the accessory gene regulator (Agr) quorum-sensing system in *S. aureus* (see section 1.3.2.2.1) (JANZON; LÖFDAHL; ARVIDSON, 1989a).

PSMs present amphipathic structure and surfactant-like properties, which favor their aggregation in oligomers facilitating bacterial spread on surfaces and biofilm formation (PERIASAMY et al., 2012a; TALBOT et al., 2001; TSOMPANIDOU et al., 2013b; WANG et al., 2011; ZAMAN; ANDREASEN, 2021). They bind non-specifically to the host cell membrane (Fig. 1.7), leading to pore-formation and membrane lysis of various cells, including monocytes, neutrophils, osteoblasts, endothelial, and epithelial cells (CHEUNG; DUONG; OTTO, 2012; FORSMAN et al., 2012; GIESE et al., 2011; KRETSCHMER et al., 2010; OLIVEIRA; BORGES; SIMÕES, 2018; RASIGADE et al., 2013; SUREWAARD et al., 2013; TALBOT et al., 2001; WANG et al., 2007).

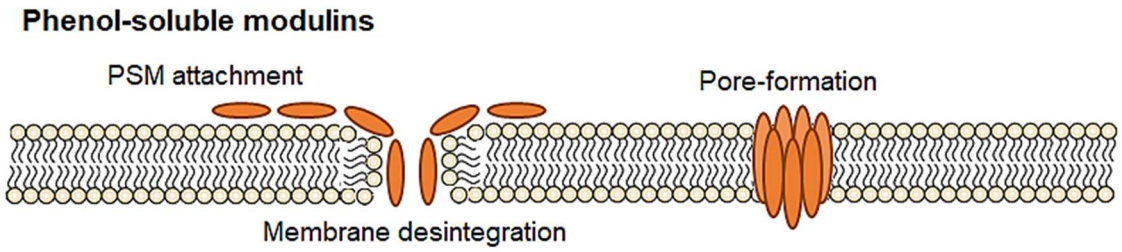

Figure 1.7. Phenol-soluble modulins (PSMs) bind to the host membrane non-specifically. PSMs aggregation in oligomers leads to pore-formation and membrane disintegration (adapted from Oliveira et al., 2018).

PSMs are also potent pro-inflammatory agents. It has been demonstrated that sensing of PSMs by human formyl peptide receptor (FPR) triggers chemotaxis and the release of pro-inflammatory cytokines (KRETSCHMER et al., 2010). Deleting the *psmα* operon abrogates neutrophil response (KRETSCHMER et al., 2010) and attenuates bacteria in a mouse bloodstream infection model (WANG et al., 2007). In addition, PSM production, especially of PSMαs, is associated with the expression of several chemokines and cytokines in human keratinocytes, suggesting their contribution to skin inflammation (DAMOUR et al., 2021). Indeed, it has already been demonstrated that δ-toxin induces mast cell degranulation, enhancing allergic skin disease (NAKAMURA et al., 2013). Finally, PSMs interfere with dendritic cell functions by turning them into "less stimulatory" cells, modulating the host's adaptive immune response, and increasing bacterial tolerance in the host (RICHARDSON et al., 2018b, 2019).

1.3.1.3.2 Exfoliative toxins

S. aureus expresses five exfoliative toxins: ETA, ETB, ETC, ETD, and ETE. They share identity sequences ranging from ~ 40 to 60%, with exception of ETC presenting only ~ 12% similarity (CAVARELLI et al., 1997; IMANISHI et al., 2019; JOHNSON et al., 1979; SATO et al., 1994; YAMAGUCHI et al., 2002). These ETs are serine proteases also known as "epidermolytic" toxins, since they promote dissolution of superficial layers of the skin by recognizing and hydrolizing desmoglein 1 (Dsg1), a cell-cell adhesion molecule (Fig. 1.8) (AMAGAI et al., 2000).

The dissociation of keratinocytes promotes skin peeling, leading to localized epidermal infections like bullous impetigo or generalized diseases such as the staphylococcal scalded skin syndrome (SSSS) (BRAZEL et al., 2021). SSSS is more

common in susceptible young children that lack neutralizing antibodies and an efficient clearance of toxins, which also explains why adults developing this disease are usually immunocompromised (BRAZEL et al., 2021; HANDLER; SCHWARTZ, 2014).

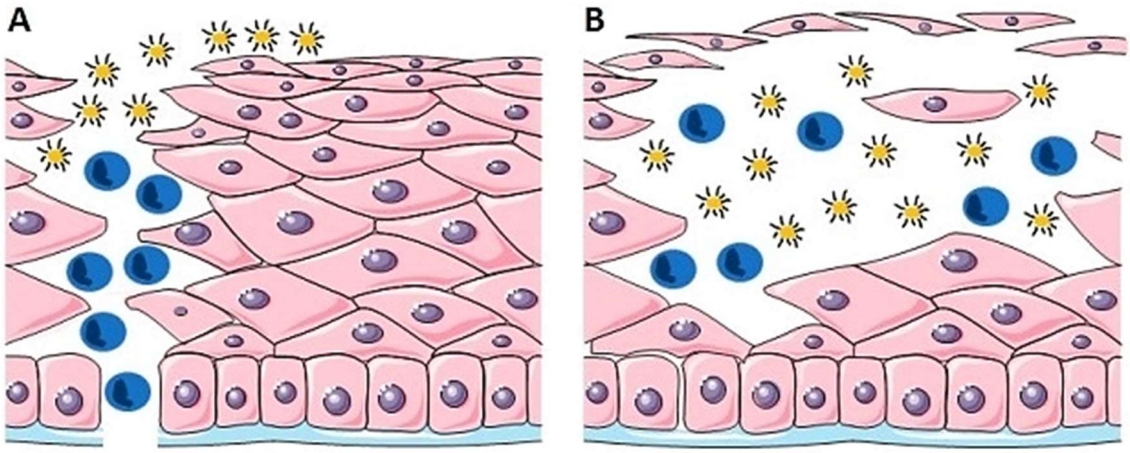

Figure 1.8. Bacterial invasion and blistering caused by ETs. A: *S. aureus* adheres and penetrates through intercellular gaps between superficial keratinocytes. B: Expansion of blisters by ETs (adapted from Oliveira et al., 2018).

1.3.1.3.3 Superantigens

Joining the arsenal of *S. aureus* exotoxins is the superfamily of superantigen-like proteins, which comprises almost 30 members, including staphylococcal enterotoxins (SEs) and the toxic shock syndrome toxin-1 (TSST-1) (HU et al., 2021). These superantigens are potent immunostimulatory molecules that can trigger excessive lymphocyte cell activation and proliferation, exacerbating inflammatory responses and endotoxic shock (LLEWELYN; COHEN, 2002).

As shown in Table 1, *S. aureus* superantigens cause several diseases in animals and humans, being TSST-1, SEB, and SEC the more recurrent (HU et al., 2021). The TSST-1 is one of the most famous toxins known to be the major, if not the only, cause of menstrual toxin shock syndrome (BERGER et al., 2019; DAVIS et al., 1980). Finally, SEs are the primary source of food contamination and poisoning (ARGUDÍN; MENDOZA; RODICIO, 2010; HU et al., 2021; LE LOIR; BARON; GAUTIER, 2003).

Table 1.1. *S. aureus* superantigens in human and life-stock animal diseases (Hu et al., 2021).

	Disease	**Superantigen**
Life-stock animal diseases	Bovine mastitis	SEC, TSST-1, SEG, SEN, SEO
	Swine sepsis	SEB, TSST-1
	Arthritis and septicemia in poultry	SEA, SEB, SEC, SEQ
Human diseases	Staphylococcal food poisoning	SEA~SEE, SEH, and SE02
	Soft tissue infection associated	TSST-1, SEB, SEC
	Post respiratory viral infection	TSST-1, SEB, SEC, SelX
	Purpura fulminans	TSST-1, SEB, SEC
	Kawasaki-like diseases	TSST-1, SEB, SEC
	Staphylococcal pneumonia	SEB, SEC, TSST-1, SelX
	Staphylococcal infective endocarditis	SEC, TSST-1
	Staphylococcal sepsis	SEA, SEC, TSST-1
	Chronic rhinosinusitis	SEA to SEE, SEG to SEO
	Atopic dermatitis	SEs, TSST-1
	Staphylococcal nonmenstrual TSS	TSST-1, SEB, SEC
	Inflammatory arthritis	SEA, SEB, SEC, TSST-1
	Autoimmune diseases	SEB, SEC, SED, TSST-1

1.3.2 Virulence regulation systems

S. aureus's success as both a commensal bacterium and a pathogen can be attributed to its complex gene regulation network essential in the adaptation to changing conditions. These regulation systems assure bacteria to sense and respond optimally to different environment niches by controlling both locally and temporally the expression of genes essential for bacterial survival upon specific stimuli to promote nutrient acquisition, adhesion, colonization, and host defense evasion. These regulatory mechanisms include sigma factors (σ), transcription regulators, the Agr quorum-sensing system, two-component systems, and regulatory RNAs (sRNAs), which will be discussed below.

1.3.2.1 Effectors of transcription

1.3.2.1.1 Sigma factors

In prokaryotes, the initiation of transcription depends on the association of the RNA polymerase (RNAP) to a specific subunit called sigma factor (σ), which together form an active holoenzyme. These σ provide promoter recognition specificity enabling the simultaneous transcription of physiologically related gene sets, also called regulons (KAZMIERCZAK; WIEDMANN; BOOR, 2005). *S. aureus* possesses four sigma factors, σ^A, σ^B, σ^S and σ^H. The σ^A is a major transcriptional regulator controlling housekeeping genes necessary for bacterial growth and survival and was first characterized in 1996 in *S. aureus* (DEORA; MISRA, 1996).

The remaining three σ are considered alternative regulators since they generally respond to altered environmental conditions. The σ^B is considered a global stress response regulator, activated upon several environmental stimuli (Table 2) (CHAN et al., 1998; SHAW et al., 2006). Moreover, σ^B also negatively regulates the Agr system, a crucial quorum-sensing regulator of *S. aureus* (see section 1.3.2.2.1) (BISCHOFF; ENTENZA; GIACHINO, 2001; LAUDERDALE et al., 2009). Finally, it has been shown that σ^S controls the expression of genes related to fitness and survival, and the σ^H factor is linked to the prophage life cycle by modulating prophage integration and excision in *S. aureus* (MILLER et al., 2012; SHAW et al., 2008; TAO; WU; SUN, 2010).

Table 1.2. Activators of the *S. aureus* sigma factors and their cellular roles (Based on Arbade, 2016).

	Activators	**Roles**
σ^A	Nutrition stress, antibiotics	Housekeeping sigma factor essential for bacterial growth
σ^B	Nutrient starvation, stationary phase, high salt, ethanol, high and low temperature, acid pH, nitrosative stress, cell wall-active agents	Plays a role in tolerance, virulence, and adaptation to chronic infections
σ^S	Starvation, DNA damage, cell wall damage, oxidative stress	Survival facing starvation, high temperatures, and detergent-induced lysis
σ^H	Phage 11 infection	Modulates prophage life cycle and bacterial virulence

1.3.2.1.2 Main transcription regulators

CcpA

CcpA is a vital regulator in Gram-positive bacteria that affects metabolism and virulence. This regulator controls the catabolism of carbon sources, directing the cell to preferential use of glucose (DEUTSCHER et al., 1995; WARNER; LOLKEMA, 2003). The CcpA repressor system operates with two components: (1) an active CcpA complex; and (2) catabolic responsive elements (*cre*), which are specifically recognized DNA sequences in the genome. In the presence of high glucose concentrations, the histidine/phosphatase HPrK phosphorylates Hpr, a protein that associates with CcpA to form an active complex able to bind *cre* and repress target gene expression (DEUTSCHER et al., 1995; WARNER; LOLKEMA, 2003). Contrary, inactive CcpA cannot bind the target DNA sequences, leaving carbon catabolism genes to be freely expressed (LEIBA et al., 2012).

In *S. aureus*, the CcpA system is also linked to antibiotic susceptibility and regulation of virulence (RUDRA; BOYD, 2020). It has been demonstrated that high glucose results in increased CcpA-dependent biofilm formation (SEIDL et al., 2008). Indeed, *ccpA* mutant presents decreased expression levels of *icaA* and *cidA* genes, which are related to elements essential for biofilm structure, such as polysaccharide intercellular adhesin (PIA) and extracellular DNA (eDNA) (see section 1.3.3.2) (IDREES et al., 2021; SEIDL et al., 2008). Moreover, CcpA also regulates the expression of essential exotoxins, such as Spa, α-hemolysin, and TSST-1, playing an important role in bacterial virulence (SEIDL et al., 2006; SEIDL; BISCHOFF; BERGER-BÄCHI, 2008).

CodY

S. aureus can synthesize the majority of amino acids necessary for cell functioning. However, why waste energy to synthesize these elements when they are available in the environment? Gram-positive bacteria rely on CodY to save energy, a transcription regulator able to sense and respond to nutrient availability (BRINSMADE, 2016; STENZ et al., 2011). When inactive, CodY presents a tetramer conformation unable to bind DNA. In contrast, the direct interaction of CodY with available guanosine-5'-triphosphate (GTP) and branched-chain amino acids (BCAA) results in an active CodY form that binds to consensus sequences in the genome to repress the expression of target genes (Fig. 1.9) (LEVDIKOV et al., 2006).

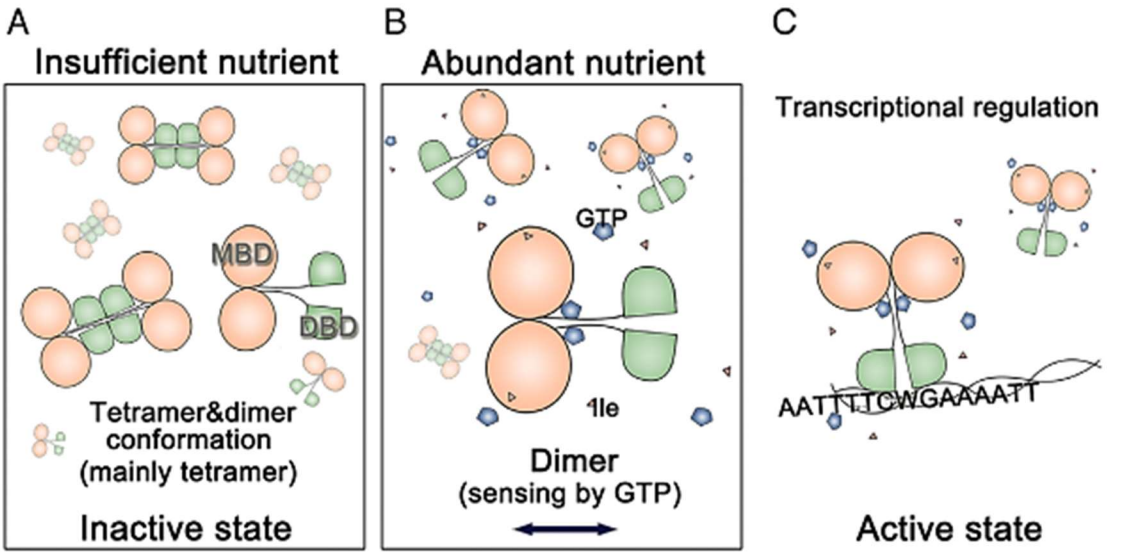

Figure 1.9. *S. aureus* CodY regulation according to nutrient availability. A: Under insufficient nutrition, CodY is inactive, presenting mainly a tetramer conformation. B: Sensing GTP by CodY in a nutrient-rich environment promotes its rearrangement to dimer conformation. C: Active CodY binds to DNA to regulate transcription. MBD (orange): metabolite binding domain; DBD (green): DNA binding domain; Ile: isoleucine (from Han et al., 2016).

In *S. aureus*, CodY controls the direct or indirect expression of more than 200 genes, including those related to amino acid metabolism, nutrient uptake, transport systems, and virulence factors (MAJERCZYK et al., 2010; RUDRA; BOYD, 2020). Generally, genes directly repressed by CodY are related to metabolism while indirect gene regulation is promoted mainly by repression of the Agr system (see section 1.3.2.2.1) (MAJERCZYK et al., 2008; POHL et al., 2009; RUDRA; BOYD, 2020). Indeed, the *S. aureus codY* mutant strain enhanced *agr* locus expression, increasing the expression of several toxins and genes related to biofilm formation (MAJERCZYK et al., 2008). CodY acts as an important bridge between central metabolism and virulence, where regulation is precisely refined. For instance, when *S. aureus* faces nutrient deprivation, the transcription of amino acid pathways is prioritized (WATERS et al., 2016). Conversely, as CodY activity drops, virulence factors that promote nutrient extraction from tissues are the last to be derepressed, ensuring nutrient acquisition (WATERS et al., 2016). These fine-tuned transitions promoted by CodY ensure *S. aureus* adaptation and survival during different steps of infection.

Sar family

The Sar family comprises ten proteins: SarA, SarR, SarS, SarT, SarU, SarV, SarX, SarZ, and MgrA (CHEUNG et al., 2008). The environmental signals triggering many of them are still unknown, with a few exceptions. It has been demonstrated that besides variations found in amino acid sequences of SarZ, MgrA, and SarA, a common region with a Cys-9 residue can undergo post-translational modifications. The phosphorylation of Cys-9 by ROS or kinase Stk1 alters protein affinity to DNA, preventing their binding to target genes (SUN et al., 2012). More research is necessary to identify triggering signals that activate other Sar proteins to understand better their roles in governing *S. aureus* gene expression.

It is well known that proteins in the Sar family are essential for *S. aureus* virulence and tolerance (CHEUNG et al., 2008; JENUL; HORSWILL, 2019). They are differentially expressed during bacterial growth, and, as illustrated in Figure 1.10, they compose a complex regulatory network in *S. aureus* as they present opposite or overlapping roles (BALLAL; RAY; MANNA, 2009). Their interaction with several target genes, other transcriptional regulators, and even activation/repression by own Sar family members work together to fine-tune gene expression in this bacterium (BALLAL; RAY; MANNA, 2009; BEENKEN et al., 2010; CHEUNG et al., 2008; CHEUNG; BAE; OTTO, 2021; JENUL; HORSWILL, 2019). Consequently, mutation of single Sar family members could result in the differential expression of hundreds of genes, as is the case of a Δ*sarA* mutant strain (ORIOL et al., 2021).

SarA is a major regulator and one of the best studied proteins in this family. SarA production is under the control of three different promoters, being P1 and P2 controlled by σ^A, and P3 by the global stress response σ^B (MANNA et al., 2018). It is responsible for the increased synthesis of fibronectin- and fibrinogen-binding proteins, enterotoxins, TSST-1, hemolysins, and genes involved in oxidative stress, capsular and biofilm formation, while it downregulates collagen binding proteins, proteases, and Spa (BALLAL; MANNA, 2009, 2010; BEENKEN; BLEVINS; SMELTZER, 2003a; BLEVINS et al., 1999; CHEUNG et al., 1992; ORIOL et al., 2021; RECHTIN et al., 1999; TROTONDA et al., 2005).

This particular combination of protein expression is given by the capacity of SarA to upregulate the Agr system and repress the other Sar family members, including itself and Rot (JENUL; HORSWILL, 2019; REYES et al., 2011). The last regulates the expression of at least 150 genes mainly by repressing proteases and toxins and activating superantigens and surface proteins (SAÏD-SALIM et al., 2003).

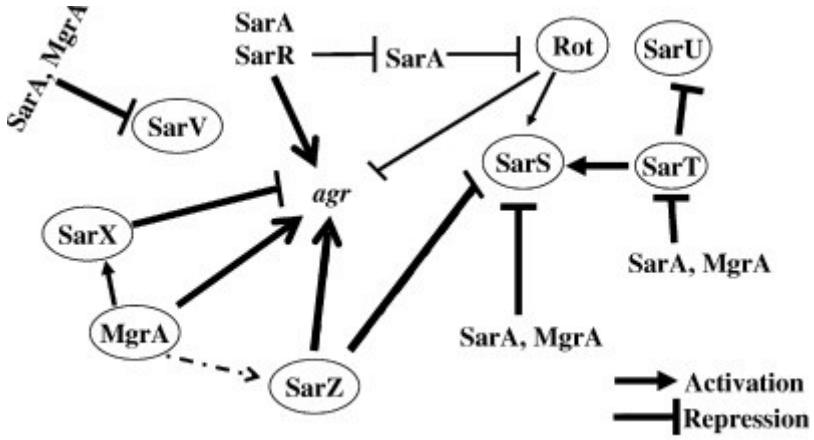

Figure 1.10. The regulatory network between the Agr system and 10 Sar proteins. Lines indicate the nature of regulation: thick, completely involved; thin, partially involved (adapted from Ballal et al., 2009).

1.3.2.2 Signal transduction systems

1.3.2.2.1 Agr quorum-sensing system

One of the most studied and important *S. aureus* global regulators is the quorum-sensing accessory gene regulator (Agr). The *arg* locus comprises two transcriptional units controlled by P2 and P3 promoters: RNAII encoding the genes *agrA*, *agrB*, *agrC* and *agrD*, and RNAIII, the major effector and regulator of *agr*-targeted genes (JANZON; LÖFDAHL; ARVIDSON, 1989a; NOVICK et al., 1995). The auto inducing peptide (AIP) encoded by *agrD* is the extracellular quorum signal, which is activated upon high-cell densities (JI; BEAVIS; NOVICK, 1995).

Figure 1.11 shows that the AIP is exported out of the cell by a transmembrane endopeptidase encoded by *agrB* (ZHANG et al., 2002). A two-component signal transduction system is triggered by the phosphorylation of the histidine kinase AgrC upon binding to AIP (LINA et al., 1998), which actives the response regulator AgrA, which in

turn binds P2, P3, and *psmα* and *psmβ* (operons) promoter regions (LE; OTTO, 2015). Interestingly, it has been shown that AIP regulation can be strain-specific since large variations observed on *agr* locus sequences lead to the expression of AIPs exerting different functions, which confers competitional advantages for specific bacterial populations (DUFOUR et al., 2002; JI; BEAVIS; NOVICK, 1997).

Agr regulation is essential to the progression of *S. aureus* infections, controlling the expression of several virulence factors (Fig 1.11). When the Agr system is suppressed at low cell densities bacteria produce surface components (e.g., Spa, fibrinogen-binding proteins) necessary to bacteria-host interactions and colonization in the first stages of infection (DUNMAN et al., 2001; LE; OTTO, 2015; TAN et al., 2018). Alternatively, high-cell densities achieved upon bacterial internalization and proliferation stimulate the Agr system and increase the expression of degrative exoenzymes and toxins (e.g., α-toxin, γ-hemolysin, leukocidins, PSMs), some of which are pro-inflammatory components (BEN ZAKOUR et al., 2008; DUNMAN et al., 2001; TAN et al., 2018).

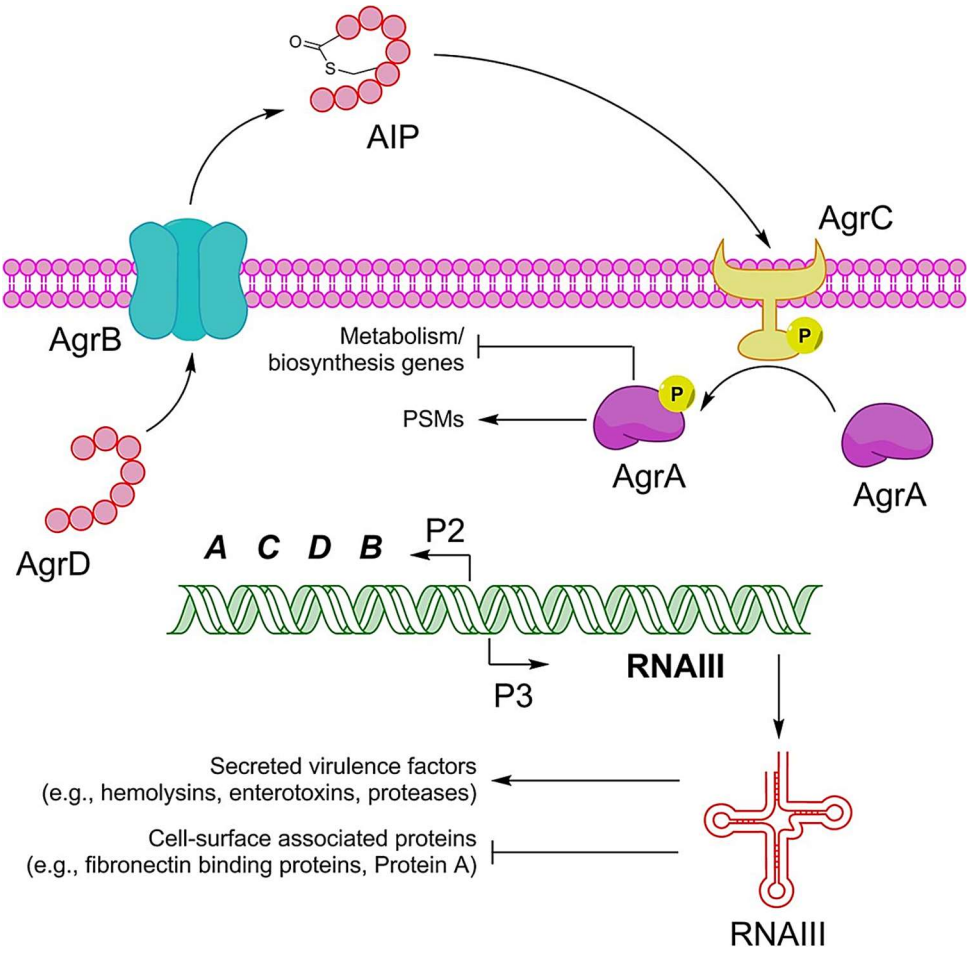

Figure 1.11. Diagram of the *S. aureus* accessory gene regulatory (Agr) quorum-sensing system (from Salam and Quave, 2018).

1.3.2.2.2 Two-component regulatory systems

S. aureus possesses several two-component regulatory systems (TCSs) that enable the bacteria to sense and rapidly respond to changing environmental conditions such as nutritional availability, temperature, osmotic pressure, antibiotics, and host effectors (BLEUL; FRANCOIS; WOLZ, 2022). The TCSs are composed of a histidine kinase (HK) and a response regulator (RR) protein that, together, promote the transduction of a signal message across the cellular membrane to coordinately regulate the cellular response (STOCK; ROBINSON; GOUDREAU, 2000).

These TCSs sensing machineries ensure the effective "connectiveness" between environmental stimuli and cellular physiology, guarantying optimal adaptation and survival. However, even though they contribute to regulating *S. aureus* optimal growth and virulence under certain conditions, it has been demonstrated that only WalRK is essential for survival (RAPUN-ARAIZ et al., 2020; VILLANUEVA et al., 2018). Table 3 displays the triggering signals and functions exerted by *S. aureus* histidine kinases, while Figure 1.12 illustrates their structures.

Table 1.3. *S. aureus* two-component system histidine kinases, their triggering signals, and functions (adapted from Bleul et al., 2022).

Histidine Kinase	Triggering signal	Function
WalK	unknown	Cell division, autolysis
HptS	Glucose-6-P	Glucose-6-P import
LytS	Membrane potential	Cell wall metabolism
GraS	Indolicidin, Mellitin, Nisin, LL-37, Colistin, Polymyxin B	Surface charge CAMP Resistance
SaeS	HNP1–3	Virulence
ArlS	Low Manganese, Low glucose	Cell wall surface proteins, Manganese homeostasis
SrrB	Reduced menaquinone	Oxidative stress
PhoR	Low Pi	Phosphate homeostasis
AirS	Oxygen	Redox sensing
VraS	Cell wall damage	Cell wall stress resistance
AgrC	AIP (quorum sensing)	Virulence
KdpD	c-di-AMP	K+ homeostasis
HssS	Hemin toxicity	Heme detoxification
NreB	Low oxygen, High NO3-	Nitrogen respiration
BraS	Bacitracin, Nisin	Bacteriocin resistance

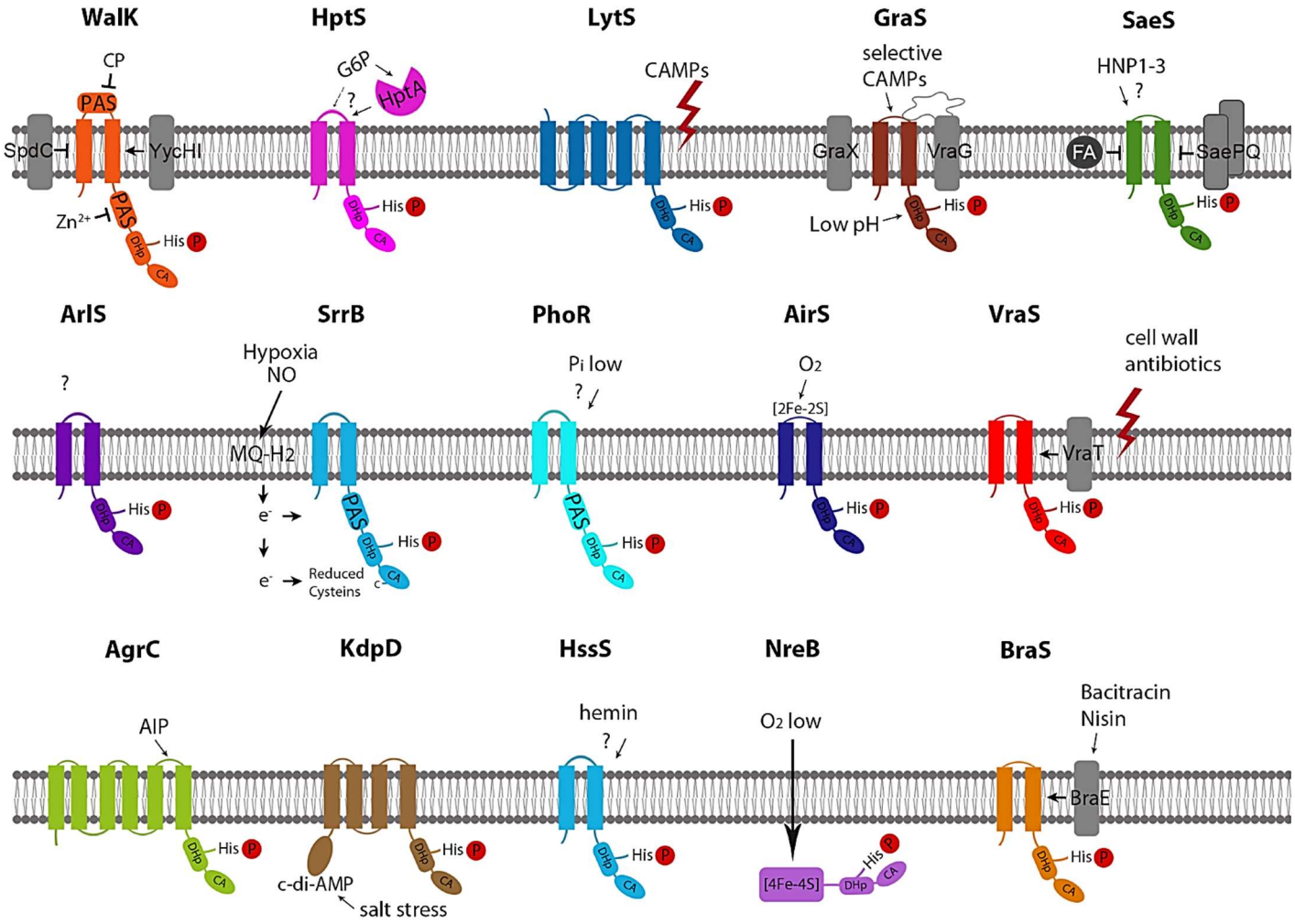

Figure 1.12. Structure and signaling mechanisms of *S. aureus* histidine kinases. CAMP: Cationic antimicrobial peptide; CP: Peptidoglycan cleavage products; FA: fatty acid (Bleul et al., 2022).

1.3.2.3 Regulatory RNAs

Regulation of gene expression is a complex process that relies on several mechanisms, some of which were previously discussed. In this section, another essential component of gene regulation will be addressed: small regulatory RNAs (sRNAs). These sRNAs are usually non-coding and compose complex regulatory networks that affect proteins and/or the structure, stability, and translation efficacy of target RNAs, allowing bacteria to rapidly respond to changing environmental conditions and stresses (FELDEN et al., 2011; HOE et al., 2013; JØRGENSEN; PETTERSEN; KALLIPOLITIS, 2020). In addition to promoting self-regulation, bacterial sRNAs can also affect host-pathogen interactions by targeting host elements during infection. (HOE et al., 2013; SVENSSON; SHARMA, 2016; WESTERMANN, 2018).

As shown in Figure 1.13, sRNAs' primary mechanism of action is their direct binding (base-pairing) to target mRNAs, which can result in their respective repression or activation. Repression can occur either by obstruction of transduction initiation or by mRNA degradation by the recruitment of RNAses. Conversely, sRNA can free the transduction initiation site to activate or prevent mRNA degradation (Fig 1.13) (SVENSSON; SHARMA, 2016). These sRNAs can be classified according to a genomic relationship with their targets, *cis*-acting sRNAs related to adjacent genes and *trans*-acting sRNAs related to genes found in different locations of the genome (STORZ; VOGEL; WASSARMAN, 2011; SVENSSON; SHARMA, 2016).

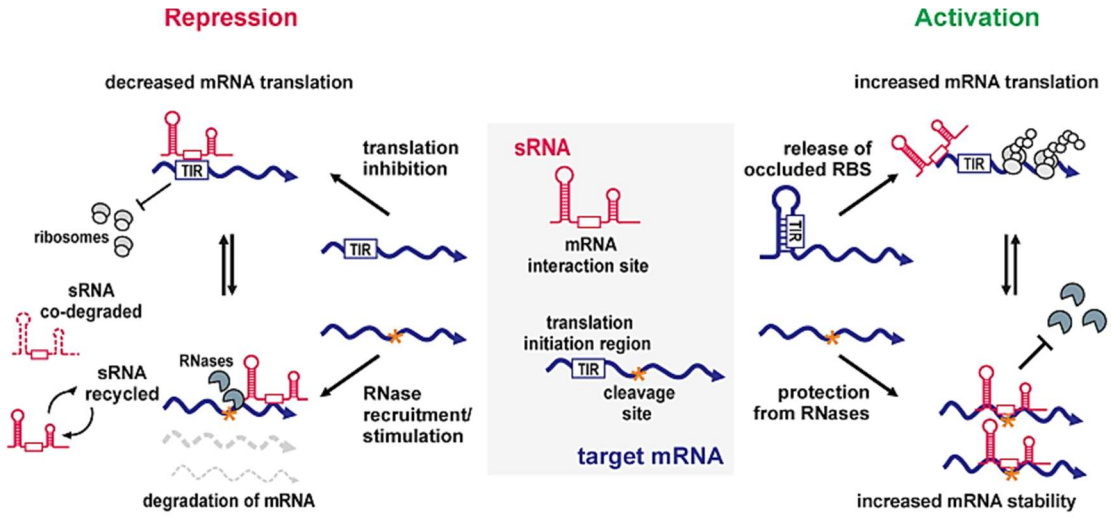

Figure 1.13. Repression of activation of mRNAs by sRNAs. Direct or indirect interactions between sRNAs (red) and mRNAs (blue) are displayed. Orange asterisks indicated potential RNase cleavage sites. TIR, translation initiation region (from Svensson and Sharma, 2016).

The interest in bacterial sRNAs has increased in the past years. Several studies using *in silico* and/or experimental approaches, such as bioinformatics and RNA-sequencing, have suggested the presence of hundreds of sRNAs in *S. aureus*, though only a few have been functionally characterized (BOHN et al., 2010; BROACH; WEISS; SHAW, 2016; CARROLL et al., 2016; GEISSMANN et al., 2009; HOWDEN et al., 2013; LIU et al., 2018b; PICHON; FELDEN, 2005).

Studying these sRNAs is challenging since their identification depends on the tested strains, experimental conditions, and methods of detection used (LIU et al., 2018b). In addition, assigning different names to the same sRNAs in different studied strains can lead to confusion, a problem recently addressed with the creation of the Staphylococcal Regulatory RNA Database (SRD) (SASSI et al., 2015). Here, we will focus on some crucial *S. aureus* sRNAs that play roles in virulence and metabolism.

1.3.2.3.1 RNAIII

RNAIII is an essential *S. aureus* regulatory RNA, being considered a paradigm due to its multifactorial functions (Table x) (FELDEN; AUGAGNEUR, 2021). Going in the opposite direction of usual sRNAs, RNAIII is relatively long (514 nucleotides) and presents a coding region for δ-hemolysin (Hld) (JANZON; LÖFDAHL; ARVIDSON, 1989b). RNAIII is also the effector molecule of the Agr quorum-sensing system that in response to high cell densities, regulates the production of several proteins by interfering in the translation of several targets (LE; OTTO, 2015; RECSEI et al., 1986) (Fig. 1.14). The RNAIII interacts with target mRNAs to either activate translation, as it is the case of hemolysin Hla and the global regulator MgrA, or to repress the production of surface proteins (e.g., Sbi) and the toxin repressor Rot (BENITO et al., 2000; BOISSET et al., 2007; CHEVALIER et al., 2010; FELDEN; AUGAGNEUR, 2021; GEISINGER et al., 2006; GUPTA; LUONG; LEE, 2015; MORFELDT et al., 1995). RNAIII activity results in the downregulation of surface elements and increased toxin secretion, a state essentially linked to the progression of staphylococcal infections.

Table 1.4. Described functions of regulatory RNAIII from *S. aureus* (adapted from Svensson and Sharma, 2016; Felden and Augagneur, 2021).

RNAIII effect	**Target**	**Encoded target function**	**Mechanism of action**
Repression	*spa*	Adhesion and immune evasion	Translation inhibition and degradation by RNaseIII
	coa	Adhesion	
	sa1000	Adhesion	
	rot	Transcription factor and toxin repressor	
	sbi	Adhesion and immune evasion	Translation inhibition
	lytM	Cell-wall metabolism and release of Spa	
	sa2353	Surface secretory antigen, function unknown	
Activation	*hla*	Alpha toxin	Translation activator
	mgrA	Transcription factor, an inhibitor of surface proteins and autolysis, and activator of capsule synthesis	Stabilization of mRNA

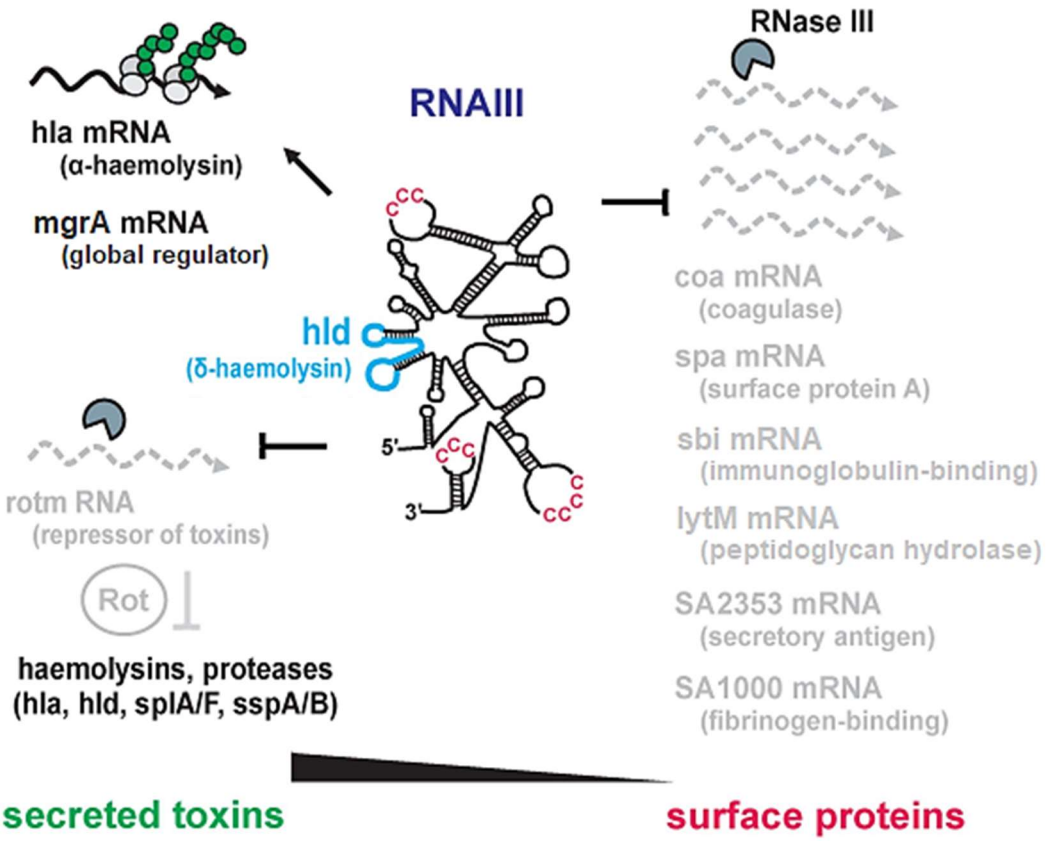

Figure 1.14. *S. aureus* regulatory RNAIII functions. RNAIII structure encodes delta-hemolysin (blue) and also presents C-rich loops (red) used to repress target elements, such as the toxin repressor Rot and genes encoding surface proteins (Coa, Spa, Sbi, LytM, SA2353, SA1000). RNAIII also directly activates Hla and MgrA mRNAs. The overall result of RNAIII activity is increased toxin expression and repression of surface proteins (adapted from Svensson and Sharma, 2016; Felden and Augagneur, 2021).

1.3.2.3.2 Rsa family

Rsa is a family of non-coding *S. aureus* RNAs identified with the help of bioinformatics and RNomic approaches, named RsaA-K and RsaOA-OX. Their nomenclature does not reflect homology but discovered order, though some names refer to the identical RNA sequences reported almost simultaneously but independently (BOHN et al., 2010; GEISSMANN et al., 2009; MARCHAIS et al., 2009). Some of these Rsa RNAs will be discussed below. Interestingly, most of them contained a C-rich sequence motif (UCCC), a signature of regulatory RNAs repressing translation initiation of target mRNAs (Geissmann et al., 2009).

RsaA

It has been shown that RsaA acts as a virulence suppressor of acute infections since a Δ*rsaA* mutant presented decreased protection against host clearance, contributing to *S. aureus* persistence (ROMILLY et al., 2014). This observation is linked to the RsaA ability to repress the synthesis of MgrA, a global transcriptional regulator involved in activating capsular synthesis and inhibiting surface proteins (LUONG; NEWELL; LEE, 2003; ROMILLY et al., 2014). The decrease of MgrA mediated by RsaA leads to disminished capsule formation and increased biofilm production, leading to the appearance of chronic infections (ROMILLY et al., 2014; TROTONDA et al., 2008). RsaA has also been shown to repress mRNAs coding enzymes linked to peptidoglycan metabolism and the anti-inflammatory protein FLIPr (TOMASINI et al., 2017).

RsaC

Oxidative stress is a common host defense against maleficent intruders, such as pathogenic bacteria. These impostors must find strategies to overcome the oxidative burst to survive, and in *S. aureus*, one of them is the sRNA RsaC. RsaC is an atypical sRNA presenting more than 1000 nucleotides, associated with the adaptation to oxidative stress in response to manganese starvation (LALAOUNA et al., 2019). As shown in Figure 1.15, RsaC is co-transcribed with a major manganese transporter (MntABC) and is suppressed in the presence of this nutrient. When facing manganese starvation, RsaC is produced and cleaved by RNAIII, resulting in a mature, stable, and active form. Active RsaC represses *sodA*, coding for a manganese-dependent superoxide dismutase, and *sarA* coding for a transcriptional repressor of *sodM* (Fig 1.15). The last is an alternative SOD enzyme that uses iron as a co-factor (LALAOUNA et al., 2019). The regulation of SOD enzymes by RsaC allows bacteria to adapt by creating a balanced interconnection between oxidative stress and metal bioavailability in the host. Indeed *S. aureus* Newman Δ*rsaC* mutant is less virulent than the wild-type strain in a murine model, showing this sRNA's critical role in *S. aureus* pathogenicity (PANTHEE et al., 2021).

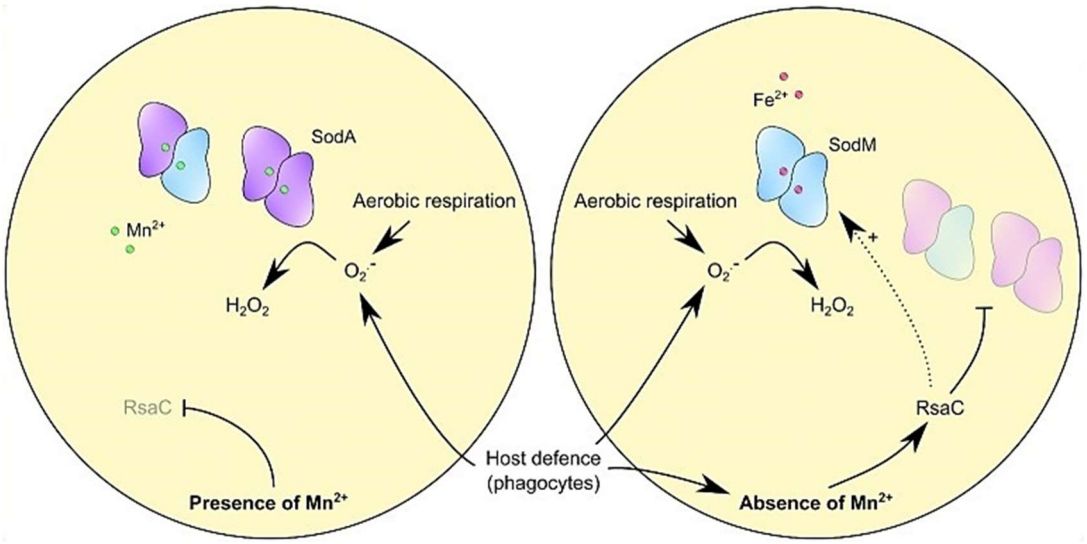

Figure 1.15. RsaD regulation in *S. aureus*. RsaC is repressed in the presence of manganese (Mn), which is used as a co-factor by SodA to detoxify cells from ROS. When facing manganese starvation, active RsaC inhibits SodA production and indirectly activates SodM via the transcriptional regulator SarA. SodM uses iron as a co-factor to replace SodA and restore the ROS detoxification pathway (adapted from Lalaouna et al., 2019).

RsaD

S. aureus RsaD is an important sRNA that regulates cell death in response to acidic stress by redirecting carbon flow. Actually, it has been previously demonstrated that *S. aureus* presents substantial cell death after aerobic growth in high glucose media (THOMAS et al., 2014). The increased acetate production from pyruvate at high glucose concentrations contributes to acetic acid formation and proton increase, resulting in lower pH and cell death (RUSSELL, 1991). Since RsaD is activated by σ^B, a global regulator that responds to weak acid stress, its role was investigated. Indeed, it was shown that RsaD inhibits the expression of acetolactate synthase (AlsS), an enzyme that consumes protons and generates acetoin from pyruvate, being considered an acid-resistance mechanism (AUGAGNEUR et al., 2020; THOMAS et al., 2014). When RsaD is absent, AlsS total activity leads to the cell's detoxification. In this way, RsaD balance control between acetate and acetoin could contribute to the regulation of cell death in high glycolytic environments (AUGAGNEUR et al., 2020). Moreover, RsaD composes a complex regulatory network, as SrrAB also activates it and is downregulated by CodY and Agr (AUGAGNEUR et al., 2020).

RsaE

Different from other RNAs in this family, RsaE is a conserved sRNA found in *Staphylococcus*, *Macrococcus*, and *Bacillus* (GEISSMANN et al., 2009). It has been demonstrated that RsaE regulates several enzymes involved in the TCA cycle, amino acid and peptide transport, metabolism of lipids and carbohydrates, and arginine degradation (Fig 1.16). More precisely, RsaE interacts and inhibits translation initiation of target mRNAs, such as *sucC* and *sucD* (subunits of succinyl-CoA), *oppB* (oligopeptide transport system, opp-3 operon), and SA0873, of unknown function (GEISSMANN et al., 2009). In addition, RsaE is overexpressed in small colony variants (SCV) (see section 1.3.3.1) by negatively regulating RNAIII, which contributes to diminished toxicity and bacterial persistence (TUCHSCHERR; LÖFFLER; PROCTOR, 2020). Similar to RsaA and RsaH, RsaE synthesis is induced under oxidate stress, osmotic stress, and acidic pH (GEISSMANN et al., 2009).

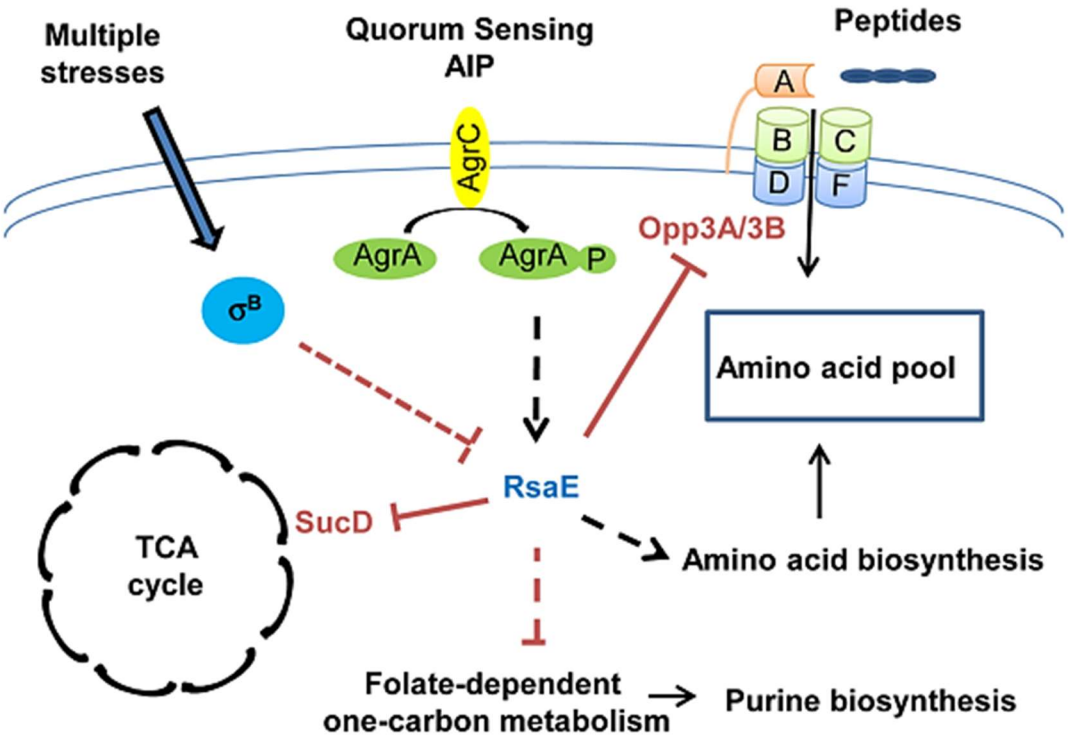

Figure 1.16. *S. aureus* RsaE regulation of central metabolism. RsaE regulates directly or indirectly the expression of several genes, including those involved in the TCA cycle, carbohydrate metabolism, amino acid synthesis, and peptide transport. Straight and dashed lines represent direct and indirect regulation, black arrows = stimulation; Red bars: represent inhibitions (Guillet et al., 2013).

1.3.2.3.3 Spr family

The sRNAs in the Spr family are found in small pathogenicity islands present along the *S. aureus* chromosome. They were named from SprA to SprG and were first identified by *in silico* analysis, their expression confirmed by Northern blot analyses (PICHON; FELDEN, 2005). Pathogenic islands are involved in *S. aureus* virulence since they comprise several genes encoding virulence factors and elements involved in antibiotic resistance. The main Spr sRNAs will be discussed below.

SprC

SprC has been linked to attenuated *S. aureus* virulence since a mutant for SprC became more virulent than the wild-type strain, even though the mutant was more efficiently phagocyted by host cells (LE PABIC et al., 2015). This sRNA disfavors bacterial resistance to oxidative stress, which is consistent with the immediate repression of SprC following phagocytosis (LE PABIC et al., 2015). SprC inhibits the expression of the Atl, an autolysin linked to bacterial cell wall remodeling and biofilm formation (DUBRAC et al., 2007). Indeed, Atl is essential to promote concealing of bacterial PGN at the cell surface to avoid host detection (ATILANO et al., 2014).Recently, the SprC regulon has been expanded by identifying at least 60 transcripts differentially expressed in an *sprC* knockout strain (ZHOU et al., 2022). Among them are the leukocidins lukE and lukD, which were previously downregulated by SprC (ZHAO et al., 2017; ZHOU et al., 2022). This multifaceted sRNA influences elements linked to several processes, including metabolism (such as purine and sugar), catalytic functions, and *S. aureus* pathogenesis (LE PABIC et al., 2015; ZHAO et al., 2017; ZHOU et al., 2022).

SprD

Contrary to SprC, SprD is a sRNA linked to enhanced virulence of *S. aureus*. It was demonstrated that bacterial virulence is abolished in a Δ*sprD* mutant strain, while mice infected with a wild-type strain die of a sepsis model. In addition, the Δ*sprD* mutant strain also failed to generate renal abscesses (CHABELSKAYA; GAILLOT; FELDEN, 2010). The virulent phenotype apported by SprD partly relies on its interaction with Sbi, an important staphylococcal surface protein involved in bacterial immune evasion. SprD represses Sbi translation by an antisense pairing, the same repressor mechanism used by RNAIII to prevent Sbi production (CHABELSKAYA; BORDEAU; FELDEN, 2014; CHABELSKAYA; GAILLOT; FELDEN, 2010).

SprX

SprX, also named RsaOR (BOHN et al., 2010), is an *S. aureus* sRNA that plays an essential role in antibiotic resistance, particularly in the class of glycopeptides such as vancomycin. SprX inhibits the production of SpoVG, a protein involved in capsule formation and antibiotic resistance (SCHULTHESS et al., 2009). Overexpression of SprX leads to direct antisense pairing and suppression of SpoVG mRNA, leading to higher bacterial susceptibility to antibiotics (EYRAUD et al., 2014). Moreover, SprX positively regulates the expression of the essential TCS WalKR involved in *S. aureus* autolysin synthesis (BUCHAD; NAIR, 2021). Indeed, SprX deletion resulted in the down-regulation of several autolysins, such as Atl, LytM, and IsaA, resulting in higher cell aggregation and reduced biofilm formation (BUCHAD; NAIR, 2021). Finally, SprX has also been associated with the production of other virulence factors, such as toxin Hld and adhesin ClfB, showing its important role in *S. aureus* pathogenesis (KATHIRVEL; BUCHAD; NAIR, 2016).

SprY

It has been recently shown that SprY is a sRNA that regulates *S. aureus* virulence by titrating RNAIII activity. As previously discussed, RNAIII is a major virulence factor regulator that relies on several functional stem loops to regulate the expression of target genes (BENITO et al., 2000; BRONESKY et al., 2016). It has been shown that SprY binds to an active region of RNAIII involved in the repression of extracellular complement binding protein Ecb and the toxin repressor Rot (Fig. 1.17) (LE HUYEN et al., 2021). Therefore, SprY activity results in a decrease in *S. aureus* toxin expression. Interestingly, SprY presents a ‘sponge-like’ activity, since it competes with RNAIII mRNA targets. SprY adjusts RNAIII function when they present comparable levels, a state that changes at high cell densities where RNAIII accumulates (Fig. 1.17) (LE HUYEN et al., 2021). This sophisticated mechanism allows bacteria to accurately adjust virulence to colonize the host without causing severe damage, giving *S. aureus* the perfect balance for a successful infection.

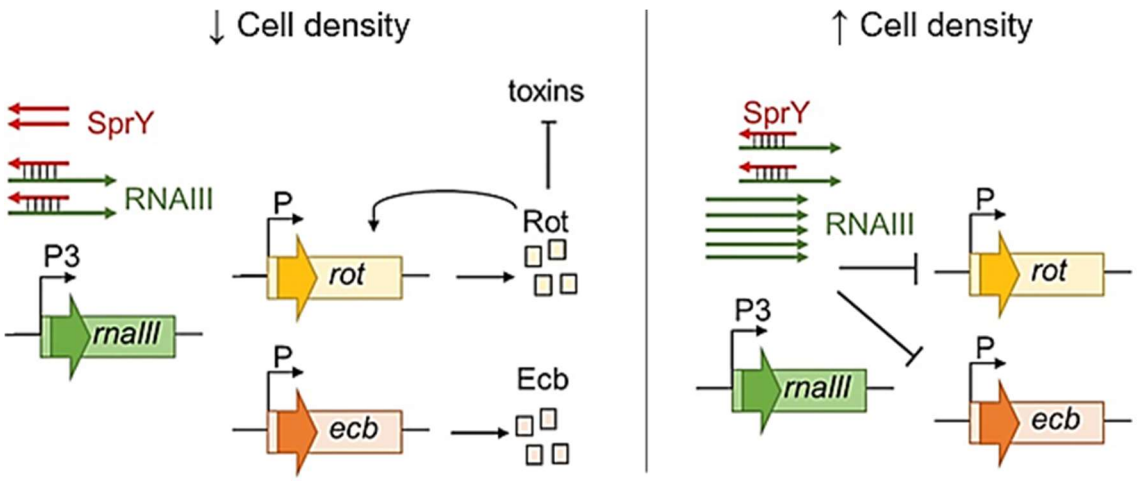

Figure 1.17. Schematic view of SprY regulation system. "Sponge-like" SprY activity is exerted when SprY sequestrates all RNAIII copies presented at low concentrations in the cell. Conversely, at high bacterial densities, SprY concentration is overcome by RNAIII, which then represses the translation of Ecb and Rot (adapted from Le Huyen et al., 2021).

Toxin-antitoxin systems

SprA and SprG/F are part of Type I toxin-antitoxin (TA) systems composed of two elements. In each of these systems, a gene encodes an antitoxin able to neutralize the action of small stable toxins that promote growth arrest or cell death (SCHUSTER; BERTRAM, 2013). They are found in most prokaryotes, and under standard conditions, toxin translation is counteracted by the antitoxin activity to ensure average bacterial growth. Contrariwise, stress conditions can contribute to antitoxin degradation, and the consequent unbalance between TA components leads to altered cellular processes such as DNA replication, translation, and cell wall synthesis (SCHUSTER; BERTRAM, 2013; SINGH et al., 2021). This cellular state triggers biofilm formation and antibiotic resistance, contributing to the appearance of 'persisters' (SINGH et al., 2021).

The SprA1/SprA1$_{AS}$ TA system is encoded by the pathogenic island vSaβ and was first described in 2012. It is composed of the toxin PepA1 and an antitoxin encoded by *srn_3590_sprA1$_{AS}$*, whose production is decreased in response to acid and oxidative stresses (Fig 1.18A) (SAYED et al., 2012). A homologous TA system, described as SprA2/SprA2$_{AS}$, is, on the other hand, present in the *S. aureus* core genome. In this case, the production of the PepA2 toxin is controlled by intracellular levels of antitoxin SprA2$_{AS}$, which responds to stringent and hyper-osmotic stresses (GERMAIN-AMIOT et al., 2019).

The SprG1/SprF1 is encoded by the pathogenic island ΦSa3PI. The antitoxin SprF1 controls traduction of mRNA SprG1, which encodes two peptides responsible for cell wall pore-formation and bacterial lysis (Fig 1.18B) (PINEL-MARIE; BRIELLE; FELDEN, 2014). In addition to its TA function, the antitoxin SprF1 also binds to ribosomes to globally reduce traduction in *S. aureus*, contributing to retarded metabolism and appearance of persistent cells (PINEL-MARIE et al., 2021). Finally, three other TA systems similar to SprG1/SprF1 are found in the *S. aureus* core genome. SprG2/SprF2 and SprG3/SprF3 systems seem to respond to oxidative and hyper-osmotic stresses to promote bacteriostasis, while SprG4/SprF4 functionally isunknown (RIFFAUD et al., 2019).

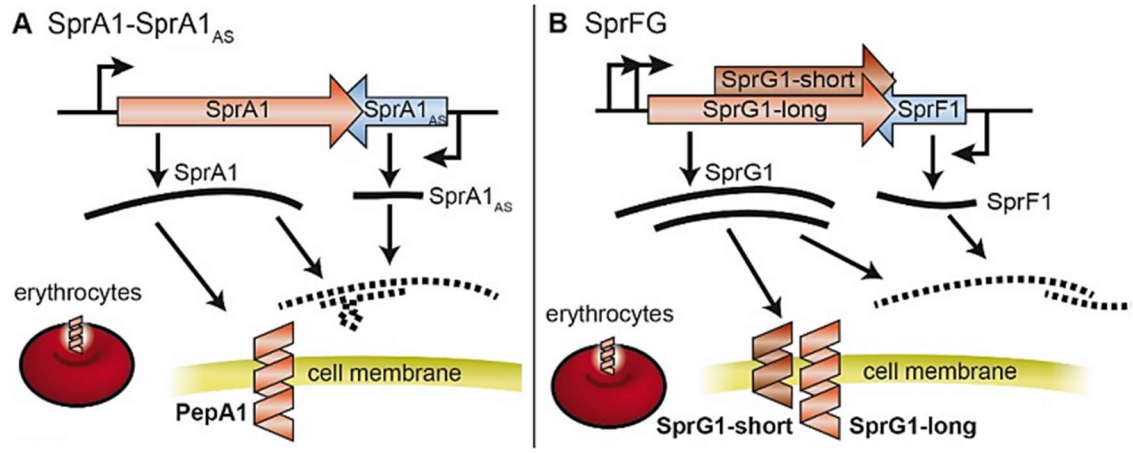

Figure 1.18. Type I toxin-antitoxin systems of *S. aureus*. RNAs from toxin and antitoxin are transcribed from convergent promoters for both (A) SprA1-SprA1$_{AS}$ and (B) SprG1/SprF1. SprG1 mRNA encodes two peptides of different lengths (short and long) (from Schuster and Bertram, 2016).

1.3.2.3.4 Teg family

Teg family is a group of almost 200 small transcripts and antisense RNAs molecules identified by RNA-sequencing that may act as sRNAs (BEAUME et al., 2010, 2011). They were named according to their discovery order for "Transcript from Experimental method from Geneva" (Teg). The regulatory function of only a few Teg RNAs has been validated.

Teg41

Teg41 is a *S. aureus* sRNA divergently transcribed from the locus encoding PSMα peptides, which present potent cytolitic activity (CARROLL et al., 2016; ZAPF et al., 2019). Prediction analysis indicated interaction between the 3' end of Teg41 and *psmα* transcripts, which was experimentally validated by the deletion of a 24-nucleotide region of Teg41 (ZAPF et al., 2019). Indeed, the modified strain presented attenuated virulence in a mouse abscess infection model, which was consistent with a 10-fold reduction in PSMα-dependent hemolytic activity (ZAPF et al., 2019). Since PSMs are potent *S. aureus* toxins, Teg41 plays a critical role in *S. aureus* virulence.

Teg49

The RNA-seq analysis by Beaume et al. (2010) pointed out that Teg48 and Teg49 reside in the promoter regions P1 and P2, respectively, of the transcriptional regulator SarA. By using knockout strains for *sarA* and *teg49* and RNA-seq, Manna et al. (2018) demonstrated that Teg49 regulates over 200 genes in a SarA-independent manner, including those linked to metabolism cell-wall synthesis, cellular processes, and virulence factors. In addition, qRT-PCR experiments validated Teg49 control of regulatory elements, such as SaeR and RbsU, and virulence factors, such as leukocidin LukF and the protease SplA (MANNA et al., 2018). Teg49 mutant strain did not impact SarA expression or biofilm formation. However, they exhibited reduced bacterial load in an abscess skin model compared to the parent strain (MANNA et al., 2018). Finally, Teg49 was also shown to repress the expression of *spn*, a gene encoding a peptide able to inhibit the catalytic activity of myeloperoxidase (MPO) of human neutrophils. In this case, the Teg49 mutant strain presented an increased resistance to MPO and killing after phagocytosis (CENGHER et al., 2022).

1.3.3 Persistence

In addition to the expression of several virulence determinants that contribute to *S. aureus* pathogenesis, this bacterium also presents other mechanisms to guarantee bacterial success. It has been shown that *S. aureus* can invade both professional and non-professional phagocyte cells, and its intracellular localization contributes to bacterial survival (FRAUNHOLZ; SINHA, 2012; HÄFFNER et al., 2020; STROBEL et al., 2016). Moreover, when facing unfavorable conditions, *S. aureus* may switch to alternative lifestyles presenting altered metabolic and physiologic states with limited or no growth (GUO et al., 2022). These altered subpopulations presenting higher tolerance to antibiotic treatment and clearance by the host immune system will be discussed below.

1.3.3.1 Small colony variants (SCVs)

Small colony variants (SCVs) are a phenotype found in subpopulations of *S. aureus* generated by the accumulation of mutations in the genome that drastically affect bacterial metabolism (TUCHSCHERR; LÖFFLER; PROCTOR, 2020). SCVs are usually non-pigmented, non-hemolytic, and present slow growth, leading to host bacterial persistence (JOHNS et al., 2015; KAHL; BECKER; LÖFFLER, 2016). Figure 1.19 summarizes the SCVs dynamics in *S. aureus* infections.

As shown, SCVs present upregulated σ^B, a transcription factor that negatively controls the Agr system leading to the diminished production of exotoxins and less aggressiviness of these subpopulations (BISCHOFF; ENTENZA; GIACHINO, 2001; LAUDERDALE et al., 2009). It has been demonstrated that σ^B is essential to developing SCVs and chronic infections, as infection with a *sigB* mutant results in complete bacterial clearance after a few days (TUCHSCHERR et al., 2015). Therefore, the SCVs phenotype represents a significant problem for *S. aureus* treatment since it can rapidly switch to the original wild-type lifestyle, being the source of recurrent and chronic infections (KAHL; BECKER; LÖFFLER, 2016; PROCTOR et al., 1995; TUCHSCHERR; LÖFFLER; PROCTOR, 2020).

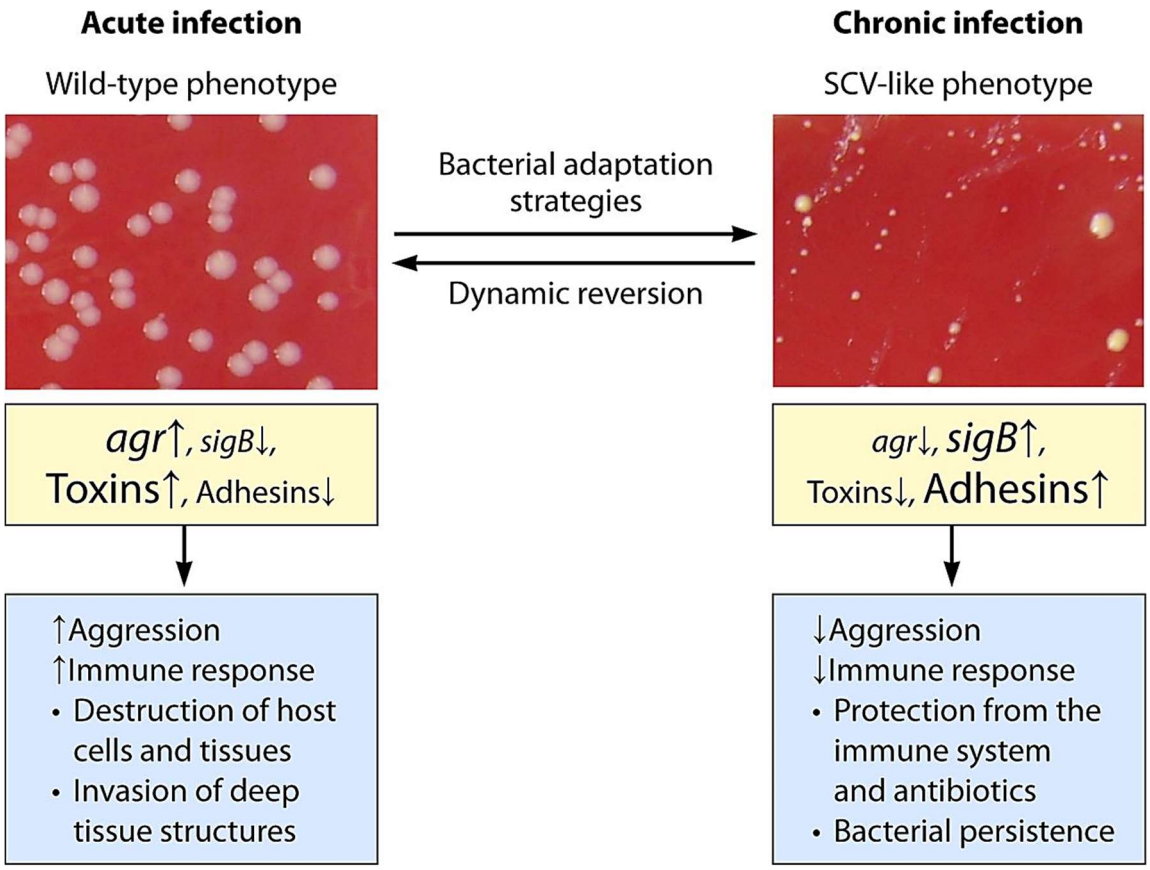

Figure 1.19. Dynamic of *S. aureus* infections and the occurrence of SCVs. Acute infection: upregulation of Agr and toxin expression promotes accentuated inflammation and bacteria invasion. Chronic infection: the appearance of SCV phenotype with reduced Agr and toxin secretion leads to diminished host immune response activation, leading to bacterial persistence. Finally, SCVs can revert to wild-type metabolism, causing reinfections (Kahl et al., 2016).

1.3.3.2 Biofilm formation

Biofilm formation is another important strategy used by *S. aureus* to persist in the environment. As a highly surface-adhering bacterium, *S. aureus* has a strong historic of prevalence on substrates, making bacterial eradication difficult (IDREES et al., 2021; MAIKRANZ et al., 2020; MERGHNI et al., 2016). Biofilms are characterized by a structure where bacterial cells are embedded in a protective matrix composed of proteins, polysaccharides, extracellular DNA (eDNA) and polymeric substances that facilitates survival in hostile and extreme environments (Fig. 1.20) (FLEMMING; WINGENDER, 2010; JOO; OTTO, 2012; ROHDE et al., 2007).

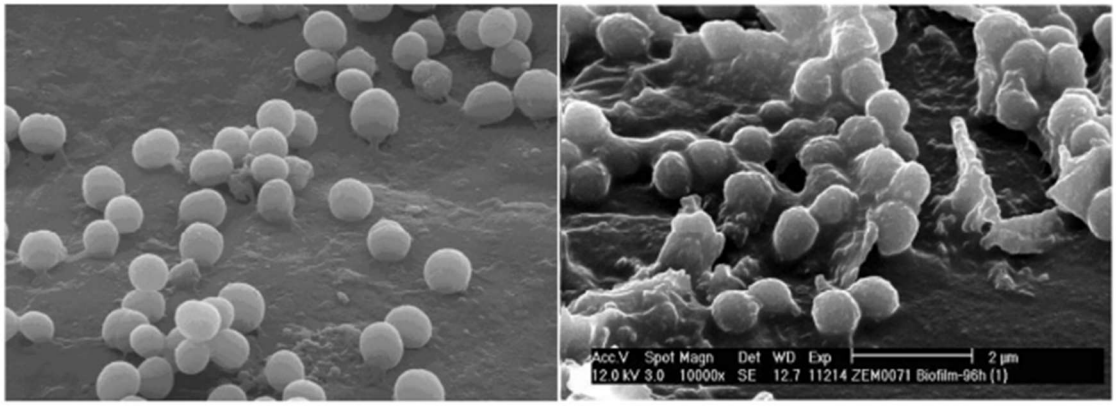

Figure 1.20. *S. aureus* from broth culture (left) and in a biofilm matrix (right) (from Geipel, 2009).

As shown in Figure 1.21, the biofilm cycle comprises the following main stages: attachment of planktonic cells to abiotic or biotic surfaces; microcolony development and biofilm maturation; and biofilm detachment and bacterial dispersal (KOSTAKIOTI; HADJIFRANGISKOU; HULTGREN, 2013; LE et al., 2014; OTTO, 2013). Bacterial cells integrating biofilm presents altered gene expression patterns and physiological properties than planktonic cells (RESCH et al., 2005). More specifically, due to the gradient of nutrients and oxygen found in this structure, at least four metabolic states were identified in the *S. aureus* biofilm matrix, including aerobic, fermentative, and dormant bacterial growing, in addition to dead cells (RANI et al., 2007).

It has been demonstrated that *S. aureus* biofilm formation is the result of a complex process involving the action of several enzymes (e.g., proteases, nucleases) and the interconnection of global regulators, such as SarA, σ^B, and Agr (LAUDERDALE et al., 2009). Indeed, it was shown that *sarA* and *sigB* mutants could not form biofilm due to elevated protease activity levels in these isogenic strains (BEENKEN; BLEVINS; SMELTZER, 2003b; MARTÍ et al., 2010). On the other hand, the Agr system activation promotes the expression of proteases and PSMs, the latter acting as surfactant-like agents to contribute to biofilm structuring detachment (Fig. 1.21) (BOLES; HORSWILL, 2008; LE et al., 2014; LE; OTTO, 2015; PERIASAMY et al., 2012a).

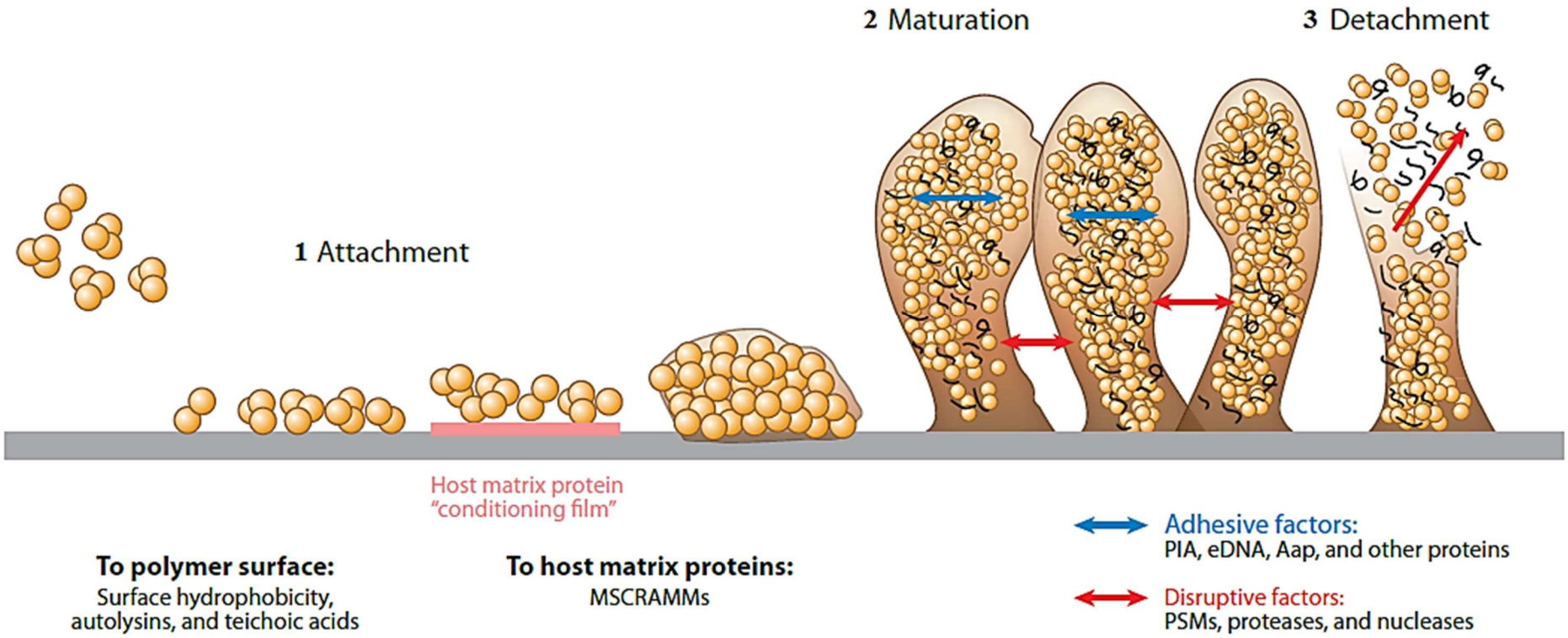

Figure 1.21. Phases of *S. aureus* biofilm formation. Bacteria attach to abiotic or biotic surfaces, where agglomeration and accumulation of adhesive molecules in the matrix form a mature biofilm. The biofilm cycle includes (1) bacterial attachment, (2) biofilm maturation, and (3) biofilm detachment. Channels on the biofilm structure are molded by the action of disruptive factors, which also contribute to biofilm detachment and bacterial dispersal. MSCRAMMs: microbial surface components recognizing adhesive matrix molecules; PIA: polysaccharide intercellular adhesin; eDNA: extracellular DNA; Aap: accumulation-associated protein; and PSMs: phenol-soluble modulins (adapted from Otto, 2013).

Biofilm development is related to several *S. aureus* diseases, such as osteomyelitis, endocarditis, cystic fibrosis, and infections associated with medical devices (e.g., catheters, prosthetic joints, heart valves) (ARCHER et al., 2011; DONLAN, 2001; REFFUVEILLE et al., 2017). *S. aureus* biofilm formation leads to the development of chronic infections due to its ability to protect bacteria against host defense mechanisms and antibiotic treatment. This phenotype leads to elevated morbidity and mortality of affected patients, being, therefore, a subject of global concern (ARCHER et al., 2011; IDREES et al., 2021; OTTO, 2018; REFFUVEILLE et al., 2017).

1.4. *Staphylococcus aureus* antibiotic resistance

1.4.1 Resistance to β-lactams

1.4.1.1 Penicillin

Penicillin was discovered in 1928 by Alexander Fleming but was first introduced to clinical use in 1941. At this time, *S. aureus* was highly susceptible to this promising antibiotic. However, the first penicillin-resistant *S. aureus* strains soon appeared (BARBER; ROZWADOWSKA-DOWZENKO, 1948; KIRBY, 1944). The resistant strains produced penicillinase (encoded by the *blaZ* gene), an enzyme able to inactivate penicillin by cleaving the β-lactam ring (KIRBY, 1944), and by late 1960 a high percentage (approximately 80%) of community or nosocomial acquired *S. aureus* infections were penicillin-resistant (CHAMBERS, 2001; LOWY, 2003).

1.4.1.2 Methicillin

In the search for new strategies to treat bacterial infections, methicillin, a semisynthetic antibiotic from the class of β-lactams, was discovered. However, shortly after the introduction of methicillin in clinical use in 1961, methicillin-resistant *S. aureus* (MRSA) strains also emerged (BARBER, 1961). The MRSA strains present resistance to nearly all beta-lactam antibiotics, including penicillins, cephalosporins, and carbapenems. The mecA gene confers this resistance in a mobile genetic element, the staphylococcal cassette chromosome SCCmecA encoding penicillin-binding-protein 2 (PBP2) (PEACOCK; PATERSON, 2015). β-lactams prevent bacteria growth by inactivating PBPs, proteins essential for modifying peptidoglycan (PGN) in the cell wall (BLUMBERG;

STROMINGER, 1974; LIMA et al., 2020; TIPPER, 1985). Since PBP2 has a low affinity to β -lactams, it can exercise the required stages for cell wall synthesis, guarantying bacterial resistance and survival when facing this class of antibiotics (HARTMAN; TOMASZ, 1984).

1.4.2 Vancomycin

Other types of antibiotics came to use as an alternative to treat staphylococcal infections. Vancomycin has been considered the golden antibiotic to treat multi-drug resistant staphylococcal infections. This antibiotic belongs to the class of glycopeptides and binds to the C-terminal D-Ala-D-Ala residue of the PGN precursor, preventing cell wall synthesis (COURVALIN, 2006; HOWDEN et al., 2010). The antibiotic pressure created by the extensive use of vancomycin to treat MRSA infections probably led to the selection of strains with reduced vancomycin susceptibility (DAUM et al., 1992; HIRAMATSUA et al., 1997). These strains, designated as vancomycin-intermediate *S. aureus* (VISA), presented mutations in genes related to the control of the cell wall biosynthesis, which resulted in thicker bacterial cell walls, requiring higher minimal inhibitory concentrations (MIC) of vancomycin (MIC ≤ 8 μg/mL) (HIRAMATSUA et al., 1997) (Hiramatsua et al., 1997).

The first report of a vancomycin-resistant *S. aureus* (VRSA) strain transpired in 2002 when a clinical US patient developed infection with an *S. aureus* strain resistant to this antibiotic (MIC > 128 μg/mL) (MCGUINNESS; MALACHOWA; DELEO, 2017; SIEVERT et al., 2002). The VRSA strains carry the *vanA* gene, one of the vancomycin genes (*vanAB*) found initially in *Enterococci* (DEPARDIEU et al., 2007; MCGUINNESS; MALACHOWA; DELEO, 2017). This gene was acquired by horizontal transfer with the transposon Tn1546 from *Enterococcus faecalis*, which forms D-Ala-D-Lac able to replace D-Ala-D-Ala in PGN synthesis, decreasing affinity to several classes of glycopeptides and promoting resistance (DEPARDIEU et al., 2007; PÉRICHON; COURVALIN, 2009). An overview of the emergence of antibiotic-resistant *S. aureus* to β-lactams and vancomycin is shown in Figure 1.22 (CHAMBERS; DELEO, 2009).

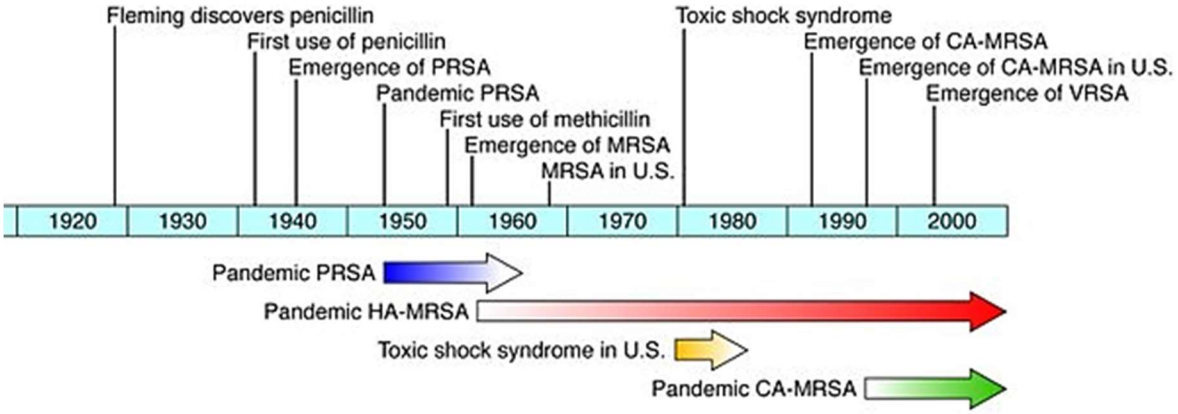

Figure 1.22. Timeline of epidemic waves and appearance of *S. aureus* antibiotic-resistant strains. Arrows show each pandemic/epidemic's approximate duration (from Chambers and DeLeo, 2009).

1.4.3 Other antibiotics

Quinolones are antibiotics that interfere with the essential DNA supercoiling required for DNA separation during replication/translation processes by inhibiting bacterial topoisomerases such as the DNA gyrase and topoisomerase IV (FÀBREGA et al., 2009). *S. aureus* strains that are quinolone-resistant present point mutations in the gene coding for essential subunits of these enzymes (e.g., GrlA and GyrA) (TANAKA et al., 2000). In addition, the expression of the efflux pump NorA is also a mechanism by which *S. aureus* has become resistant to this class of antibiotics (COSTA et al., 2019; UBUKATA; ITOH-YAMASHITA; KONNO, 1989).

1.5 *Staphylococcus aureus* prophylaxis

1.5.1 Diagnosis

Diagnosis of *S. aureus* can be achieved using a combination of several methods. Biochemical assays (catalase and coagulase activities), light microscopy (Gram staining), and isolation in selective media (mannitol salt agar) are commonly applied (FOSTER, 1996; KATEETE et al., 2010; ZURITA; MEJÍA; GUZMÁN-BLANCO, 2010). Rapid diagnosis tests such as fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR) amplification are also employed (BRAKSTAD; AASBAKK; MAELAND, 1992;

JEYASEKARAN et al., 2011; OLIVEIRA et al., 2002; ZURITA; MEJÍA; GUZMÁN-BLANCO, 2010). Antimicrobial assays are conducted in clinical samples to identify the strain susceptibility and potential treatments to fight infection (ZURITA; MEJÍA; GUZMÁN-BLANCO, 2010). Commercial test kits are also available to detect *S. aureus* and its enterotoxins in food samples (REDDY; SRIRAMA; DIRISALA, 2017).

1.5.2 Control

In healthcare facilities, it is vital to observe specific guidelines to control and avoid *S. aureus* dissemination. Firstly, screening patients for surveillance is an essential routine task that must include information about the patient's admission, demographics, and types of infection (e.g. site of the specimen, where and when infection was acquired) (COIA et al., 2006). Healthcare staff members should also be routinely tested to detect potential carries that could contribute to the dissemination of *S. aureus* in nosocomial settings. Secondly, antimicrobial sensitivity screening is critical to selecting the suitable treatment to avoid the inappropriate dosage or use of antibiotics, which could contribute to resistance acquisition via selective pressure (COIA et al., 2006). Additionally, in the case of invasive procedures such as surgeries, decolonization of nasal, throat, and skin with topical antibiotics (e.g., mupirocin) and antiseptic detergents are performed to reduce the risk of infections (COIA et al., 2006). Finally, good practices for infection control such as patient isolation, dedicated staff members, decontamination of facilities, and rigorous hygiene procedures must be followed to avoid *S. aureus* cross-contamination and dissemination (COIA et al., 2006).

1.5.3 Treatment

S. aureus infections are mainly treated with antibiotics. However, *S. aureus*'s resistance to multiple antimicrobial classes in hospitals and community makes its treatment a global concern. Therefore, efforts to find new immunotherapy approaches to tackle resistant *S. aureus* are of great interest. Nevertheless, historical data have shown that *S. aureus* genome plasticity gives it a high ability to rapidly acquire resistance to new antibiotics after their first use (BARBER, 1961; BARBER; ROZWADOWSKA-DOWZENKO, 1948; KIRBY, 1944; SRINIVASAN; DICK; PERL, 2002). Considering the high cost and little success in antibiotic development, together with rising bacterial antibiotic resistance, alternatives have been sought in the field of vaccines and therapeutic antibodies.

1.5.4 Prevention

A potential vaccine to generate protective immunity against *S. aureus* infection could be achieved by eliciting three primary immune responses, as summarized in Figure 1.23 (BAGNOLI; BERTHOLET; GRANDI, 2012). However, despite endless efforts in the past decades, simple based immunotherapy approaches seem not effective to fight *S. aureus*. This ineffectiveness could be explained by the nature of this bacterium, which can hijack the host immune response by different mechanisms, including the inhibition of complement activation, prevention of opsonization and phagocytosis, and lysis of white blood cells, among others (see section 1.3).

Attempts to achieve passive or active immunization against *S. aureus* have tested a variety of antigens mainly involved in pathogenesis, such as toxins (α-toxin, Panton-Valentine Leucocidin (PVL), enterotoxin A and B), surface elements (capsule polysaccharide (CP), wall teichoic acid (WTA)), adhesins (SdrD, IsdA), immune evasion proteins (SdrE, polysaccharide intercellular adhesin (PIA)), and survival mediators (e.g., IsdB for iron uptake) (OTTO, 2011; REDDY; SRIRAMA; DIRISALA, 2017). They will be discussed below.

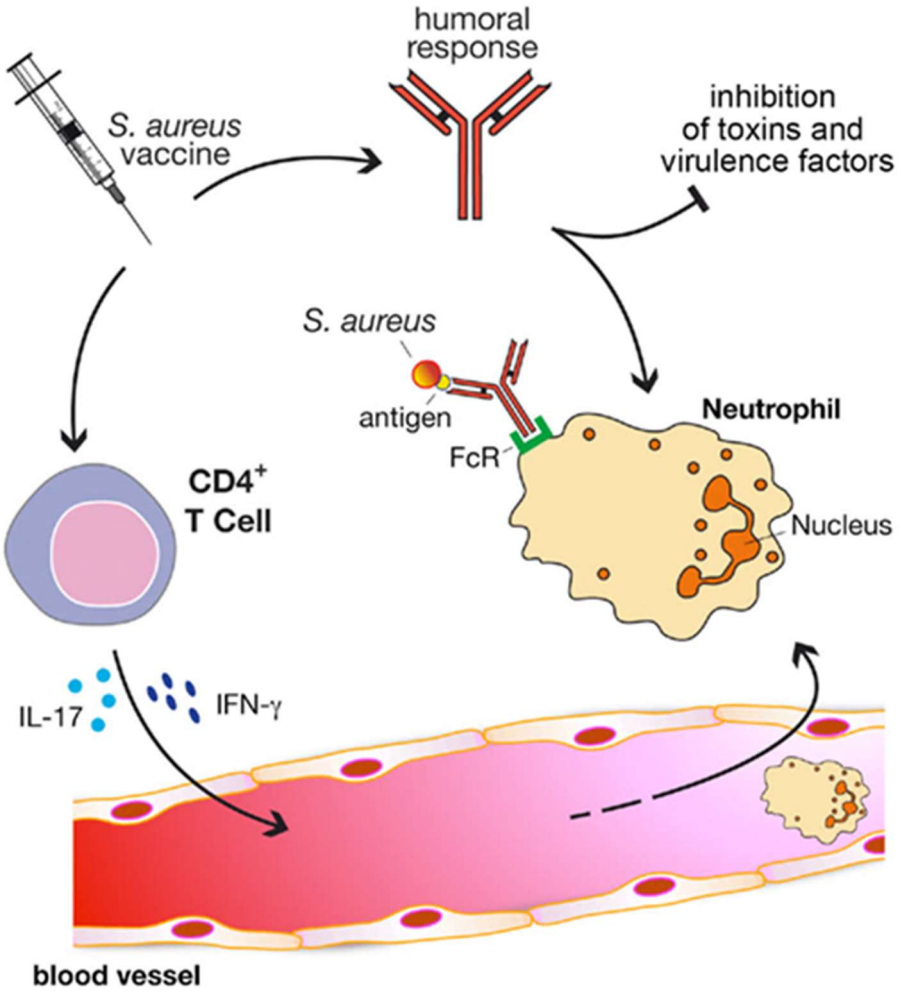

Figure 1.23. A model of an effective vaccine against *S. aureus*. Three primary immune responses elicit protective immunity: (1) antibodies to directly inhibit virulence factors; (2) antibodies from promoting opsonization; and (3) cell-mediated activation and recruitment of phagocytic cells to the site of infection (from Bagnoli et al., 2012).

1.5.4.1 Passive immunization

An emerging alternative to fight *S. aureus* infections is the use of therapeutic antibodies. Most attempts in this field envisage the neutralization of virulence determinants such as toxins (ANSARI et al., 2019; CHEUNG; OTTO, 2012). To illustrate that, numerous studies reported the role of anti-α-hemolysin antibodies in preventing or improving pneumonia and skin infections in murine models (KENNEDY et al., 2010; RAGLE; WARDENBURG, 2009; TKACZYK et al., 2012; WARDENBURG; SCHNEEWIND, 2008). However, the use of antibody therapy in humanized trials has not been decisive in

mitigating *S. aureus* infections (SPEZIALE; PIETROCOLA, 2021; SPEZIALE; RINDI; PIETROCOLA, 2018).

Since *S. aureus* possesses a large repertoire of virulence factors, often with redundant roles, antibody immunotherapy must target several elements to succeed. Furthermore, molecular variations in *S. aureus* strains may also affect the broad efficacy of such a tool. Therefore, strategies targeting virulence regulators may be promising. Indeed, inhibition of the Agr quorum sensing system may offer an alternative for suppressing several virulence factors. Contrariwise, dysfunction of the *arg* system is associated with increased biofilm formation and persistent *S. aureus* bacteremia (FOWLER et al., 2004; VUONG et al., 2000), factors that must be carefully considered if using Agr as an anti-*S. aureus* vaccine target.

1.5.4.2 Active immunization

Regarding active immunization, though continuous efforts have been made to produce an effective vaccine against *S. aureus*, there are, to date, no successful products (ANSARI et al., 2019; OTTO, 2011). Since predictive data from animal models is limited (JUNHEE SEOK et al., 2013; LÖFFLER et al., 2010), most vaccine candidates have failed in clinical trials, having only two proceeding to phase III. For instance, the vaccine Merck V710 based on the iron uptake protein IsdB presented promising results in animal models (KUKLIN et al., 2006). However resulted in increased mortality in vaccinated surgery patients (FOWLER et al., 2013; MCNEELY et al., 2014). Another vaccine, the StaphyVax, targeting type 5 and 8 capsular polysaccharides, conferred partial immunity against bacteremia in the first 40 weeks, but protection diminished over time as antibody levels decreased (SHINEFIELD et al., 2002).

Potential alternatives for developing next-generation *S. aureus* vaccines were recently proposed (CHOI et al., 2015; JIANG et al., 2019; O'BRIEN; MCLOUGHLIN, 2019; PRADOS-ROSALES et al., 2014a; WANG et al., 2018b). One is using extracellular vesicles (EVs) as a vaccine platform for immunization. The properties, roles, and possible therapeutical applications of EVs will be discussed in the next section.

Extracellular vesicles

1.6 Overview of extracellular vesicles

Extracellular vesicles (EVs) are lipid bilayered packages released by living cells in the extracellular milieu. The secretion of these spherical particles is part of an evolutionarily conserved mechanism found in individuals across Eukaryotes, Archaea, and Bacteria (DEATHERAGEA; COOKSONA, 2012; GILL; CATCHPOLE; FORTERRE, 2019). Despite being classified in several types depending on their size, origin, and formation process, all of them can carry biomolecules such as lipids, proteins, metabolites, and nucleic acids (GILL; CATCHPOLE; FORTERRE, 2019). The vesicle formation and release process has been disregarded for a long time since it was seen solely as machinery for expelling unwanted material from the cells. Nowadays, EVs are recognized as a mechanism of cell-to-cell communication (VAN NIEL et al., 2022).

It has been shown that EVs can interact with local or distant target cells to deliver messages. Their lipid bilayer structure provides a safe environment for the secretion and transport of sensitive materials, such as RNA, protecting them from external degradation (GILL; CATCHPOLE; FORTERRE, 2019; GUILLAUME; D'ANGELO; RAPOSO, 2018). Moreover, the direct delivery of their content to target cells ensures rigorous and specific distribution of information to fulfill their dedicated functions (MATHIEU et al., 2019). Indeed, it is well established that EV cargo delivery affects the physiology of recipient cells. Moreover, several studies suggested that the EV content results from active, selective packing processes, which later will guarantee the orchestration of cell communication (ANAND et al., 2019; SALAM; QUAVE, 2018; STAHL; RAPOSO, 2019). All of these properties make EVs vital players in cell functioning and physiology.

1.6.1 Eukaryotic EVs

The existence of eukaryotic EVs has been known for several decades. However, pioneer studies and techniques could still not correctly identify and differentiate vesicle classes, resulting in overlapping terms to designate these particles over the years. Today there are several known processes for vesicle formation in eukaryotes, categorized into three main groups: exosomes, microvesicles, and apoptotic bodies (Figure 1.24) (WAQAS et al., 2022).

The first is produced during endosomal maturation when multivesicular bodies (MVBs) formed as a result of intraluminal vesicle generation fuse to the plasma membrane to release the also called exosomes (30-150 nm) (HARDING; HEUSER; STAHL, 1983; JOHNSTONE et al., 1987; WAQAS et al., 2022). The second group comprises particles budding directly from the plasma membrane ranging from 50 to 1000 nm, designed as microvesicles (DANG et al., 2020; TRICARICO; CLANCY; D'SOUZA-SCHOREY, 2017; WOLF, 1967). Finally, morphological changes in cells undergoing apoptosis, such as cytoskeleton rupture and cell shrinkage, promote the generation of apoptotic bodies, larger particles ranging from 50 to 5000 nm (DANG et al., 2020; KERR; WYLLIE; CURRIE, 1972).

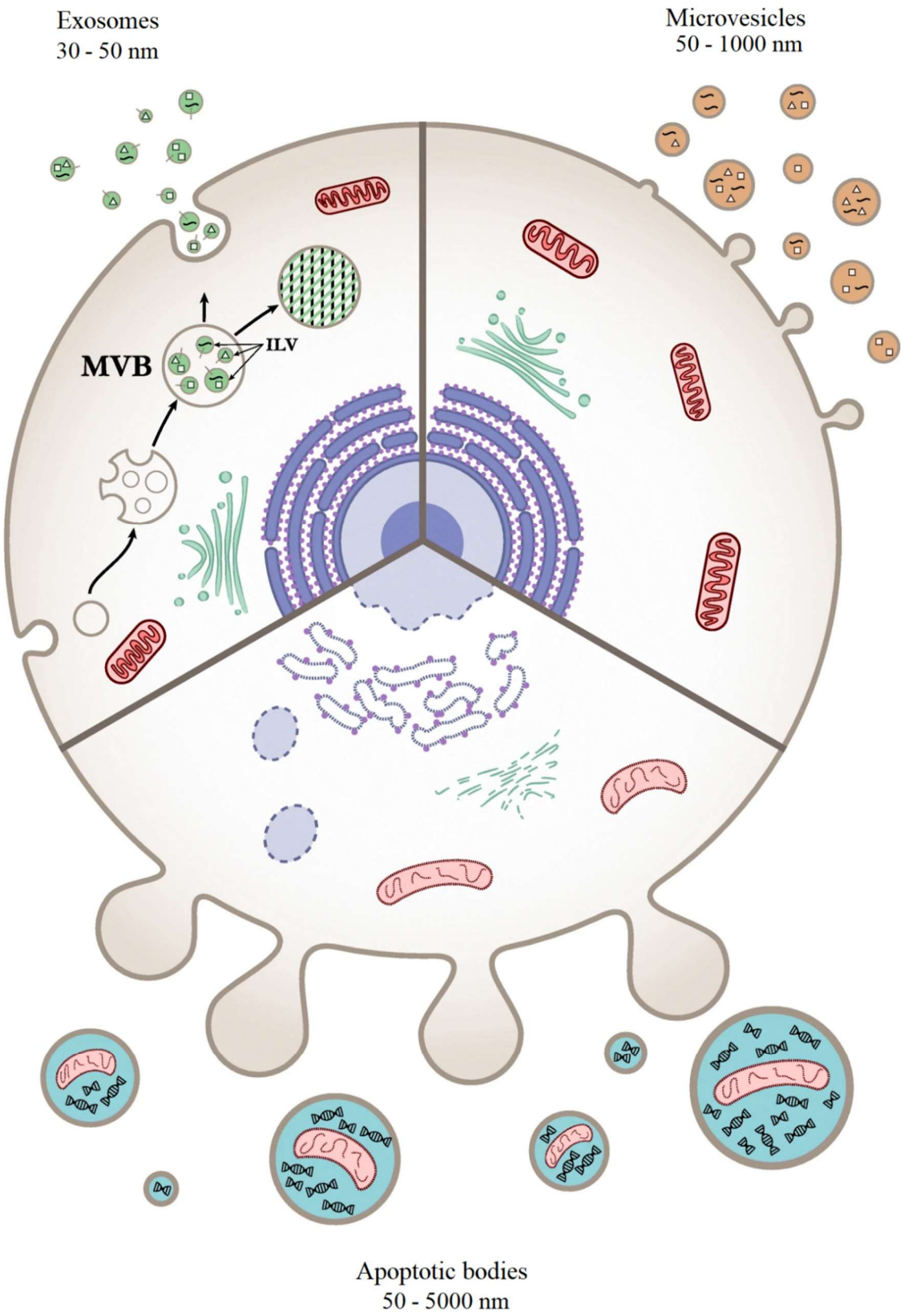

Figure 1.24. Biogenesis of vesicles in eukaryotic cells. The formation of intraluminal vesicles (ILV) during endosomal maturation generated multivesicular bodies (MVBs) that fuse to the plasma membrane to release exosomes. Microvesicles and apoptotic bodies are produced through blebbing of the membrane (adapted from Mohan et al., 2020).

1.6.2 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 (WORK; KNOX; VESK, 1966). In Gram-negative bacteria, vesiculation occurs from the budding out of the outer membrane (OM) that captures components in the periplasm. This process forms nanoparticles called outer membrane vesicles (OMVs), which are released in the extracellular milieu (Fig. 1.25) (SCHWECHHEIMER; KUEHN, 2015).

On the other hand, Gram-positive bacteria lack an outer membrane and have a thicker peptidoglycan (PGN) cell wall (Fig 1.25), which was regarded as a barrier to EV release. Although observations of EVs released by *Bacillus cereus* and *B. subtilis* date back to 1990 (DORWARD; GARON, 1990), the first complete description of Gram-positive bacterial EVs was reported much later, in 2009, when Lee and collaborators characterized EVs released by *S. aureus* (LEE et al., 2009). Ever since, other studies confirmed EV production 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 (BROWN et al., 2014; JEON et al., 2017; JIANG et al., 2014; KIM et al., 2016; LI et al., 2017; LÓPEZ et al., 2012; OLAYA-ABRIL et al., 2014; PRADOS-ROSALES et al., 2014a; RIVERA et al., 2010; RODOVALHO et al., 2020; WAGNER et al., 2018).

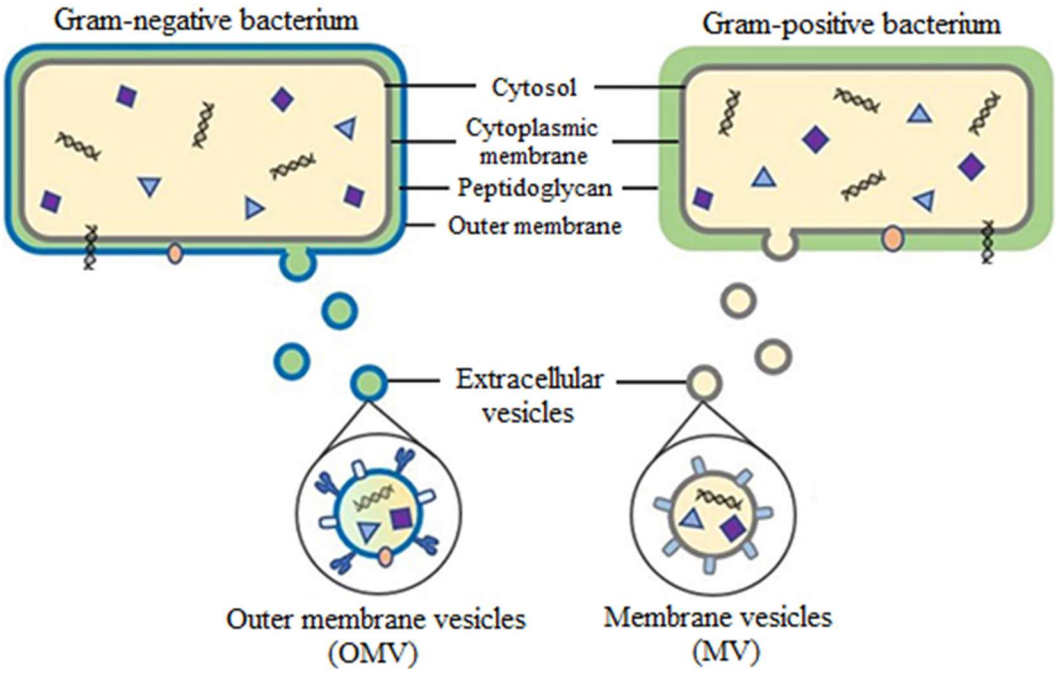

Figure 1.25. Biogenesis of bacterial extracellular vesicles (adapted from Yang et al., 2018).

Since *S. aureus* is the object of this study, in the next section, several aspects regarding the EV field and their impact on the lifestyle of this bacterium will be discussed. This section has already been published as a book chapter (LUZ et al., 2021a), and the version found in this thesis has been updated with the latest research.

1.7 EVs and their role in *Staphylococcus aureus* resistance and virulence

1.7.1 General considerations

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 (GURUNG et al., 2011; HONG et al., 2014; JEON et al., 2016; LEE et al., 2009, 2013; TARTAGLIA et al., 2018, 2020). In some cases, they trigger the production of cytokines and promote tissue inflammation (HONG et al., 2011; JUN et al., 2017; KIM et al., 2019, 2012).

As EVs are also regarded as potential vehicles for biotechnological and clinical applications, such as the development of vaccines (CHOI et al., 2015; DAVENPORT et al., 2008; WANG et al., 2018b; YUAN et al., 2018), 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 (Figure 1.26).

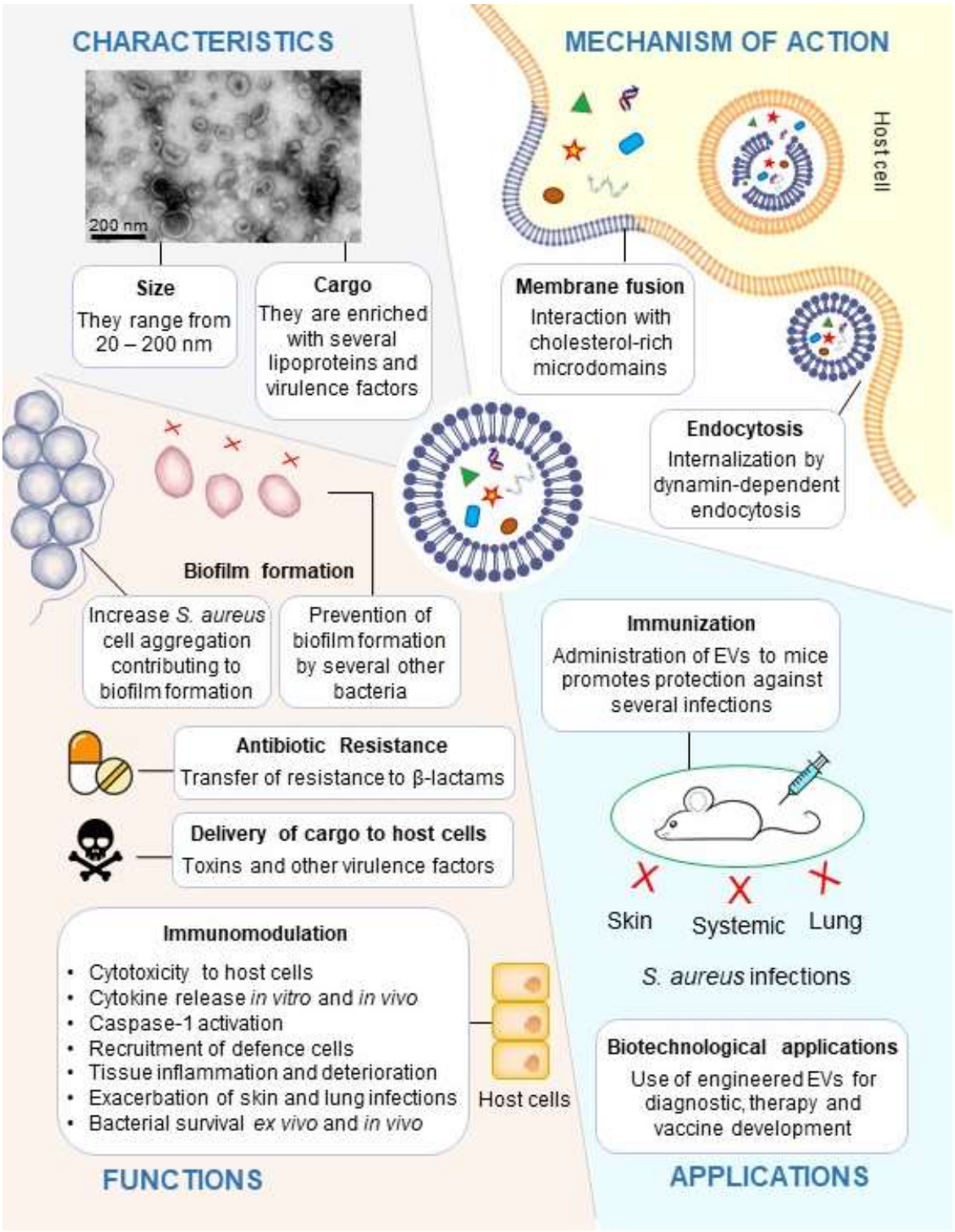

Figure 1.26. General features of *S. aureus* EVs (from Luz et al., 2021a).

1.7.2 Biogenesis

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 were proposed to explain EVs production, including one or a combination of many processes (HAURAT; ELHENAWY; FELDMAN, 2015). It has been proposed that the accumulation of molecules in the periplasm space alters turgor pressure, promoting OMV release (MCBROOM; KUEHN, 2007; ZHOU et al., 1998). In another model, lipid structure and topology alterations could lead to membrane curvature modifications, resulting in vesicle bubbling from the outer membrane (ELHENAWY et al., 2016). On the contrary, EVs biogenesis is still poorly understood in Gram-positive bacteria due to the recent discovery of EV release by these microorganisms (COELHO; CASADEVALL, 2019; LEE et al., 2009). Notably, efforts have been made to understand how EVs can get through the thick PGN layer in the Gram-positive bacteria's cell wall.

In *S. aureus*, phenol-soluble modulins (PSMs) were 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 (CHEUNG et al., 2014; LI et al., 2014b; PESCHEL; OTTO, 2013) (see section 1.3.1.3.1). The staphylococcal PSMs were reported to have cytolytic and membrane-damaging activities, be pro-inflammatory, participate in biofilm formation, and be responsible for mobilizing lipoproteins from the staphylococcal cytoplasmic membrane and the export of cytoplasmic proteins (BJÖRNSDOTTIR et al., 2017; HANZELMANN et al., 2016; LAABEI et al., 2014; PERIASAMY et al., 2012b; TSOMPANIDOU et al., 2013a).

Since *S. aureus* EVs are generally enriched for 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 the size and number of EVs recovered from the culture supernatant (WANG et al., 2018b). Similarly, *psmα* deletion in the *S. aureus* AH1263 strain also resulted in a sharp decrease in EV production (BRIAUD et al., 2021). Another study with strain USA300 revealed striking differences in EV production between the wild-type and a Δ*psmα3* mutant (SCHLATTERER et al., 2018), 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* (SCHLATTERER et al., 2018). 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 (WANG; EAGEN; LEE, 2020). Interestingly, it has been also demonstrated that a substitution mutation in sigma factor σ^B enhances EV release by *S. aureus*. This mutation reduces σ^B capacity to bind to the promoter region of *nuc* thermonuclease, resulting in its direct down-regulation. The role of *nuc* in EV release was further validated in an *S. aureus* strain deleted for this gene. However, its precise role in EV production is still unclear (LIU et al., 2018a).

EV production also varies according to strain variability. In 2020, Tartaglia *et al*. reported that EVs derived from five *S. aureus* strains from different host origins (human, bovine and ovine) presented significant differences in EV concentration and size (Tartaglia et al. 2020). Similarly, a study comparing five *S. aureus* mastitis-isolates also identified differences in EV production and particle size distribution (SAENZ-DE-JUANO et al., 2022). Finally, EVs derived from a laboratory-adapted and two clinical *S. aureus* strains also differed in morphology, production, and size particle distribution (BITTO et al., 2021a). These data reinforce that inter-strain variabilities also play an essential role in EVs' biogenesis and production.

1.7.3 Vesicle cargo composition

Different molecules may be incorporated into EVs during their biogenesis: nucleic acids, proteins, lipids, and metabolites (BROWN et al., 2015; GILL; CATCHPOLE; FORTERRE, 2019; KIM et al., 2015; WATANABE, 2016). However, most studies on *S. aureus* EV cargo composition 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 (LEE et al., 2009). 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 as penicillin-binding proteins PBP1, PBP2, and PBP3, and β-lactamase (LEE et al., 2009). 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 have characterized the EV protein content of other *S. aureus* strains, revealing from 90 to 617 identified proteins, including numerous virulence factors (Table 1.5).

Table 1.5. *S. aureus*-EVs characterization and functions.

Strain	No. of proteins	Function	Ref.
01ST93		non-cytotoxic to host cells (Hep-2)	(JEON et al., 2016)
03ST17	143	non-cytotoxic to host cells (Hep-2, HaCaT)	(JEON et al., 2016; JUN et al., 2017)
		cytotoxic to host cells (HaCaT)	(KWON et al., 2018)
		immunomodulation *in vitro* and *in vivo* (e.g., ↑ IL-1β, IL-6, IL-8, TNF-α, and MCP-1)	(JUN et al., 2017; KWON et al., 2018)
		mast cell recruitment and exacerbation of skin inflammation	
06ST1048	143	cytotoxic to host cells (Hep-2)	(GURUNG et al., 2011; JEON et al., 2016)
		delivery of Spa protein through EVs (Hep-2)	(GURUNG et al., 2011)
8325-4		induction of the MAPK pathway (THP-1 and MLE-12)	(AN et al., 2019)
		cytotoxicity to host cells (HeLa)	(THAY; WAI; OSCARSSON, 2013)
		hemolytic activity	(AN et al., 2019; THAY; WAI; OSCARSSON, 2013)
8325-4 Δ*hla*		low cytotoxic to host cells (HeLa)	(THAY; WAI; OSCARSSON, 2013)
		weaker induction of MAPK pathway (THP-1 and MLE-12)	(AN et al., 2019)
834	255	immunomodulation *in vitro*, *in vivo* and *ex vivo* (↑ IL-1β, IL-4, IL-5, IL-6, IL-17A, TNF-α, IgE) induce hypersensitivity in a mouse infection model	(ASANO et al., 2021)
AH1263		EVs from 40°C culture, but not 34°C and 37°C were cytotoxic (human and rabbit erythrocytes) EVs from 34°C, but not 37°C and 40°C were cytotoxic to THP-1 cells	(BRIAUD et al., 2021)
AH1263 Δ*psmα*		non-cytotoxic (human and rabbit erythrocytes)	(BRIAUD et al., 2021)
AH1263 Δ*hla*		non-cytotoxic (human and rabbit erythrocytes)	(BRIAUD et al., 2021)

ATCC 12600	390	protective against vaginal HIV-1 infections *in vitro* and *ex vivo*	(COSTANTINI et al., 2022)
ATCC 14458	90	ND	(LEE et al., 2009)
		cytotoxic to host cells (HaCaT) immunomodulation *in vivo* (↑ IL-1β and IL-6, ↓ TNF-α)	(HONG et al., 2014)
		immunomodulation *in vitro* and *in vivo* (e.g., ↑ IL-6, INF-γ, MIP-1α, eotaxin)	(HONG et al., 2011)
		immunomodulation *in vitro* and *in vivo* (e.g., ↑ IL-6, TNF-α, IL-12, INF-γ)	(KIM et al., 2012)
		induce skin and lung inflammation in mice	(HONG et al., 2011, 2014; KIM et al., 2012)
		protective against lung infections	(CHOI et al., 2015)
		transfer of resistance to β-lactams	(LEE et al., 2013)
ATCC 25923		Cytotoxic to host cells (HaCaT) immunomodulation *in vitro* (↑ IL-1β, IL-6, TNF-α, IL-8, and MCP-1)	(KWON et al., 2019)
		prevention of biofilm formation by other bacteria	(IM et al., 2017)
ATCC 6538		non-cytotoxic to host cells (HDMECs) induce recruitment of monocytes (THP-1) immunomodulation *in vitro* (e.g., ↑ E-selectin, ICAM1 and VCAM1, IL-6)	(KIM et al., 2019)
BWMR22		exogenous EVs from vancomycin-treated culture promote *S. aureus* aggregation	(HE et al., 2017)
BPH 2760		immunomodulation *in vitro* (↑ IL-6, IL-8, MCP-1)	(BITTO et al., 2021a)
BPH 2900		immunomodulation *in vitro* (↑ IL-8, MCP-1)	(BITTO et al., 2021a)
C-29		immunomodulation *in vitro* (↑ IL-1β, IL-6, IL-8, TNF-α, and MCP-1)	(YAMANASHI et al., 2022)
CCUG64514 CCUG64516 CCUG64520 CCUG64520		cytotoxic to host cells (THP-1) immunomodulation *in vitro* (↑ IL-8, IL-10, MCP-1, MMP-9, DDIT4, and ↓ Bcl-2)	(ZABOROWSKA et al., 2021)
CI1449		exogenous EVs confer bacterial resistance to whole blood killing	(ANDREONI et al., 2019)

JE2	180	cytotoxic to host cells (A549 lung cells, HL60 neutrophils, human leukocytes, THP-1 cells, human macrophages MΦ, rabbit erythrocytes) immunomodulation *in vitro* (↑ IL-1β, IL-18, and caspase-1 activation)	(WANG et al., 2018b, 2021b; WANG; EAGEN; LEE, 2020)
JE2 Δ*arg*, Δ*sae*, Δ*lukAB*, Δ*lukSF-PV,* Δ*hla*		decreased cytotoxicity and immunomodulation	(WANG et al., 2021b; WANG; EAGEN; LEE, 2020)
JE2-Δ*agr* Δ*spa*	212	non-cytotoxic to host cells (human leukocytes, A549, HL60, and rabbit erythrocytes) non-protective against lethal sepsis	(WANG et al., 2018b)
JE2 Δ*agr* Δ*spa* pHla$_{H35L}$-LukE		non-cytotoxic to host cells (human leukocytes, A549, HL60, and rabbit erythrocytes) protective against lethal sepsis	(WANG et al., 2018b)
JE2 Δ*lgt*	198	decreased cytotoxicity to host cells (human macrophages) defective in the induction of IL-1β, IL-18, and IL-6, and caspase-1 activation *in vitro*	(WANG; EAGEN; LEE, 2020)
LAC LAC Δ*psmα1-4* LAC Δ for all 5 leukocidins		cytotoxic to human neutrophils moderate decreased in cytotoxic against human neutrophils non-cytotoxic to human neutrophils	(WANG et al., 2021b)
M060	153	cytotoxic to host cells (Hep-2, COS-7, and HaCaT) immunomodulation *in vitro* (↑ IL-1β, IL-6, TNF-α, IL-8, and MCP-1)	(JEON et al., 2016; KWON et al., 2019) (KWON et al., 2019)
M5512VL		immunomodulation *in vitro* (↑ TNF-α)	(SAENZ-DE-JUANO et al., 2022)
MSSA476	LB[1]: 131 BHI[2]: 617	exogenous EVs promote bacterial survival *ex vivo* and *in vivo* (human whole blood and neutrophils)	(ASKARIAN et al., 2018)
MW2	168	ND	(TARTAGLIA et al., 2020)
N305*	222	non-cytotoxic to host cells (PS and MAC-T)	(TARTAGLIA et al., 2018)

		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	(TARTAGLIA et al., 2020)
NCTC 6571		immunomodulation *in vitro* (↑ IL-8, MCP-1)	(BITTO et al., 2021a)
Newman		immunomodulation *in vitro* (↑ IL-6, MIP-2, TNF-α, and IFN-β mRNA)	(KOPPARAPU et al., 2021; RODRIGUEZ; KUEHN, 2020)
O11*	164	ND	(TARTAGLIA et al., 2020)
O46*	171	ND	(TARTAGLIA et al., 2020)
RF122*	160	ND	(TARTAGLIA et al., 2020)
RN4220	92	ND	(HE et al., 2017)
RN4220 Δ*agr*	119	engineered EVs protect mice against viral infections	(HE et al., 2017)
S23	1260	ND	(WANG et al., 2021a)
ST541		transfer of resistance to ampicillin	(LEE et al., 2022)
ST692	[3]: 137 [4]: 156	transfer of resistance to β-lactams	(KIM et al., 2020)
USA300		immunomodulation *in vitro* and *in vivo* (↑ IL-8, TNF-α, IgM, total IgG, IgG1, IgG2a, and IgG2b)	(ASKARIAN et al., 2018; SCHLATTERER et al., 2018; STAUDENMAIER et al., 2022)
		in vitro induction of neutrophil recruitment and NET formation	(STAUDENMAIER et al., 2022)
		in vitro enhancement of skin colonization	
		protective against systemic and skin infections	(ASKARIAN et al., 2018)

*, animal isolates; ND, Not determined; [1], Luria-Bertani Medium [2], Brain Heart Infusion Medium; [3], optimal condition; [4], the sub-inhibitory concentration of ampicillin

Note: Production of EVs was also demonstrated for *S. aureus* strains ATCC 35556 (FRASSINETTI; FALLENI; DEL CARRATORE, 2020), ATCC 700699 (GURUNG et al., 2011), M5702 (SAENZ-DE-JUANO et al., 2022), Mastidis (SAENZ-DE-JUANO et al., 2022), NRS135 (ANDREONI et al., 2019), $NRS77_{phage}$ (ANDREONI et al., 2019), $RN4220_{phage}$ (ANDREONI et al., 2019), RN6390 (LEE et al., 2013), SH1000 (Briaud et al., 2021), TSST-1 103D (GURUNG et al., 2011), and UAMS-1 (Briaud et al., 2021), however, proteomic or functional characterization were not performed.

As shown in Table 1.5, *S. aureus* EVs comprise several proteins. The numbers of proteins vary from one study to another because of the proteomic approaches and growth conditions. Sometimes EV proteome comprises up to 24% of the whole bacterial predicted proteome. It is expected that different protein detection methods may give divergent results; indeed, some studies have evidenced such variations. Lee et al. (2009) identified 41 and 84 proteins with In-gel and In-solution digestion methods, respectively, with only 35 proteins being shared by both sets of proteins (LEE et al., 2009). In another study, Askarian et al. (2018) demonstrated that 43 and 286 proteins are exclusively identified when using either In-solution and Lipid-Based Protein Immobilization (LPI) methods, respectively (ASKARIAN et al., 2018). 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 study by Tartaglia et al. (2020) characterized and compared the proteome of EVs derived from several *S. aureus* strains using the same experimental approach. 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 (TARTAGLIA et al., 2020). 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 (TARTAGLIA et al., 2020). 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 uncovered (TARTAGLIA et al., 2020).

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 (BAGER et al., 2013; KIM; EDWARDS; FENSELAU, 2016). However, several studies showed strong evidence that protein cargo sorting is a selective regulated process in both Gram-negative and Gram-positive bacteria (BONNINGTON; KUEHN, 2014; BROWN et al., 2015; TARTAGLIA et al., 2020). 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 (MCMAHON et al., 2012). As another example, *Porphyromonas gingivalis*

OMVs also exclude proteins abundant in the OM and are enriched with several virulence factors (HAURAT et al., 2011; VEITH et al., 2014).

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 (BROWN et al., 2014). *Mycobacterium bovis* and *Mycobacterium tuberculosis* are also enriched with several lipoproteins, some of which can modulate the host response in a TLR2-dependent fashion, contributing to mycobacterial virulence (PRADOS-ROSALES et al., 2014a).

Several studies demonstrated that *S. aureus* EV cargo comprises secreted, cell wall-anchored, membrane, and cytoplasmic proteins. The latter being their most abundant component (ASKARIAN et al., 2018; LEE et al., 2009; TARTAGLIA et al., 2018, 2020). This feature is interesting since it is the unique known pathway of Gram-positive bacteria to secrete cytoplasmic proteins, which lack any export signals. Moreover, compared to whole-cell proteome, *S. aureus* EVs are also enriched with virulence factors, extracellular proteins, and lipoproteins (LEE et al., 2009; TARTAGLIA et al., 2020). 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 (LEE et al., 2009). A study by Yamanashi et al. (2022) demonstrated that enterotoxin A was selectively packed at EVs compared to *S. aureus* C-29 producing cells (Yamanashi et al., 2022). Furthermore, secreted virulence factors such as coagulases, β-lactamase, and hemolysins were also enriched (Lee et al., 2009). Finally, comparative proteomics revealed that lipoproteins of five *S. aureus* clinical and animal isolates accounted for approximately 20% of the EV content, corresponding to only 2.5% of the predicted proteome (TARTAGLIA et al., 2020). These data show that some protein populations are enriched in *S. aureus* EVs, and they reinforce the hypothesis that the selection of protein cargo is a mechanism common in *S. aureus*. 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 (TARTAGLIA et al., 2020).

As mentioned earlier, data regarding the characterization of other staphylococcal EVs components 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. (2018) 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 (SCHLATTERER et al., 2018). In another study, the Fourier Transform InfraRed spectroscopy (FTIR) approach showed that administration of the antibiotic vancomycin induced chemical changes in *S. aureus* EVs, including the reduction of carbohydrate yield in comparison to untreated cells (HE et al., 2017).

Regarding nucleic acids, Bitto et al. (2021) used DNA stain SYTO-61 and Agilent Bioanalyser to demonstrate that the *S. aureus* strains 6571, 2760, and 2900 carry DNA and RNA (BITTO et al., 2021a). Rodriguez and Kuehn (2020) 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 (RODRIGUEZ; KUEHN, 2020). In a study by Andreoni et al. (2019), quantification with the PicoGreen dsDNA kit also revealed the association of DNA molecules to *S. aureus* EVs (ANDREONI et al., 2019). Finally, recent transcriptomic analyses provided a partial characterization of the *S. aureus* EV RNA content, focusing mainly on sRNAs (JOSHI et al., 2021a) and the variation of the EV RNA cargo according to different growth temperatures (BRIAUD et al., 2021). However, further investigations are necessary better to characterize the nucleic acid content of *S. aureus* EVs.

Regarding strain-dependent EV composition variability, a study by Bitto et al. demonstrated that, while DNA and peptidoglycan (PGN) EV quantities were similar between a laboratory-adapted and two clinical *S. aureus* strains, significant differences were observed in the amount of RNA and protein detected (BITTO et al., 2021a). Indeed, SDS-PAGE revealed a different protein profile between EVs, and Agilent analyses confirmed variation in RNA cargo, especially of potential sRNAs (BITTO et al., 2021a). Moreover, differences detected by Western blot using peptidoglycan antibodies suggest that variations in cross-bridging, tertiary structure, and acetylation may also exist in inter-strain EVs (BITTO et al., 2021a). Regarding inter-species content cargo, analysis has shown that *S. aureus* EVs present significantly higher amounts of DNA than *Helicobacter pylori* and *Pseudomonas aeruginosa*, while the RNA content was significantly higher only when compared to *H. pylori* (BITTO et al., 2021b).

1.7.4 EVs functions

First considered “trash bags” to remove unwanted molecules from cells, nowadays, it is well-established that EVs play essential roles in 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) 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 1.5).

Studies demonstrated that *S. aureus* EVs could be cytotoxic and 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 (HONG et al., 2014). 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 mutants lacking several pore-forming toxins (PFTs) (WANG; EAGEN; LEE, 2020). In another study Thay et al. (2013) showed that *S. aureus* EVs contributed to HeLa cell cytotoxicity and erythrocyte lysis in a dose-dependent manner (THAY; WAI; OSCARSSON, 2013). 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 (THAY; WAI; OSCARSSON, 2013).

In vivo experiments conducted by Hong et al. (2014) 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 (HONG et al., 2014). 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 (HONG et al., 2014). In another study, analysis has shown that EVs derived from the *S. aureus* strain AH1263 grown at 40°C but not at 34°C and 37°C promoted human and rabbit erythrocytes cell lysis, probably due to the high levels of PSMα and Hla toxins found in EVs recovered at 40°C (BRIAUD et al., 2021). Together, these findings highlight the critical role of EVs in host cell death during staphylococcal infections.

Besides delivering toxins to host cells, *S. aureus* EVs were shown to play an important role in antibiotic resistance. Lee et al. (2013) demonstrated that biologically active BlaZ, a β-lactamase protein, is present inside *S. aureus* EVs. 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* (LEE et al., 2013). In a more recent report by Kim et al. 2020, 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 strains against six different β-lactam antibiotics in a dose-dependent manner (KIM et al., 2020). Remarkably, not only *S. aureus* EVs can transiently protect susceptible bacteria against antibiotic treatment, it has been demonstrated that *E. coli* strain RC85 exposed to MRSA ST541-derived EVs acquired long-term antibiotic resistance (LEE et al., 2022). The treated *E. coli* strain RC85-T presented higher MIC levels against several β-lactam antibiotics. Moreover, EVs derived from RC85, and RC85-T strains presented differences in particle size and protein composition, being β-lactamase activity 12-fold higher in RC85-T EVs. Accordingly, only RC85-T EVs were able to protect susceptible *E. coli* against ampicillin (LEE et al., 2022).

Regarding biofilm formation, it has been shown that adding exogenous EVs purified from the culture supernatant of strain BWMR22 grown in the presence of a sub-inhibitory concentration of vancomycin increased *S. aureus* adhesion and cell aggregation, contributing to biofilm formation (HE et al., 2017). In another study, applying *S. aureus* EVs to polystyrene surfaces reduced biofilm formation by several other pathogenic bacteria, including *Acinetobacter baumannii*, *Enterococcus faecium*, and *Klebsiella pneumonia* (IM et al., 2017). 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 (IM et al., 2017). This conversion of surface properties confers a vital competitive advantage that could explain the prevalence of *S. aureus* as a nosocomial pathogen. Conversely, studies have already demonstrated that *P. aeruginosa*-derived OMVs inhibit *S. aureus* growth and biofilm formation (KADURUGAMUWA; BEVERIDGE, 1996; ZHAO et al., 2022).

Various studies also demonstrated the role of *S. aureus* EVs on immunomodulation or their contribution to the induction of several infections. Detection of *S. aureus* EVs in house dust led Kim et al. (2012) 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 (KIM et al., 2012). In a study with mouse cells *in vitro*, EVs derived from the *S. aureus* 834 methicillin-resistant strain stimulated a pro-inflammatory response via TLRs 2, 4, and 9, resulting in increased expression of IL-6, TNF-a, IFN-γ, and IL-1β (ASANO et al., 2021). Zaborowska et al. (2021) have shown that EVs derived from *S. aureus* and *S. epidermidis*, the main causing agents of osteomyelitis, could activate NF-κB and promote the expression of pro- and anti-inflammatory genes such as IL-8, IL-10, and MCP-1. Interestingly, TLR3 was activated in response to EVs derived from both staphylococcal species, while TLR2 and TLR4 remained unchanged. Moreover, *S.*

aureus EVs presented significantly higher concentration yields and stronger cytolytic activity when compared to *S. epidermidis* EVs (ZABOROWSKA et al., 2021).

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 (HONG et al., 2011, 2014). In addition, *S. aureus* EVs were also shown to exacerbate inflammation in an AD mouse model (JUN et al., 2017). Topical application of *S. aureus* EVs resulted in severe eczematous dermatitis, skin thickening, and a massive infiltration by inflammatory and mast cells. These symptoms were not observed when animals were treated with lysed EVs (JUN et al., 2017). In another study, *in vitro* analyses 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 (KIM et al., 2019).

Staudenmaier et al. (2022) demonstrated that USA300 EVs increased keratinocyte IL-8 and TNF-a release, similar to cytokine levels induced by live bacteria. Moreover, USA300 EVs also induced neutrophil recruitment and NET formation, which was closely related to the Spa protein packed into EVs (STAUDENMAIER et al., 2022). Indeed, a *spa* mutant could not induce IL-8 expression while still inducing TNF-a. Moreover, EVs from the USA300 *spa* mutant presented a reduced binding ability to keratinocytes compared to wild-type strains (STAUDENMAIER et al., 2022). Interestingly, EVs derived from commensal *Staphylococcus* strains (*S. epidermidis*, *S. lugdunensis,* and *S. hominis*) could protect against *S. aureus* adherence to keratinocytes, which was not true for EVs released by *S. aureus* itself (STAUDENMAIER et al., 2022). In another report, EVs derived from the three *S. aureus* strains (2760, 2900, and 6571) induced the expression of IL-8 and MCP-1 in A549 cells, as well as live bacteria (BITTO et al., 2021a). Interestingly, although live bacteria from the three strains induced IL-6 production, only EVs derived from the 2760 strain did so. Finally, EVs from all three strains were shown to induce autophagosome formation in A549 cells and to be degraded by the autophagy route (BITTO et al., 2021a).

Studies by Wang et al. demonstrated that EVs derived from the *S. aureus* JE2 strain are cytotoxic against A549, rabbit erythrocytes, and HL60 neutrophils. They can activate TLR2 signaling of NLRP3 inflammasomes in human macrophages through K+ efflux and apoptosis-associated speck-like protein (ASC) recruitment (WANG et al., 2021b; WANG;

EAGEN; LEE, 2020). Interestingly, EVs from Δ*arg*Δ*saeRS* presented decreased cytotoxicity, absence of caspase-1 activation, and a drop in IL-1β and IL-18 levels released by human macrophages (WANG et al., 2021b; WANG; EAGEN; LEE, 2020). Similarly, a mutation in a gene involved in lipidation and maturation of lipoproteins (Δ*lgt*) also reduced their ability to induce caspase-1 activation and cytokine release (WANG; EAGEN; LEE, 2020). Finally, they also evaluated the effect of cytolytic elements such as leukocidins and PSMs in EVs. They showed that a LAC mutant strain for Δ*psmα*1-4 presented a modest decrease in cytotoxicity, while the LAC strain lacking all five leukocidins exhibited a minor cytotoxic effect, showing that this last class of pore-forming toxins (PFT) are mainly responsible for host cell lysis induced by *S. aureus* EVs (WANG et al., 2021b).

Rodriguez and Kueh 2020 demonstrated that nucleic acids associated with *S. aureus* EVs are immunomodulatory. They identified DNA and RNA populations associated with EVs derived from the Newman strain and provided evidence that these nucleic acids are delivered into host endosomal compartments (RODRIGUEZ; KUEHN, 2020). *In vitro* experiments showed that murine macrophages exposed to EVs presented a strong IFN-β mRNA expression after 3 hours of stimulation. 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 (RODRIGUEZ; KUEHN, 2020). These results were corroborated when the exposition of TLR3−/−, TLR7−/−, and TLR9−/− mouse macrophages to EVs reflected a substantial decrease in IFN-ß mRNA expression (RODRIGUEZ; KUEHN, 2020).

Another study with *S. aureus* Newman strain demonstrated that its derived EVs stimulate the production of the pro-inflammatory components IL-6, MIP-2, and TNF-α at both mice macrophages and splenocytes, being the first two TLR2-dependent (KOPPARAPU et al., 2021). Interestingly, the Newman EVs' pro-inflammatory activity towards mice cells was attributed to lipoproteins since EVs derived from a Δ*lgt* mutant induced significantly lower levels of IL-6 and MIP-2 (KOPPARAPU et al., 2021). Additionally, they showed that mice knee joints injected with EVs derived from Δ*lgt* mutant strain presented less pronounced arthritis when compared to mice injected with wild-type EVs, reinforcing the role of lipoproteins in the pro-inflammatory effect exerted by *S. aureus* EVs *in vivo* (KOPPARAPU et al., 2021).

As described above, most studies regarding *S. aureus* EVs have focused mainly on clinical human isolates, and to date, there are only two reports describing the biological functions of EVs derived from an *S. aureus* animal strain. Tartaglia et al. (2018) 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. 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 (TARTAGLIA et al., 2018). Another report also evaluated the effect of EVs derived from the *S. aureus* M5512VL mastitis isolate on primary bovine mammary epithelial cells (pbMECs). While heat-killed bacteria increased the production of IL-6, TLR2, and TNF-α, EVs induced only TNF-α after 24 h stimulation (SAENZ-DE-JUANO et al., 2022).

Altogether, these data indicate that *S. aureus* EVs can interact with and modulate host cell immune response, suggesting that EVs can play an essential role in staphylococcal pathogenesis. However, a recent study with EVs derived from Gram-negative and Gram-positive bacteria demonstrated that different protein assays significantly vary the given EV protein content. More importantly, stimulation of host cells with equivalent protein quantities assessed by these different methods resulted in divergent experimental outcomes, such as IL-8 responses (BITTO et al., 2021b). These results highlight the importance of EV purification and quantification methods in studying their biological and immunological functions. Therefore, these critical parameters must be carefully considered to standardize the EVs study field.

1.7.5 EVs delivery to host cells

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 efficiently delivering components in a concentrated manner. Gurung et al. (2011) 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.

Other studies confirmed this EVs role. For instance, disrupted EVs produced by *S. aureus* ATCC 25923 strain was four times less cytotoxic than intact EVs (KWON et al., 2019). Again, whole and lysed EVs derived from strain 03ST17 were cytotoxic and pro-inflammatory; however, these properties were more intense when EVs were intact (JUN et al., 2017; KWON et al., 2019). Nevertheless, in some cases, EV integrity does not influence their cytotoxic properties, as is the case of *S. aureus* M060 EVs, which in both intact and disrupted states present same cytotoxicity levels towards HaCaT cells (KWON et al., 2019). 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.

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 (CARUANA; WALPER, 2020). 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, lipid-raft-, caveolin- or clathrin-mediated endocytosis (MULCAHY; PINK; CARTER, 2014).

Studies showed that *S. aureus* EVs could interact with host cells via a cholesterol-rich membrane microdomain. The cholesterol-sequestering agent Filipin III prevents EV membrane fusion and cargo delivery into host cells (GURUNG et al., 2011; THAY; WAI; OSCARSSON, 2013). 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 (WANG; EAGEN; LEE, 2020). This finding is supported by a recent report by Rodriguez and Kueh (2020), where macrophages exposed to *S. aureus* Newman EVs had a substantial decrease in IFN-β mRNA expression when cells were also pretreated with dynasore. They also provided visual evidence through molecule labeling and confocal microscopy that EV-associated RNAs are efficiently delivered into macrophages (RODRIGUEZ; KUEHN, 2020). Both membrane fusion and endocytosis depend on the integrity of EVs and may explain why intact EVs usually present higher cytotoxicity since they allow direct delivery of concentrated components into host cells, enhancing cell damage and immunomodulation. Although these 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.

1.7.6 EVs environmental modulation

Besides intrinsic bacterial factors, several external factors were also shown to modify EV production. Differences in growth phases and temperatures were reported to affect *S. aureus* EV size, concentration, and environmental stresses (BRIAUD et al., 2021; WANG et al., 2021b). For instance, Briaud et al. (2021) observed that *S. aureus* strain AH1263 released bigger-sized EVs at 34°C and 40°C compared to 37°C conditions (BRIAUD et al., 2021). In addition, more AH1263 EVs are recovered at 15 h cultures compared to 6 h and 3 h growth phases (BRIAUD et al., 2021). In another study by Wang et al. (2021), the analysis demonstrated that iron-depletion, ethanol, and oxidative stress conditions enhanced *S. aureus* JE2 EV production, while EV release was reduced in response to osmotic stress (WANG et al., 2021b).

Regarding antibiotics, it has been shown that *S. aureus* exposure to 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 (WANG et al., 2018b) and 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 (ANDREONI et al., 2019). Again, the addition of the β-lactam ampicillin increased *S. aureus* EV production in a dose-dependent manner, corresponding to a 22.4-fold increase at 64 μg/mL concentration (KIM et al., 2020). 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 (VOLLMER; BLANOT; DE PEDRO, 2008). β-lactams 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 a looser PGN matrix structure, allowing vesicles to cross the cell wall with less resistance, generating particles in higher numbers and sizes. The correlation between vesicle release and PGN cross-linking has also been reported for Gram-negative bacteria (DEATHERAGE et al., 2009; SCHWECHHEIMER; KUEHN, 2015).

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 (ASKARIAN et al., 2018). 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 (ASKARIAN et al., 2018). Regarding the effect of antibiotics, proteomics identified 156, and 137 proteins in EVs derived *S. aureus* ST692 strain cultured in the presence and absence of a sub-inhibitory concentration of ampicillin, respectively, while only 67 proteins were shared by both conditions (KIM et al., 2020). In another study, proteomic analysis of EVs derived from MRSA S23 strain with and without sublethal concentrations of the β-lactam imipenem detected a total of 1260 proteins, from which 861 were differentially expressed between the two conditions (WANG et al., 2021a). 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 protein ratio relative to carbohydrates (HE et al., 2017).

Previous reports have demonstrated that PSMα production increases with temperature (Bastock et al., 2021), and its association *S. aureus* EV release (Schlatterer et al., 2018). In a study by Wang et al. (2021), significant higher amounts of JE2 EV protein yields were found at 30°C when compared to bacteria grown at 37°C or 40°C (Wang et al., 2021b). Conversely, a study by Briaud et al. (2021) found that protein and lipid concentrations of EVs derived from *S. aureus* AH1263 strain increases following the rise in growth temperatures of the tested conditions 34°C, 37°C and 40°C (Briaud et al., 2021). The observed effect of temperature in *S. aureus* EVs was also true for Newman, MW2, and SH1000 strains, but not UAMS-1, which is a low PSMα producer (Zielinska et al., 2011; Briaud et al., 2021).

Further analysis by Briaud et al. (2021) also provided a deep characterization of the impact of growth temperatures on the protein and RNA EV content of the *S. aureus* AH1263 strain. Proteomic analyses detected 923 proteins at 34°C, 899 at 37°C, and 645 at 40°C, 598 of which were common to all conditions (BRIAUD et al., 2021). Interestingly, although protein diversity decreased in EVs recovered from higher temperatures, they observed that virulence factors were more abundant at 40°C, including several toxins (BRIAUD et al., 2021). Regarding EV RNAs, no differences were observed in RNA amounts in the lumen of EVs from the three conditions, while extracellular RNA was higher in EVs derived from 34°C cultures when compared to 37°C and 40°C (BRIAUD et al., 2021). Finally, the

transcriptomic analysis revealed that AH1263 EVs carry rRNAs, mRNAs, sRNAs, and tRNAs, and significative differences in RNA abundance are detected in EVs derived from 34°C and 37°C conditions (BRIAUD et al., 2021). This study highlights how minor temperature variations affect *S. aureus* EV content.

Finally, EV content can also be impacted by a combination of intrinsic and external factors. For instance, Andreoni et al. (2019) 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. 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 elucidate better EV biogenesis and cargo selection in *S. aureus* and other bacteria.

1.7.7 EVs strain specificity

Cytotoxicity and immunomodulation of EVs towards host cells vary according to the *S. aureus* strain, growth conditions, and host cell line studied since both can have specific characteristics. As virulence factors vary from an *S. aureus* strain to another or according to the environment, so does the cargo of EVs. This variation affects cytotoxicity and host cell response to EVs contact. It was demonstrated that the presence of α-hemolysin in EVs is directly related to hosting cell death, and EVs from α-hemolysin-negative strains have very low or no cytotoxic effect on different cell types (HONG et al., 2014; THAY; WAI; OSCARSSON, 2013). Similarly, EVs from the 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 (JEON et al., 2016). It was also demonstrated that EVs from *S. aureus* ATCC 25923 induce a stronger immune response in HaCaT cells than M060 EVs at the same concentrations (KWON et al., 2019). Finally, a recent study evaluated the cytolytic effect of EVs derived from the *S. aureus* strain AH1263 grown under different growth temperatures. They found that EVs derived from 40°C cultures but not from 34°C and 37°C promoted human and rabbit erythrocytes cell lysis (BRIAUD et al., 2021). In contrast, 34°C EVs were cytotoxic against THP-1 cells, unlike EVs derived from 37°C and 40°C conditions (BRIAUD et al., 2021). These differences can be attributed to variations observed in the EV cargo across the tested growth temperatures (BRIAUD et

al., 2021). These data show how strain variability and growth conditions affect EVs' functions towards host cells.

Distinct cell lines used *in vitro* may have dissimilar responses that reflect specific host cells-EVs interactions, resulting in variable cytotoxicity and immunomodulation levels. For instance, 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 (ASKARIAN et al., 2018). In another study, *S. aureus* JE2 EVs were shown to be less cytotoxic to airway epithelial cells (A549) than to erythrocytes and neutrophil-like HL60 cells (WANG et al., 2018b). In another example, exposure to ATCC 14458 *S. aureus* EVs, alveolar macrophages produced TNF-α and IL-6, while A549 cells produced only IL-6 (KIM et al., 2012). Together, these findings show that EVs' role in host cell toxicity and immune response is strongly affected by the variations strain-dependent EV cargo, and to disparities in molecular and physiological characteristics of the host cell types.

1.7.8 Applications of *S. aureus*-EVs

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, making them 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 (GERRITZEN et al., 2017; JIANG et al., 2019; PENG; YIN; WANG, 2020). 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 (KIM et al., 2013). 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 to elicit a cross-protection against different strains (CHOI et al., 2017).

Several studies have already reported the use of EVs for immunization against *S. aureus* infections, revealing its potential in vaccine design. For instance, Irene et al. used engineered 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% to 20% of the total protein content (IRENE et al., 2019). The

engineered OMVs conferred significant protection against mouse sepsis and kidney and skin *S. aureus* infections (IRENE et al., 2019). 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 (ASKARIAN et al., 2018).

Another report demonstrated that the 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 pro-inflammatory mediators such as TNF-α, IL-6, and IL-12, suggesting the induction of adaptive immunity (CHOI et al., 2015). As expected, intramuscular administration with three doses of > 5 μg of ATCC 14458 EVs resulted in 100% protection against challenges with a lethal dose of bacteria in a mouse pneumonia model, with a reduction of bacterial colonization, pneumonia, and production of cytokines (CHOI et al., 2015). 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 (CHOI et al., 2015).

Another example of *S. aureus* EVs' application as a vaccine was shown by Wang et al. (2018) (Fig 1.27). EVs were purified from the JE2 Δa*gr*Δ*spa* strain containing a plasmid coding for non-toxic Hla and LukE toxins under the control of the *spa* promoter, whose activity is enhanced in the absence of the *arg* quorum sensing system. They demonstrated that recombinant non-toxic Hla and LukE are immunogenic, and engineered EVs carrying these detoxified cytolysins protected mice against lethal sepsis infection (WANG et al., 2018b). However, despite all the examples of EVs' protective effect against staphylococcal infections, *S. aureus* EVs may also induce hypersensitivity instead of protection. Asano et al. (2021) demonstrated that mice stimulated with *S. aureus* 834-derived EVs died on day 1 post-infection challenge with *S. aureus*-MRSA, while untreated mice died gradually between days 3 and 8 post-infection (ASANO et al., 2021).

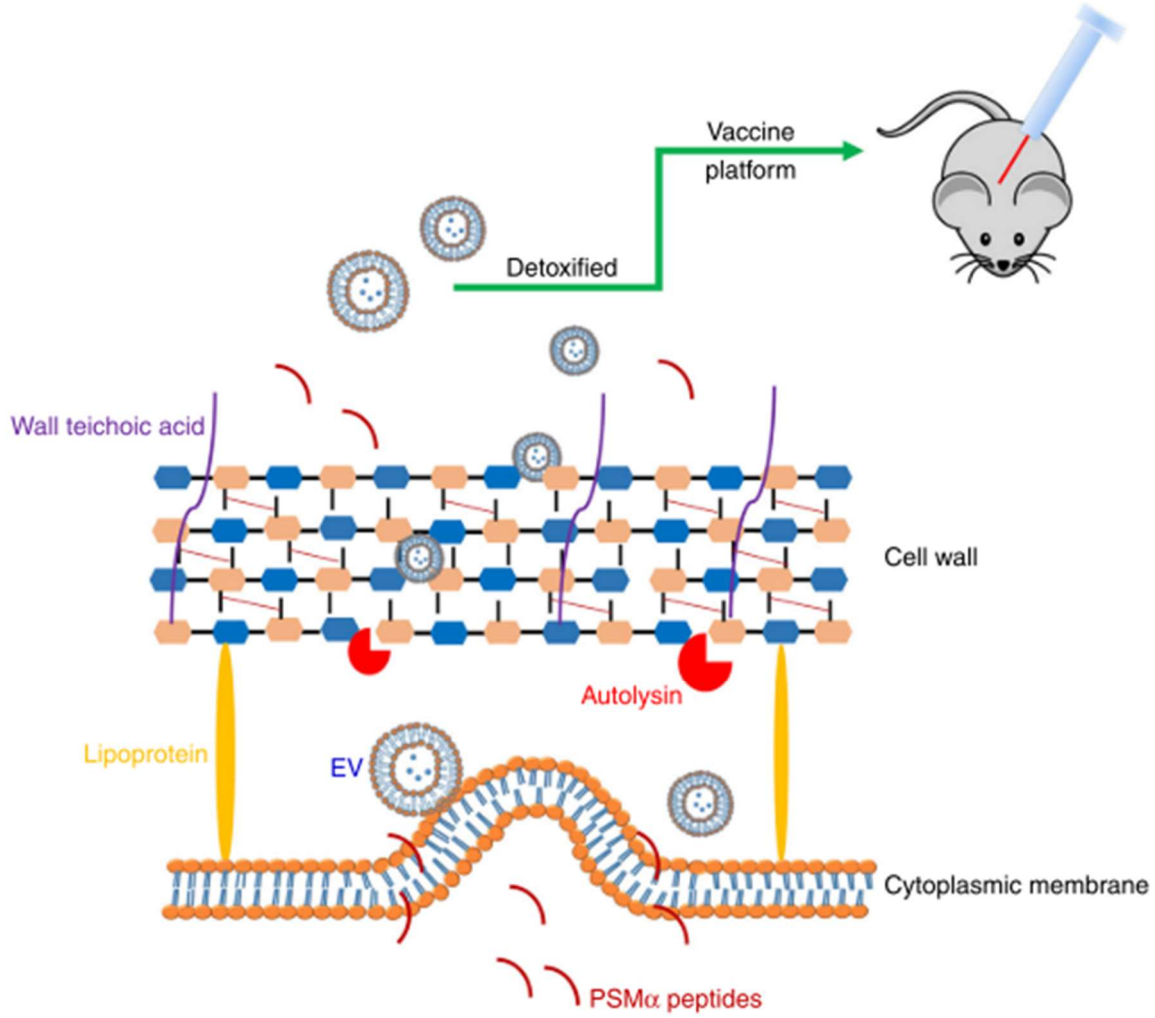

Figure 1.27. *Staphylococcus aureus* PSMα peptides promote membrane disruption and EVs release. Autolysins hydrolyze peptidoglycan, particularly at cell division sites, facilitating EV liberation. Engineered EVs with reduced virulence factor loads open the possibility of host immunization with detoxified particles, presenting protective efficacy in *S. aureus* models of infection (from Wang et al., 2018).

Regarding the use of *S. aureus* EVs to fight other infections, Yuan et al. (2018) 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. Major components of *S. aureus* EVs were fused to tag sequences that incorporated viral antigens, generating PdhB-FLAG and Eno-FLAG proteins associated with envelope E domain III, the primary protective domain for the prevention of dengue virus (DENV) (YUAN et al., 2018). These heterologous viral antigens were successfully integrated into EVs, which induced antibodies against four DENV serotypes and protected mice against lethal challenges with DENV-2 (YUAN et al., 2018). Finally, it was recently demonstrated

that *S. aureus*-derived EVs, but not EV-free supernatants, could protect against HIV-1 infection *in vitro* and *ex vivo* in a dose-dependent manner (COSTANTINI et al., 2022). This protection was attributed to the inhibition of HIV-1 cell receptor interactions by protein components exposed on the EVs surface since EV proteinase K treatment abolished protection (COSTANTINI et al., 2022).

1.7.9 Conclusion

As addressed here, the study of *S. aureus* EVs is an exciting area of growing interest due to this bacterium's clinical and veterinary importance. EVs transport various biomolecules, including lipids, metabolites, proteins, and nucleic acids, and have reportedly been associated with bacterial survival and host-pathogen interactions. Despite the recent discovery of EVs released by Gram-positive bacteria, *S. aureus* is, to date, one of the best-documented bacteria in this field. Nevertheless, several research questions remain open to elucidate these particles' roles better.

First, EVs biogenesis is still poorly understood in Gram-positive bacteria even though recent studies showed *S. aureus*-EVs biogenesis could be affected by a range of intrinsic and external factors, such as PSMs, autolysins, and environmental conditions, such as antibiotics. These factors result in different EV production yields, sizes, compositions, and functions. Although EV content reflects mainly the physiological state of the producing cells, several studies evidenced the relative enrichment of some components related to bacterial survival and virulence inside *S. aureus* EVs, suggesting that selective cargo sorting exists. Since the EV cargo determines their biological functions, clarifying which components are selected and how is crucial to understanding their role in pathogenesis and using them as a platform for the development of molecular delivery systems.

Second, most studies on *S. aureus* EVs have focused on proteomes, while other components have been disregarded, mainly DNA and RNA. As well as proteins, nucleic acid cargo could play essential roles in *S. aureus* survival and pathogenesis. They could be associated with horizontal gene transfer for antibiotic resistance and regulation of host gene expression by sRNAs, as reported in other bacteria. A recent study evidenced the presence of nucleic acid cargo in *S. aureus* EVs and their immunomodulatory roles towards host cells. However, these elements were not extensively characterized, and more research is necessary to elucidate their composition and functions.

Third, the physiological role of *S. aureus* EVs remains elusive. Staphylococcal EV cargo includes several virulence factors, which were shown to induce skin and pulmonary inflammations. Also, several studies demonstrated that, in most cases, delivering intact EVs is crucial to cytotoxicity and host cell response, reinforcing that these membranous particles have a crucial role in protecting bioactive compounds for proper delivery. Although several functions of *S. aureus* EVs have been reported and demonstrated *in vitro*, *ex vivo*, and *in vivo*, and in various infection contexts, 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, however, this phenomenon is still unknown.

Finally, their ability to induce a host immune response has raised 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. More importantly, engineered EVs carrying non-toxic virulence factors were shown to be immunogenic and non-cytotoxicity, making them safe vehicles for immunization. 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.

Chapter 2. Context and aim of the Ph.D. project

2.1 Context and research question

As presented in the literature review, *S. aureus* is a successful opportunistic pathogen able to survive in several niches and infect various hosts causing a wide range of diseases. This bacterium uses many strategies to hijack the host defense, notably by expressing a redundant repertoire of virulence factors that help bacterial cells evade the immune response. Interestingly, recent studies have shown the role of *S. aureus* EVs on bacterial physiology and pathogenesis. These EVs were shown to carry essential elements involved in bacterial persistence and survival, including adhesins, toxins, and β-lactamases. More interestingly, data suggest that virulence factors are enriched in *S. aureus* EVs, as observed for several other pathogens.

To date, *S. aureus* EVs were primarily characterized by proteomic approaches, leaving a significant gap in the knowledge of other EV components, such as nucleic acids. Recently, the study of RNAs, including small regulatory RNAs (sRNAs), have gained attention due to their intra and interspecies gene-regulatory and immunomodulatory roles. The characterization of the EV RNA content has been reported in some bacteria. However, data about RNA cargo of *S. aureus* EVs is still lacking. Moreover, most reports regarding staphylococcal EVs functions have focused on clinical strains, as the knowledge about EVs derived from *S. aureus* animal isolates have been neglected. Finally, considering bacterial EVs seem to present enriched cargo, recent studies rarely compare their effects with live bacteria. Therefore, it is still unclear if released EVs exert the same functions as bacteria in the context of infection.

Here, we hypothesized that *S. aureus*-derived EVs carry not only proteins, but also RNAs (including sRNAs), that may be selectively packed to exert specific functions. In this context, we postulate that *S. aureus* EVs, including those from animal strains, can modulate the host immune response differently from that of live bacteria.

2.2 Objectives and strategy

This work aims the study of *S. aureus*' EVs, including their biophysical and content characterization (protein and RNA), and further investigation of the molecular mechanisms involved in EVs-bacteria and EVs-host interactions (mainly of animal models), including host cellular and immune responses. For this, an interdisciplinary approach was used, including nanoparticle characterization techniques, functional assays, and omic approaches such as transcriptomics and proteomics to evaluate the features and biological roles of *S. aureus*-derived EVs.

Two *S. aureus* strains were used in this study. The first is the laboratory *S. aureus* model strain HG003, which is widely used in studies of antibiotic resistance. This work provides the first exploratory work of EVs derived from this strain, providing EV RNA and protein content characterization and the study of their impact on the host immune response. The second strain, the mastitis model *S. aureus* Newbould 305 (N305), was chosen to perform a deeper investigation of their role in host-pathogen interactions. The protein content and the first insights into *S. aureus* N305-derived EVs' impact on immunomodulation were published in 2018 (TARTAGLIA et al., 2018). Here, we expand this knowledge.

This work has been divided into several steps to investigate the proposed hypothesis. Figure 2.1 provides a general picture of the strategy used to complete the following specific objectives:

1. Isolation and purification of EVs from *S. aureus* HG003 strain grown under different environmental conditions by sucrose density gradient.
2. Description of *S. aureus* HG003 EVs biophysical characteristics (size, concentration, morphology) from different environmental conditions.
3. Characterization of EVs RNA composition of HG003 EVs from different environmental conditions through RNA-Seq.
4. Characterization of EVs protein composition of HG003 EVs from different environmental conditions.
5. Evaluation of the intra-species effect of *S. aureus* HG003-derived EVs.
6. Investigation of the impact of *S. aureus* HG003-derived EVs on the gene expression of different human cell lines.

7. Isolation and purification of EVs derived from *S. aureus* N305 strain by sucrose density gradient.

8. Evaluation of gene expression profile of bovine mammary epithelial cells (bMECs) stimulated *in vitro* with N305 EVs at different exposure times through RNA-seq.

Objectives 1-3 are addressed in Chapter 3, which presents an original research article reporting the complete physical characterization and the RNA content description of EVs derived from *S. aureus* HG003 strain grown under different environmental conditions.

Objective 4 is addressed in Chapter 4, which presents an original research article describing the protein content of *S. aureus* HG003 strain and its derived EVs grown under different environmental conditions.

Objectives 5 and 6 are addressed in Chapter 5, where additional results show the impact of the addition of HG003 EVs on *S. aureus* growth and in the gene expression of three different human cell lines.

Objectives 7 and 8 are addressed in Chapter 6, where sequencing analysis gives a deep comprehension of the eukaryotic immune response after exposure to *S. aureus* N305 EVs, giving new insights into their roles in staphylococcal pathogenesis.

The general conclusions and perspectives of this work are presented in Chapter 7.

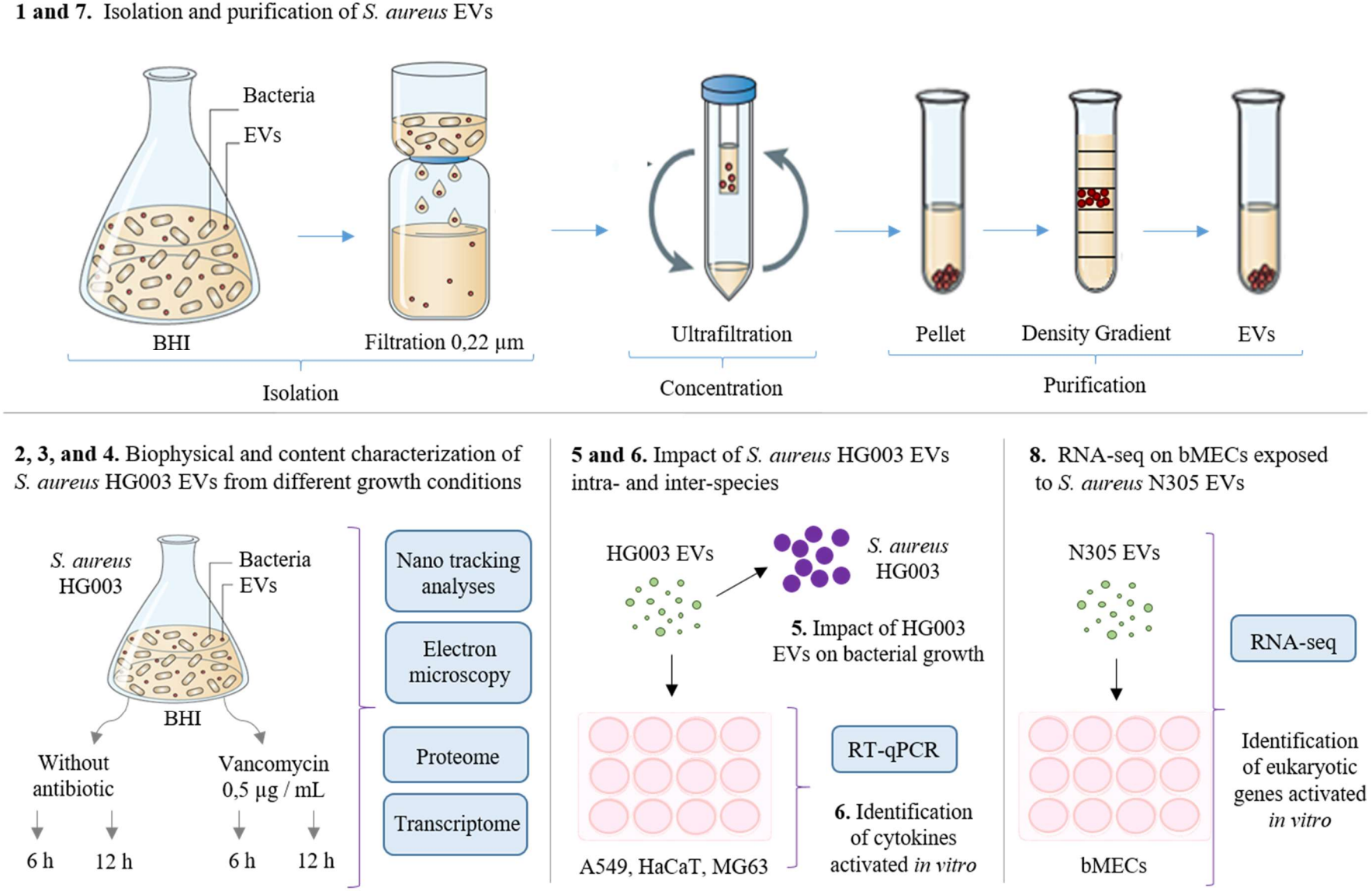

Figure 2.1 Experimental strategy used in this PhD thesis.

Chapter 3. Original research article 1

Environmental Plasticity of the RNA Content of *Staphylococcus aureus* Extracellular Vesicles

In the next section will you find attached an original article published in Frontiers in Microbiology (impact factor 5.59) on 11 March 2021, Section Infectious Agents and Disease. https://doi.org/10.3389/fmicb.2021.634226

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ORIGINAL RESEARCH
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Environmental Plasticity of the RNA Content of *Staphylococcus aureus* Extracellular Vesicles

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The roles of bacterial extracellular vesicles (EVs) in cell-to-cell signaling are progressively being unraveled. These membranous spheres released by many living cells carry various macromolecules, some of which influence host-pathogen interactions. Bacterial EVs contain RNA, which may serve in communicating with their infected hosts. *Staphylococcus aureus*, an opportunistic human and animal pathogen, produces EVs whose RNA content is still poorly characterized. Here, we investigated in depth the RNA content of *S. aureus* EVs. A high-throughput RNA sequencing approach identified RNAs in EVs produced by the clinical *S. aureus* strain HG003 under different environmental conditions: early- and late-stationary growth phases, and presence or absence of a sublethal vancomycin concentration. On average, sequences corresponding to 78.0% of the annotated transcripts in HG003 genome were identified in HG003 EVs. However, only ~5% of them were highly covered by reads (≥90% coverage) indicating that a large fraction of EV RNAs, notably mRNAs and sRNAs, were fragmented in EVs. According to growth conditions, from 86 to 273 highly covered RNAs were identified into the EVs. They corresponded to 286 unique RNAs, including 220 mRNAs. They coded for numerous virulence-associated factors (*hld* encoded by the multifunctional sRNA RNAIII, *agrBCD*, *psmβ1*, *sbi*, *spa*, and *isaB*), ribosomal proteins, transcriptional regulators, and metabolic enzymes. Twenty-eight sRNAs were also detected, including *bona fide* RsaC. The presence of 22 RNAs within HG003 EVs was confirmed by reverse transcription quantitative PCR (RT-qPCR) experiments. Several of these 286 RNAs were shown to belong to the same transcriptional units in *S. aureus*. Both nature and abundance of the EV RNAs were dramatically affected depending on the growth phase and the presence of vancomycin, whereas much less variations were found in the pool of cellular RNAs of the parent cells. Moreover, the RNA abundance pattern differed between EVs and EV-producing cells according to the growth conditions. Altogether, our findings show that the environment shapes the RNA cargo of the *S. aureus* EVs. Although the composition of EVs is impacted by the physiological state of the producing cells, our findings suggest a selective packaging of RNAs into EVs, as proposed for EV protein cargo. Our study shedds light to the possible roles of potentially functional RNAs in *S. aureus* EVs, notably in host-pathogen interactions.

Keywords: membrane vesicle, small regulatory RNA, virulence factors, vancomycin, RNA-Seq, extracellular vesicle, RsaC, RNAIII

INTRODUCTION

The release of extracellular vesicles (EVs) by living cells is a well-established phenomenon required for intercellular communications and *trans*-kingdom interactions (Brown et al., 2015; Toyofuku, 2019). These spherical membranous particles vary from 20 to 300 nm in diameter and contain macromolecules such as nucleic acids, proteins, lipids, and small metabolites. Initially considered to be trash bags to eliminate unwanted material outside of the cells, they are now widely recognized as protective delivery shuttles of bioactive molecules from donor to recipient cells (Brown et al., 2015; Kim et al., 2015; Gill et al., 2019). The functional characterization of bacterial EVs is of interest due to their capacities to affect bacteria-host cell interactions and bacterial pathogenesis (Kaparakis-Liaskos and Ferrero, 2015; Tsatsaronis et al., 2018). Although the formation of outer membrane vesicles (OMVs) in Gram-negative bacteria was early documented in 1966 (Work et al., 1966), the formation of such structures was disregarded in Gram-positive bacteria until recently. The production of EVs by a Gram-positive bacterium, *Staphylococcus aureus*, was demonstrated in 2009 and, ever since, numereous studies confirmed EV release by other Gram-positive bacteria (Lee et al., 2009, 2013a; Rivera et al., 2010; Prados-Rosales et al., 2011; Brown et al., 2014; Olaya-Abril et al., 2014; Kim et al., 2016a; Liu et al., 2018b).

Staphylococcus aureus commonly colonizes the skin or nasal tract of vertebrates, without causing disease (Wertheim et al., 2005). However, it is also one of the main opportunistic pathogen in humans, and a frequent cause of multi-drug resistant nosocomial infections (Ziebuhr, 2001). *S. aureus* is responsible for a wide array of diseases, ranging from minor infections in soft tissues to life-threatening diseases, such as sepsis, meningitis, and pneumonia (Salgado-Pabón and Schlievert, 2014; Tong et al., 2015). The type and severity of infections depend on strain-specific virulence factors, mostly expressed from accessory genetic elements (Gill et al., 2011). Secreted and surface-exposed *S. aureus* virulence factors weaken the host immune response, leading to bacterial immune evasion and pathogenesis (Foster, 2005). EVs could be a vehicle for secretion and surface-display of these molecules and, accordingly, recent studies indicate that *S. aureus* EVs carry important bacterial survival and virulence factors, such as β-lactamases, toxins, and proteins involved in adhesion to host cells (Lee et al., 2009; Gurung et al., 2011; Jeon et al., 2016; Askarian et al., 2018; Tartaglia et al., 2018, 2020; Wang et al., 2018).

Biologically active β-lactamase in *S. aureus* EVs can confer a transient resistance against ampicillin to surrounding sensible bacteria (Lee et al., 2013b). Furthermore, the presence of α-hemolysin inside EVs accelerates host cell death (Thay et al., 2013; Hong et al., 2014), and EV-associated exfoliative toxin A (ETA) induces a characteristic toxicity onto human epithelial cells (Jeon et al., 2016). Moreover, *S. aureus*-derived EVs facilitate the induction and exacerbation of skin and pulmonary inflammations (Hong et al., 2011, 2014; Kim et al., 2012; Jun et al., 2017). EVs-associated molecules can be more efficient than cytoplasmic proteins to elicit an immune response and host-cell toxicity (Hong et al., 2014). In response to antibiotics exposures, EVs increase *S. aureus* adhesion and cell aggregation, and contribute to biofilm formation (He et al., 2017). Recent data highlight the importance of EVs in staphylococcal pathogenesis since EVs derived from various human and animal strains of *S. aureus* share a conserved EV proteome (Tartaglia et al., 2020).

The vast majority of functional studies on bacterial EVs, however, challenged their proteome. Regarding the presence of DNAs and RNAs in EVs, most studies have been conducted on Gram-negative bacteria (Perez Vidakovics et al., 2010; Blenkiron et al., 2016; Koeppen et al., 2016; Bitto et al., 2017; Choi et al., 2017; Malabirade et al., 2018; Yu et al., 2018; Han et al., 2019). OMV-associated RNAs can include messenger RNAs (mRNA), transfer RNAs (tRNA), ribosomal RNAs (rRNA), or small regulatory RNAs (sRNA; Biller et al., 2014; Ghosal et al., 2015; Ho et al., 2015; Sjöström et al., 2015; Blenkiron et al., 2016; Koeppen et al., 2016; Choi et al., 2017; Dauros-Singorenko et al., 2018; Liu et al., 2018a; Malabirade et al., 2018; Tsatsaronis et al., 2018; Frantz et al., 2019). EV-associated RNA cargo, notably sRNAs, can influence host-pathogen interactions, cell-to-cell communications, and bacterial pathogenesis (Dauros-Singorenko et al., 2018; Tsatsaronis et al., 2018; Lee, 2019; Ahmadi Badi et al., 2020; Lécrivain and Beckmann, 2020). For instance, OMVs from *Pseudomonas aeruginosa* can transfer an sRNA into the human airway cells, resulting in IL-8 decrease (Koeppen et al., 2016). Likewise, transfection of OMV-associated sRNAs from the periodontal pathogens *Aggregatibacter actinomycetemcomitans*, *Phorphyromonas gingivalis*, and *Trepanema denticola* into human cells reduced host interleukine release (Choi et al., 2017). The presence of RNAs within Gram-positive EVs has been reported for fewer species (Resch et al., 2016; Dauros Singorenko et al., 2017; Frantz et al., 2019; Rodriguez and Kuehn, 2020). Interestingly, Frantz et al. (2019) recently reported that the EV-associated *rli*32 sRNA of *Listeria monocytogenes* can trigger the induction of a type I IFN response in host cells. This finding supports that Gram-positive EVs can also participate to host-pathogen interactions by dedicated vesicular RNAs. Data about RNA cargo in EVs released by *S. aureus* are scarce, with only two recent reports. While the first provided a partial RNA profile of *S. aureus* MSSA476 EVs without functional analyses (Joshi et al., 2021), the second showed that the uncharacterized RNA content of *S. aureus* Newman EVs likely stimulate the potent IFN-β response observed in cultured macrophage cells (Rodriguez and Kuehn, 2020).

As far as we know, our work is the first example that provides a detailed RNA profile associated to EVs from a reference clinical *S. aureus* strain, HG003. The staphylococcal EV RNA cargo was unveiled by high-throughput RNA sequencing from purified EVs after release by cells grown under various environmental conditions. They include early- and late-stationary growth phases, with or without a sublethal concentration of vancomycin, an antibiotic used to treat multidrug-resistant infections and that influences *S. aureus* EV biogenesis and functions (Hsu et al., 2011; He et al., 2017). The RNA cargo from the EVs was analyzed and compared to the RNA content of the HG003 parental cells.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

The *S. aureus* strain used in this work was the model strain HG003 (Herbert et al., 2010), a NCTC8325 derivative, isolated in 1960 from a sepsis patient. HG003 contains functional *rsbU* and *tcaR* genes, two global regulators that are missing in the NCTC8325 parent strain. The HG003 genome is well documented (Sassi et al., 2014), and this strain is widely used as a reference to investigate staphylococcal regulation and virulence (Liu et al., 2018a). HG003 strain was pre-inoculated in BHI broth and grown overnight at 37°C under 150 rpm/min agitation, and then inoculated 0.1% in 500 ml of fresh BHI (125 rpm/min, at 37°C) on a 1 L Scott flask. Bacterial cultures were retrieved after 6 h and 12 h for early- and late-stationary phases, respectively, in the presence or absence of a sub-inhibitory concentration (0.5 μg/ml) of vancomycin (**Supplementary Figure S1**).

S. aureus EVs Isolation and Purification

Cultures were submitted to EVs isolation and purification, as previously described (Tartaglia et al., 2018, 2020). In brief, for each condition 1 L of bacterial cell culture was centrifuged at 6,000 × *g* for 15 min and filtered through 0.22 μm Nalgene top filters (Thermo Scientific). Then, the culture supernatant fraction was concentrated around 100-fold using the Amicon ultrafiltration systems (Millipore) with a 100kDa filter, and ultra-centrifuged for 120 min at 150,000 × *g* to eliminate the soluble proteins. Next, the suspended pellet was applied to a discontinuous sucrose gradient (8–68%) and ultra-centrifuged at 100,000 × *g* for 150 min. Fractions containing EVs were recovered and washed in TBS (150 mM NaCl; 50 mM Tris-Cl, pH 7.5) for final ultra-centrifugation at 150,000 × *g* (120 min). At last, EVs were suspended in cold TBS and kept at −80°C until use.

EVs Visualization by Electron Microscopy

Negative staining electron microscopy was performed as previously described (Rodovalho et al., 2020) to investigate the shape and integrity of purified EVs. EVs samples were diluted, and solutions containing between 10^{10} and 10^{11} particles per ml were analyzed. For this, samples were applied to glow-discharged copper EM grids (FF200-Cu) for 30 s, followed by excess solution removal with filter paper. The same process was repeated with 2% uranyl acetate, and samples were observed with a Jeol 1400 transmission electron microscope (JEOL Ltd.), operating at 120 kV.

Determination of EVs Sizes and Concentrations

Nanoparticle Tracking Analysis (NTA) using an sCMOS camera and a Blue488 laser (Nano Sight NS300) was performed to assess EVs size and concentration. For that, samples were diluted into TBS to achieve optimal concentration and submitted to a constant flux generated by a syringe pump (speed 50), at 25°C. Results were retrieved from 5 × 60 s videos recorded with camera level at 15 and threshold at 5, while other parameters were adjusted as necessary.

RNA Extraction From *S. aureus* HG003 Whole Cells and Its Derived EVs

RNA extraction was carried out as similar as possible for both cell and EV samples. Bacterial RNA extraction was performed from 10 ml culture pellet. The samples were mixed with glass beads in 300 μl lysis buffer (0.5% SDS w/v, 30 mM sodium acetate; 1 mM EDTA) and 400 μl phenol (acid buffered at pH 5.0) at 65°C. Mechanical lysis was accomplished with 2 cycles of 30 s in Precellys at 6,500 rpm. For EV sample RNA extraction, particles isolated from the equivalent of 800 ml bacterial culture were mixed with 300 μl of lysis buffer and 400 μl phenol at 65°C, the same volumes used for cell RNA extraction. Since EVs lack the thick layer of peptidoglycan (PGN) found in the bacterial cell wall, mechanic lysis was not necessary and was achieved with lysis buffer. EV and EV-producing cell samples were incubated for 10 min at 65°C, being homogenized by vortex every minute. Next, samples were centrifuged during 10 min 13,000 rpm, 4°C, and the upper phase was recovered to a new tube. All samples were mixed with additional 400 μl of phenol at 65°C, and the previous steps were repeated. Then, 400 μl of phenol:chloroform 1:1 was added, followed by two times addition of 400 μl pure chloroform, repeating the step of upper phase recovery, mixture and centrifugation (5 min at 13,000 rpm, 4°C). Subsequently, 1.5 volumes of ice-cold 100% ethanol and 10% volume of NaAc were added and the mix was stored at −20°C overnight. Samples were centrifuged at 13,000 rpm for 30 min at 4°C, and the pellets were washed twice with 1 ml of cold 70% ethanol. Finally, the pellets were dried with a SpeedVac concentrator for 2 min and dissolved in RNase-free water. The quality and quantity of the RNAs were verified by Nano Drop, agarose gel, and Bioanalyzer (Agilent). Samples were kept at −80°C until use. No RNase treatment was applied to bacterial cell or to EV samples before RNA extraction.

RNA Sequencing

The RNA samples were sent to ViroScan3D® (Lyon, France) for DNA removal, ribosomal RNA depletion and RNA sequencing. Total RNA samples were submitted to a DNase treatment with RNase-Free DNase Set (Qiagen) according to manufacturer's instructions. Then, the samples quantified using the Quantifluor RNA system (Promega), and qualified using RNA Nano Chip on Bioanalyzer 2100 (Agilent) for the EV-producing cell samples, and on the SS RNA system on Fragment Analyzer (AATI) for the EVs samples. RNA samples were then submitted to the standard protocol Ovation Universal Prokaryotic RNA-Seq, Nugen, Anydeplete rRNA, library preparation. RNA quantity used for library preparation are displayed in **Supplementary Table S1**. The quality of libraries was assessed with the Quantifluor DNA system (Promega) and qualified with the HS-NGS system on Fragment Analyzer (Aati). The insert mean size of the libraries was 0.34 kp for the EV-producing cell samples, and 0.45 kp for

the EV samples (**Supplementary Table S1**). Sequencing was performed with Illumina, NextSeq500, 75 cycles, single-read, High Output. For each experimental condition, three biological replicates were sequenced. EV-producing cell samples ranged from 9 to 27 million pair-end reads per sample, and EV samples ranged from 30 to 67 million pair-end reads per sample. Reads mapping to the reference genome ranged from 8 to 26 million, and from 0.38 to 27 million reads for EV-producing cells and EV samples, respectively. Basic statistics of the RNA-Seq data are displayed in **Supplementary Table S1**.

Transcriptome Analysis

The reads were cleaned and trimmed with Trim-Galore (Martin, 2011) using the default parameters. Reads were mapped with Bowtie2 (Langmead and Salzberg, 2013) in local mode against two staphylococcal genomes used as references: the NCTC 8325 (NC_007795.1) reference genome with sRNA annotation from SRD (Sassi et al., 2015) and the HG003 genome (GCA_000736455.1) for the non-annotated genes in NCTC8325 genome. Genes were counted with FeatureCounts (Liao et al., 2014) with the strand, the multi mapping, and the overlapping options.

A list of differentially expressed RNAs was obtained by EdgeR (Robinson et al., 2009) embedded in SARTools (Varet et al., 2016). The threshold of statistical significance was set to 0.05, with the adjustment method of Benjamini-Hochberg. RNA coverage was calculated with Bedtools coverage (Quinlan and Hall, 2010). RNAs with ≥ 90% coverage in at least one EV condition were kept for further indepth analysis. RNA coverage visualization was performed with the Integrative Viewer Software (IGV; Thorvaldsdóttir et al., 2013) on a log scale.

Subcellular location prediction was performed with SurfG+ (Barinov et al., 2009). Clusters of Orthologous Groups (COGs) and KEGG categories were obtained using the eggNOG-mapper v2 web tool (Huerta-cepas et al., 2017, 2019). Functional enrichment analysis was performed with g:Profiler web-server (Raudvere et al., 2019; Reimand et al., 2019). A maximum value of p 0.05 was set as a threshold for significative categories.

A timepoint clustering study was conducted with the R package maSigPro (Conesa et al., 2006; Nueda et al., 2014) on highly covered EV RNAs with normalized counts by EdgeR. In this analysis vancomycin treatment is not taken into consideration. The threshold of statistical significance was set to 0.05, with the adjustment method of Benjamini-Hochberg.

RT-qPCR

Reverse transcription quantitative PCR (RT-qPCR) was used to validate RNA-seq results. EVs were isolated from the cell-free supernatants of three new independent *S. aureus* cultures at late-stationary growth phases (12 h) in the absence of vancomycin. EV RNAs were purified as mentioned above. Around 1.5 µg of RNAs was treated with DNAse I (Amplification Grade, Invitrogen) according to manufacturer's instructions. cDNA synthesis was performed with the high capacity cDNA Reverse Transcription kit (Applied Biosystems). The primers used for quantitative PCR (qPCR) are listed in **Supplementary Table S2** and were designed using eprimer3 software (EMBOSS). qPCR was carried out in a 16 µl volume containing 15 ng cDNA, specific primers (300 nM), and 8 µl IQ™ SYBR Green Supermix (Bio-Rad). Reactions were run on a CFX96 real-time system (Bio-Rad, France) using the following cycling parameters: DNA polymerase activation and DNA denaturation 95°C for 5 min, 40 cycles of denaturation at 94°C for 15 s, and extension at 60°C for 30 s. Melting curve analysis was included to check the amplification of single PCR products. Samples setups included biological triplicates and technical duplicates as well as negative controls corresponding to qPCR reactions performed without cDNA (cDNA negative control) and from RT reactions obtained from EV RNAs without reverse transcriptase (RT negative control). Results were analyzed with the GFX Manager software and Ct values were determined. Results with Ct equal or above 40 were considered negative and only experiments with $\Delta Ct \geq 4$ between negative controls and RT samples were considered.

RESULTS

S. aureus HG003 Produces EVs in Different Growth Conditions

Extracellular vesicles secreted by HG003 were isolated from the cell-free supernatants of bacterial cultures at early- and late-stationary growth phases (6 and 12 h, respectively), as well as in the absence (V-) and presence (V+) of a sublethal concentration of vancomycin (0.5 µg/ml). For that purpose, we used centrifugation, filtration, and density gradient ultracentrifugation, the standard method for EV isolation and purification at high purity (Yamada et al., 2012; Dauros Singorenko et al., 2017). EV homogeneity and integrity were evaluated by both negative staining electron microscopy and by NTA. Electron micrographs of purified EVs revealed typical nano-sized vesicular structures, with cup-shaped forms in all tested conditions (Raposo and Stoorvogel, 2013; **Figure 1A** and **Supplementary Figure S2**). NTA analyses showed a typical profile of particles for all EV samples (**Figure 1B** and **Supplementary Figure S2**). A significant increase of approximately 55% in EV diameter was observed in those purified from late-stationary phase cultures, compared to early-stationary phase cultures (for both 6V− vs. 12V− and 6V+ vs. 12V+), whereas no significant difference was observed in the absence or presence of vancomycin (6V− vs. 6V+ and 12V− vs. 12V+, **Figure 1C**). EV yield is essentially similar at the two growth phases, irrespective to the presence/absence of vancomycin (**Figure 1D**). In summary, *S. aureus* HG003 releases EVs with variable diameters depending on the growth phase. A sublethal concentration of vancomycin, however, does not impact the EV morphology, concentration, or diameter.

The *S. aureus* EVs Harbor all RNA Functional Classes

Total RNA was extracted from HG003 EVs to investigate their compositions. The quality of the RNA preparations was checked and validated, and the samples sequenced. RNA-seq data were

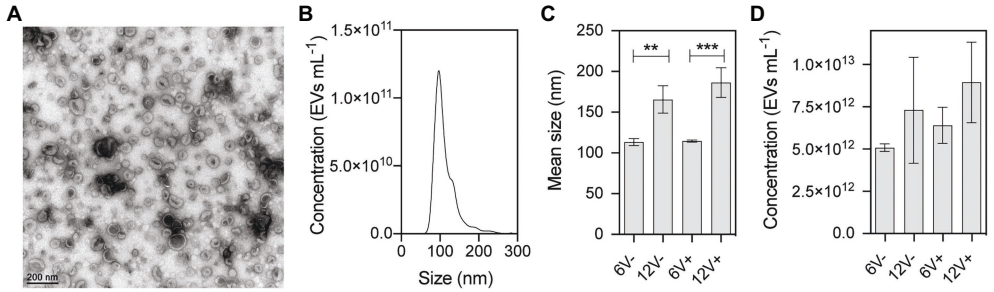

FIGURE 1 | Physical characterization of purified *Staphylococcus aureus* HG003-secreted extracellular vesicles (EVs). **(A)** Representative electron microscopy image of negatively stained HG003 EVs. **(B)** Representative graph of the EV size distribution. **(C)** Mean EV sizes. **(D)** EV yields. Data were obtained from three independent EV replicates. Asterisks indicate statistical significance (one-way ANOVA followed by Tukey's multiple comparisons test: $^{**}p < 0.01$; $^{***}p < 0.001$). Early- and late-stationary growth phases (6 and 12, respectively) in the absence (V–) or presence (V+) of vancomycin.

compared between the different growth conditions, and with those obtained from parental HG003 cells that produced the EVs in each condition (i.e., the EV-producing cells). Around 2649 ± 238 RNAs annotated in the HG003 genome were identified in the EVs according to growth conditions, with an average count of over five reads per RNA in each condition, whereas 3120 ± 35 annotated RNAs were identified within the EV-producing cells (**Supplementary Tables S3** and **S4**). All the four main RNA functional classes (tRNAs, rRNAs, mRNAs, and sRNAs) were identified in both the purified EVs and the EV-producing cells (**Figure 2A**). In both the EVs and the EV-producing cells, ~84% of the mapped RNAs corresponded to protein-coding genes (mRNAs). The sRNAs were the second most abundant mapped RNA class (~12% of the reads). The remaining 4% of the mapped RNAs are tRNAs and residual rRNAs. Note that most of the rRNAs were voluntarily removed during the RNA purification. Most of the annotated mRNAs, tRNAs, and residual rRNAs were identified in the EV-producing parental cells (from 86 to 96%), while this value dropped to 79.8 ± 2.7% for sRNAs (**Figure 2B**). These percentages were slightly lower for EV samples (from 72 to 89%), although they remained high for the sRNAs (59.1 ± 10.2%). All these RNAs detected in the purified EVs prompted us to check their coverages, to evaluate their integrity.

The *S. aureus* Purified EVs Contain Both Fragmented and Intact RNAs From Various Functional Classes

For the EV-producing cells, the median values of mRNAs, tRNAs, and residual rRNAs coverages were between 95 and 100%, and were 74 and 92% for the sRNAs (**Figure 3A** and **Supplementary Table S4**), implying that those RNAs were mainly intact, and not degraded. The coverage profile was drastically different for the RNAs recovered from the purified EVs. While the coverage of the residual rRNAs varied from 83 to 92%, the median coverage values for the other RNA functional classes ranged from 61 to 92% for the tRNAs, 15 to 47% for the mRNAs, and 4 to 20% for the sRNAs (**Figure 3A**). These lower coverages suggested that a substantial fraction of mRNAs and sRNAs were fragmented in the EVs compared to the EV-producing cells. To analyze potentially functional RNAs in EVs, only RNAs with a coverage ≥90% were considered for further analysis (**Supplementary Table S5**). The distribution of the newly filtered RNAs was depicted in **Figures 3B**,**C**. Such a harsh quality criterion impacted mainly the RNAs from the EVs, and particularly mRNAs and sRNAs. Only 3.5 ± 2.7 and 3.4 ± 1.2% of annotated mRNAs and sRNAs, respectively, were identified within EVs with such a threshold, while 67.9 ± 7.2% and 34.9 ± 5.0% of annotated mRNAs and sRNA were identified, respectively, for EV-producing cells (**Figure 3C**). Compared to the parental cells, the EVs were slightly depleted into mRNAs (68.0 ± 7.0% for the EVs vs. 88.2 ± 0.3% in the EV-producing cells) but, interestingly, were enriched for the other RNA functional classes including the sRNAs (14.3 ± 3.2% for the EVs vs. 8.6 ± 0.4% in the EV-producing cells, **Figure 3B**).

Functional Characterization of the RNAs From the EVs

According to experimental conditions, from 86 to 273 RNAs with a ≥90% coverage and an average count of over 5 reads per RNA were identified within EVs from *S. aureus* HG003 (**Figure 4A** and **Supplementary Table S5**). They corresponded to 286 unique RNAs and were either mRNAs (220), tRNAs (28), residual rRNAs (10), and sRNAs (28). The presence of some of these transcrits associated with HG003 EVs and corresponding either to mRNAs or sRNAs was confirmed by RT-qPCR on RNAs extracted from three independent biological replicates (**Figure 5**). Among the mapped mRNAs, most were implicated in translation, ribosomal structure and biogenesis (17.5%, COG J), energy production and conversion (13.6%, COG C), carbohydrate transport and metabolism (COG G, 7.9%), transcription (5.2%, COG K), and cell wall/membrane/envelope biogenesis (5.2%, COG M; **Figure 4B**). Several COG and KEGG categories were notably enriched ($p < 0.05$) in the EVs compared to the EV-producing bacteria (**Figure 4C**). mRNAs expressing proteins with a cytoplasmic location prediction were more represented in the EVs (79.5%) than into the producing cells (72.9%; **Figure 4D**). Interestingly, EVs contained several mRNAs coding for virulence-associated proteins such as the immune evasion protein A and Sbi, the Atl autolysin,

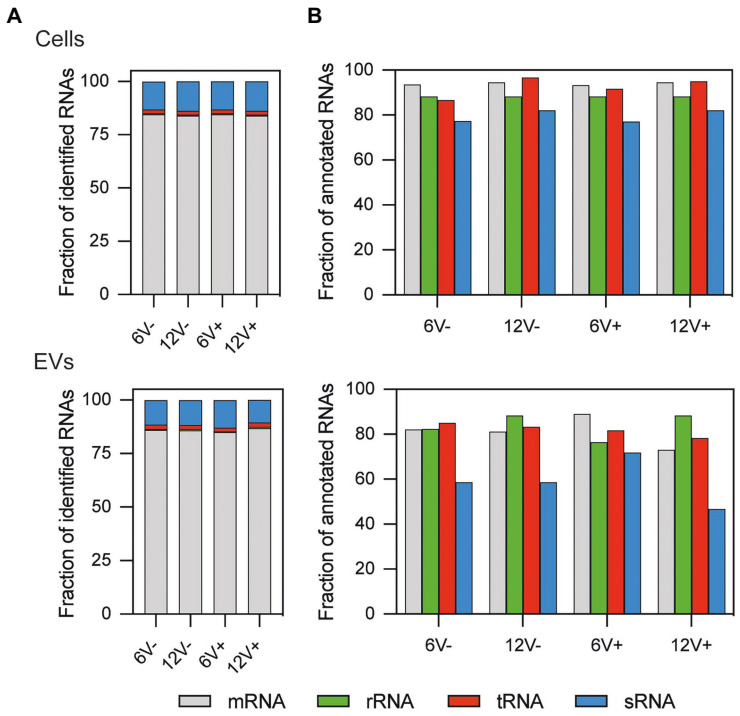

FIGURE 2 | Relative RNA composition of *S. aureus* HG003 and its secreted EVs. Individual colored bars represent the relative amount of each RNA class for mapped reads **(A)** and annotated RNAs **(B)**. RNA-Seq data is the average of three independent replicates. Number of reads have been normalized with EdgeR. RNA classes are defined from the *S. aureus* genome annotation NCTC8325/HG003. Early- and late-stationary growth phases (6 and 12, respectively) in absence (V–) or presence (V+) of vancomycin.

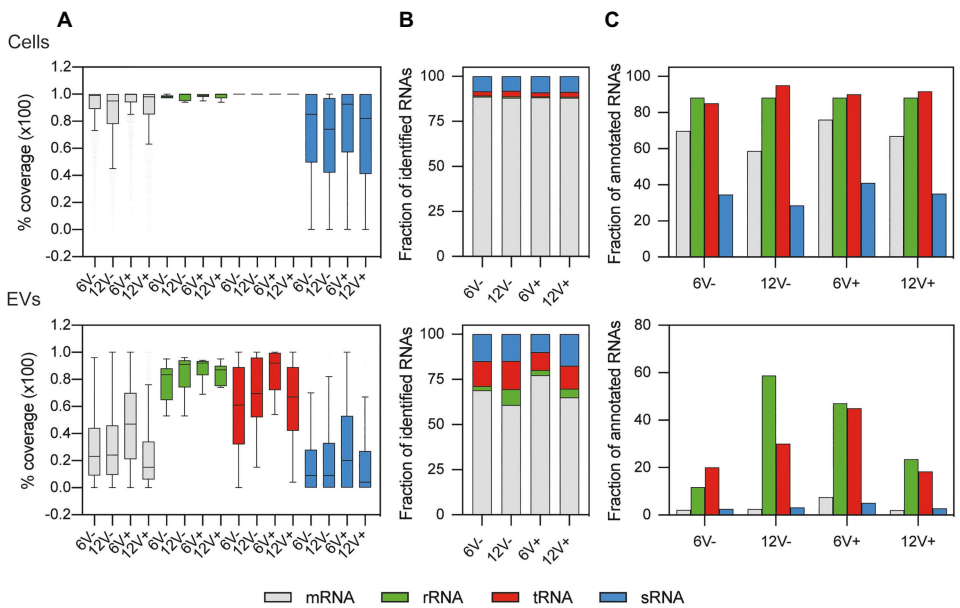

FIGURE 3 | Relative composition of highly covered RNAs from *S. aureus* HG003 and its secreted EVs. Colors represent the relative amount of each RNA class. **(A)** Percentage of RNA median coverage. Distribution of newly filtered RNAs with ≥90% coverage were plotted for **(B)** mapped reads **(C)** and annotated RNA. Number of reads have been normalized with EdgeR. RNA classes are defined from the *S. aureus* genome annotation NCTC 8325/HG003. Early- and late-stationary growth phases (6 and 12, respectively) in absence (V–) or presence (V+) of vancomycin.

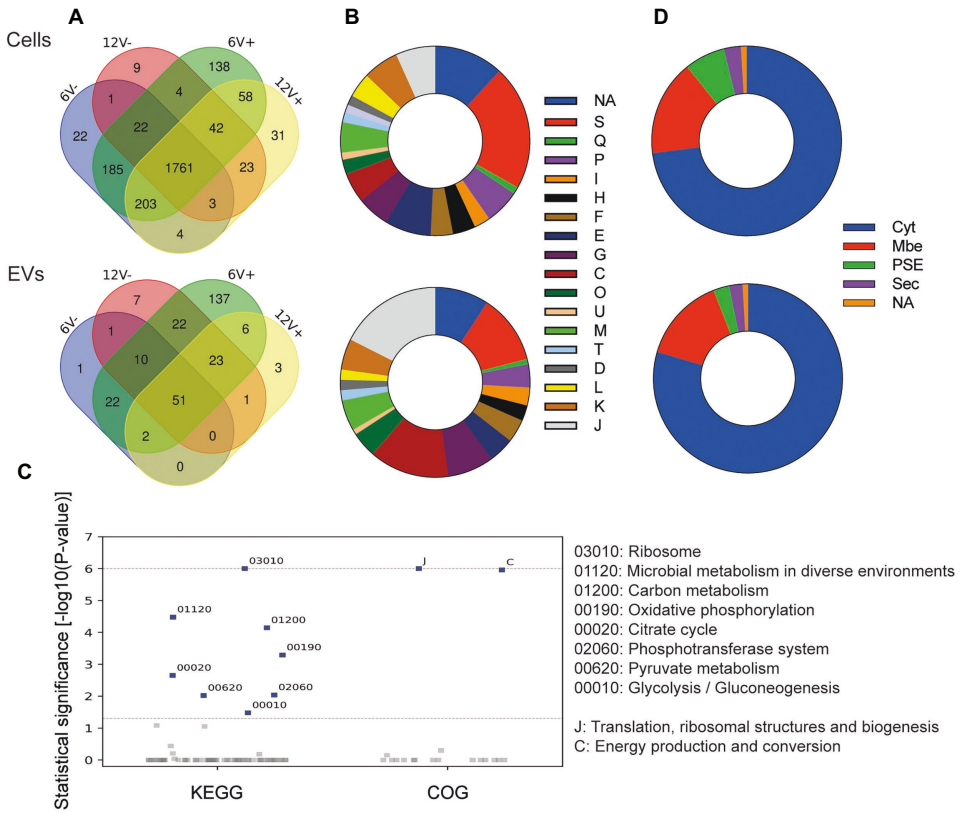

FIGURE 4 | *Staphylococcus aureus* HG003 EV RNA cargo and its modulation by different growth conditions. **(A)** Venn diagrams of RNA composition in EV-producing cells (upper panel) and EVs (lower panel) from different growth conditions. Early- and late-stationary growth phases (6 and 12, respectively) in absence (V-) or presence (V+) of vancomycin. **(B)** Prediction of Clusters of Orthologous Groups (COG) categories for mRNAs: NA, not predicted; S, function unknown; Q, secondary metabolites biosynthesis, transport, and catabolism; P, inorganic ion transport and metabolism; I, lipid transport and metabolism; H, coenzyme transport and metabolism; F, nucleotide transport and metabolism; E, amino acid transport and metabolism; G, carbohydrate transport and metabolism; C, energy production and conversion; O, post-translational modification, protein turnover, and chaperones; U, intracellular trafficking, secretion, and vesicular transport; M, cell wall/membrane/envelope biogenesis; T, signal transduction mechanisms; V, defense mechanisms; D, cell cycle control, cell division, chromosome partitioning; L, replication, recombination and repair; K, transcription; J, translation, ribosomal structure and biogenesis. **(C)** COG and KEGG categories enriched ($p < 0.05$) in EVs compared to EV-producing cells. **(D)** Subcellular localization of proteins encoded by mRNAs as predicted by SurfG+: Cyt, cytoplasmatic; Mbe, membrane; PSE, surface-exposed; Sec, secreted; NA, Not predicted.

the Hld δ-hemolysin encoded by the multifunctional sRNA RNAIII, the PSMβ1 Phenol Soluble Modulin, the FntA iron-storage ferritin, and the MntABC iron ABC transporter. Among the 20 tRNAs annotated in the genome, 15 were identified into the EVs ($tRNA^{His}$, $tRNA^{Asn}$, $tRNA^{Glu}$, $tRNA^{Arg}$, and $tRNA^{Asp}$ were absent). Five copies of the 16S and 23S rRNAs were also detected, implying that our rRNA depletion procedure was incomplete. Finally, 28 annotated potential sRNAs were detected within EVs, and among the 50 or so *bona fide* sRNAs defined for the HG003 strain (Liu et al., 2018a), only RsaC was identified with a ≥90% coverage in this study. Note that despite encoding the highly covered Hld transcript, the sRNA RNAIII presented only 71% gene coverage and therefore was excluded from analysis. Around 196 out of the 286 EV-associated RNAs colocalized at the same loci onto the HG003 chromosome, to form 42 clusters of 2 to 29 contiguous genes that were experimentally shown to belong to the same transcriptional units (Mäder et al., 2016; **Supplementary Table S6**). Among these transcriptional units, 17 displayed a RNA-Seq coverage ≥90% across the entire operon in both the EVs and the EV-producing bacteria. **Figure 6** illustrates the sequencing coverage of various contiguous genes within the EVs and the EV-producing cells. Long mRNA operons, up to ~14,000 nucleotides, were detected as fully covered by reads into the purified EVs, supporting the presence of highly covered RNAs and operons as full-length transcripts.

EV RNA Composition Varies With Growth Conditions

The RNA composition of the purified EVs was compared between early- and late-stationary phases, and with or without vancomycin. Eighteen percentage (n = 51) of all detected RNAs with ≥90% coverage were common to all the EV samples (**Figure 4A** and **Supplementary Table S5**), implying that the RNA content of the EVs highly varied according to the growth conditions. The percentage of RNAs shared by all the EV-producing cell samples, however, was much higher (70%, n = 1761). The shared RNAs among the EVs included mRNAs expressing virulence factors

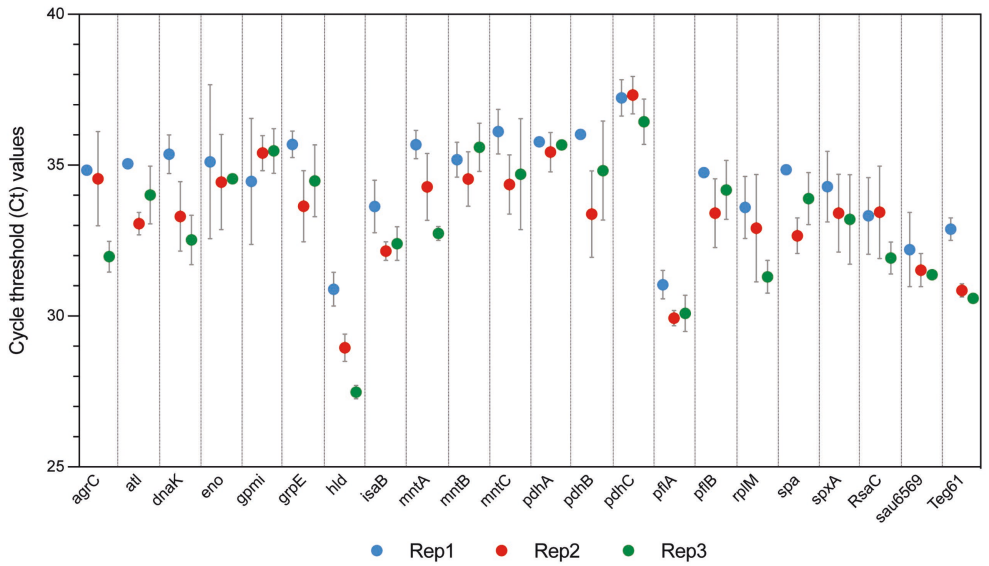

FIGURE 5 | Reverse transcription quantitative PCR (RT-qPCR) validation of *S. aureus* HG003 EV RNAs. RT-qPCR experiments were performed from RNAs extracted from EV samples isolated from the cell-free supernatants of three independent *S. aureus* cultures at late-stationary growth phases (12 h) in the absence of vancomycin (Rep1, Rep2, and Rep3). Quantitative PCR (qPCR) successfully amplified the coding-sequence of 19 mRNAs, and 3 sRNAs. Samples setups included biological triplicates (Rep1, Rep2, and Rep3) and technical duplicates as well as negative controls corresponding to qPCR reactions performed without cDNA, and from RT reactions performed without reverse transcriptase enzyme. Ct values are expressed as mean ± SD from two independent technical replicates performed in triplicates.

(Atl and Spa), metabolic enzymes (pyruvate dehydrogenase and cytochrome c oxidase complexes, glycolytic enzymes) and transcriptional regulators (SpxA, CggR, and GlnR), as well as RNAs involved in translation (ribosomal proteins, rRNAs, and tRNAs; **Supplementary Table S5**). The 51 common RNAs also included 9 potential sRNAs, notably RsaC involved in *S. aureus* oxidative stress adaptation and nutritional immunity (Lalaouna et al., 2019).

For both the EVs and EV-producing cells, more RNAs were detected at 6 h (275 and 2443 for EVs and EV-producing cells, respectively) than at 12 h (126 and 2161 for EVs and EV-producing cells, respectively, **Figure 4A**). Likewise, more RNAs were detected in the EVs in the presence (277 and 2,474 for EVs and EV-producing cells, respectively) than in the absence of vancomycin (140 and 2,279 for EVs and EV-producing cells, respectively; **Figure 4A**), indicating that the antibiotic modifies the RNA cargo of the EVs. These results also highlighted that the growth phase and the antibiotic stress impacted mostly the RNA content of the EVs, but much less that of the parental cells. Indeed, when we considered the RNAs detected in only one condition (i.e., specific RNAs), their fractions were higher in the EVs than in EV-producing cells, and that for all the tested conditions. For example, 58% of RNAs found within EVs at 6 h were specific to this condition, while specific RNAs represented only 14% of all RNAs detected at 6 h in the EV-producing cells.

EV RNA Abundance Varies With Growth Conditions

In addition to the qualitative variations observed, significant differences (Padj < 0.05) in EV RNA abundance between the experimental conditions were also detected (**Supplementary Table S7**). Among the 286 EV-associated RNAs, 110 were differentially abundant between two conditions. Variations were detected at all times and in the absence or presence of vancomycin, although the growth phase appeared to have a greater impact on RNA abundance (75 and 64 differentially abundant RNAs were detected between early- and late-stationary phase with or without vancomycin, respectively), than the antibiotic stress (8 and 9 differentially abundant RNAs were detected between presence and absence of vancomycin in early- and late-stationary phase, respectively; **Supplementary Table S7**). A selection of RNAs with a modulation of their abundance according to the growth conditions is displayed in **Figure 7**. The most modulated RNAs into EVs produced from the two growth conditions were mRNAs coding for virulence-associated factors, such as *agrB*, *agrC*, *agrD*, *psmB1*, and *hld* with a 30- to 1300-fold change, two potential annotated sRNAs, srn_0560, and srn_1000 with a 16- and 190-fold change, respectively, $tRNA^{Gly}$ (SAOUHSC_T00025) and $tRNA^{Thr}$ (SAOUHSC_T00054), with fold changes greater than 16. Among the differentially abundant RNAs according to the growth phase, 32 were detected both in presence and absence of vancomycin, with similar fold changes highlighting their reproducible variations into EVs across different environmental conditions (**Figure 7** and **Supplementary Table S7**).

Differentially expressed RNAs were also detected for the EV-producing cells when their expression was compared between early- and late-stationary phase both in absence (n = 136) and in presence of vancomycin (n = 147), which was expected since bacterial transcription differs qualitatively

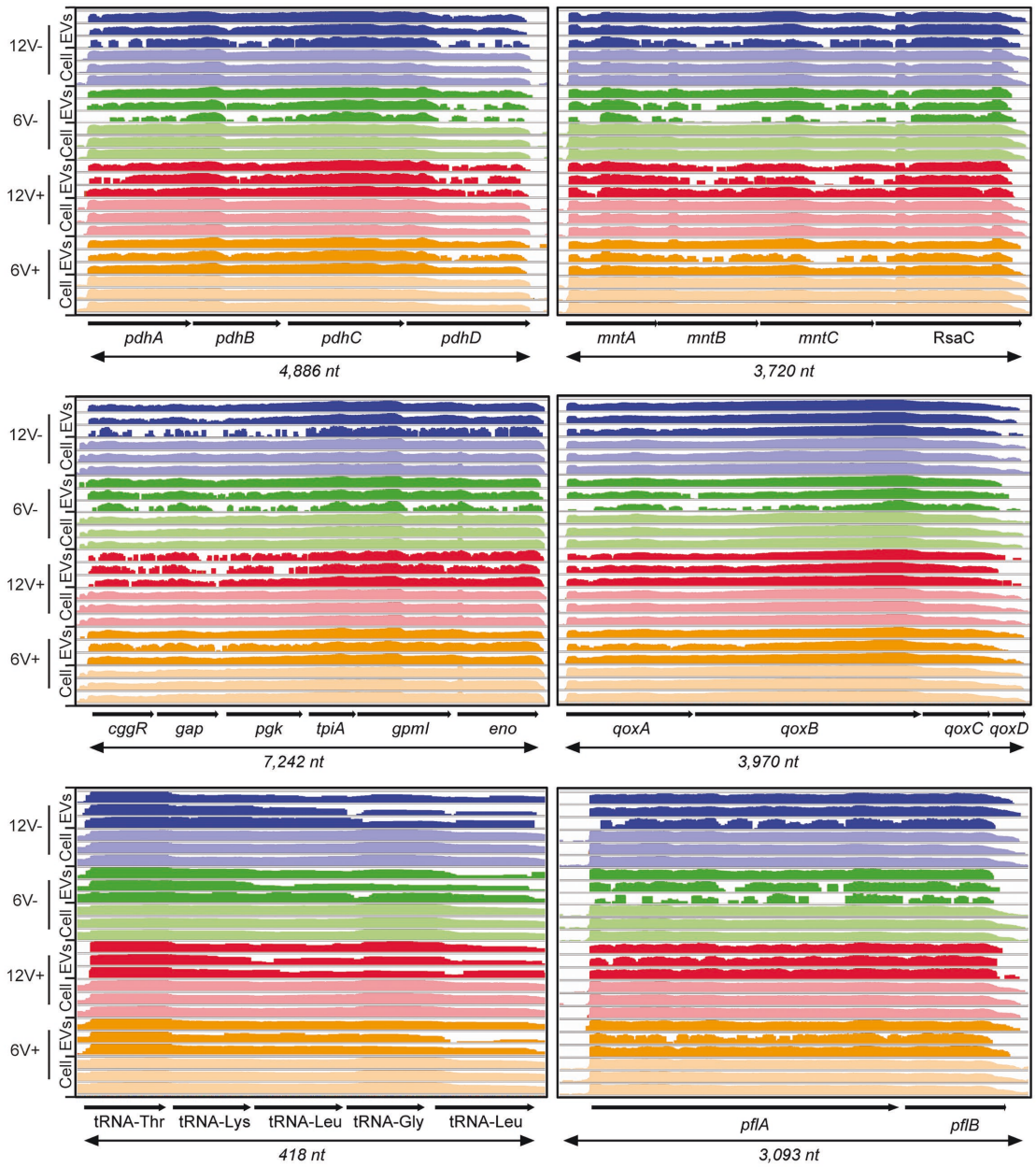

FIGURE 6 | HG003 EVs contain long mRNA transcripts. Comparison between operon coverages between EV-producing cell and EV samples in different conditions. Early- and late-stationary growth phases (6 and 12, respectively) in absence (V-) or presence (V+) of vancomycin. RNA coverage is visualized with Integrative Viewer Software (IGV) in log scale.

and quantitatively when facing different growth conditions (**Supplementary Table S8**). Note that no significantly differentially expressed RNAs were detected according to the presence of vancomycin. As observed previously for the EV RNA content, the growth conditions, particularly the growth phase, impacted mostly the RNA abundance of the EVs, but much less than of the parental cells. Indeed, 38% of RNAs detected within EVs displayed changes in their abundance between conditions, while the fraction of modulated RNAs counted for only 2% of the RNAs in the EV-producing cells. The abundance pattern of several RNAs differed between the EVs and the EV-producing cells according to the growth conditions (**Figure 7**). While some RNAs such as *agrBCD*, *psmβ1*, and *hld* mRNAs displayed the same variations of their abundance pattern in EVs and EV-producing cells regardless the growth conditions. Others, such as *spa* and RsaC, were differentially abundant between the EVs and the EV-producing cells.

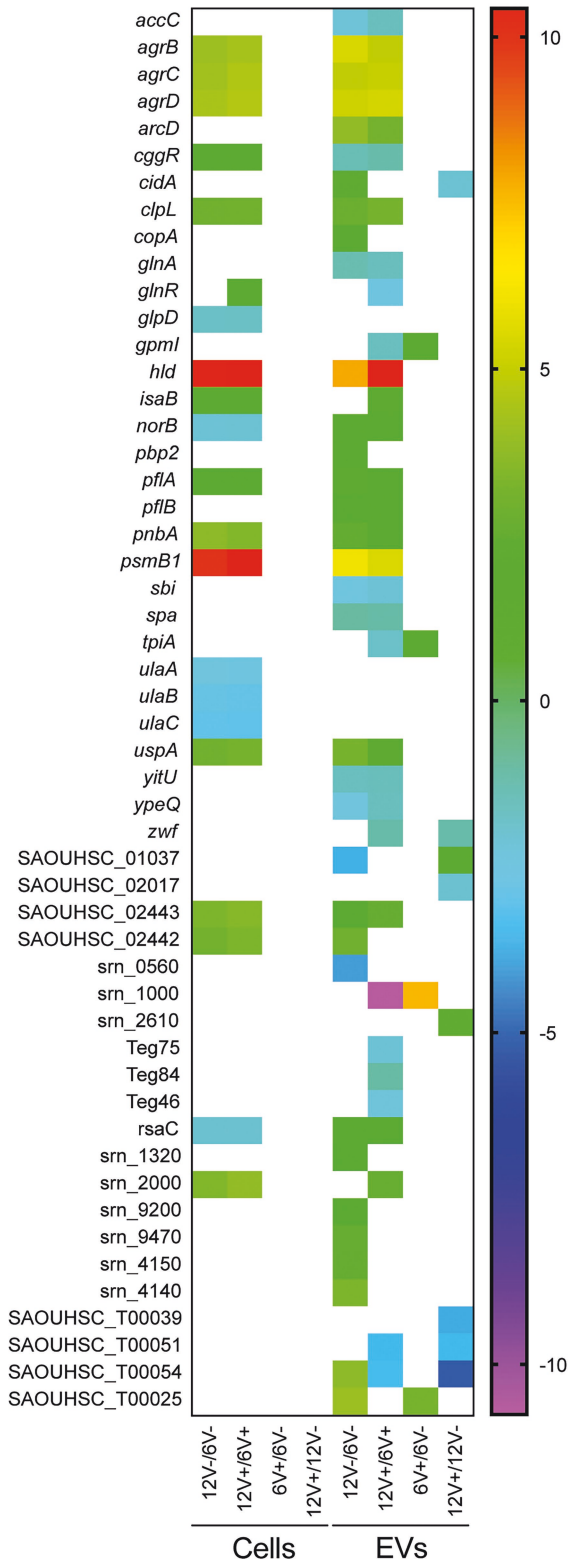

FIGURE 7 | Differential RNA abundance between the EV-producing cells and the EVs. Colored square shows different RNA abundance patterns between

(Continued)

FIGURE 7 | EV-producing cells and EVs with a log2 fold change. Comparisons comprised data with at least one of the two samples containing ≥90% coverage. The log2 fold change is displayed as colored squares from −2 (purple) to 10 (red). Early- and late-stationary growth phases (6 and 12, respectively) in absence (V−) or presence (V+) of vancomycin.

Timepoint Clustering Analysis Reveals Different RNA Abundance Profiles Between the EVs and EV Producing Cells

To evaluate the influence of the growth phase on EV and EV producing cell RNA composition, a negative binomial-based approach, with the R package maSigPro (Conesa et al., 2006) was applied. Briefly, maSigPro provides a differentially expressed transcript analysis of serial data between experimental groups (e.g., EV and EV producing cells). maSigPro was applied to the 286 highly covered EV RNAs and identified 91 RNAs with significant temporal profile changes (Padj < 0.05). RNAs were clustered according to their expression profiles (**Supplementary Table S9**). **Figure 8** shows the six RNA clusters obtained. Three clusters grouped transcripts with a similar expression profile between EV and EV-producing cells: cluster 4 with 8 RNAs (including, e.g., *agrBD* and *arcC2*) and cluster 3 with 18 RNAs (including, e.g., *hld* and *psmβ1*) contained more abundant transcripts over time in both EVs and EV-producing cells, whereas cluster 6 with 14 RNAs (including, e.g., *fusA*, *tuf*, and *secY1*) contained less abundant transcripts at 12 h than at 6 h in both. Interestingly, the three other clusters grouped RNAs that showed opposite expression profiles over time in EVs and EV-producing cells. Cluster 1 with 10 RNAs (including, e.g., RsaC and *pdhA*), and cluster 2 with 24 RNAs (including, e.g., *ldh1*, *qoxABC*, and *rpoBC*) grouped, similarly, more abundant transcripts at 12 h in EVs, and less abundant transcripts at 12 h in EV-producing cells. On the contrary, cluster 5 with 17 RNAs (including, e.g., *ccgR* and *sbi*) grouped less abundant transcripts in EVs and more abundant transcripts in EV-producing cells at 12 h. Altogether, this analysis highlighted that the transcript expression pattern could temporally differ between EVs and EV-producing cells.

DISCUSSION

Extracellular vesicles are universal carriers of macromolecules including extracellular RNAs all along from bacteria, archaea, and fungi to protists. Recent investigations on bacterial EV biogenesis, release, and trafficking showed their functional importance for bacterial communication and survival (Tsatsaronis et al., 2018). Information regarding *S. aureus* EV RNA cargo, however, is lagging behind. Here, we report the first exploratory work on EVs released by *S. aureus* HG003, the characterization of its EV RNA cargo under different conditions, and an indepth transcriptomic comparison between the EVs and the EV-producing cells.

Environmental conditions, such as growth phase and environmental stresses, reportedly influence the production, content and functions of EVs (Tashiro et al., 2010; Kim et al., 2016b; Orench-Rivera and Kuehn, 2016; Askarian et al., 2018;

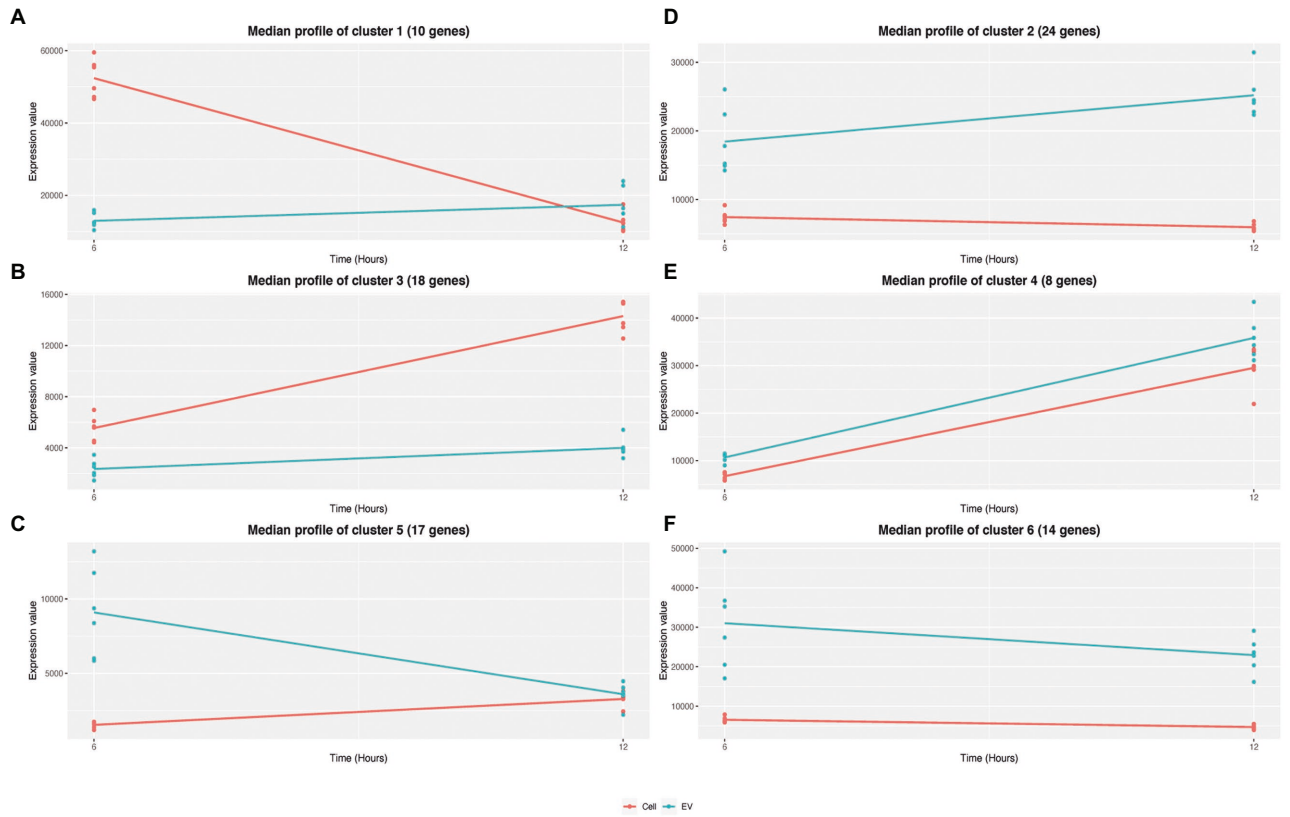

FIGURE 8 | Timepoint clustering of expression profiles between EVs and EV-producing cells. The analyses were performed with the R package maSigPro from normalized counts by EdgeR. Statistical significance ($p < 0.05$) was adjusted with Benjamini-Hochberg method. Median expression values are calculated at 6 and 12 h for EVs (blue) and EV-producing cells (red) for the six clusters (from **A** to **F**).

Yun et al., 2018; Andreoni et al., 2019). Here, to investigate the impact of environmental changes on *S. aureus* EV production, the selected conditions were the early- and late-stationary growth phases, with or without a sublethal concentration of vancomycin that does not impact growth. Differences were observed regarding EV sizes. EVs derived from the late-stationary growth phase were larger than those collected during the early-stationary phase. This can be due to cell-wall morphology and peptidoglycan structure that are characteristics of the growth stage in *S. aureus* (Zhou and Cegelski, 2012). A correlation between the degree of peptidoglycan cross-linking to the cell wall stiffness and EVs release was observed for both Gram-negative and Gram-positive bacteria (Zhou et al., 1998; Deatherage et al., 2009; Schrempf and Merling, 2015; Schwechheimer et al., 2015; Wang et al., 2018). Notably, sub-inhibitory concentrations of penicillin decreases peptidoglycan cross-linking, triggering an increase in *S. aureus* EV yields and sizes (Wang et al., 2018). Here, we show that sublethal concentrations of vancomycin, an antibiotic that also targets peptidoglycan synthesis in *S. aureus*, does not impact either EV morphology or EV production yields, but does change their RNA content in terms of composition and abundance. Vancomycin affects *S. aureus* EV activity starting at 1 μg/ml (He et al., 2017). The sub-inhibitory vancomycin concentration used here (0.5 μg/ml) is probably too low to detect any changes in EV morphology and production.

All RNA classes are detected by RNA-Seq within HG003-derived EVs. These include rRNAs, which were still detected, suggesting that the rRNA depletion carried out here was incomplete. In the absence of filtering by coverage of sequencing data, on average, 78.0 ± 7.0% of the annotated transcripts in HG003 genome were present in the EVs (91.8 ± 1.0% for EV-producing cells). Of these, a large portion of mapped RNAs corresponded to mRNAs. These results are consistent with RNA-Seq data obtained with similar criteria from OMVs in *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) that harbor around 73% of the annotated transcripts including up to 86% of mRNAs according to growth conditions (Malabirade et al., 2018). A recent study addressing the sRNA content of EVs also pointed out that mRNAs are the more abundant RNA species in EVs derived from *S. aureus* strain MSSA476 after rRNA depletion (Joshi et al., 2021). On the contrary, studies with *Escherichia coli* revealed that EVs were enriched mainly with short RNAs, such as tRNAs (Ghosal et al., 2015; Blenkiron et al., 2016). Nevertheless, these variations may be a result of different RNA extraction and library preparation protocols, or simply correspond to singular characteristics of EVs derived from different bacterial species. As expected, data

filtering of RNAs with ≥90% coverage decreased the number of detected RNAs. However, the RNA content of the EVs, particularly the mRNAs and sRNAs (only 5.1 ± 2.8% of EV RNAs initially detected are still identified after the filtering), was much more affected than that of the EV-producing cells (69.0 ± 7.90% of EV-producing cell RNAs are still detected after the filtering). The low RNA coverage in the HG003 EVs might perhaps reflect the absence of transcription within EVs and, thus, the progressive degradation of a substantial fraction of the EVs-associated RNAs after their formation and/or during their purification. These findings are consistent with a recent report showing that the predominant RNA type in EVs from *S. aureus* Newman is <300-nucleotide long (Rodriguez and Kuehn, 2020). The presence of numerous processed or degraded RNAs could be a common feature of the bacterial EVs, as Gram-negative *S. Typhimurium* OMV-associated RNAs are also processed or degraded (Malabirade et al., 2018). Since one of the primary physiological functions attributed to EVs is the removal of unwanted materials from cells, such as misfolded or degraded proteins (McBroom and Kuehn, 2007), the *S. aureus* EVs may also help removing the degraded RNAs from the bacteria (Groot and Lee, 2020).

Two hundred and eighty-six highly covered RNAs can be identified within HG003 EVs. That number of highly covered EV RNAs varies according to growth conditions, from 86 in late-stationay growth phase with vancomycin to 273 in early-stationay growth phase also with vancomycin. As expected, all the EV transcrits are also detected in the EV-producing cells. Among them, 51 transcripts are shared by EVs collected in all tested conditions. The 286 highly covered EV RNAs encompass short transcripts, such as tRNAs (~75 nucleotides), mRNAs (*psmβ1*, 135 nucleotides; *agrD*, 141 nucleotides), and some sRNAs (Teg84, 79 nucleotides; Teg46, 124 nucleotides). Yet, these highly covered EV RNAs also comprise long transcripts, including 15 mRNAs with lengths >2,000 nucleotides (e.g., *pbp2*, *copA*, *rpoB*, *rpoC*, and *atl*). 67% of these RNAs are organized into 42 gene clusters that are co-transcribed in *S. aureus* (Mäder et al., 2016). Among them, 17 are full-length transcripts across entire operons in HG003 EVs, with lengths up to ~14,000 nucleotides. As observed for *S. Typhimurium* OMV RNAs (Malabirade et al., 2018), these findings support that highly covered RNAs are present as full-length transcripts. These RNAs belong to all annotated classes of RNAs. The mRNAs from the EVs encode proteins involved in transcription, translation, energy production and conversion, carbohydrate metabolism, and cell wall biogenesis. In addition to these housekeeping functions, EVs also harbors mRNAs encoding virulence-associated proteins, such as the *agr* operon responsible of quorum-sensing, autolysin Atl, protein A (Spa), immunoglobulin-binding protein (Sbi), immunodominant staphylococcal antigen B (IsaB), δ hemolysin (Hld) encoded by the multifunctional sRNA RNAIII, and the PSMβ1 phenol-soluble modulin, as well as several iron acquisition systems. Besides, most tRNA species are detected within the EVs, as well as residual rRNAs and 28 annotated sRNAs, including *bona-fide* RsaC involved in *S. aureus* oxidative stress adaptation and nutritional immunity (Lalaouna et al., 2019). Note that RsaC and RNAIII were also detected within *S. aureus* EVs from strain MSSA476 (Joshi et al., 2021), suggesting their wider occurence in staphylococcal EVs.

The presence of full-length, functional RNAs in EVs raises the question of their biological roles. EVs are produced to transport bioactive molecules to interact and communicate with other cells. So far, most studies on *S. aureus* EVs investigated the protein cargo. Therefore, the broad spectrum of activities associated with *S. aureus*-derived EVs was related to their protein content (Jeon et al., 2016). In some *S. aureus* strains, EVs carry β-lactams that confer transient resistance to ampicillin-susceptible *E. coli* and *S. aureus* (Lee et al., 2013b). Likewise, mycobactin-containing *Mycobacterium tuberculosis* EVs can deliver iron to strains deficient for iron-uptake (Prados-Rosales et al., 2014). The delivery of full-length mRNAs *via* EVs to the surrounding bacterial cells, notably those involved into energetic and metabolic functions, could improve their responses to environmental stimuli to fasten their adaptation. Likewise, rRNAs and tRNAs could boost translation in EV recipient bacterial cells and improve their fitness. In bacteria, sRNAs fine tune target gene expression, usually at the posttranscriptional level in response to changes in the environment, including antibiotic resistance and tolerance (Mediati et al., 2021). Most bacteria encode dozens of sRNAs that are transcribed as independent transcripts or processed from mRNAs. The presence of sRNAs with regulatory roles within *S. aureus* EVs could be a relocation strategy in other surrounding bacteria that need more of these sRNAs for adaptation and infection spreading and/or to coordinate bacterial group adaptation, activities and behaviors. The transfer of RsaC could enhance the concentration of that riboregulator to other *S. aureus* cells when intracellular, especially helping survival within the phagolysosome if the *S. aureus* EVs are internalized by the host cells together with the bacteria. Transfer of functional RNAs to bacterial cells that do not encode the corresponding genes in their genome could also be part of a transient horizontal phenotype acquisition, which could be of use during infection to disseminate specific virulence-associated factors through the bacterial community. Finally, beside interactions between bacterial cells, *S. aureus* EV associated RNAs, notably sRNAs, may be involved in the host-pathogen interactions (Eberle et al., 2009; Li and Chen, 2012; Furuse et al., 2014; Sha et al., 2014; Koeppen et al., 2016; Westermann et al., 2016; Choi et al., 2017; Frantz et al., 2019; Han et al., 2019; Lee, 2019; Rodriguez and Kuehn, 2020). The 28 potential annotated sRNA detected within HG003 EVs are potential candidates for further functional characterization, especially during *S. aureus*-host cell interactions. *S. aureus* secreted EVs elicit immune responses that mimic those of the EV-producing cells (Gurung et al., 2011; Hong et al., 2011, 2014; Kim et al., 2012, 2019; Thay et al., 2013; Choi et al., 2015; Jeon et al., 2016; Jun et al., 2017; Askarian et al., 2018; Tartaglia et al., 2018; Wang et al., 2018, 2020; Rodriguez and Kuehn, 2020). Strikingly, within their RNA cargo, several mRNAs encode immunomodulatory proteins, as Sbi, Spa and PSMβ1, and may participate into the immune response triggered by the protein cargo if they are ultimately translated. Yet, such functions remain to be demonstrated for the RNA cargo of

S. aureus EVs. mRNAs expressing PSMβ and hemolysin δ toxins from the EVs, if translated into recipient bacteria or host cells, could perhaps facilitate staphylococcal intracellular survival, but this hypothesis should be experimentally challenged.

The RNA cargo of HG003 EVs, in both identity and abundance, depends on the growth conditions. Similarly, the EV RNA cargo of *S. Typhimurium* is also sensitive to environmental changes indicating that it reflects the bacterial adaptation to its environment (Malabirade et al., 2018). It could be a faster way to transfer information of changes perceived by one cell to surrounding cells even before they sensed the environmental stimuli in order to quickly promote group adaptation. We found, however, that the vancomycin treatment had less impact on RNA abundance compared to the growth phase. Although the composition of HG003 EVs represented the intracellular state of the bacterial transcriptome through global packaging, two main findings, however, reinforce the concept of a potential selective packaging of RNAs into EVs, as proposed for its protein cargo (Haurat et al., 2011; Cahill et al., 2015; Tartaglia et al., 2020). First, we measured an enrichment for several functional and subcellular localization RNA categories in EVs when compared to EV-producing cells. Second, the relative abundance of several RNAs between two environmental conditions was different in the EVs and the EV-producing cells. Other studies also found that some RNA populations were enriched in EVs from Gram-negative and Gram-positive pathogenic bacteria (Ghosal et al., 2015; Koeppen et al., 2016; Resch et al., 2016; Malabirade et al., 2018; Malge et al., 2018; Han et al., 2019; Langlete et al., 2019; Zhang et al., 2020). This notably includes sRNAs, which can play regulatory activity in the host (Koeppen et al., 2016; Malabirade et al., 2018; Langlete et al., 2019; Zhang et al., 2020). The enrichment of RNAs associated with bacterial diseases in EVs derived from many pathogenic bacteria reinforces the physiopathological role of these structures in host-pathogen interaction and host cell invasion, which could be borne by their RNA cargo as well as by their protein cargo. The selective mechanisms of EV RNA content packaging have not yet been elucidated. It has been proposed that RNA packing into EVs could depend on RNA size and location (eg., nearby EVs formation site), as well as on their affinity for other molecules (eg., membrane proteins; Langlete et al., 2019). Nevertheless, such enrichment results should be interpreted with carefulness. Indeed, they could also reflect a difference in RNA half-lives between EVs and EV-producing cells, as well as a difference in RNase activity (Langlete et al., 2019; Lécrivain and Beckmann, 2020), pointing out that RNAs with longer half-lives could be protected from degradation, leading to an artifactual accumulation in the EVs over time.

In summary, our exploratory work provides novel insights in *S. aureus* EVs by the characterization of its RNA cargo and paves the way for further functional studies. Mainly, it sheds light on the possible roles of EV RNA cargo in intra- and inter-species communication, in the virulence and pathogenesis of *S. aureus*, and as trash bags for degraded RNAs. The study of bacterial EV RNA cargo is an emerging area of research. Evidently, as with all emerging fields, each advance raises further questions: Are the full-length RNAs in HG003 EVs functional, and do they possess similar functions than in the bacterial cytoplasm? What are the rules for RNA sorting into HG003 EVs? What are the roles and functions of the *S. aureus* EV RNA cargo? These exciting questions, among others, should be adressed in further studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/ena, https://www.ebi.ac.uk/ena/browser/view/PRJEB40502.

AUTHOR CONTRIBUTIONS

BSRL, BF, SC, YL, VA, and EG conceived and designed the experiments. VA and EG supervised the study. BSRL and SC performed the experiments. AN performed computational analysis. BSRL, BF, SC, VRR, AN, and EG analyzed the data. VA, YL, and EG contributed to funding acquisition. BSRL and EG wrote the original draft. All authors contributed to data interpretation, drafting the manuscript, critically revising the manuscript, and approving its final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.634226/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter 4. Original research article 2

Impact of environmental conditions on the protein content of *Staphylococcus aureus* and its derived extracellular vesicles

In the next section will you find attached an original article published in Microorganisms (impact factor 4.92) on 9 September 2022, Special Issue "Characterization and Molecular Analysis of Staphylococcal Species: Their Impact on Colonization and Infections". https://doi.org/10.3390/microorganisms10091808

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Impact of Environmental Conditions on the Protein Content of *Staphylococcus aureus* and Its Derived Extracellular Vesicles

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Abstract: *Staphylococcus aureus*, a major opportunistic pathogen in humans, produces extracellular vesicles (EVs) that are involved in cellular communication, the delivery of virulence factors, and modulation of the host immune system response. However, to date, the impact of culture conditions on the physicochemical and functional properties of *S. aureus* EVs is still largely unexplored. Here, we use a proteomic approach to provide a complete protein characterization of *S. aureus* HG003, a NCTC8325 derivative strain and its derived EVs under four growth conditions: early- and late-stationary growth phases, and in the absence and presence of a sub-inhibitory concentration of vancomycin. The HG003 EV protein composition in terms of subcellular localization, COG and KEGG categories, as well as their relative abundance are modulated by the environment and differs from that of whole-cell (WC). Moreover, the environmental conditions that were tested had a more pronounced impact on the EV protein composition when compared to the WC, supporting the existence of mechanisms for the selective packing of EV cargo. This study provides the first general picture of the impact of different growth conditions in the proteome of *S. aureus* EVs and its producing-cells and paves the way for future studies to understand better *S. aureus* EV production, composition, and roles.

Keywords: membrane vesicle; exosome; virulence factors; vancomycin; proteomics; communication; adaptation

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1. Introduction

Staphylococcus aureus is a Gram-positive bacterium acting both as a commensal and as an important opportunist pathogen. This versatile bacterium causes a broad spectrum of diseases ranging from minor to severe infections in several mammalian species, particularly humans and cattle [1–3]. The ability of *S. aureus* to colonize a diversity of hosts and niches in its hosts as well as to cause a vast array of diseases is reflected by the expression of numerous virulence factors, such as adhesins, toxins, and other elements that are capable of promoting host cell invasion, evasion, and pathogenesis [4–7]. Several virulence factors are secreted in the extracellular milieu, while others are surface-exposed in the *S. aureus* cell wall. Therefore, extracellular vesicles (EVs) are vehicles for transporting and delivering these elements. Indeed, this long-known phenomenon was first described in Gram-positive bacteria in 2009, when Lee et al. reported EV release by *S. aureus* [8].

EVs can be described as biological bubbles for intercellular communication that are released by cells in all domains of life [9–11]. Even though they were first judged as simple cell trash, they are currently recognized as essential elements in cell-to-cell interactions [12–15]. EVs are spheres that are formed of a lipid bilayer ranging from 20 to 300 nm that wraps

and protects biological compounds such as nucleic acids, metabolites, and proteins from the action of external elements [9,11]. These bioactive molecules gain a ticket to a bubble ride in EVs, which can travel through close or distant sites from where they were produced to exert their functions [16,17]. Bacterial EVs play roles in bacterial physiology, resistance, competition, and host-pathogen interactions [13,18–22].

The study of EVs is an emerging field in medical and veterinary medicine of *S. aureus* since several reports have highlighted their contribution to cargo delivery, signaling, and cell–cell communication, contributing to physiological and pathological processes [23]. Indeed, *S. aureus* EVs carry several virulence factors (e.g., β-lactamases, toxins, adhesins) that exert essential functions, including the transfer of antibiotic resistance to susceptible bacteria, host cell death, immunomodulation, and exacerbation of inflammatory processes [8,24–38].

During infection, *S. aureus* undergoes several physiological states (e.g., growth phases) and stresses (e.g., antibiotic treatment) that affect bacterial metabolism, gene expression, and consequently, EV production and composition. Indeed, significant differences in particle size and/or concentration were reported in response to different conditions, such as growth media [39], growth phases [40], and growth temperatures [27,40], as well as after exposure to environmental stresses, such as iron-depletion, ethanol, oxidative, osmotic stresses [27], and antibiotics [41–43]. Concerning EV composition, a recent study by Briaud et al. demonstrated that temperature variations affect the EV protein and RNA cargo quantitatively and qualitatively, which is also reflected in different EV cytolytic activities towards host cells [40]. Culture media, growth phase, and antibiotic treatment were also shown to alter *S. aureus* EV content [39,43–45]. However, studies comparing the impact of different environmental conditions on both *S. aureus* producing cells and its derived EVs are still lacking.

In this context, this work aims to explore the protein content of *S. aureus* strain HG003 and its derived EVs under the influence of four conditions: early- and late-stationary growth phases, and in the absence and presence of a sub-inhibitory concentration of vancomycin. The protein cargo was compared between producing cells and EVs, shedding light on how the environment influences *S. aureus* EV cargo packing.

2. Materials and Methods

2.1. Bacterial Strains and Cultures

The *S. aureus* strain that was used in this work was the model strain HG003 [46], a derivative of NCTC8325 that was isolated in 1960 from a sepsis patient. HG003 contains functional *rsbU* (coding for an activator of the sigma factor B) and *tcaR* (coding for an activator of protein A transcription) genes, two global regulators missing in the NCTC8325 parent strain. The HG003 genome is well documented [47] and this strain is widely used as a reference to investigate staphylococcal regulation and virulence [48]. Here, *S. aureus* HG003 was grown in four experimental conditions: early- and late-stationary growth phases (6 and 12 h, respectively), an in the absence (V−) and presence (V+) of a sub-inhibitory concentration of vancomycin (0.5 µg/mL) that do not affect the HG003 growth [44]. Note that the minimum inhibitory concentration (MIC) of vancomycin for HG003 strain is 0.5 mg/mL [49]. Pre-inoculums and cultures were grown overnight in BHI broth at 37 °C under 150 rpm/min agitation. The bacterial growth was determined by the measurement of the optical density at 600 nm.

2.2. EVs Isolation and Purification

EVs were isolated and purified as previously described [44]. Briefly, 1 L of bacterial cell culture was centrifuged at 6000× *g* for 15 min and filtered through 0.22 µm Nalgene top filters (Thermo Scientific, Waltham, MA, United States). Then, the culture supernatant fraction was concentrated around 100-fold using the Amicon ultrafiltration systems (Millipore, Burlington, MA, United States) with a 100 kDa filter and ultra-centrifuged for 120 min at 150,000× *g* to eliminate the soluble proteins. Next, the suspended pellet was applied to a

discontinuous sucrose gradient (8–68%) and ultra-centrifuged at 100,000× *g* for 150 min. The fractions containing EVs were recovered and washed in TBS (150 mM NaCl; 50 mM Tris-Cl, pH 7.5) for final ultra-centrifugation at 150,000× *g* (120 min). At last, the EVs were suspended in cold TBS and kept at −80 °C until use. The quality of the EV samples (i.e., homogeneity, integrity, and reproducibility) was assessed by transmission electron microscopy (TEM), Nano Tracking Analysis (NTA) and SDS-PAGE.

2.3. Physical Characterization of EVs

The EVs' size and concentration were determined by NTA (NanoSight NS300, Malvern Panalytical, Worcestershire, United Kingdom) as previously described [44]. The EVs were negatively stained with 2% uranyl acetate and analyzed with JEOL 1400 Electron Microscope (Jeol, Tokyo, Japan) as previously described [44].

2.4. Proteome of S. aureus HG003 and Its Derived Vesicles

2.4.1. Protein Extraction and Visualization

The same bacterial cultures were used for EV and whole-cell (WC) protein extraction. Aliquots of 10 mL of bacterial cells were centrifuged and rinsed twice with 10 mL of TBS buffer and suspended in 10 mL of lysis solution (1.52 g Tris-HCL, 0.03 g SDS, 0.3 g DTT, pH 7.5, 10 mL H_2O). For WC, mechanic lysis was performed with Precellys (6500 rpm, 2 × 30 s), and protein extracts were recovered after 30 min centrifugation at 14,000× *g* rpm. Since EVs lack cell walls, lysis is achieved with a lysis solution. Using silver staining, the bacterial WC and EV protein profiles were visualized by SDS-PAGE [50,51].

2.4.2. Protein Identification and Quantification

For NanoLC-ESI-MS/MS analysis, three independent biological replicates of WC and EV protein extracts (approximately 30 µg per sample) were resolved using 12% SDS-PAGE and Biosafe Blue Coomassie coloration. Then, gel sections were cleansed with acetonitrile and ammonium bicarbonate solutions and dried under a SpeedVac concentrator (SVC100H, Savant Instruments Inc, New York, NY, United States). The samples were submitted to overnight in-gel trypsinization at 37 °C [52,53]. Peptide separation and detection by mass spectrometry were performed according to Tarnaud et al. [54]. The X!TandemPipeline software [55] was used to identify peptides (maximum e-value of 0.05) from the MS/MS spectra. The peptides were searched against the two genomes sequence of *S. aureus* NCTC8325 and HG003 (GenBank accessions no. NC_007795.1 and GCA_000736455.1). The database search parameters were specified as follows: trypsin cleavage was used and the peptide mass tolerance was set at 10 ppm for MS and 0.05 Da for MS/MS. Methionine oxidation and serine or threonine phosphorylation were selected as a variable modifications. For each peptide that was identified, a minimum e-value that was lower than 0.05 was considered to be a prerequisite for validation. A minimum of two peptides per protein was imposed, resulting in a protein false discovery rate (FDR) < 0.5% for peptide and protein identifications. Note that a protein was considered present in a given condition when it was detected in at least two out of three biological replicates. Each peptide that was identified by tandem mass spectrometry was quantified using the free MassChroQ software (MassChroQ 2.2.21, PAPPSO, Jouy-en-Josas, France) [56] before data treatment and statistical analysis under R software (R 3.2.2, The R Foundation for Statistical Computing, Vienna, Austria). A specific R package called 'MassChroqR' was used to automatically filter dubious peptides for which the standard deviation of their retention time was longer than 30 s and to regroup peptide quantification data into proteins. For XIC-based quantification, normalization was performed to take account of possible global quantitative variations between LC-MS runs. The peptides that were shared between the different proteins were excluded automatically from the dataset as well as peptides that were present in fewer than 85% of the samples. Missing data were then imputed from a linear regression based on other peptide intensities for the same protein [57]. Analysis of variance was used to determine proteins with significantly different abundances between our two culture conditions.

2.4.3. Proteomic Analysis

Subcellular location and lipoproteins were predicted using SurfG+, PSORTb, and PRED-LIPO [58–60]. The eggNOG-mapper v2 web tool was used to retrieve clusters of orthologous groups (COG) and KEGG protein categories [61]. Venn diagrams were obtained using Draw Venn Diagram [62] and volcano plots were conceived using VolcaNoseR [63]. Functional enrichment analyses were performed with the g: Profiler web server with the g: SCS multiple testing correction methods [64]. An input list of WC and EV proteins was compared to a custom-made Gene Matrix Transposed (GMT) file representing the theoretical proteome of *S. aureus* HG003 to identify statistically significant enriched COG and KEGG pathway terms (significance threshold of 0.05).

3. Results

3.1. Proteome of S. aureus HG003 and Its Derived EVs

Mass spectrometry analyses were performed to investigate the impact of different growth conditions on the proteome of the laboratory *S. aureus* HG003 strain and its derived EVs. WC and EV samples were recovered from early- and late-stationary growth phases (6 and 12 h, respectively) in the absence (V−) and presence (V+) of a sub-inhibitory concentration of vancomycin. Note that these same bacterial cultures were previously employed to characterize the RNA profile of HG003 and its derived EVs [44]. As previously reported by Luz et al., the EV preparation showed typical particle shape and size when analyzed by TEM and NTA (Figure 1A,B) [44]. The SDS-PAGE approach revealed a homogeneous protein profile between conditions in each group (Figure 1C); however, the EV protein profile was specific when compared to WC (Figure S1).

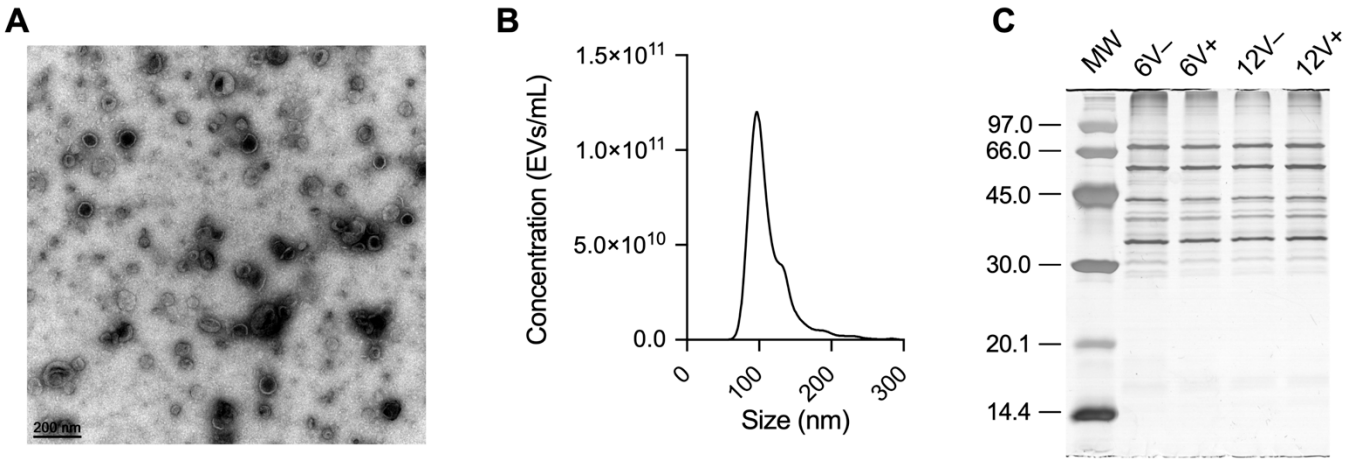

Figure 1. *S. aureus* HG003 EV characterization. (**A**) Cup-shaped EVs by transmission electron microscopy (TEM). (**B**) Monodisperse profile revealed by nanoparticle tracking analysis (NTA). (**C**) Protein profile of EV samples resolved in 12% SDS-PAGE. Molecular weight (MW) standards are indicated in kDa. Early- and late-stationary growth phases (6 and 12 h, respectively) in the absence (V−) or presence (V+) of vancomycin.

3.2. Protein Composition and Functional Characterization of S. aureus HG003 and Its EVs

Nano LC-ESI-MS/MS analyses identified 1111 unique proteins in all conditions, of which 967 were found in WC samples, and 556 were found in EVs samples (Table S1). Principal component analysis (PCA) was first performed on peptide quantification values to assess the consistency of the proteomics data (Figure S2). The PCA scatter plot showed that samples from WC and EVs formed separate clusters. All the WC samples formed a unique cluster, while the EV samples were grouped into two clusters depending on whether the EVs were produced at 6 or 12 h. The impact of vancomycin availability on EV protein composition seemed to be limited compared to the incubation time. The PCA confirmed

the consistency between biological replicates under each condition and pointed out the high impact of growth conditions on EV protein composition compared to WC.

Then, analyses were performed to investigate the composition and functions that were associated with WC and EV proteomes. The predictions of subcellular localization revealed that cytoplasmic proteins composed from 71 to 84% of the WC proteome, while these values dropped down to 42–55% for EVs (PSORTb, *n* = 668 vs. 236; SurfG+, *n* = 808 vs. 287; PRED-LIPO, *n* = 814 vs. 309) (Figure 2A–C). In contrast, WC presented less membrane proteins (6–10%) when compared to EVs (23–39%) (PSORTb, *n* = 133 vs. 220; SurfG+, *n* = 58 vs. 128; PRED-LIPO, *n* = 99 vs. 177) (Figure 2A–C). Interestingly, EVs seemed enriched with surface-exposed proteins (PSE) (SurfG+, *n* = 108 vs. 67) and lipoproteins (SurfG+, *n* = 44 vs. 23; PRED-LIPO, *n* = 41 vs. 22) (Figure 2B,C). Remarkably, proteins that were identified only in EVs (*n* = 144) corresponded mainly to membrane (*n* = 75) and PSE (*n* = 45) proteins, according to SurfG+ analysis.

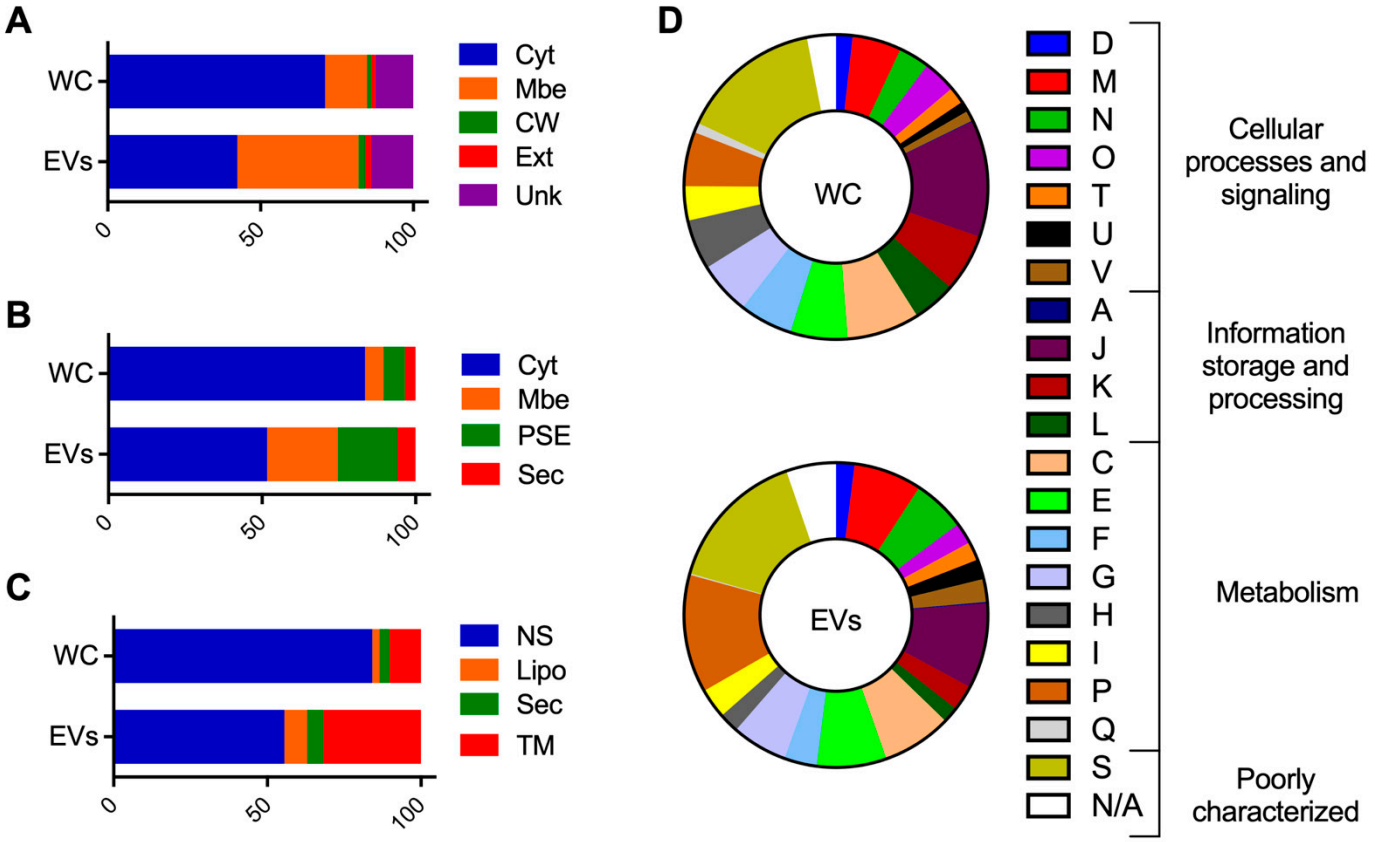

Figure 2. Comparative proteome analysis of *S. aureus* HG003 WC and EVs. The prediction of subcellular locations of proteins with PSORTb (**A**), SurfG+ (**B**), and PRED-LIPO (**C**), and the prediction of COG functional categories of proteins with eggNOG-mapper (**D**). Cyt, cytoplasmatic; Mbe, Membrane; CW, cell wall; Ext, extracellular; Unk, unknown; PSE, surface-exposed; Sec, secreted; NS, no signals found; Lipo, lipoprotein; TM, transmembrane; COG functional categories: D, Cell cycle control, cell division, chromosome partitioning; M, cell wall/membrane/envelope biogenesis; N, cell motility; O, post-translational modification, protein turnover and chaperones; T, signal transduction mechanisms; U, intracellular trafficking, secretion and vesicular transport; V, defense mechanisms; A, RNA processing and modification; J, translation, ribosomal structure, and biogenesis; K, transcription; L, replication, recombination, and repair; C, energy production, and conversion; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport, and catabolism; S, function unknown; NA, not available.

Cellular processes that were related to COG also differed between WC and EV proteomes (Figure 2D). Generally, fewer EV proteins belonged to COG categories that were associated with "information, storage, and processing" when compared to WC (13.6% vs. 23.2%). On the other hand, COG categories that were related to "cellular processes and signaling" and "metabolism" accounted for more proteins in EVs (23.6% and 42.2%) compared to WC samples (17.8% and 40.8%). Remarkably, some COGs were twice more represented in EVs when compared to the WC, such as intracellular trafficking, secretion, and vesicular transport (U, 2.1% vs. 1.1%), defense mechanisms (V, 2.4% vs. 1.1%), and inorganic ion transport and metabolism (P, 12.5% vs. 5.7%) (Figure 2D). Note that similar results from the composition and functions of WC and EV proteomes were obtained when each growth condition was analyzed individually (Figures S3–S6).

Functional enrichment analysis relative to the theoretical proteome of *S. aureus* HG003 also identified different enriched COG and KEGG categories for WC and EVs (adjusted p-value < 0.05). While both shared proteins that were related to metabolism, translation, and energy production, EVs were exclusively enriched with proteins that were linked to cell wall biogenesis, two-component systems, and transport systems (Figure 3). In summary, the vesicular proteome differs from that of WC, showing less cytoplasmic and more surface-exposed-, membrane-, and lipoproteins, being ~25% exclusive to EVs. Additionally, COG/KEGG enrichment analyses reinforce that functional protein profiles that are found in EVs significantly differ from those of the WC.

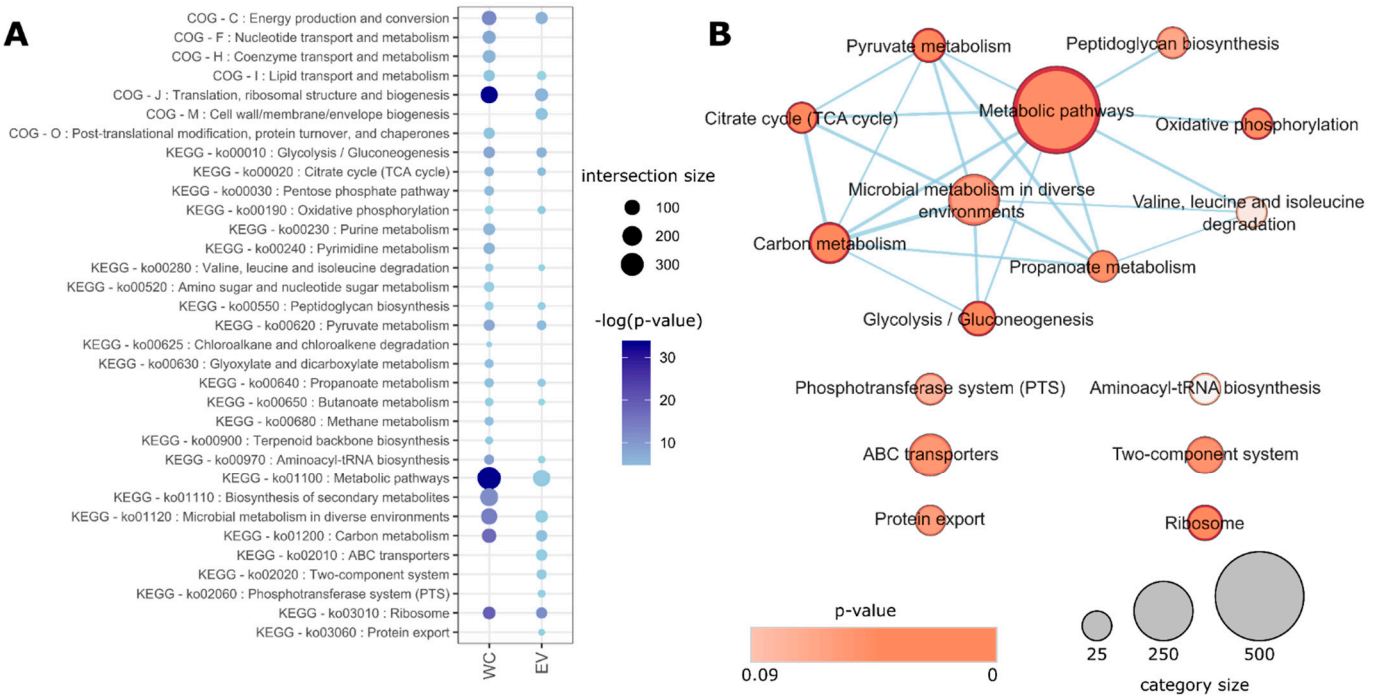

Figure 3. KEGG and COG categories that are enriched in *S. aureus* HG003 WC and EV proteomes. (**A**) Significantly enriched COG and KEGG terms that were associated with WC and/or EV groups. Darker colors represent more significant p-values (in logarithm scale) and bubble sizes represent the number of proteins in that category that were identified in the respective sample (the intersection size). (**B**) Enrichment map for EV-related KEGG terms, showing significant functional categories, their total sizes, and relationships (protein sharing).

3.3. Cellular and EV Protein Composition Varies with Growth Conditions

The WC and EV protein compositions were compared between early- and late-stationary phases, in the absence or presence of vancomycin. From 1111 unique proteins identified with Nano LC-ESI-MS/MS experiments, 555 and 144 were exclusive to WC and EVs, respectively (Figure 4A, Table S1). Proteins that were identified only in EVs

included the enzymes thermonuclease (Nuc) and lipase (Lip), the adhesin Eap, and the Hld toxin. Although the different environmental conditions that were tested in this study resulted in several variations in protein content, the EV core proteome was represented by 340 proteins (61.1%), while this portion was higher for the WC core proteome (78.8%, *n* = 762) (Figure 4B,C). Proteins that were shared by EVs in all conditions comprised of virulence factors (Atl, Sbi, EbpS, Lip), metabolic enzymes (pyruvate dehydrogenase complex PdhABCD, Enolase), survival elements (SirA, FhuD1, FtnA), and resistance proteins (VraS, FmtA, PBPs).

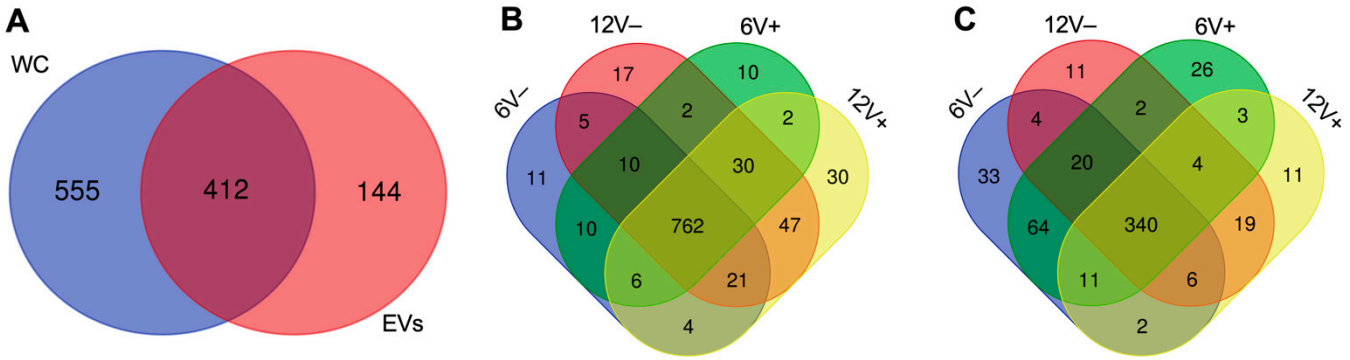

Figure 4. *S. aureus* HG003 WC and EV proteomes are differently modulated by growth conditions. Venn diagrams of all proteins that were identified in HG003 WC and EVs (**A**), and proteins that were identified in different growth conditions in WC (**B**) and EVs (**C**). Early- and late-stationary growth phases (6 and 12 h, respectively) in absence (V−) or presence (V+) of vancomycin.

Similar to a recent report that was published by our group on the RNA content of HG003 EVs in the same conditions, EV protein loads were higher at 6 h than at 12 h (515 vs. 433 proteins identified) [44]. EV proteins that were found at 6 h (*n* = 123), but not at 12 h, included autolysins (Sle1 and AcmB), the immunogenic protein SsaA, and the toxin LukH. Remarkably, proteins that were exclusive to EV samples at 12 h (*n* = 41) included Hld and PSMβ1. On the other hand, WC presented more proteins at 12 h than at 6 h (936 vs. 873). The analysis identified Pbp4, SepF, and GroES among those that were exclusive to WC at 6 h (*n* = 31), while RsfS, linked to ribosomal activity, the transcription regulator FapR, and the virulence factors Emp, SCIN, and LukG were present only in late-stationary growth phase samples (*n* = 94). Our data indicated a moderate antibiotic effect in both groups (EVs = 508 vs. 516; WC = 934 vs. 925, for the presence and absence of vancomycin, respectively). In EVs, 40 proteins were identified only in the presence of vancomycin (e.g., IsaB, FhuD1), with only 3 proteins that were found at both 6V+ and 12V+ conditions: the ribosomal protein RplQ, the cation/H+ antiporter MnhG, and the putative PitA protein that were linked to inorganic phosphate transport. Among the 48 EV proteins that were found only in the absence of vancomycin, 24 belonged to metabolic-related COG groups. In WC, the division protein DivlC belonged to the group of 33 proteins that were found only in the absence of vancomycin, while proteins that were related to DNA repair such as Nfo and Fpg were among the 42 proteins that were found only in the presence of vancomycin.

3.4. Cellular and EV Protein Abundance Varies with Growth Conditions

Quantitative analysis provided interesting information on the state of the cell and its derived EVs in different growth conditions (Table S2). Regarding to the growth phase, 64 and 110 proteins were differently abundant in WC samples in the absence and presence of vancomycin, respectively. In WC samples without vancomycin, 25 proteins were more abundant at 6 h (e.g., Atl, IsaA, VicK), while 39 were more abundant at the late-stationary growth phase (e.g., IsaB, ModA, MreC) (Figure 5A). In the presence of vancomycin, 57 WC proteins were more abundant at 6 h (e.g., IsaA, PrkC, DltD), while 53 were more abundant

at 12 h (e.g., Asp23, CcpA, IsaB, MreC) (Figure 5B). Compared to the WC samples, the protein content of EVs were less impacted by the growth phase, with only 23 and 9 proteins differently abundant in the absence and presence of vancomycin, respectively (Figure 5C,D). In both conditions, the virulence factor regulator SarR was more abundant at 6 h, and proteins AtpG, GlpF, TreP, and YqeZ were more abundant at 12 h (Figure 5C,D). In the absence of vancomycin, SitA, TagF1, and PTS transporters were among the 16 proteins that were richer at 12 h, while FusA was less abundant (Figure 5C). The presence of vancomycin hardly affected the protein abundance in either WC or EVs, with only a few exceptions. In WC, HemB and the lactonase Drp35 were less abundant at 6V− when compared to 6V+, presenting log2 fold change (log2FC) values of −2.14 and −5.01, respectively (Table S2). In EVs, proteins that were more abundant in the presence of vancomycin at 12 h were the polypeptide cell division protein DivIVA (log2FC = 3.36) and the S-ribosylhomocysteine lyase LuxS (log2FC = 3.20) (Table S2).

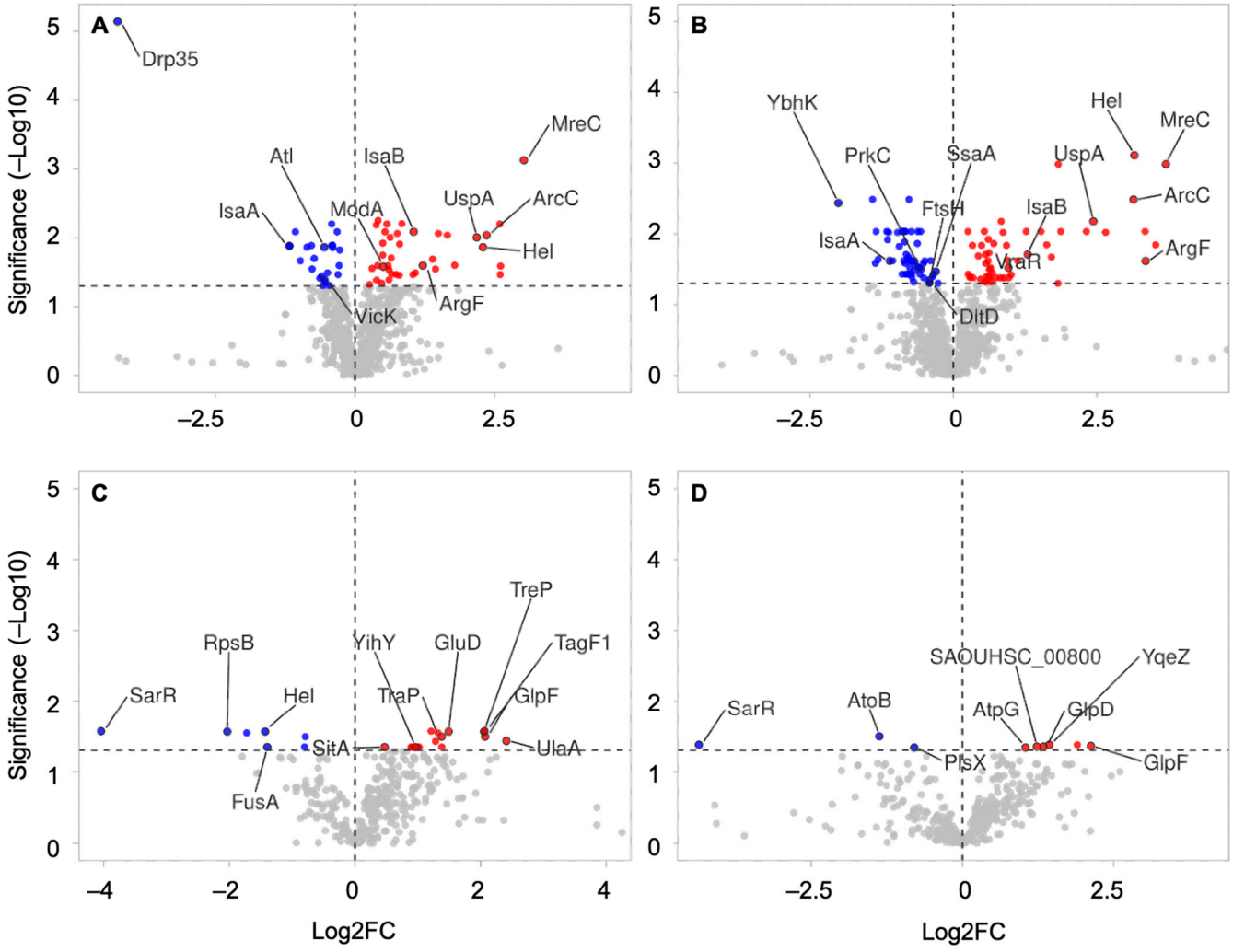

Figure 5. Volcano plot showing differently abundant proteins between growth phases. The graphs provide the log2 fold change (log2FC) ratios between 12 h and 6 h (12/6) conditions from WC without vancomycin (**A**), WC with vancomycin (**B**), EVs without vancomycin (**C**), and EVs with vancomycin (**D**). The vertical and horizontal dotted lines show the FC and threshold of significance (adjusted *p*-value < 0.05), respectively. The results are plotted in dots on a logarithmic scale, with negative log2FC in blue, positive log2FC in red, and nonsignificant values in grey.

Astonishing differences were mainly observed in the relative protein abundance between WC and its derived EVs. Table 1 displays the top ten more and less abundant

proteins in EVs relative to WC in different conditions. Ribosomal components and other proteins that were related to translation and transduction were generally less abundant in EVs when compared to WC (Table S2). Conversely, key virulence factors that were related to adhesion and colonization, resistance, and survival were more abundant in *S. aureus* HG003-derived EVs than in the bacteria. Some proteins were more abundant in EVs whatever the condition that was tested (e.g., Atl, CamS, CydB, DltD, FatB, FecB, FtsH, HtrA, LtaS, ModA, SitA, YidC) (Figure 6, Table S2). Other virulence factors such as the adhesin EbpS, the protein kinase PkrC, the multidrug resistance efflux pump EmrA, ABC transporters (e.g., AdcA, FhuD), the quinol oxidase complex QoxAB, and PBPs were more abundant in EVs in multiple conditions (Figure 6, Table S2).

Table 1. The top ten most abundant and less abundant proteins in EVs compared to WC in all conditions that were tested. Ratios correspond to the relative abundance of proteins (log2FC) between EVs and WC (EV/WC) conditions at 6 h without vancomycin (6V−), 6 h with vancomycin (6V+), 12 h without vancomycin (12V−), and 12 h with vancomycin (12V+).

6V−		**6V+**		**12V−**		**12V+**	
Protein	**Ratio**	**Protein**	**Ratio**	**Protein**	**Ratio**	**Protein**	**Ratio**
RplV	−3.08	Hup	−4.35	RpsJ	−3.62	Ctc	−3.38
RpsJ	−2.91	RplK	−3.91	RplM	−3.23	RplQ	−3.24
RpsI	−2.91	RplQ	−3.85	RpsE	−3.17	RpsE	−3.07
TraP	−2.87	Ldh	−3.27	RplR	−3.13	Ldh	−3.04
RplO	−2.82	PyrH	−2.88	RplL	−3.09	AtoB	−2.99
RpsE	−2.56	Ddh	−2.68	RplS	−2.58	RplM	−2.54
Ald	−2.43	RplL	−2.66	RpsK	−2.51	Pgk	−2.37
RplS	−1.78	RpsQ	−2.65	RpsD	−2.48	RpsD	−2.36
GluD	−1.52	RpsI	−2.41	Ddh	−2.4	Ddh	−2.31
RpsC	−1.29	RplO	−2.25	Pgk	−2.17	AdhP	−2.17
Atl	3.61	CyoA	3.14	Atl	4.38	* 00356	5.06
NupC	3.79	Pbp4	3.78	FatB	4.66	LpdA	5.1
CamS	4.01	ModA	4.02	SrtA	4.71	RecN	5.12
YidC	4.11	SrtA	4.05	SpsB	4.78	* 00717	5.16
* 00356	4.41	AgcS	4.61	PonA	5.18	SitA	5.22
SitA	4.56	PdhB	4.8	TcyA	5.21	SpsB	5.25
TcyA	4.64	TcyA	4.86	* 02587	5.34	TcyA	5.34
PdhB	4.67	LpdA	4.94	FhuD	5.38	FhuD	5.42
FhuD	5.05	* 00356	5.17	SitA	5.45	* 02587	5.5
LpdA	5.13	* 02650	6.13	PdhC	6.54	* 02650	5.83

* = SAOUHSC_ (locus tag). Example: SAOUHSC_00356.

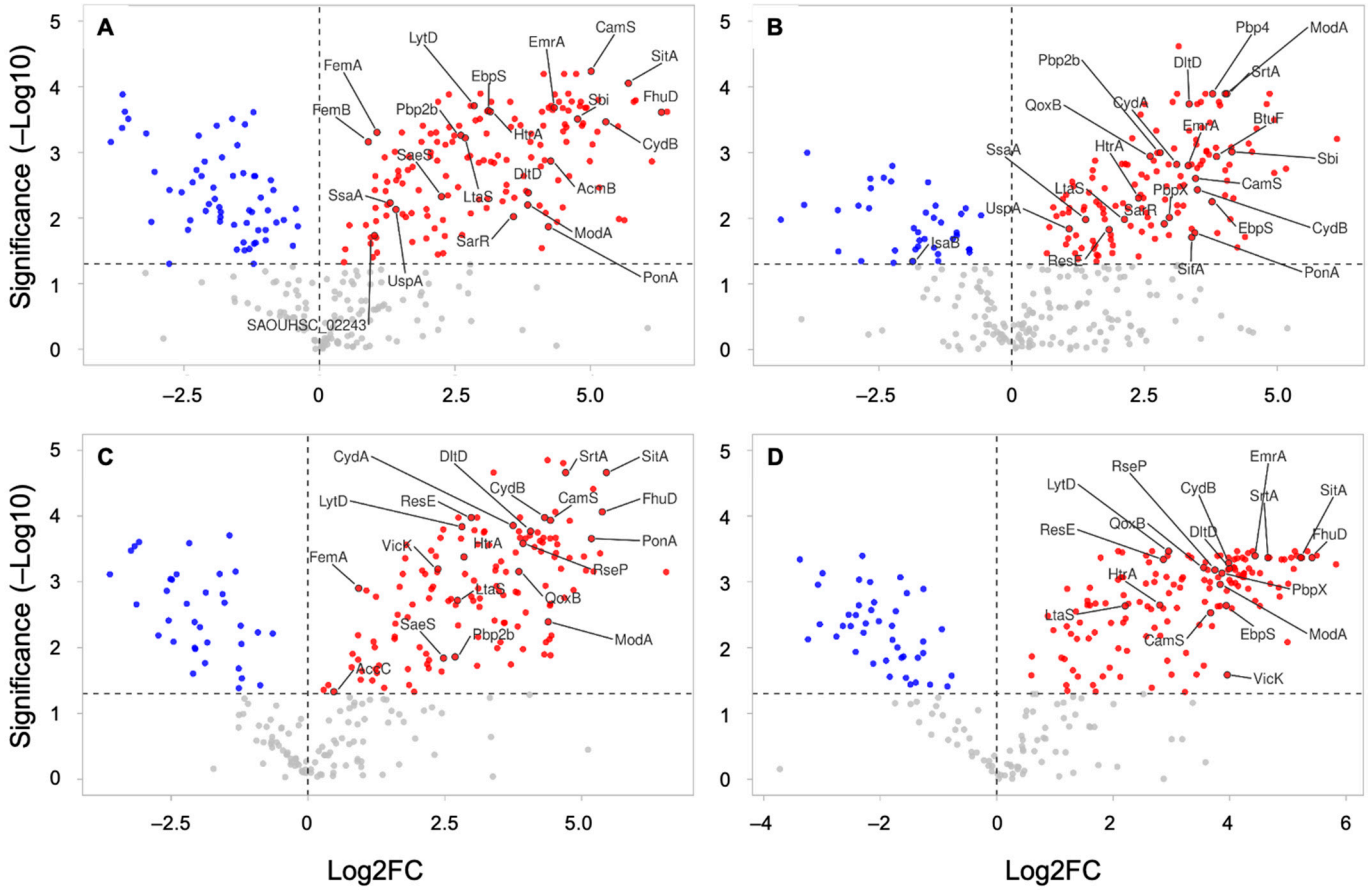

Figure 6. Volcano plots showing the relative protein abundance between *S. aureus* WC and its derived EVs. The graphs provide protein log2 fold change (log2FC) ratios between EVs and WC (EV/WC) conditions at 6 h without vancomycin (6V−) (**A**), 6 h with vancomycin (6V+) (**B**), 12 h without vancomycin (12V−) (**C**), and 12 h with vancomycin (12V+) (**D**). The vertical and horizontal dotted lines show the log2FC and significance threshold (adjusted *p*-value < 0.05), respectively. The results are plotted on a logarithmic scale, with negative log2FC in blue, positive log2FC in red, and nonsignificant values in grey.

Some differences that were observed in the relative protein abundance between EVs and WC seemed to be affected by growth conditions. For instance, the immunoglobulin-binding protein Sbi, the secretory antigen SsaA, and the transcriptional regulator SarR were relatively more abundant in EVs than WC only at 6 h, while no significant differences were observed at 12 h (Figure 6). On the other hand, the histidine kinase VicK and the protein SecA were relatively enriched in EVs only at 12 h. Some differences were also observed regarding to the presence of vancomycin. PBP4 was relatively more abundant in EVs than WC at 6V+ vs. 6V− (log2FC = 3.78), while PBPX was more abundant in EVs compared to WC at V+ vs. V− in both growth conditions (Log2FC at 6 h = 2.96 and Log2FC at 12 h = 3.87). The moonlighting protein enolase was less abundant in EVs only in the presence of vancomycin (6V+ and 12V+), while EF-Tu and GAPA1 were less abundant in EVs than in the WC at 12 h (V− and V+). Our data showed that the protein content and abundance of *S. aureus* HG003 and its derived EVs vary according to the growth conditions. Moreover, important virulence factors were differentially abundant in WC and EVs, suggesting the selective packing of proteins into HG003 EVs.

3.5. Comparison between Protein and RNA Content of HG003 EVs

Comparative analyses between previously published HG003 EV RNome and proteomic data showed that of a total of 220 mRNAs [50], 116 (52.7%) were not found in

the corresponding protein form in HG003 EVs. Another 88 mRNAs (40%) were present in HG003 EVs in at least one of the four conditions in their corresponding protein form. Finally, 16 elements (7.3%) were present in all conditions that were tested in both RNA and protein forms, including Eno, DnaK, Atl, QoXB, SecY, FusA, and PdhABC (Table 2).

Table 2. *S. aureus* HG003 elements that were found in EVs in both RNA and protein form in all the conditions that were tested.

Locus Tag	Product	Gene	SurfG+	COG
SAOUHSC_00994	bifunctional autolysin precursor, putative	*atl*	Sec	M
SAOUHSC_01683	molecular chaperone DnaK	*dnaK*	Cyt	O
SAOUHSC_00799	phosphopyruvate hydratase	*eno*	Cyt	G
SAOUHSC_00529	elongation factor G	*fusA*	Cyt	J
SAOUHSC_00206	L-lactate dehydrogenase	*ldh1*	Cyt	C
SAOUHSC_01040	pyruvate dehydrogenase α subunit, putative	*pdhA*	Cyt	C
SAOUHSC_01041	pyruvate dehydrogenase β subunit, putative	*pdhB*	Cyt	C
SAOUHSC_01042	branched-chain α-keto acid dehydrogenase	*pdhC*	Cyt	C
SAOUHSC_00796	phosphoglycerate kinase	*pgk*	Cyt	F
SAOUHSC_01002	quinol oxidase AA3, subunit II, putative	*qoxA*	PSE	C
SAOUHSC_01001	quinol oxidase, subunit I	*qoxB*	Memb	C
SAOUHSC_02478	50S ribosomal protein L13	*rplM*	Cyt	J
SAOUHSC_02506	30S ribosomal protein S3	*rpsC*	Cyt	J
SAOUHSC_00528	30S ribosomal protein S7	*rpsG*	Cyt	J
SAOUHSC_00527	30S ribosomal protein S12	*rpsL*	Cyt	J
SAOUHSC_02491	preprotein translocase subunit SecY	*secY1*	Memb	U

4. Discussion

Since the first observation describing the EV release by *S. aureus* in 2009 [8], various studies have reported the EV production and secretion by several human clinical isolates and by strains that were isolated from other mammalian species [44]. The study of EVs biogenesis, release, and cargo sorting provides insight into the importance of these particles for bacterial physiology and survival, in addition to a better understanding of how EVs exert their functions [19,65–67]. However, to date, few *S. aureus* EV studies have considered the impact of different environmental conditions on vesicle production and composition [27,39,40,43,68]. A previous report by our group provided the first extensive characterization of the *S. aureus* HG003 EV RNome under four growth conditions: early- and late-stationary growth phases, and in the absence and presence of a sub-inhibitory concentration of vancomycin (0.5 µg/mL) [44]. Here, we used a proteomic approach to investigate how these conditions impact HG003 EV protein content. We also provide the first comparison of protein composition and abundance between EVs and their producing bacterial cells.

Our data indicated that HG003 EV proteome composition differed from that of the producing cells, presenting a higher proportion of membrane, surface-exposed and lipoproteins, and less cytoplasmic proteins compared to bacteria. Nevertheless, cytoplasmic proteins composed almost 50% of the EV proteome, an interesting feature that allows the secretion of proteins lacking export signals. The proportions that were observed here have already been reported in a study comparing EVs that were derived from five different *S. aureus* clinical and animal isolates, which presented similar protein compositions [25]. In addition to the different proportions that were observed in protein subcellular localization, HG003 EVs also presented exclusive features. We found that one-quarter of the total EV pro-

teins were not detected in the bacterial proteome, although they were analyzed under the same conditions. It has already been suggested that Gram-negative bacteria manage to 'exclude' from EVs proteins that are abundant in the outer membrane (OM), which are usually associated with outer membrane vesicles (OMVs) biogenesis and composition [65,69,70]. In fact, EVs that are derived from various Gram-positive bacterial species are especially loaded with virulence factors [71–74]. Here, we demonstrate that most HG003 EV exclusive elements are membrane-associated or surface-exposed proteins, including the adhesin Eap and the δ-hemosylin toxin, which are key staphylococcal virulence factors. These differences in protein identification suggest differential cargo yield in EVs and bacteria, which may help EVs exert specific functions during infection.

Variations in the protein content of both WC and EVs were detected in different conditions. Regarding the growth phase, proteins that are exclusive to WC at 6 h included SepF, linked to cell division and GroES, a co-chaperonin that is essential in assisting protein folding [75,76]. These proteins are related to active dividing cells, corresponding to a bacterial population in the exponential growth phase. In contrast, the presence of the RsfS ribosomal silencing protein [77] and the FapR repressor of membrane lipid biosynthesis [78] at 12 h indicates *S. aureus* arrested cell division. In EVs, we found that immunogenic proteins such as SsaA are present only at the early-stationary growth phase. It has already been demonstrated that pre-inflamed lungs allow bacteria to hijack and resist host defense mechanisms in allergic asthma [79,80]. In this line, the presence of immunostimulatory molecules in EVs at lower cell densities could help to activate host cells even before bacterial arrival at distant sites, favoring *S. aureus* survival and persistence. It is also remarkable to note that LukH is present in EVs only at 6 h and in WC at all conditions, while LukG is found only in WC at 12 h. It has been demonstrated that individual components of LukH and LukG cannot promote host cell lysis, yet they each induce high levels of IL-8 release [81]. EVs may serve as vehicles to induce the host immune response at early infection states, while functional pore-forming toxin LukGH appears only later. Accordingly, Hld and PSMβ1 are exclusive to EVs at 12 h, which corresponds to the known toxin production by bacteria at stationary phases [6,82]. These pore-forming toxins promote host cell death and immunomodulation [83,84], a role that could be complementary played by EVs.

Regarding to the impact of antibiotics on the protein cargo, we observed that Nfo and Fpg proteins that were linked to DNA repair were identified in WC only in the presence of vancomycin. Accordingly, it has already been demonstrated that SOS response in *S. aureus* is slightly upregulated when facing cell wall inhibitory antibiotics [85,86]. Curiously, the putative inorganic phosphate PitA transporter that is found in EVs only in the presence of vancomycin has been previously associated with *S. aureus* tolerance to several antibiotics, including vancomycin [87,88].

Globally, our data showed that the conditions that were tested here had a more pronounced impact on the EV protein composition than on the WC. These results show that EV cargo is not an exact reflection of the protein cargo of producing cells. Slight variations applied to EVs may serve as a fine-tuning mechanism to help the bacterial population rapidly and efficiently respond to external conditions. Yet, despite the variations that were found, the EV core proteome still carries proteins that are linked to virulence (e.g., Ebps, Sbi), metabolism (e.g., Eno, PdhABCD), survival (e.g., FhuD), and resistance (FmtA, PBPs). Many of these elements are also found across several *S. aureus* strains [23,25] and may play active roles in the infection and pathogenesis of this bacterium.

Interestingly, differences in protein abundance were observed when comparing growth phases in WC and EVs. In WC, the Asp23 protein was more abundant at 12 h V+ compared to 6 h V+, but not in the absence of antibiotics. Accordingly, a 10-fold expression increase of the *asp23* gene has been shown after treatment with vancomycin [89]. The fact that Asp23 is more abundant at 12 h may reflect the accumulation of this protein after exposure to this antibiotic. In EVs, PTS transporters were more abundant at the late-stationary growth phase. Considering that bacteria face nutriment stress at high cell densities, EVs could help bacteria to improve sugar uptake. Finally, the impact of the absence and presence of

antibiotics on protein abundance in the same conditions (either 6 h or 12 h) was scarce. In WC, the protein Drp35 was more abundant at 6 h in the presence of vancomycin. Accordingly, this protein acts in cells with perturbed membrane integrity, and its activation has been consistently reported in response to certain antibiotics, including vancomycin [90]. Moreover, it was interesting to note that LuxS was more abundant in EVs at 12 h in the presence of vancomycin. LuxS is involved in the synthesis of autoinducer 2 (AI-2), an essential element of the *S. aureus* agr quorum-sensing (QS) system [91]. Dysfunctional agr has been associated with glycopeptide intermediate-level resistant *S. aureus* (GISA) [92,93], and the use of QS inhibitors increases *S. aureus* susceptibility to vancomycin [94,95]. In addition, the QS also contributes to biofilm structuring and detachment through the activation and expression of proteases and other molecules with surfactant-like properties, such as PSMs [82,96–98]. In light of this, EVs that are derived from the stationary growth phase containing exclusive elements such as PSMβ1 may play a role in biofilm formation. It has already been shown that EVs that are purified from *S. aureus* BWMR22 strain that are grown in the presence of a sub-inhibitory concentration of vancomycin are able to increase bacterial adhesion and cell aggregation, contributing to *S. aureus* biofilm formation [99]. Interestingly, *S. aureus* EVs' ability to increase surface hydrophilicity confers a competitive advantage by reducing biofilm formation by several other pathogenic bacteria, including *Acinetobacter baumannii*, *Enterococcus faecium*, and *Klebsiella pneumonia* [100]. Together, our data suggests that EVs may play a role in response to vancomycin stimuli and in biofilm formation through QS. The role of EVs in the physiology and fitness of the *S. aureus* global population may be addressed in future studies.

To our surprise, the most remarkable differences that were observed in this study were related to the relative protein abundance in EVs and bacteria. Even though ribosomes are highly abundant in cells [101], we found that proteins that are related to translation were relatively less abundant in EVs when compared to bacteria, while key virulence factors were more abundant. Highly loaded proteins in EVs included those that are involved in adhesion, colonization, survival, resistance, and modulation of the host immune response. One example is the Sbi protein, which was relatively more abundant in EVs at 6 h. This immunoglobulin-binding protein could interfere with complement activation, opsonophagocytosis and neutrophil killing [102,103]. In fact, it has been proposed that surface-localized Sbi protein in *S. aureus* ATCC14458 EVs may favor binding to host cells [8]. Interestingly, PBPX and PBP4 were relatively more abundant in EVs compared to bacteria only in the presence of vancomycin. We could speculate that the information that is perceived by bacteria in response to slight stimuli may somehow be transferred into EVs without largely affecting bacterial composition. These modified EVs could act as vehicles to promote bacterial resistance to antibiotics, among other important functions. Indeed, recent studies demonstrated that antibiotic stress contributes to the increased loading of β-lactam degrading proteins in *S. aureus* EVs, which protect ampicillin-susceptible bacteria in a dose-dependent manner [24,43].

Finally, although similar tendencies were observed between the HG003 EV protein and RNA content [44], such as EVs being more loaded at 6 h than 12 h, matches in coding mRNAs and proteins were not obvious. More than half of HG003 EV mRNAs [44] were not found in their corresponding protein forms. Some exceptions include the autolysin Atl, the moonlighting protein Eno, the elongation factor FusA, and the chaperone DnaK, which are present in all conditions in both RNA and protein forms. Their constant appearance in the EV proteome of several *S. aureus* strains and other species, including Gram-negative bacteria, suggests their role in EV biogenesis [25]. Indeed, Atl has already been associated with EVs release in *S. aureus* [41], while chaperones were shown to participate in selecting and packing proteins into EVs in Gram-negative bacteria [104,105].

5. Conclusions

This work provides a general proteomic picture of EVs that were derived from the *S. aureus* HG003 strain under different growth conditions. Additionally, comparing the

EV proteome to that of producing cells in the same conditions brought new insights into *S. aureus* EV characteristics. Our data demonstrate that HG003 EV content and abundance are modulated by the environment and differ from that of bacterial cells, supporting the hypothesis that EV release is a mechanism that is ruled by selective cargo packing. New and exciting studies will continue to unveil the processes governing *S. aureus* EV biogenesis and production, and their role in bacterial physiology and pathogenesis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10091808/s1, Figure S1: Protein profile of *S. aureus* HG003 WC and EVs. SDS-PAGE (12% gel) showing the protein profile of triplicates of WC (**A**) and EVs (**B**) in each condition. Molecular weight (MW) standards are indicated in kDa. Early- and late-stationary growth phases (6 and 12 h, respectively) in the absence (V−) or presence (V+) of vancomycin.; Figure S2: Principal component analysis (PCA) of peptide abundance in samples under each condition and across biological replicates. The percentages of variance that is associated with each axis are displayed. − and + corresponds to conditions in the absence and presence of vancomycin, respectively; Figure S3. Comparative proteome analysis of *S. aureus* HG003 WC and EVs at 6 h without vancomycin (6V−). Prediction of sub-cellular locations of proteins with PSORTb (**A**), SurfG+ (**B**), and PRED-LIPO (**C**), and the prediction of COG functional categories of proteins with eggNOG-mapper (**D**). Cyt, cytoplasmatic; Mbe, membrane; CW, cell wall; Ext, extracellular; Unk, unknown; PSE, surface-exposed; Sec, secreted; NS, no signals found; Lipo, lipoprotein; TM, transmembrane; COG functional categories: D, Cell cycle control, cell division, chromosome partitioning; M, cell wall/membrane/envelope biogenesis; N, cell motility; O, Post-translational modification, protein turnover and chaperones; T, signal transduction mechanisms; U, Intracellular trafficking, secretion and vesicular transport; V, defense mechanisms; A, RNA processing and modification; J, translation, ribosomal structure and biogenesis; K, transcription; L, replication, recombination, and repair; C, energy production and conversion; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport, and catabolism; S, function unknown; NA, not available; Figure S4. Comparative proteome analysis of *S. aureus* HG003 WC and EVs at 6 h with vancomycin (6V+). Prediction of subcellular locations of proteins with PSORTb (**A**), SurfG+ (**B**), and PRED-LIPO (**C**), and prediction of COG functional categories of proteins with eggNOG-mapper (**D**). For legend, please refer to Figure S3; Figure S5. Comparative proteome analysis of *S. aureus* HG003 WC and EVs at 12 h without vancomycin (12V−). Prediction of subcellular locations of proteins with PSORTb (**A**), SurfG+ (**B**), and PRED-LIPO (**C**), and prediction of COG functional categories of proteins with eggNOG-mapper (**D**). For legend, please refer to Figure S3; Figure S6. Comparative proteome analysis of *S. aureus* HG003 WC and EVs at 12 h with vancomycin (12V+). Prediction of subcellular locations of proteins with PSORTb (**A**), SurfG+ (**B**), and PRED-LIPO (**C**), and prediction of COG functional categories of proteins with eggNOG-mapper (**D**). For legend, please refer to Figure S3; Table S1: Characteristics of proteins of *Staphylococcus aureus* strain HG003 and their detection by mass spectrometry in the whole cell (WC) and extracellular vesicles (EVs) fractions.; Table S2: Comparative analysis of the relative abundance of proteins that were identified in *Staphylococcus aureus* strain HG003 whole cell (WC) and extracellular vesicles (EVs) fractions.

Author Contributions: Conceptualization, B.S.R.d.L., S.C., Y.L.L., V.A.d.C.A. and E.G; Methodology, B.S.R.d.L., S.C., Y.L.L. and É.G.; Investigation, B.S.R.d.L., V.B.-B. and S.C.; Data curation, A.N. and J.J.; Formal analysis, B.S.R.d.L., J.J., V.d.R.R., A.N. and É.G.; Visualization, B.S.R.d.L., V.d.R.R., A.N. and É.G.; Project administration, V.A.d.C.A. and É.G.; Funding acquisition, V.A.d.C.A., Y.L.L. and É.G.; Writing—original draft preparation, B.S.R.d.L. and É.G.; Writing—review and editing, B.S.R.d.L., V.d.R.R., S.C., V.B.-B., J.J., A.N., Y.L.L., V.A.d.C.A. and É.G. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The mass spectrometry proteomics data can be found at [106].

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Chapter 5. Additional results – Part 1

Intra-species and inter-kingdom impact of *Staphylococcus aureus*-derived extracellular vesicles

5.1 Introduction

Staphylococcus aureus is a successful opportunistic pathogenic bacterium with global impact (TONG et al., 2015). Despite commonly colonizing healthy humans, this bacterium can develop a wide range of diseases when conditions are favorable (SAKR et al., 2018; VAN BELKUM et al., 2009a; WERTHEIM et al., 2005a). Human diseases comprise several acute and chronic infections (e.g. bacteremia, pneumonia, osteomyelitis, skin and soft tissue manifestations), and toxin-induced syndromes (FISHER; OTTO; CHEUNG, 2018; LOWY, 1998; MISHRA; YADAV; MISHRA, 2016; SILVERSIDES; LAPPIN; FERGUSON, 2010; SUNDERKÖTTER; BECKER, 2015).With the rise in antibiotic resistance in recent years, treatment against *S. aureus* is becoming increasingly unavailable (O'BRIEN; MCLOUGHLIN, 2019). Therefore, a better understanding of bacterial physiology and pathogenesis is essential in searching for strategies to fight staphylococcal-associated infections.

Special attention has recently been given to bacterial extracellular vesicles (EVs). EVs are nanoparticles composed of bilayered lipidic membranes released in the extracellular environment by living cells from all kingdoms (DEATHERAGEA; COOKSONA, 2012; GILL; CATCHPOLE; FORTERRE, 2019). These daughter particles carry components their parent cells produce, including biomolecules such as nucleic acids, metabolites, and proteins (STAHL; RAPOSO, 2019; VAN NIEL; D'ANGELO; RAPOSO, 2018). It has been shown that *S. aureus* produces EVs exerting several functions, such as the transfer of transient antibiotic resistance to susceptible bacteria (KIM et al., 2020; LEE et al., 2022, 2013), the prevention of biofilm formation by other bacterial species (IM et al., 2017), immunomodulation, and infection exacerbation (ASANO et al., 2021; BITTO et al., 2021a; CHEN et al., 2011; HONG et al., 2014; JUN et al., 2017; KIM et al., 2012; LUZ et al., 2021a; TARTAGLIA et al., 2018; WANG et al., 2018b, 2021b; WANG; EAGEN; LEE, 2020).

Transcriptomic and proteomic analyses revealed that EVs from the *S. aureus* HG003 strain contain several RNAs and proteins involved in bacterial metabolic processes and

virulence (see chapters 3 and 4). These elements raises the question of the possible HG003 EV roles in bacterial fitness and host-pathogen interactions. To date, the impact of *S. aureus* EVs on the physiology of the population itself remains largely unexplored. In addition, considering that host cells with distinct characteristics respond in a different way to interaction with *S. aureus*, only a few studies explored the ability of EVs to distinctively stimulate diverse host cell lines (ASKARIAN et al., 2018; KIM et al., 2012; WANG et al., 2018b). More importantly, it is also unclear if released EVs exert different functions from that of live EV-producing bacteria. In this context, we decided to investigate if *S. aureus* HG003-derived EVs could impact populational bacterial growth and if they exert immunomodulatory stimuli similar to live bacteria towards different host cells. HG003 EVs were added to cultures of *S. aureus* HG003 strain and three human cell lines: keratinocytes, osteoblasts, and lung epithelial cells. In the last case, the infection with live bacteria was conducted for comparison. The data presented here bring insights into the importance of EVs in *S. aureus* physiology and how they can impact the development of different infections.

5.2 Material and Methods

5.2.1 Bacterial strain and culture

The *S. aureus* HG003 used in this work derives from NCTC 8325, isolated from a sepsis patient in 1960. HG003 contains functional *rsbU* and *tcaR* global regulators lacking in the parent strain (Herbert et al., 2010) and is widely used in staphylococcal regulation and virulence studies (Liu et al.,2018a). HG003 pre-cultures were grown in Brain-Heart Infusion (BHI) broth overnight at 37°C under 150 rpm/min agitation and then inoculated 0.1% in fresh BHI (at 37°C, 125 rpm/min). Bacterial growth was assessed by optical density at 600 nm (OD_{600}) and cultures were treated after 12 h for EV extraction.

5.2.2 EVs isolation, purification, and characterization

EVs were isolated and purified as previously described (LUZ et al., 2021b). Briefly, bacterial culture was centrifuged at 6,000 × g for 15 min and filtered through 0.22 μm Nalgene top filters (Ref.: 295-3345, Thermo Scientific). Then, the supernatant was concentrated around 100-fold using the Amicon ultrafiltration systems (Ref.: 2659661, Merck Millipore) with a 100kDa filter and ultra-centrifuged for 120 min at 150,000 × g to eliminate the soluble proteins. Next, the suspended pellet was applied to a discontinuous sucrose gradient (8–68%) and ultra-centrifuged at 100,000 × g for 150 min. Fractions

containing EVs were recovered and washed in TBS buffer (150 mM NaCl; 50 mM Tris-Cl, pH 7.5) for final ultra-centrifugation at 150,000 × g (120 min). At last, EVs were suspended in cold TBS and kept at - 80°C until use. Physical EV characterization was performed using Nano Tracking analyses (NTA) (Nano Sight NS300, Malvern Panalytical) and Electron microscopy (TEM) as previously described (LUZ et al., 2021b).

5.2.3 EV impact on *S. aureus*

To evaluate the impact of the addition of supplementary EVs on the *S. aureus* population, the BioTek Synergy HTX (Agilent) 96-well plate reader was used. Different concentrations of HG003 EVs (1e+9, 1e+10, and 5e+10) were added to *S. aureus* HG003 culture grown in three different media: BHI, RPMI containing 10% BHI, and NZM. The OD_{600} was measured every 10 min for over 20 h.

5.2.4 EVs impact on the host

5.2.4.1 Eukaryotic cell cultures

Eukaryotic cell lines were incubated at 37°C in a humidified incubator (5% CO_2), and cultured in different media according to cell line necessities. A549 lung epithelial cells (CLS Cell Lines Service, 300114, GmbH, Germany) were cultured in Kaighn's modification of HAM's F12 medium (Ref.: 21127022, Gibco), while HaCaT human keratinocytes (CLS Cell Lines Service, 300493, GmbH, Germany) and osteoblast-like MG-63 cells (LGC Standards, Teddington, UK) were cultured in Dulbecco's modified Eagle medium (DMEM) (Ref.: 11965092, Gibco). Both culture media were supplemented with fetal bovine serum (FBS) (Ref.: A4766801, Gibco) at 10% final concentration. Cells were regularly subcultured after achieving confluent monolayers (80%) with the help of Trypsin/EDTA (Ref.: T4174, Merck).

5.2.4.2 Cell proliferation assays

The assess viability, cell lines were seeded in 96-well plates at 2e+4 per well, where they were exposed to triton X-100 at 0.01% (positive control) and different EV concentrations (1e+8, 5e+8, and 1e+9 per well) during 24 h. Cell proliferation was evaluated using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega), according to the manufacturer's instructions. Xenius (SAFAS, Monaco) microplate reader was used to read absorbance (490 nm).

5.2.4.3 Expression levels of target genes

Several genes were chosen to evaluate the host immune response to *S. aureus* HG003 and its derived EVs. A549, HaCaT, and MG-63 cell lines were seeded in their respective media at 2e+5 cells per well in 12-well plates to achieve a confluent monolayer. Then, cells were washed twice with DPBS (Sigma-Aldrich) and exposed to either TBS (negative control), live *S. aureus* HG003 (100:1), or EVs (100.000:1) for 4 h or 24 h. The live *S. aureus* group and its respective negative control were washed three times with DPBS after 2 h exposition to avoid cell monolayer destruction. Then, the appropriate media of each cell line containing 100 μg/mL gentamicin was added to inactivate extracellular bacteria until the end of incubation (2 h for 4 h infection, or 22 h for 24 h infection). All groups contained three biological replicates. After an incubation period, cells were retrieved with RNeasy Lysis Buffer (Qiagen) containing β-mercaptoethanol, which were kept at - 80°C until use. RNA extraction was performed with RNeasy® Mini kit (Qiagen) with on-column DNA digestion (Qiagen), and cDNA was synthesized with qScript cDNA Synthesis kit (Quantabio) according to the manufacturer's instructions.

Table 5.1 displays the list of primers used in this study, which were retrieved from the literature or the Harvard PrimerBank platform (SPANDIDOS et al., 2008, 2010; WANG; SEED, 2003). All high-throughput qPCR reactions were performed using the large-scale gene expression platform "Human and Environmental Genomics" (Rennes, France), using the Takara SmartChip Real-time PCR system (Takara, Mountain View, CA, USA). PCR mixtures are processed in 5184-well plates with a reaction volume of 100 nL containing 1 ng/mL cDNA final concentration and primers at 500 nM. For each primer set, a no template control was included. Amplification conditions were as follows: 5 min at 95 °C followed by 45 cycles of 10 sec at 95 °C, 30 sec at 60 °C, and 30 sec at 72 °C, followed by 1 min at 95 °C and 1 min at 60 °C. Each sample was submitted to qPCR reactions in technical triplicates, and the melting curve of each PCR product was used to assess amplification specificity. qPCR results were analyzed using SmartChip qPCR Software (v 2.8.6.1). The housekeeping genes TOP1, GAPDH, and PPIA were the more stable according to RefFinder (XIE et al., 2012) and were used as reference genes for normalization. Genes considered significantly differentially expressed corresponded to those with a *P*-value < 0.05 (Student's t-test) compared to the control.

Table 5.1 List of primers used in this study.

Primer	Amplicon Size (nt)	Sequence 5' - 3'	Reference
β-actin F	117	AACGGCTCCGGCATGTGCAA	Wang et al., 2018
β-actin R		CTTCTGACCCATGCCCACCA	
B2M_F	248	GAGGCTATCCAGCGTACTCCA	PrimerBank ID: 37704380c1
B2M_R		CGGCAGGCATACTCATCTTTT	
GAPDH_F	101	CTGGGCTACACTGAGCACC	PrimerBank ID: 3784378404907c304907c2
GAPDH_R		AAGTGGTCGTTGAGGGCAATG	
HPRT1_F	131	CCTGGCGTCGTGATTAGTGAT	PrimerBank ID: 164518913c1
HPRT1_R		AGACGTTCAGTCCTGTCCATAA	
PGK1_F	137	GAACAAGGTTAAAGCCGAGCC	PrimerBank ID: 183603937c2
PGK1_R		GTGGCAGATTGACTCCTACCA	
PPIA F	184	GACCCAACACAAATGGTTCC	Deplanche et al., 2015
PPIA R		TCGAGTTGTCCACAGTCAGC	
RPL13A_F	135	GCCATCGTGGCTAAACAGGTA	PrimerBank ID: 14591905c1
RPL13A_R		GTTGGTGTTCATCCGCTTGC	
TOP1_F	135	AAGGTCCAGTATTTGCCCCAC	PrimerBank ID: 19913404c2
TOP1_R		ATTCATGGTCGAGCATTTTTGC	
YWHAZ_F	179	TGTAGGAGCCCGTAGGTCATC	PrimerBank ID: 208973243c2
YWHAZ_R		GTGAAGCATTGGGGATCAAGA	
IL1β_F	132	ATGATGGCTTATTACAGTGGCAA	PrimerBank ID: 27894305c1
IL1β_R		GTCGGAGATTCGTAGCTGGA	
IL6_F	149	ACTCACCTCTTCAGAACGAATTG	PrimerBank ID: 224831235c1
IL6_R		CCATCTTTGGAAGGTTCAGGTTG	
IL8_F	112	ACTGAGAGTGATTGAGAGTGGAC	PrimerBank ID: 10834978a1
IL8_R		AACCCTCTGCACCCAGTTTTC	
IL17A_F	151	AGATTACTACAACCGATCCACCT	PrimerBank ID: 27477085c2
IL17A_R		GGGGACAGAGTTCATGTGGTA	
IL18_F	186	GCTGCTGAACCAGTAGAAGAC	Rozwadowska et al., 2007
IL18_R		CCGATTTCCTTGGTCAATGAAGA	
RANTES_F	135	ATCCTCATTGCTACTGCCCTC	Kauts et al., 2013
RANTES_R		GCCACTGGTGTAGAAATACTCC	
MCP-1 F	150	TCGCTCAGCCAGATGCAAT	Jun et al., 2013
MCP-1 R		TGGCCACAATGGTCTTGAAG	
NFKβ1_F	122	CACAAGGCAGCAAATAGACG	Zhao et al., 2018
NFKβ1_R		GAGTTAGCAGTGAGGCACCA	
TNFα_F	220	CCTCTCTCTAATCAGCCCTCTG	PrimerBank ID: 25952110c1
TNFα_R		GAGGACCTGGGAGTAGATGAG	
TLR2_F	163	ATCCTCCAATCAGGCTTCTCT	Malvandi et al., 2011
TLR2_R		ACACCTCTGTAGGTCACTGTTG	

5.3 Results

5.3.1 HG003 EV impact on *S. aureus* growth

As previously described by our group, HG003 EVs carry several RNAs and proteins linked to metabolism and virulence (see Chapters 3 and 4). More importantly, many of these elements were shown to be relatively enriched in EVs compared to parent cells. We hypothesized that *S. aureus* EVs could affect bacterial growth. As shown in Figure 5.1, the addition of intact HG003 EVs impacted bacterial growth according to the media broth. While the addition of supplementary HG003 EVs did not alter *S. aureus* HG003 growth in BHI rich medium (Fig 5.1A), they improved bacterial growth in a dose-dependent manner in both NZM and RPMI + 10% BHI media (Fig 5.1BC). This is the first evidence showing the positive impact of the addition of intact supplementary *S. aureus* EVs on bacterial populational growth.

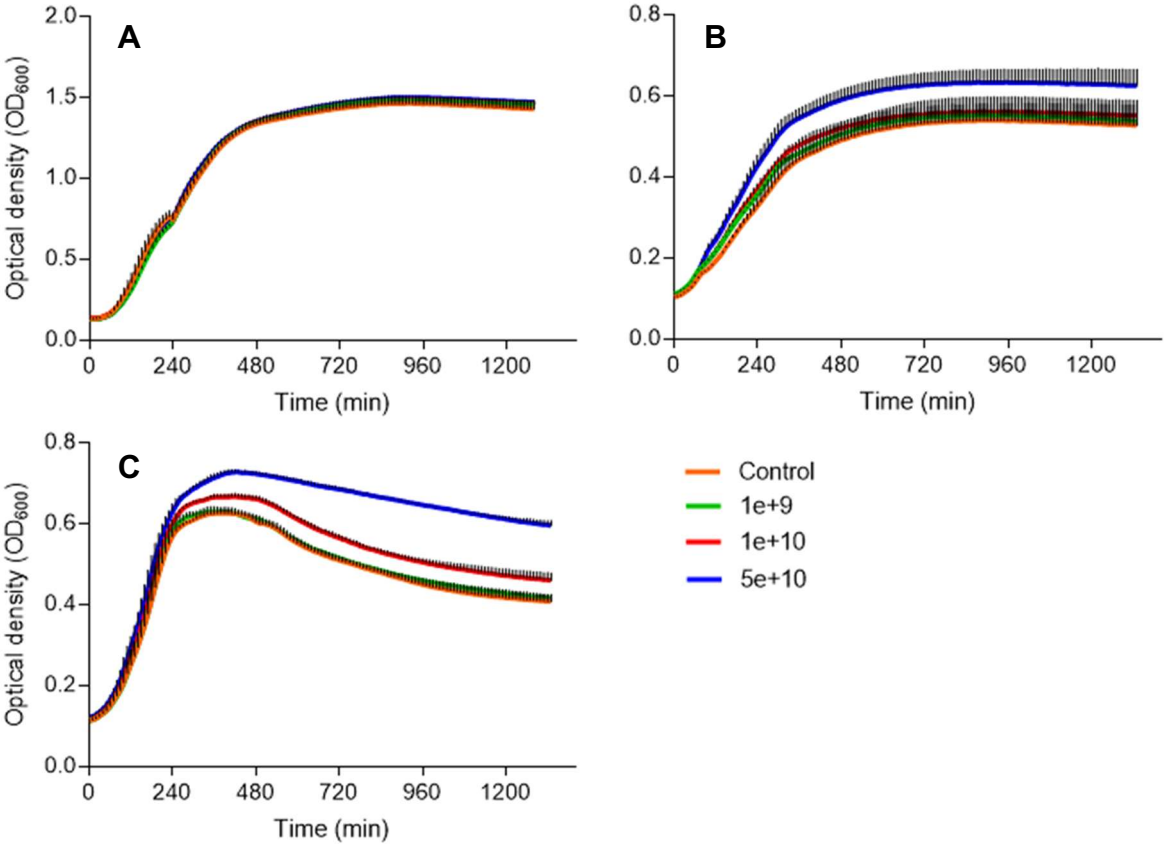


Figure 5.1 Impact of *S. aureus* HG003 EVs on population growth. Three different concentrations of HG003 EVs (1e+9, 1e+10, and 5e+10) were added to *S. aureus* HG003 strain grown in BHI (**A**), NZM (**B**), and RPMI containing 10% BHI (**C**). BioTek plate reader was used to measure OD_{600} every 10 min for over 20 h to evaluate the impact of EV addition bacterial growth.

5.3.2 HG003 EV impact on the host

In order to investigate if HG003 EVs affect host cell viability, MTS cell proliferation assays were performed. For this, the eukaryotic cell lines A549 (pulmonary), HaCaT (keratinocyte), and MG-63 (osteoblast) were exposed to different concentrations of HG003 EVs (1e+8, 5e+8, and 1e+9). Triton at 0.01 % was used as a positive control. As shown in Figure 5.2, HG003 EVs were not cytotoxic to any of the cell lines in the tested conditions.

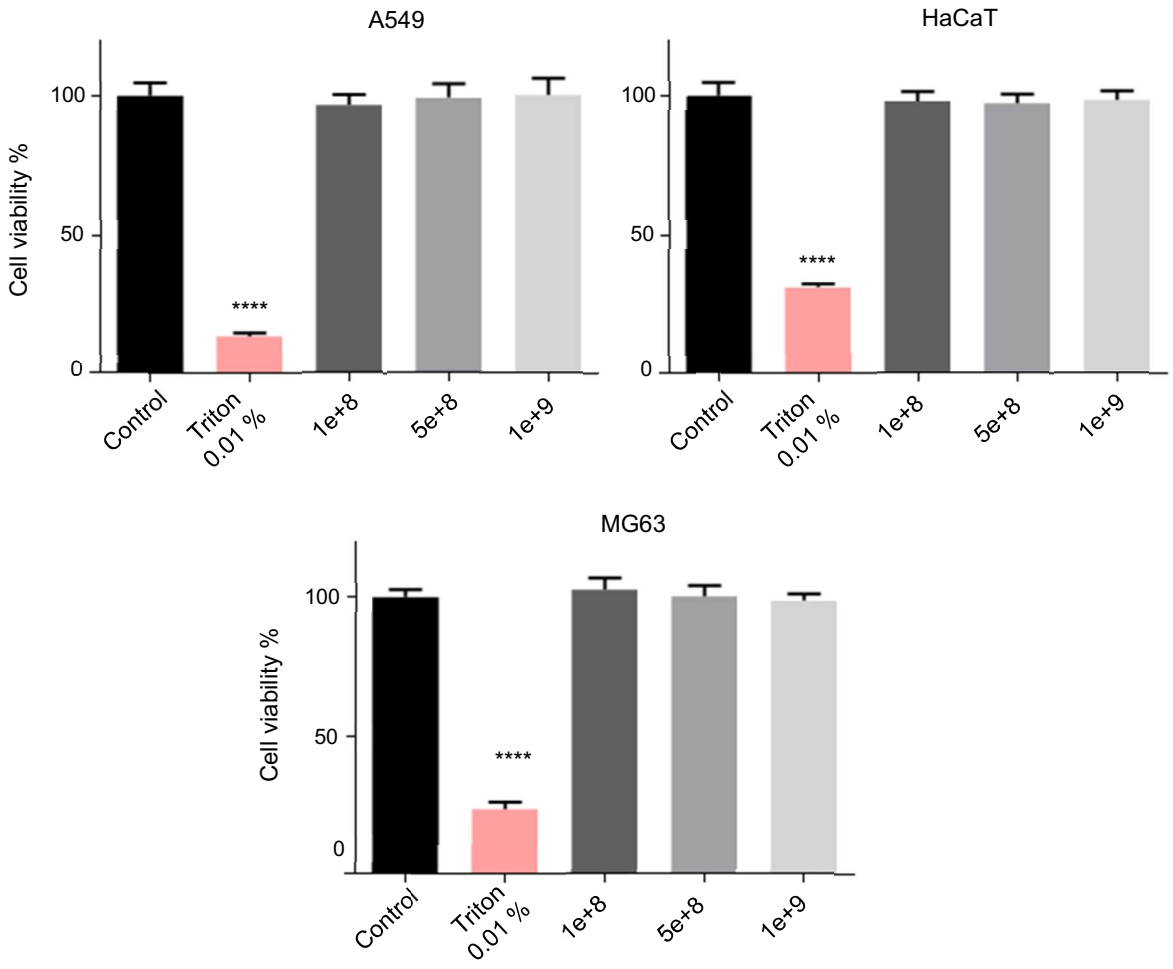


Figure 5.2 *S. aureus* HG003 EVs are not cytotoxic against different host cell lines. Viability percentage of A549, HaCaT and MG-63 after treatment with TBS (negative control), Triton 0.01 % (positive control), and different HG003 EV concentrations (1e+8, 5e+8 and 1e+9). ANOVA with Tukey's multiple comparison test: ****$P < 0.0001$.

Since HG003 EVs were not cytotoxic toward the tested eukaryotic cell lines, RT-qPCR analyses were performed to evaluate the expression of genes coding for important cytokines after exposition to EVs. Incubation with live *S. aureus* HG003 was also conducted for comparison. As observed in Figure 5.3, after 4 h and 24 h post-infection (pi), *S. aureus* HG003 and its derived EVs induced the expression of several genes involved in the host immune response. Interestingly, gene activation varied between the cell lines tested, and some differences were also observed between exposure to bacteria or EVs (Fig 5.3).

In A549 lung cells, the expression of IL-8 and MCP-1 genes was induced by bacteria at both 4 and 24 hpi, while EVs upregulated them only at 24 hpi. NFkβ1 and TNF-α genes were induced by bacteria at both times, while their expression remained unchanged in the presence of EVs. Regarding HaCaT cells, EVs induced the expression of the MCP-1 gene at 4 hpi and IL-8 and MCP-1 genes at 24 hpi. The bacteria positively affected the expression of IL-8, IL-6, IL-1β TLR2, and MCP1 genes at 4 hpi and IL-8, IL-18, IL-1β, and RANTES genes at 24 hpi. Finally, EVs and bacteria-induced the expression of more genes in MG63 cells than in the two other cell lines. EVs induced the expression of IL-1β and MCP-1 genes at 4 hpi and IL-8, IL-6, and MCP-1 genes at 24 hpi. Likewise, the bacteria-induced the expression of IL-8, IL-6, IL-1β, NFkβ1, TLR2, and MCP-1 genes at 4 hpi and IL-8, IL-6, IL-1β, RANTES, and MCP-1 genes at 24 hpi. Of note, genes induced by both groups in the same condition generally present higher expression levels after exposure to live bacteria when compared to EVs.

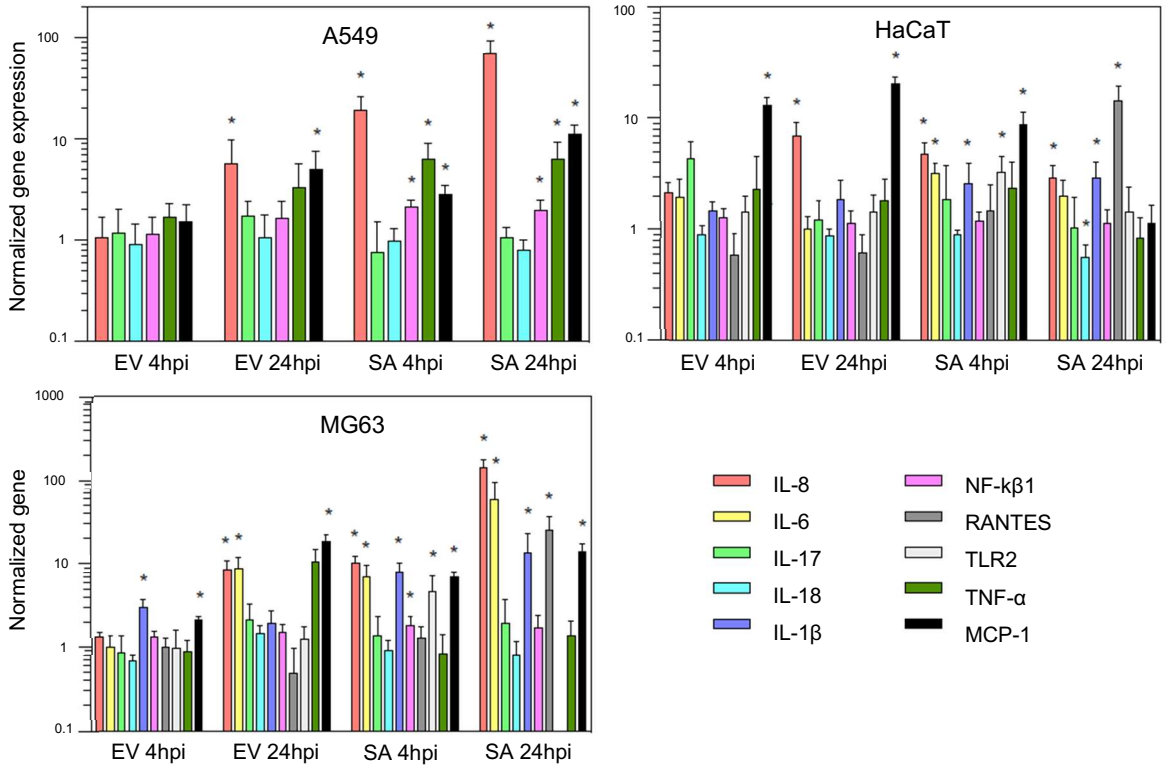


Figure 5.3 Induction of host pro-inflammatory cytokines. Gene expression of A549, HaCaT, and MG-63 cells exposed to *S. aureus* HG003 and its derived EVs during 4 h and 24 h relative to control.

5.4 Discussion

Even though the knowledge surrounding the functions exerted by *S. aureus* EVs has rapidly increased over the past years, many questions remain open to exploration. Studies on how EVs influence *S. aureus* physiology are still scarce, and reports addressing host-pathogen interactions rarely investigate the effects of EVs on distinct host cell lines or if these effects are different from those exerted by producing cells. In this work, we investigated how the addition of HG003 EVs could affect bacterial population itself or host-pathogen interactions in different cell lines and *in vitro* infection models.

As shown in Figure 5.1, the addition of HG003 EVs had a positive impact on bacterial growth, which was media-dependent. The growth of *S. aureus* remained unchanged in BHI broth even after adding different concentrations of supplementary HG003 EVs. BHI is a rich medium that favors *S. aureus* optimal growth. On the other hand, a dose-dependent increase in bacterial growth was observed in *S. aureus* cultures grown in restrictive media NZM and

RPMI + 10 % LB following supplementation with HG003 EVs. We previously demonstrated that HG003 EVs carry several RNAs and proteins involved in transcription, translation, carbohydrate metabolism, and energy production and conversion (See Chapters 3 and 4). They include carbohydrate and iron transporters (e.g., PtsG, FhuC), pyruvate dehydrogenase, cytochrome c oxidase complexes (e.g., PdhABC, CydAB), and glycolytic enzymes (e.g., GAPDH, Eno). We could hypothesize that HG003 EVs may transfer RNAs and / or proteins involved in metabolic and energetic functions to recipient cells, contributing to their nutrition and improved cell functioning, especially in starved bacterial cells. This could explain why no differences were observed in BHI cultures, while HG003 EVs exerted a positive effect on bacterial growth of *S. aureus* cultured in restrictive media. Indeed, a study by Askarian et al. (2018) demonstrated that EV supplementation of *S. aureus* MSSA476 cells grown in LB and BHI did not affect bacterial growth (ASKARIAN et al., 2018), supporting our hypothesis.

It has already been shown that outer membrane vesicles (OMVs) derived from the marine cyanobacterium *Prochlorococcus* can support the growth of heterotrophic bacterial cultures as their sole nutrient source (BILLER et al., 2014; SCHWECHHEIMER; KUEHN, 2015). OMVs produced by *Bacteroides* are also public goods that allow the distribution of hydrolases that promote the breakdown of complex polysaccharides, which serve as nutrient sources for other intestinal microbiota species (Rakoff-Nahoum et al., 2014). In another example, EVs produced by *Mycobacterium tuberculosis* facing iron restriction are enriched with siderophores, which serve as donors to iron-starved bacteria (PRADOS-ROSALES et al., 2014b). As highlighted, bacterial EVs can play several intra- and inter-species physiologic roles, having a significant impact on bacterial community functioning. Our data show that *S. aureus* EVs optimize bacterial growth in restrictive conditions through a yet unknown mechanism. Further experiments evaluating the effect of lysed *S. aureus* EVs may help elucidate if they serve only as nutriment sources, if they actively transfer elements that may affect cell functioning (e.g., RNAs and proteins involved in the quorum sensing system), or both.

There are still few studies addressing the role of *S. aureus* EVs *in vivo* (ASANO et al., 2021; ASKARIAN et al., 2018; HONG et al., 2011, 2014; JUN et al., 2017; KIM et al., 2012; KWON et al., 2018; TARTAGLIA et al., 2018), and currently EV functions are mainly addressed *in vitro* via cell culture approaches. Although many studies have already reported the cytotoxic effect of EVs derived from different *S. aureus* strains towards host cells, here, HG003 EVs were non-toxic against A549, HaCaT and MG63 in the conditions tested. Nevertheless, some studies demonstrated varied cytoxicity levels of EVs derived from *S.*

aureus subsp. *aureus* Rosenbach MSSA476 and *S. aureus* JE2 strains towards distinct host cell lines (ASKARIAN et al., 2018; WANG et al., 2018b). Therefore, we cannot exclude the possibility that HG003 EVs may be cytotoxic to other cell types. This question may be addressed in future studies. Despite the absence of cytotoxicity, HG003 EVs induced the expression of pro-inflammatory cytokines in all cell lines tested, as well as live bacteria. Interestingly, despite the absence of previous reports addressing the effect of *S. aureus* EVs on MG63 cells, here we demonstrate that HG003 EVs stimulate the expression of IL-1β, IL-6, IL-8, and MCP-1 genes. Accordingly, recent transcriptomic analysis demonstrated that MG63 cells infected with *S. aureus* SA113, an NCTC 8325 derivative, present upregulation of all the genes induced by HG003 EVs (NICOLAS et al., 2022). In addition to these genes, we found that live *S. aureus* HG003 also activates NF-kβ, TLR2, and RANTES gene expression, being the last two genes also stimulated by *S. aureus* SA113 strain in MG63 osteoblasts (NICOLAS et al., 2022). These data suggest that *S. aureus* EVs play an active role in the context of osteomyelitis.

Regarding the study of staphylococcal EVs in lung infections, few reports to date explored A549 cells. A study showed that EVs derived from the *S. aureus* JE2 strain were cytotoxic toward A549 cells (WANG et al., 2018b), which differs from the non-cytotoxic properties of HG003 EVs observed here. These differences may be attributed to variations in the cargo of EVs released by these two strains (Chapter 4, Wang et al., 2018b). In another report, Kim et al. (2012) demonstrated that *S. aureus* ATCC14458 EVs did not induce expression of TNF-α in A549 cells, similar to our results. In contrast, IL-6 production was increased after exposition to ATCC14458 EVs (KIM et al., 2012), unlike the findings obtained here. Note that IL-6 RNA gene expression was evaluated 4 h and 24 h post-infection in this study, while Kim et al. (2012) measured protein levels by ELISA after 24 h of exposition (KIM et al., 2012). Differences in *S. aureus* strains and experimenta conditions or may explain these variations in cytokine detection. At last, we provide for the first time data regarding the significantly increased expression of IL-8 and MCP-1 genes by A549 cells in response to *S. aureus* EVs. We also found that, in addition to these genes, live bacteria also induced the expression of NF-kβ and TNF-α. Accordingly, activation of IL-8 and NF-kβ in A549 cells by *S. aureus* supernatants has been previously demonstrated (SACHSE et al., 2006). These results reinforce that *S. aureus* EVs may impact lung inflammations. Future studies are necessary to understand better EVs roles in the context of staphylococcal pneumonia.

Finally, we also investigated how EVs affect HaCaT keratinocytes, one of the best-studied cell lines in the *S. aureus* EV field. EVs derived from different *S. aureus* strains exert dissimilar consequences on HaCaT cell proliferation, presenting both cytotoxic (HONG et al., 2014; KWON et al., 2018, 2019) and non-cytotoxic properties (ASKARIAN et al., 2018; JUN et al., 2017; YAMANASHI et al., 2022). Non-cytotoxicity is the outcome we observed here in our experimental conditions. Regarding host gene expression, we demonstrated that HaCaT exposition to HG003 EVs induced the expression of both IL-8 and MCP-1 genes. Previous studies have shown that HaCaT cells may release IL-1β, IL-6, IL-8, MCP-1, MIP-1α, and/or TNF-α depending on the *S. aureus* strain EV source (JUN et al., 2017; KWON et al., 2018, 2019). However, all cited studies evaluated gene expression after 6 h of stimulation, which is different from the conditions used here. Our data also showed that live bacteria increase the expression of IL-1β, IL-6, IL-18, TLR2, and RANTES, in addition to those induced by HG003 EVs. The induction of proinflammatory genes by HG003 EVs in HaCaT keratinocytes strengthens the current knowledge of their role in the induction and/or exacerbation of skin infections (HONG et al., 2011, 2014; JUN et al., 2017; KIM et al., 2012; KWON et al., 2018; STAUDENMAIER et al., 2022).

Overall, A549 lung cells were the least affected, while MG63 presented the higher number of activated genes following exposition to live bacteria. HG003 EVs stimulated the expression of IL-8 and MCP-1 in all three cell lines, while IL-1β and IL-6 were activated only in MG63 osteoblasts. It has already been reported that, after exposure to *S. aureus* ATCC 14458 EVs, alveolar macrophages produced TNF-α and IL-6 while A549 cells produced only IL-6 (KIM et al., 2012), reinforcing the hypothesis that *S. aureus* EVs exert different effect on distinct host cells. It is interesting to note that not all genes induced by *S. aureus* are also induced by HG003 EVs. Similarly, Bitto et al. (2021) demonstrated that although *S. aureus* strains 2760, 2900, and 6571 induced IL-6 expression in A549 cells, only EVs derived from the 2760 strain also induced this cytokine (BITTO et al., 2021a). Finally, genes activated by both groups generally present lower expression levels after EV stimulation. These data lead us to hypothesize that released EVs evolved to exert specific host stimulation, functioning as fine-tuned systems in host-pathogen interactions. In fact, lung pre-inflammation helps bacteria to hijack and resist host defense mechanisms in an allergic asthma model (MUES; CHU, 2020; NAVARRO et al., 2010). Considering that EVs are small particles able to travel to distant sites, their immunostimulatory properties could pave the way for the construction of a favorable environment for *S. aureus* survival during infection.

In summary, here we provide the first evidence that *S. aureus* EVs optimize bacterial growth in restrictive conditions. It is possible that *S. aureus* EVs may serve as nutrient sources for starved bacteria. However, we cannot exclude the possibility that EVs provide enzymatic elements to improve cell functioning. Moreover, the induction of pro-inflammatory genes by HG003 EVs sheds light on their role in the context of various infections. More importantly, their distinctive stimulation patterns from live bacteria may be fine-tuned mechanisms to modulate the host immune response, guaranteeing *S. aureus* pathogenesis and success.

Chapter 6. Additional results – Part 2

Remodeling of the bovine mammary epithelial cell transcriptome in response to *Staphylococcus aureus* extracellular vesicles

6.1 Introduction

Staphylococcus aureus is an important bacterium that brings significant consequences for both the medical and veterinary sectors. It is a pathobiont that commonly colonizes the nasal tract of healthy adults, a state that can evolve into a multitude of clinical infections, which range from moderate to lethal outcomes (KLUYTMANS; VAN BELKUM; VERBRUGH, 1997; SALGADO-PABÓN; SCHLIEVERT, 2014; SIVARAMAN; VENKATARAMAN; COLE, 2009; TONG et al., 2015). *S. aureus* is also an important etiological agent in veterinary medicine, causing several manifestations and economic losses in animal farming of in pigs, poultry and ruminants. Since milk is a source of great importance for the world food economy, staphylococcal infections in cows, sheep, and goats affect directly the dairy sector (ABRIL et al., 2020). *S. aureus* is the leading causing agent of mastitis, an inflammation of the mammary gland that alter the quantity and quality of milk produced by affected animals, resulting in significant economic losses for the milk production and potential food poisoning in the dairy products (ARGUDÍN; MENDOZA; RODICIO, 2010; LE LOIR; BARON; GAUTIER, 2003).

Currently, prevention and treatment against *S. aureus* infections are still difficult due to the absence of effective vaccines and the rising of antibiotic-resistant strains (BAGNOLI; BERTHOLET; GRANDI, 2012; CHAMBERS; DELEO, 2009; FOSTER, 2017). Therefore, the search for a better understanding of staphylococcal infections is a continuous effort. The first description of extracellular vesicles (EVs) released by *S. aureus* in 2009 opened the door to a new field of investigations (LEE et al., 2009). Since then, several reports have provided the characterization of EVs derived from various *S. aureus* strains, demonstrating their potential roles in infection (LUZ et al., 2021a). However, most studies have focused on human clinical strains, with only a few reports addressing EVs derived from other *S. aureus* animal isolates. One of them, conducted by Tartaglia et al. (2018), demonstrated that the *S. aureus* Newbould 305 (N305) mastitis bovine isolate releases EVs able to induce the expression of IL-8 *in vitro*, and to promote tissue inflammation, deterioration, and local

cytokine production, *in vivo* (TARTAGLIA et al., 2018). Nevertheless, a deep investigation of the role of N305 EVs is still lacking.

In that sense, this work aims to deepen the knowledge on the role of *S. aureu*s EVs in the context of non-human infections, namely mastitis. The RNA-seq approach was used to assess eukaryotic genes and signaling pathways modulated by N305 EVs in bovine mammary epithelial cells (bMECs) *in vitro*. This work broadens the knowledge about *S. aureus* EVs in host-pathogen interactions, providing brand new evidence of the contribution of staphylococcal EVs to mastitis pathogenesis.

6.2 Material and Methods

6.2.1 Bacterial strain and culture

In this work, the *S. aureus* mastitis model strain, Newbould 305 (ATCC 29740), hereafter referred to as *S. aureus* N305, was chosen to further investigate EV mechanisms of action in host-pathogen interactions. *S. aureus* N305 pre-inoculums and cultures were grown overnight in BHI broth at 37°C under 150 rpm/min agitation. Bacterial growth was determined by measurement of optical density at 600 nm (OD_{600}). Cultures were harvestedafter 15 h, and EV isolation and purification were performed as follows.

6.2.2 EVs isolation, purification, and characterization

EVs were isolated and purified as previously described (LUZ et al., 2021b). Briefly, bacterial culture was centrifuged at 6,000 × g for 15 min and filtered through 0.22 μm Nalgene top filters (Ref.: 295-3345, Thermo Scientific). Then, the supernatant was concentrated around 100-fold using the Amicon ultrafiltration systems (Ref.: 2659661, Merck Millipore) with a 100kDa filter and ultra-centrifuged for 120 min at 150,000 × g to eliminate the soluble proteins. Next, the suspended pellet was applied to a discontinuous sucrose gradient (8–68%) and ultra-centrifuged at 100,000 × g for 150 min. Fractions containing EVs were recovered and washed in TBS buffer (150 mM NaCl; 50 mM Tris-Cl, pH 7.5) for final ultra-centrifugation at 150,000 × g (120 min). At last, EVs were suspended in cold TBS and kept at - 80°C until use. Physical EV characterization was performed using Nano Tracking analyses (NTA) (Nano Sight NS300, Malvern Panalytical) and Electron microscopy (TEM) as previously described (LUZ et al., 2021b).

6.2.3 Impact of N305 EVs on the host immune response

The RNA-seq approach was chosen to evaluate bovine mammary epithelial cells (bMECs) response to living *S. aureus* N305 and EVs *in vitro* (Fig. 6.1). Relevant genes activated *in vitro* will be measured by quantitative RT-qPCR in murine mammary gland tissue samples exposed to N305 EVs to confirm EV mechanisms of action (Fig. 6.1). The *in vivo* experiment was performed previously (TARTAGLIA et al., 2018). Tissue samples are stored at -80°C.

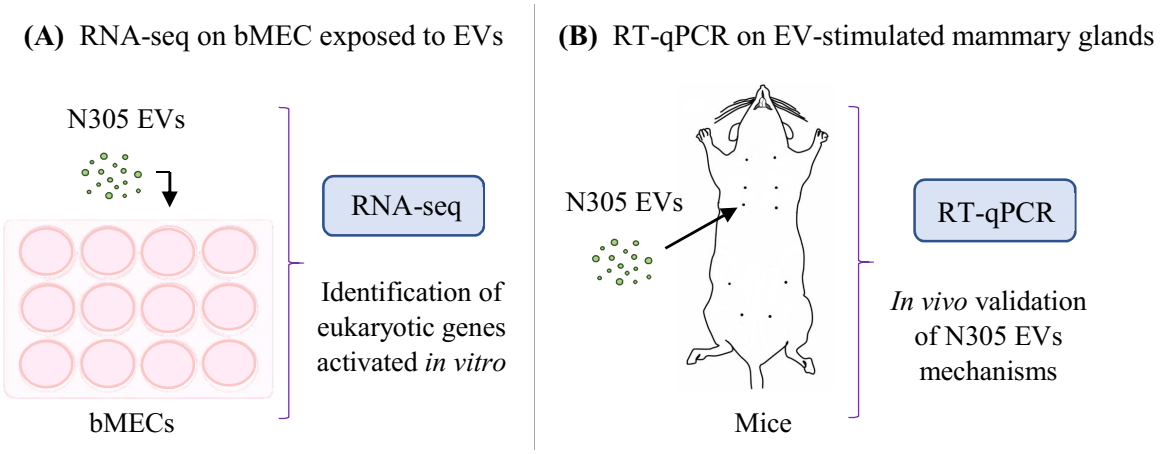


Figure 6.1 Schematic representation of the experimental strategy used to study the mechanisms of action of EVs derived from *S. aureus* N305.

6.2.3.1 bMEC culture conditions

The bovine mammary epithelial cell (bMEC) line PS (INRA, Tours, France) was incubated at 37°C in a humidified incubator (5% CO_2) in Advanced DMEM/F12 media with required supplements, as previously described (ROUSSEL et al., 2015). After achieving a confluent monolayer (80%), PS cells were treated with 0.05% trypsin (PAN-Biotech), and $2.0x10^5$ cells per well were grown for 24 h in fresh DMEM in a 12-well cell culture plate, followed by 16 h growth in DMEM supplemented with Fetal Bovine Serum (FBS) (Ref.: A4766801, Gibco). Cells were washed twice and then exposed to either TBS buffer or EVs (MOI 100.000:1) for 3 h, 4 h, 8 h, and 24 h to establish a kinetics of gene expression induced by EVs. For positive control, PS cells were exposed to living *S. aureus* N305 (MOI 100:1) for 3 h.

6.2.3.2 bMEC cell proliferation assays

To assess cell viability, bMEC cells were seeded in 96-well plates at 2 x 10^4, where they were exposed to triton X-100 at 0.01% (positive control) and different EV concentrations (1e+8, 5e+8, and 1e+9 per well) during 24 h. Cell proliferation was evaluated using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega), according to the manufacturer's instructions. Xenius (SAFAS Monaco) microplate reader was used to read absorbance (490 nm).

6.2.3.3 bMEC RNA-seq sequencing and analyses

Total RNA was extracted using RNeasy mini-kit (Qiagen) and treated with a RNAse-free DNA Removal Kit (Invitrogen) to remove residual genomic DNA according to the manufacturer's instructions. Samples were sent to NovoGene (Université de Rennes 1, Rennes, France) for library preparation and sequencing. Briefly, a total amount of 1 µg RNA per sample was used as input material, and sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA). Library quality was assessed on the Agilent Bioanalyzer 2100 system. Then, library preparations were sequenced on an Illumina platform, and paired-end reads were generated. Transcriptomic analyses were performed using the R software, Bioconductor packages (ANDERS; HUBER, 2010; LOVE; HUBER; ANDERS, 2014), and the SARTools package (VARET et al., 2016). Normalization and differential analysis was carried out according to the DESeq2 model and package.

6.2.3.4 RT-qPCR *in vivo* validation in murine mammary gland tissues

Ongoing analyses are being conducted to identify relevant genes activated by N305 EVs *in vitro*, such as pro-inflammatory cytokines and other genes involved in mastitis pathogenesis. Soon, EV-mediated modulation of host tissue gene expression will be validated on animal tissues retrieved from a previous experimental infection on mice with *S. aureus* N305 EVs (TARTAGLIA et al., 2018). For this, tissue samples will be submitted to RNA extraction, DNAse treatment, and cDNA synthesis. Finally, primers targeting relevant genes will be synthesized to validate by RT-qPCR the main host pathways modulated by N305 EVs.

6.3 Results

6.3.1 Exploration of bMEC transcriptome raw data

To investigate the impact of N305 EVs on the host immune response, bMEC cells were exposed to EVs during different times: 3 h, 4 h, 8 h and 24 h. bMEC cells exposed to live *S. aureus* N305 for 3 h were used as a positive control. Total RNAs were extracted from bMEC cells, and RNA samples were sequenced using the Illumina sequencing platform. To assess consistency of the transcriptomic data, principal component analysis (PCA) and hierarchical cluster analysis (HCA) were performed based on normalized abundances of each gene to obtain an overview of each condition. Figure 6.2 and 6.3 shows that the PCA scatter plot and HCA dendrogram plot indicated that biological triplicates clustered together. Note that biological triplicate samples related to the exposure of cells to EVs seemed to be more dispersed than control and live bacteria samples (Fig. 6.2). PCA also showed that control, live bacteria, and EV samples formed three distinct cluster groups showing the impact of exposure to EVs and live bacteria on bMEC gene expression when compared to the control conditions. This also indicated that EVs and live bacteria had very different effects on bMEC gene expression. Finally, control and EV samples at 3, 4, and 8 h seemed to be more closely related than those at 24 h (Fig. 6.2 and 6.3). PCA and clustering analyses confirmed the consistency between biological replicates under each condition and the distinct impacts of EVs and live bacteria on bMEC cells. Finally, in order to make the number of read counts comparable among samples, DESeq2 data normalization was performed (Fig. S1).

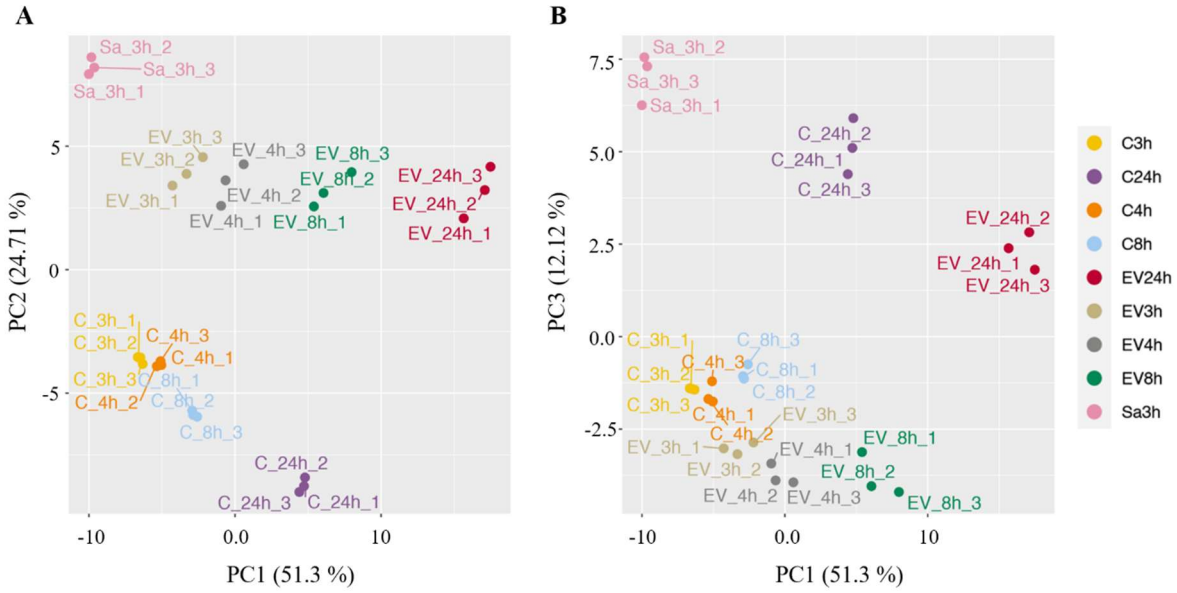


Figure 6.2 First two components of a Principal Component Analysis, with percentages of variance associated with each axis.

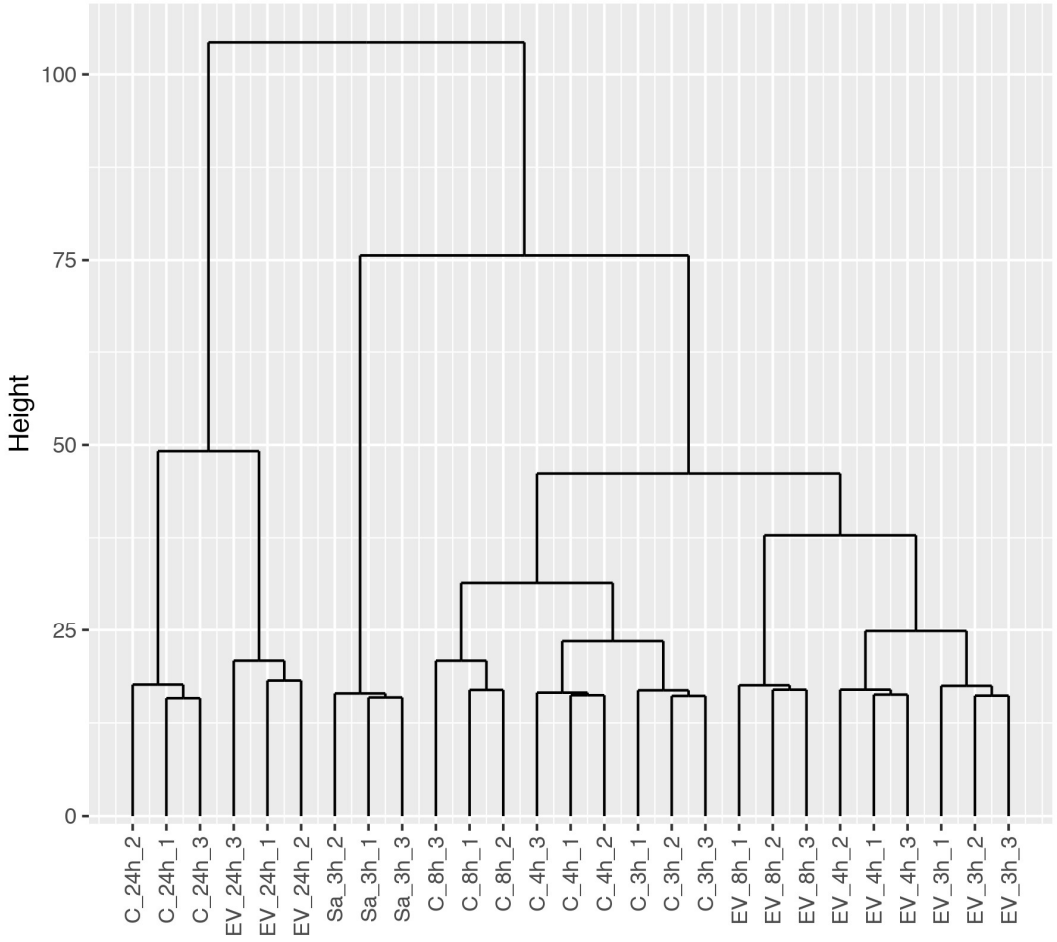


Figure 6.3 Cluster dendogram.

6.3.2 Transcriptional profiling of bMECs in response to *S. aureus* and its derived EVs

Differential expression analyses were then performed on normalized data to identify genes with a modulated expression in each condition. Exposure of bMECs to N305 EVs statistically affected (adj. *p*-val < 0.05) the expression of 3,774 protein-coding genes in all conditions, with 508, 476, 1,248, and 3,071 differentially expressed (DE) genes at 3 h, 4 h, 8 h and 24 h, respectively (Tables 6.1, S1-4). Note that the number of genes modulated by N305 EVs increased over time and that 161 genes were found DE at all time points after exposure to N305 EVs (Fig. 6.4A, Table S5). Live *S. aureus* affected the expression of thousands of genes at 3 h, with 2,313 down-regulated genes and 2,237 up-regulated genes (adj. *P*-val < 0.05) (Table 6.1, S6).

Table 6.1 Up-, down- and total number of differentially expressed (DE) genes in bMECs after exposure to *S. aureus* and EVs at various time points (adj. *P*-val < 0.05).

	All DE genes			**DE genes with |log2 FC| ≥ 0.5**		
Condition	Down	Up	Total	Down	Up	Total
EV 3 h vs. Control 3 h	139	369	508	3	113	116
EV 4 h vs. Control 4 h	152	324	476	10	100	110
EV 8 h vs. Control 8 h	459	789	1248	13	219	232
EV 24 h vs. Control 24 h	1315	1756	3071	85	433	518
S. aureus 3 h vs Control 3 h	2313	2237	4550	343	530	873

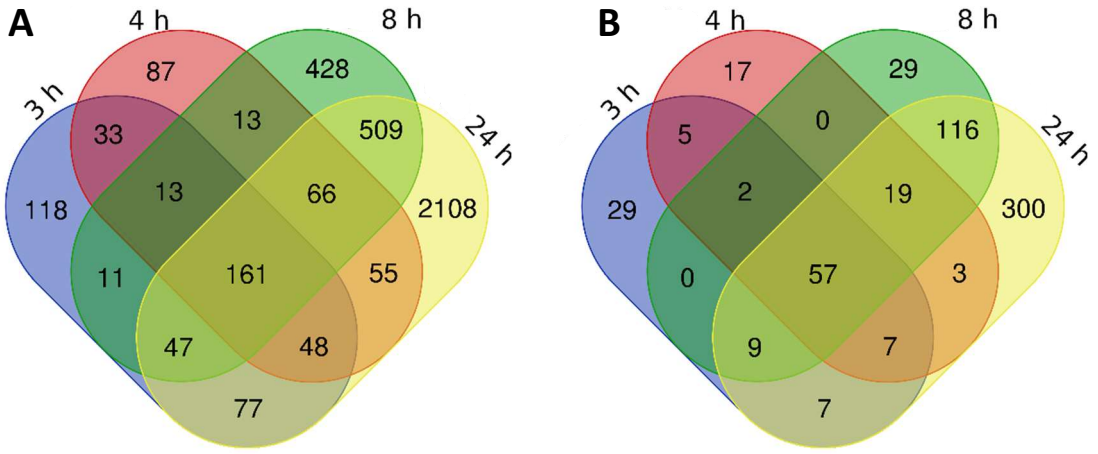


Figure 6.4 Venn diagrams of bMEC differentially expressed (DE) genes in response to N305 EVs at different times. **(A)** All DE genes; and **(B)** DE genes with |log2FC| ≥ 0.5. Adj. *P*-val < 0.05.

The number of DE genes considerably dropped down when taking into account only those with a threshold log2 fold change -0.5 > log2FC > 0.5 (|FC|≥2) (Table 6.1). In this context, 116, 110, 232, and 518 bMECs genes are modulated by N305 EVs after 3 h, 4 h, 8 h, and 24 h exposure, respectively (Table S1-4). Note that DE genes were mainly upregulated by the presence of EVs. Among them, 57 were common to all conditions (Fig. 6.4B, Table 6.2, S5), including genes linked to inflammatory pathways and important cytokines, such as IL-1β, IL-6, TNF-α, NF-κB, CCL20, CCL22, CXCL3, CXCL5, and CXCL8. A 3-hour exposure of bMECs to live *S. aureus* N305 resulted in the modulation of 873 genes with |log2FC| ≥ 0.5 (Table 6.1, S6). The lists of the top up- and down-regulated genes in bMECs in each condition are displayed in Tables 6.3 and 6.4. In addition, 168 bMEC genes were modulated by both *S. aureus* N305 and its EVs from at least one exposure time. Among them, 30 genes were DE in all conditions tested, and overall, gene induction levels for these genes were higher in EV conditions compared to live *S. aureus* (Table S7).

Interestingly, 67 genes modulated by live bacteria after 3 h exposure were also modulated by EVs in the same condition (Fig. 6.5A, Table S8), including CCL20, CCL22, CXCL1 (KC), CXCL5, IL-1β, IL-6, IL-8, and TNF-α. These commonly regulated genes included those with similar and different expression levels, depending on the source of stimulation (EVs or live bacteria). For instance, while TLR10 gene was similarly activated by both groups, IL-1β, TNF-α, CXCL1 (KC), and CXCL8 genes were more activated by N305 EVs when compared to live bacteria (log2FC TLR10 = 1.09 vs 1.39; IL-1β = 5.59 vs 2.77; TNF-α = 2.95 vs 1.60; CXCL1 = 2.90 vs 1.70; and CXCL8 = 4.38 vs 2.93 for N305 EVs and *S. aureus*, respectively) (Table S8). Conversely, the PLAT gene, encoding plasminogen activator, and EGR1 related to early growth response were less activated by N305 EVs than by live bacteria (log2FC PLAT = 0.56 vs. 1.98; EGR1 = 0.55 vs. 2.42 for N305 EVs and *S. aureus*, respectively) (Table S8). Notably, 49 genes were uniquely modulated by N305 EVs at 3 h (Fig 6.5A), including activators of inflammatory responses such as APOL-3, CD83, GPR84, and ICAM1 (Table S9). Seventy-eight percent of these genes (n = 38) were also found DE by EVs in at least one other exposure time, and 55% (n = 27) were modulated by EVs in all conditions demonstrating the specific impact of EVs on bMEC cells.

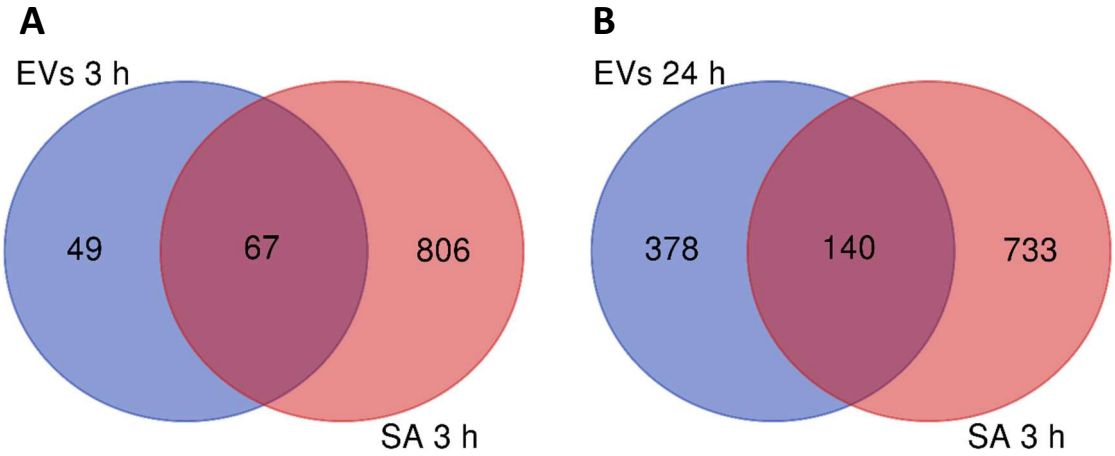


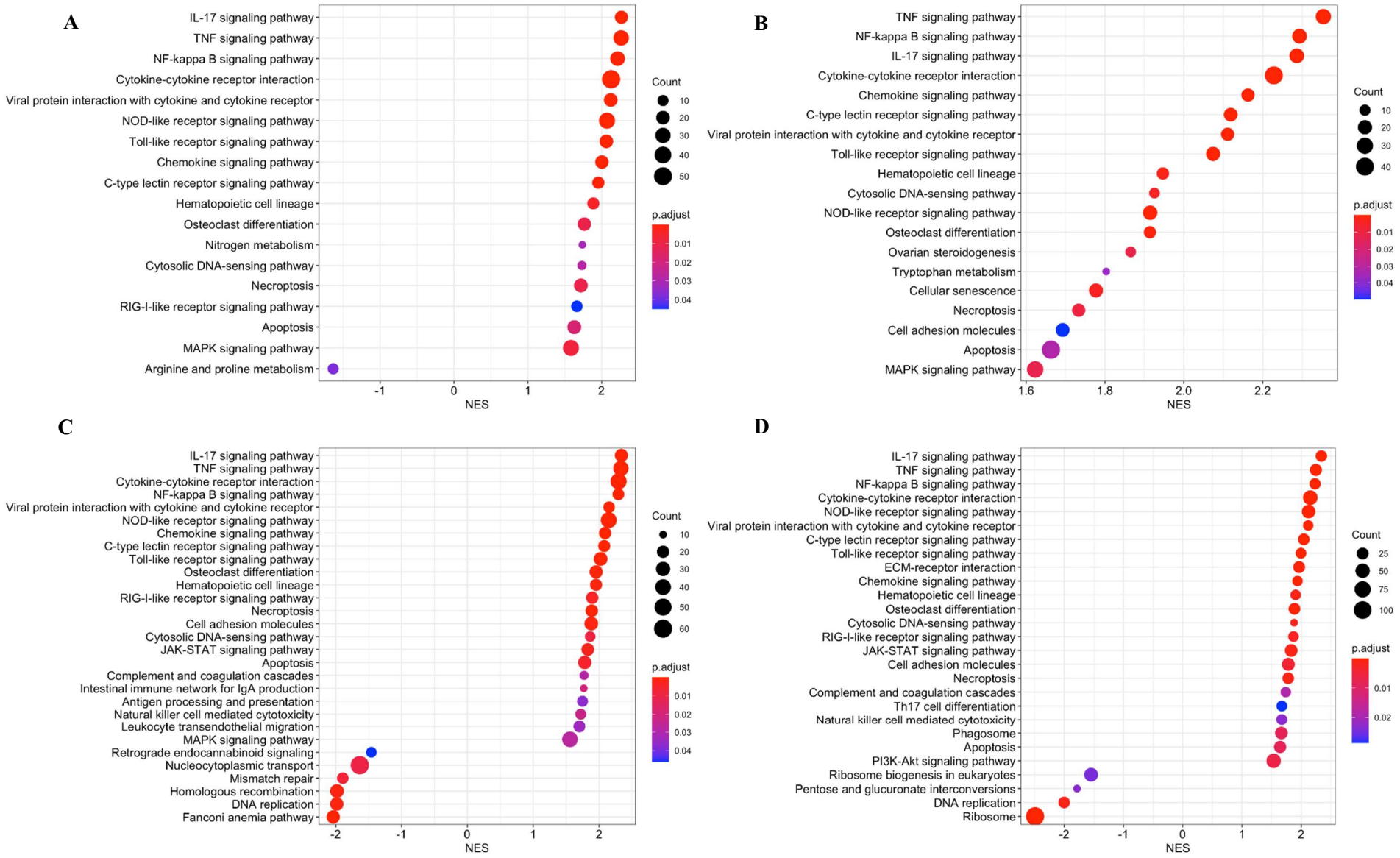
Figure 6.5 Venn diagrams of bMEC differentially expressed (DE) genes in response to N305 EVs and live *S. aureus* (SA). **(A)** EVs 3 h vs SA 3 h; and **(B)** EVs 24 h vs SA 3 h. Adj. *p*-val < 0.05. Threshold: |log2FC| ≥ 0.5.

Finally, regarding the 518 genes modulated by N305 EVs at 24 h (|log2FC| > 0.5), approximately one-quarter of them (n = 140) were also modulated by *S. aureus* at 3 h (|log2FC| > 0.5) (Fig 6.5B, Table S10), suggesting that EVs stimulation is slower in comparison to live bacteria. Among these 140 genes, there are those also activated by N305 EVs at 3 h (e.g., IL-1β, IL-6, IL-8, TNF-α), interleukin IL-33, C5AR2 involved in complement activation, and several genes involved in host cell immune response (e.g., NCF4, TCIM), and cell proliferation (e.g., PDGFB, FGF1). These preliminary results suggest that *S. aureus* N305 EVs may stimulate the host immune response with different intensities, exposure times, and by different routes.

6.3.3 Gene-Set Enrichment Analysis (GSEA) based on the KEGG database

In order to better interpret gene expression data in association with biological processes or molecular functions, gene-set enrichment analysis (GSEA) was performed using the KEGG tool. Results are given as normalized enrichment scores (NES). Overall, 41 significantly enriched KEGG pathways were induced by N305 EVs at all times (Fig. 6.6), which were organized in five functional families: cellular processes (4 pathways), environmental information (9 pathways), genetic information processes (7 pathways), metabolism (4 pathways), and organismal systems (17 pathways). Interestingly, more than half (n = 22) of enriched pathways belonged to signal transduction, immune system, and cell growth and cell death. Note that N305 EVs continuously activate several pathways, including

IL-17, TNF, NF-kβ, and *Chemokine signaling pathways, NOD-* and *Toll-like receptor signaling pathways, Cytokine-cytokine receptor interaction pathway, Cytosolic DNA-sensing pathway, Apoptosis* and *Necroptosis* (Fig 6.6). Moreover, while *EVs activated MAPK signaling pathway* after 3 h, 4 h, and 8 h exposure, *JAK-STAT signaling pathway* appeared only after 8 h and 24 h EV stimulation (Fig 6.6). Finally, the analysis revealed that inhibition of pathways related to genetic information processes appeared only after long-term stimulation with N305 EVs. For instance, *Mismatch repair* and *Homologous recombination* were repressed at 8 h, *Ribosome* and *Ribosome biogenesis* at 24 h, while *DNA replication* was inhibited at both times (Fig 6.6CD).


A
IL-17 signaling pathway
TNF signaling pathway
NF-kappa B signaling pathway
Cytokine-cytokine receptor interaction
Viral protein interaction with cytokine and cytokine receptor
NOD-like receptor signaling pathway
Toll-like receptor signaling pathway
Chemokine signaling pathway
C-type lectin receptor signaling pathway
Hematopoietic cell lineage
Osteoclast differentiation
Nitrogen metabolism
Cytosolic DNA-sensing pathway
Necroptosis
RIG-I-like receptor signaling pathway
Apoptosis
MAPK signaling pathway
Arginine and proline metabolism
-1
0
1
2
NES
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10
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TNF signaling pathway
NF-kappa B signaling pathway
IL-17 signaling pathway
Cytokine-cytokine receptor interaction
Chemokine signaling pathway
C-type lectin receptor signaling pathway
Viral protein interaction with cytokine and cytokine receptor
Toll-like receptor signaling pathway
Hematopoietic cell lineage
Cytosolic DNA-sensing pathway
NOD-like receptor signaling pathway
Osteoclast differentiation
Ovarian steroidogenesis
Tryptophan metabolism
Cellular senescence
Necroptosis
Cell adhesion molecules
Apoptosis
MAPK signaling pathway
1.6
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C
IL-17 signaling pathway
TNF signaling pathway
Cytokine-cytokine receptor interaction
NF-kappa B signaling pathway
Viral protein interaction with cytokine and cytokine receptor
NOD-like receptor signaling pathway
Chemokine signaling pathway
C-type lectin receptor signaling pathway
Toll-like receptor signaling pathway
Osteoclast differentiation
Hematopoietic cell lineage
RIG-I-like receptor signaling pathway
Necroptosis
Cell adhesion molecules
Cytosolic DNA-sensing pathway
JAK-STAT signaling pathway
Apoptosis
Complement and coagulation cascades
Intestinal immune network for IgA production
Antigen processing and presentation
Natural killer cell mediated cytotoxicity
Leukocyte transendothelial migration
MAPK signaling pathway
Retrograde endocannabinoid signaling
Nucleocytoplasmic transport
Mismatch repair
Homologous recombination
DNA replication
Fanconi anemia pathway
-2
-1
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Count
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p.adjust
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D
IL-17 signaling pathway
TNF signaling pathway
NF-kappa B signaling pathway
Cytokine-cytokine receptor interaction
NOD-like receptor signaling pathway
Viral protein interaction with cytokine and cytokine receptor
C-type lectin receptor signaling pathway
Toll-like receptor signaling pathway
ECM-receptor interaction
Chemokine signaling pathway
Hematopoietic cell lineage
Osteoclast differentiation
Cytosolic DNA-sensing pathway
RIG-I-like receptor signaling pathway
JAK-STAT signaling pathway
Cell adhesion molecules
Necroptosis
Complement and coagulation cascades
Th17 cell differentiation
Natural killer cell mediated cytotoxicity
Phagosome
Apoptosis
PI3K-Akt signaling pathway
Ribosome biogenesis in eukaryotes
Pentose and glucuronate interconversions
DNA replication
Ribosome
-2
-1
0
1
2
NES
Count
25
50
75
100
p.adjust
0.01
0.02

Figure 6.6 Dot plots of biological processes and molecular functions associated with bMEC DE genes after exposure to N305 EVs. Enriched modulated KEGG pathways in bMEC cells after 3 h (**A**), 4 h (**B**), 8 h (**C**), and 24 h (**D**) exposure to N305 EVs relative to respective controls.

Twelve KEGG pathways were significantly enriched in bMEC cells after 3 h exposure to *S. aureus* relative to control, which was organized in four functional families: cellular processes (4 pathways), genetic information processes (1 pathway), metabolism (1 pathway), and organismal systems (6 pathways). Note that the number of enriched pathways by *S. aureus* was smaller when compared to those modulated by N305 EVs at 3 h (18 pathways) (Fig 6.6A). In addition, most pathways modulated by live bacteria at 3 h were modulated by N305 EVs in the same condition, except for *Sulfur metabolism, Cell adhesion molecules,* and *Ribosome biogenesis in eukaryotes*. Enriched pathways induced by both EVs and live bacteria included those linked to signal transduction and immune system functional families, such as *IL-17, TNF,* and *Chemokine signaling pathways, NOD-* and *Toll-like receptor signaling pathways,* and *Cytokine-cytokine receptor interaction pathway* (Fig 6.6A, Fig 6.7). Interestingly, pathways enriched only by N305 EVs at 3 h were: *NF-kβ* and *MAPK signaling pathway, Nitrogen metabolism, Hematopoietic cell lineage, Cytosolic DNA-sensing pathway, Necroptosis,* and *Apoptosis* (Fig 6.6A).

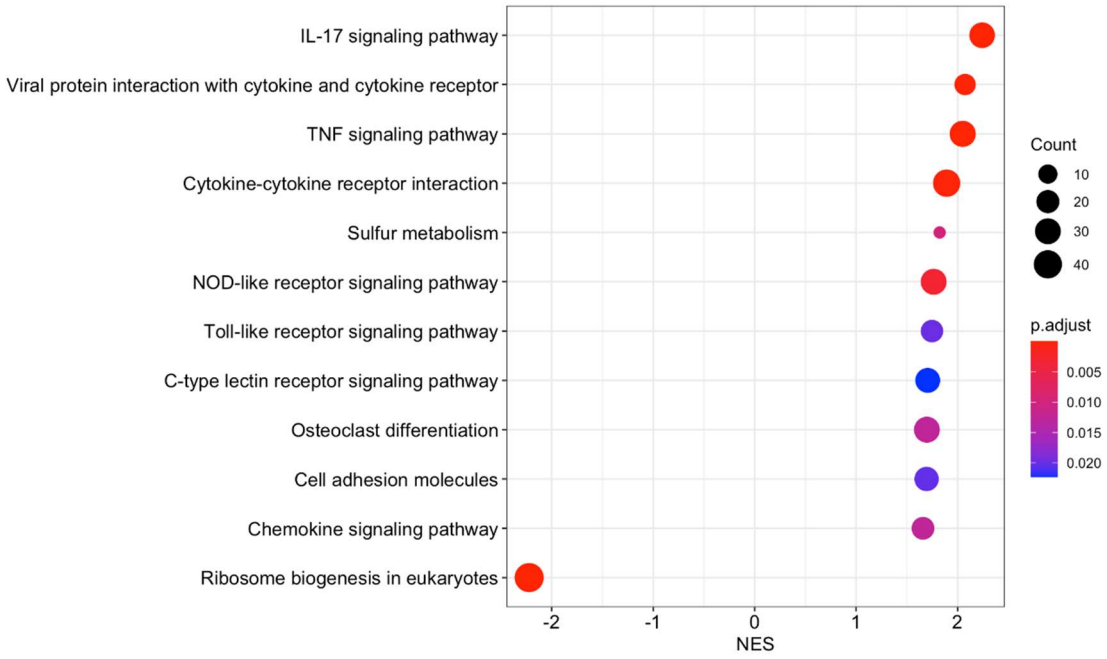

Figure 6.7 Dot plot of biological processes and molecular functions associated with bMEC DE genes after exposure to *S. aureus* N305. Enriched modulated KEGG pathways at *S. aureus* 3 h relative to control 3 h.

6.4 Discussion

The knowledge on *S. aureus* EVs is rapidly increasing due to their biological importance and their potential for developing new therapeutic strategies to fight staphylococcal infections. Nevertheless, most studies have focused on human clinical strains, which leaves a significant gap in the understanding of *S. aureus* EVs in critical veterinary diseases, such as mastitis (LUZ et al., 2021a). Economic losses caused by this disease have a substantial impact on the dairy industry, and a better comprehension of mastitis is the first step to finding concrete solutions for this food sector, as well as for animal health, welfare, and to actually reduce antibiotic usage in dairy herds. In 2018, Tartaglia and collaborators demonstrated that EVs derived from the *S. aureus* N305 bovine isolate induced pro-inflammatory response *in vitro* and *in vivo*, in addition to promoting tissue inflammation and deterioration in mice mammary glands (TARTAGLIA et al., 2018). Nevertheless, much information is still lacking in this field. Thus, this work aimed to better characterize the role of *S. aureus* EVs on the host immune response in the context of mastitis.

Bovine mammary epithelial cells (bMECs) play a critical role during intramammary infections by recognizing pathogen-associated molecular pattern molecules (PAMPs) and secreting cytokines and chemokines responsible for the activation of host immune responses, such as neutrophil recruitment (GRAY et al., 2005; RAINARD; RIOLLET, 2006). Here, we used a RNA-seq approach to deeply characterize the gene expression of bMEC stimulated with EVs derived from *S. aureus* N305, a model strain in bovine mastitis studies (PETON et al., 2014), at different exposure times: 3 h, 4 h, 8 h, and 24 h. Three hours exposure of bMECs to live *S. aureus* N305 was also performed in order to compare EV and live bacteria effects on gene expression. Principal component analysis and hierarchical clustering analysis validated the consistency of transcriptomic data and revealed the distinct impact of *S. aureus* N305 and its derived EVs on bMEC gene expression.

Regarding N305 EVs, we found that the number of differentially expressed (DE) genes increased over time, totalizing 3,774 protein-coding genes. From all DE genes, 161 were found in all conditions, from which 57 genes presented $|\log2FC| \geq 0.5$ ($|FC| \geq 2$). They included IL-1β, IL-6, TNF-α, and CXCL8 (IL-8), all of which were previously reported to be induced by EVs derived from different *S. aureus* strains (LUZ et al., 2021a). RT-qPCR experiments previously conducted by our group demonstrated that live *S. aureus* N305 and its derived EVs induced the expression of IL-1β, IL-8, and TNF-α in bMECs 3 h post-stimulation (TARTAGLIA et al., 2018). These results are corroborated by our present data

using a different approach. In contrast, while β-defensin (DEFβ1) was previously found induced by both live bacteria and EVs after 3 h (TARTAGLIA et al., 2018), here only *S. aureus* stimulated a slight expression of DEFβ1 (log2FC = 1.8). A study with another mastitis isolate, *S. aureus* M5512VL, also revealed induction of TNF-α by EVs on primary bovine mammary epithelial cells (pbMECs) after 24 h stimulation (SAENZ-DE-JUANO et al., 2022). Curiously, although there are several reports showing activation of CCL2 (MCP-1) (BITTO et al., 2021a; JUN et al., 2017; KWON et al., 2018, 2019) and CCL3 (MIP-1α) (HONG et al., 2011) genes by *S. aureus* EVs, they were not DE in none of the conditions tested here. This may be explained by differences in bacteria and host cells of each study.

The exposure of bMECs to *S. aureus* N305 during 3 h resulted in 4,550 DE genes, of which 873 genes presented $|\log2FC| \geq 0.5$. Among them, several genes were also modulated by N305 EVs at 3 h. Remarkably, bMEC expression of IL-1β, a master inflammation cytokine, was 7 times higher after exposure to N305 EVs compared to live bacteria. Similarly, a study by Wang et al. 2020 demonstrated that *S. aureus* JE2-derived EVs activate the NLRP3 inflammasome in human macrophages leading to IL-1β release (WANG; EAGEN; LEE, 2020). Released EVs may favor bacterial survival by setting up pre-inflamed sites that allow *S. aureus* to hijack the host defense, as previously shown in an allergy model (MUES; CHU, 2020; NAVARRO et al., 2010). Other genes whose induction levels were also more significant in bMECs after exposure to N305 EVs included TNF-α, IL-8, and CXCL1 (KC), with the last two genes being crucial chemotaxis elements. Accordingly, previous experiments with mice mammary glands evidenced that N305 EVs induce mainly chemotaxis responses compared to live bacteria, contributing to the migration of defense cells to the site of infections (TARTAGLIA et al., 2018). Since *S. aureus* is a versatile bacterium able to infect and persist within various host cell types, including professional phagocytes (KUBICA et al., 2008; VOYICH et al., 2005; ZAATOUT; AYACHI; KECHA, 2020), we could hypothesize that the recruitment of immune cells by EVs may favor bacterial internalization and survival. Finally, one example of a gene presenting lower expression (2.5 times) in N305 EVs when compared to a 3-hour *S. aureus* exposure is the plasminogen activator (PLAT), which, upon stimulation, triggers the high proteolytic enzyme plasmin leading to tissue damage and bacterial invasiveness (JIN et al., 2004; PEETERMANS et al., 2014). One could suggest that reduced plasmin activation by EVs may avoid exacerbated tissue damage. Since the outcome of *S. aureus* infections is highly dependent on the equilibrium between bacterial performance and host immune response, our data suggest that released EVs contribute to the control and development of pathogenesis.

Our data also revealed that 49 genes were modulated by N305 EVs at 3 h, but not by *S. aureus* in the same condition. They included CD40, CD80, and ICAM-1, being the first two membrane receptors involved in T-cell activation and inflammatory amplification. It has been previously demonstrated that T-cell activation may favorize *S. aureus* in several infections (MCLOUGHLIN et al., 2006; PARKER et al., 2015; SCHMALER et al., 2011; TZIANABOS et al., 2000). In contrast to the previously mentioned genes which presented high expression levels (e.g., IL-1β, IL-8), CD40 and CD80 were only slightly induced by EVs. Another exclusive EV modulated gene was ICAM-1, an intercellular adhesion molecule present on epithelial cells able to interact with phagocyte-surface integrins to promote neutrophil extravasation during tissue inflammation (YANG et al., 2005). The EV-mediated ICAM-1 induction was observed at all exposure times. The staphylococcal protein Eap directly binds ICAM-1 and inhibits these interactions, resulting in neutrophil misfunction (CHAVAKIS et al., 2002). Interestingly, recent proteomic analysis with *S. aureus* strain HG003 revealed that Eap protein was detected in HG003 derived EVs, but not in the whole cell proteome in the same conditions (See Chapter 4). However, this protein seems to be absent from N305 EVs (TARTAGLIA et al., 2018). Activation of specific genes by EVs may indicate that they play important roles in the *S. aureus* pathogenesis, and their contribution to infection must be further investigated.

So far, the ability of N305 EVs to modulate the host immune response presented here is in accordance with previous proteomic data showing that their cargo comprises several proteins involved in *S. aureus* pathogenesis. N305 EV cargo includes adhesins (e.g., Sbi, EbpS), toxins (e.g., Hld, Phenol-soluble globulins, PSMs), and moonlight proteins (e.g., Atl, Eno) involved in adherence to host tissues, host cell damage, and host immune evasion (TARTAGLIA et al., 2018). Considering that EVs may travel long distances, the adequate orchestration of the host immune response may benefit *S. aureus* dissemination during infection. Overall data showing numerous genes stimulated by N305 EVs throughout all conditions, such as IL-1β, IL-8, and TNF-α, suggest that EVs continuously activate host cells, promoting unceasing inflammatory stimulation. Indeed, previous experiments evaluating bMEC immune response after 8 h continuous exposure to N305 EVs revealed significant increased expression of IL-1β, IL-8, and TNF-α genes. In comparison, 2 h EVs exposure followed by an additional 6 h incubation in a culture medium without EVs (totalizing 8 h) completely abrogated their expression (data not shown). Alternatively, a comparison of the bMEC genes modulated by *S. aureus* at 3 h and by N305 EVs at 24 h (threshold: $|\log2FC| \geq 0.5$) found 140 common features, suggesting that some host responses to N305 EVs are

delayed when compared to bacteria. One of these genes is IL-33, a member of the IL-1 family of cytokines involved in chemokine production and neutrophil recruitment (CAYROL; GIRARD, 2018). IL-33 expression is associated with wounding healing and antimicrobial defense against bacterial skin infections caused by *S. aureus* (LI et al., 2014a; YIN et al., 2013). Again, our data evidence the potential role of released EVs on the precise adjustment of host responses.

Finally, we observed that even though bMEC exposure to *S. aureus* for 3 h induced almost 90% more genes than N305 EVs in the same condition, GSEA revealed that EV stimulation significantly activated more KEGG pathways than live bacteria, suggesting their strong stimulatory effect. Moreover, even though Apoptosis and Necroptosis pathways were not activated after exposure to *S. aureus* at 3 h, they were induced by N305 EVs in all conditions tested. In this line, specific properties of EVs may trigger host elements necessary for successful *S. aureus* infections. Indeed, it has been shown that purified α-hemolysin is less cytotoxic than when associated with EVs, and, while the first induced apoptotic cell death, EV-associated α-hemolysin induced necrosis (HONG et al., 2014). At last, host pathways related to genetic information processes, such as *DNA replication* and *Mismatch repair*, were inhibited after long-exposure to N305 EVs. Deplanche et al. (2015) demonstrated that *S. aureus* PSM-α induces delayed G2/M phase transition, which is associated with increased intracellular bacterial replication, host DNA damage, and cell senescence (DEPLANCHE et al., 2015, 2019). PSM-α is part of N305 EV cargo (TARTAGLIA et al., 2018) and may also contribute to the senescence of infected cells, a strategy used by pathogenic bacteria to establish infections (HUMPHREYS; ELGHAZALY; FRISAN, 2020).

Overall, the dynamics of bMEC exposure to N305 EVs at different time-points revealed that they modulate the host immune response with different intensities, exposure periods, and by routes different from those of live bacteria, suggesting that *S. aureus* EVs are fine-tuning particles functioning for the perfect balance in host-pathogen interactions. The conditions used here also revealed new chemokines and cytokines modulated by *S. aureus*-derived EVs, including CCL20 (MIP-3α), CCL22 (macrophage-derived chemokine), CXCL3 (GRO3), and CXCL5. The validation of these results in mice mammary gland tissues exposed to N305 EVs by RT-qPCR are currently ongoing to confirm their mechanisms of action *in vivo*. This study uncovered new *S. aureus* EV properties, their potentially functional roles, and provides brand-new knowledge on the role of staphylococcal EVs in the context of host-pathogen interactions and in mastitis pathogenesis.

Table 6.2 List of common DE genes modulated by N305 EVs at all times with a |log2FC| ≥ 0.5.

Gene name	log2 fold change (log2FC)			
	EVs 3 h	**EVs 4 h**	**EVs 8 h**	**EVs 24 h**
BIRC3	1.402 (2.643)	1.381 (2.605)	1.662 (3.164)	1.88 (3.682)
CA4	1.824 (3.54)	1.054 (2.076)	0.914 (1.884)	1.723 (3.302)
CAPN14	0.53 (1.444)	0.722 (1.649)	1.185 (2.273)	1.15 (2.219)
CCL20	3.796 (13.887)	3.711 (13.098)	3.967 (15.639)	3.377 (10.391)
CCL22	3.163 (8.955)	4.219 (18.618)	3.721 (13.186)	4.926 (30.398)
CCN3	0.674 (1.595)	0.763 (1.697)	1.407 (2.653)	2.27 (4.823)
CD40	0.752 (1.685)	1.207 (2.309)	1.81 (3.505)	1.662 (3.166)
CD83	4.339 (20.238)	3.851 (14.429)	3.445 (10.888)	3.352 (10.212)
CDKN1A	0.894 (1.858)	1.048 (2.067)	1.722 (3.299)	2.2 (4.596)
CFB	0.841 (1.791)	1.589 (3.008)	2.978 (7.879)	3.4 (10.555)
CSF2	1.57 (2.969)	1.798 (3.477)	1.749 (3.362)	2.957 (7.766)
CX3CL1	2.135 (4.394)	2.231 (4.695)	2.963 (7.8)	3.044 (8.248)
CXCL3	2.363 (5.146)	2.041 (4.115)	2.103 (4.296)	1.675 (3.193)
CXCL5	2.403 (5.289)	2.785 (6.891)	3.397 (10.537)	2.88 (7.364)
CXCL8	4.384 (20.873)	3.848 (14.404)	4.283 (19.47)	3.487 (11.215)
CYP1A1	4.504 (22.686)	4.112 (17.287)	1.664 (3.169)	2.465 (5.522)
DNAJB1	0.654 (1.574)	0.742 (1.672)	0.848 (1.8)	0.641 (1.56)
GPR84	3.057 (8.325)	3.751 (13.467)	6.744 (107.153)	3.867 (14.589)
GPRIN2	1.452 (2.737)	1.577 (2.983)	2.189 (4.56)	2.531 (5.779)
GRO1	2.902 (7.474)	2.639 (6.23)	2.649 (6.274)	2.152 (4.444)
HS3ST1	0.786 (1.725)	0.877 (1.836)	1.087 (2.125)	1.238 (2.359)
HSD3B1	0.742 (1.672)	1.121 (2.175)	2.28 (4.856)	2.301 (4.929)
ICAM1	1.105 (2.151)	1.22 (2.33)	2.064 (4.183)	2.185 (4.549)
IER3	0.723 (1.651)	0.789 (1.728)	1.048 (2.068)	1.26 (2.394)
IL1A	2.566 (5.921)	2.718 (6.58)	2.874 (7.332)	2.884 (7.383)
IL1B	5.591 (48.189)	5.702 (52.071)	5.628 (49.456)	6.198 (73.397)
IL1R2	2.538 (5.809)	2.512 (5.703)	3.802 (13.947)	2.585 (6.001)
IL6	2.363 (5.143)	2.557 (5.884)	2.965 (7.806)	2.679 (6.404)
IRAK2	0.532 (1.446)	0.774 (1.71)	0.898 (1.863)	0.936 (1.913)
IRF1	0.706 (1.631)	0.925 (1.898)	1.374 (2.592)	1.011 (2.015)
LIF	2.576 (5.963)	2.679 (6.402)	3.587 (12.019)	3.704 (13.029)
LRRC4	0.671 (1.592)	0.89 (1.853)	1.032 (2.044)	2.035 (4.098)
LTB	0.589 (1.504)	0.643 (1.561)	1.528 (2.883)	1.515 (2.858)
MAFF	0.899 (1.865)	0.88 (1.84)	1.593 (3.016)	1.659 (3.159)
MAP3K8	0.844 (1.796)	0.956 (1.94)	1.099 (2.141)	1.211 (2.316)
MMP1	0.814 (1.758)	1.122 (2.177)	1.939 (3.836)	3.054 (8.305)
MMP1.2	0.625 (1.542)	0.959 (1.944)	1.811 (3.51)	2.861 (7.267)
MMP9	0.601 (1.516)	0.617 (1.533)	1.747 (3.356)	3.197 (9.17)
NCF1	1.498 (2.825)	2.086 (4.246)	3.056 (8.315)	3.168 (8.986)
NFKBIA	1.258 (2.391)	1.378 (2.6)	1.565 (2.958)	1.567 (2.964)

NFKBIZ	1.401 (2.642)	1.381 (2.604)	1.186 (2.276)	1.381 (2.605)
NPPC	1.479 (2.788)	1.354 (2.555)	2.246 (4.745)	1.049 (2.069)
NRP2	0.73 (1.659)	0.809 (1.752)	1.144 (2.21)	1.747 (3.356)
NUPR1	0.594 (1.51)	0.735 (1.665)	1.488 (2.805)	1.371 (2.587)
PGLYRP2	2.172 (4.506)	2.514 (5.711)	3.999 (15.986)	3.409 (10.619)
PTGS2	0.729 (1.657)	1.012 (2.017)	1.069 (2.098)	2.139 (4.404)
RASGEF1B	0.55 (1.464)	0.535 (1.449)	0.841 (1.792)	1.073 (2.104)
RELB	0.672 (1.593)	0.955 (1.938)	1.431 (2.696)	1.122 (2.177)
RND1	1.021 (2.029)	1.391 (2.623)	1.779 (3.431)	1.968 (3.912)
RNF125	0.596 (1.512)	1.039 (2.055)	1.644 (3.125)	1.652 (3.143)
SAA3	2.069 (4.196)	2.596 (6.047)	4.743 (26.786)	5.867 (58.356)
SKINT1	1.29 (2.445)	2.058 (4.165)	3.465 (11.046)	2.605 (6.084)
STEAP4	1.453 (2.739)	1.8 (3.483)	3.717 (13.151)	4.085 (16.972)
TNF	2.954 (7.751)	2.846 (7.192)	3.434 (10.804)	3.023 (8.127)
TNFAIP3	2.318 (4.987)	2.165 (4.485)	2.376 (5.191)	2.105 (4.301)
TNFSF15	1.447 (2.727)	1.406 (2.649)	1.994 (3.984)	1.895 (3.718)
ZC3H12A	0.809 (1.753)	0.878 (1.838)	1.13 (2.189)	1.291 (2.448)

Table 6.3 List of top down-regulated genes in each condition.

EVs 3 h		**EVs 4 h**		**EVs 8 h**		**EVs 24 h**		**SA 3 h**	
Gene	log2FC	Gene	log2FC	Gene	log2FC	Gene	log2FC	Gene	log2FC
CRNN	-1,72	CRYAB	-2,46	GPRC5A	-1,39	ZNF181	-2,42	LRRC3	-2,60
PDE9A	-0,78	JAK3	-1,11	CACNA1A	-1,02	F2	-2,20	MEOX1	-2,51
GAB2	-0,67	SALL2	-0,64	SLC2A12	-0,74	MGC137036	-2,06	NLRC3	-2,43
DLX3	-0,49	STRA6	-0,62	POU2AF1	-0,67	DMRTA1	-1,97	RIPPLY1	-2,27
INHBB	-0,46	WNT11	-0,59	FBXL2	-0,65	SBK2	-1,70	TRIM63	-2,21
MFAP3L	-0,46	ZNF827	-0,54	TRNP1	-0,64	FBXO43	-1,64	RASL11B	-2,15
PROM1	-0,42	LMCD1	-0,53	CILP2	-0,60	BMP2	-1,61	PITPNM2	-2,11
ARHGAP24	-0,39	CHRM4	-0,53	CYP4F2	-0,56	STOX1	-1,57	GIPC2	-2,06
IL20RA	-0,39	ADAMTS3	-0,47	THRSP	-0,56	GPR19	-1,55	WNT9A	-1,96
CCN1	-0,38	ADAMTSL1	-0,46	ZNF268	-0,51	PLB1	-1,47	TMEM74B	-1,83
FRMPD1	-0,38	CCN2	-0,46	CDHR1	-0,50	KRT75	-1,26	SCHIP1	-1,77
PRAG1	-0,38	SERPINB9	-0,45	RMI2	-0,46	KIF26A	-1,23	SYTL5	-1,75
ADAMTS14	-0,36	PADI4	-0,42	FGFRL1	-0,44	GSTA1	-1,15	NCF4	-1,72
RNF39	-0,35	PADI3	-0,42	TUBA1D	-0,44	RIPPLY1	-1,03	RORC	-1,67
ZBTB42	-0,35	SPNS2	-0,41	LRRC15	-0,43	MYRFL	-0,99	LOXL4	-1,55
LFNG	-0,34	KRT80	-0,39	SULF1	-0,41	ELN	-0,99	ANKRD1	-1,51
SH3BP4	-0,34	COL3A1	-0,38	MCM8	-0,40	CYP2R1	-0,98	ABCG1	-1,48
MAB21L4	-0,32	SPHK1	-0,35	PLEKHG1	-0,39	CCDC153	-0,96	RASGEF1B	-1,42
CCN2	-0,31	MTX3	-0,35	LIX1	-0,39	CPE	-0,95	ZC3H12D	-1,36
CDK6	-0,31	ADAMTS14	-0,34	BARX2	-0,39	SPON1	-0,89	LAPTM5	-1,35

Table 6.4 List of top-up-regulated genes in each condition.

EVs 3 h		**EVs 4 h**		**EVs 8 h**		**EVs 24 h**		**SA 3 h**	
Gene	log2FC	Gene	log2FC	Gene	log2FC	Gene	log2FC	Gene	log2FC
IL1B	5,59	IL1B	5,70	SLC6A12	6,91	SLC6A12	6,69	FAM217A	5,72
APOL-3	4,80	CCL22	4,22	GPR84	6,74	IL1B	6,20	CYP1A1	4,60
CYP1A1	4,50	CYP1A1	4,11	ADORA2A	6,11	SAA3	5,87	WNT4	4,57
CXCL8	4,38	CD83	3,85	BCL2A1	6,05	BCL2A1	5,53	RSAD2	4,07
CD83	4,34	CXCL8	3,85	IL36RN	5,88	VNN1	5,30	ZNF536	3,75
CCL20	3,80	GPR84	3,75	IL1B	5,63	M-SAA3.2	5,08	ANKRD37	3,48
ZNF536	3,50	CCL20	3,71	VCAM1	4,81	CCL22	4,93	MYO16	3,24
CCL22	3,16	TNF	2,85	SAA3	4,74	PTGIR	4,86	EVI2A	3,13
GPR84	3,06	CXCL5	2,79	CXCL8	4,28	TAP	4,45	CXCL8	2,93
TNF	2,95	IL1A	2,72	PGLYRP2	4,00	IL36RN	4,34	CCL20	2,85
GRO1	2,90	LIF	2,68	M-SAA3.2	3,99	STEAP4	4,09	FOSB	2,82
LIF	2,58	BCL2A1	2,64	CCL20	3,97	GPR84	3,87	IL1B	2,77
IL1A	2,57	GRO1	2,64	IL1R2	3,80	CYRIA	3,79	MEDAG	2,66
IL1R2	2,54	SAA3	2,60	CCL22	3,72	CCL5	3,78	GFPT2	2,62
MELTF	2,53	IL6	2,56	STEAP4	3,72	OLR1	3,73	CRISPLD2	2,57
CXCL5	2,40	PGLYRP2	2,51	LIF	3,59	LIF	3,70	SLCO4A1	2,56
CXCL3	2,36	IL1R2	2,51	ASB9	3,52	PLEKHS1	3,58	XYLT1	2,54
IL6	2,36	SLC6A12	2,51	SKINT1	3,47	CXCL8	3,49	MELTF	2,50
TNFAIP3	2,32	PLEKHS1	2,48	CD83	3,45	FBP2	3,47	SYNE3	2,50
PGLYRP2	2,17	CX3CL1	2,23	TNF	3,43	DNAJC5B	3,45	SDC2	2,49

Chapter 7. General conclusions and perspectives

7.1 Thesis' contribution

This thesis project provides the comparison of the transcriptomic and proteomic profile of *S. aureus* HG003 and its derived EVs in different environmental conditions: early- and late-stationary growth phases, and in the absence and presence of a sub-lethal concentration of vancomycin. We found that tested conditions did not significantly affect EV concentration, however, EVs released at late-stationary phase were larger than EVs released at early-stationary phase. Regarding transcriptome analysis, this is first work extensively investigating the RNA content and profile of *S. aureus* EVs, and also the first of this kind in Gram-positive bacteria. We demonstrated that HG003 EVs carry all RNA classes, which corresponded to a considerable portion of annotated transcripts for the HG003 strain. However, most of these RNAs transcripts were fragmented, with only a small percentage (5%) of highly covered RNAs. Among the highly covered RNAs there are 10 residual rRNAs, 28 sRNAs, 28 tRNAs, and 220 mRNAs, many of which belonged to the same transcriptional units in *S. aureus*. Interestingly, metabolic elements and important virulence factors were found as mRNAs, as well as in the protein form in HG003 EVs. Finally, although the tested conditions had a more pronounced impact in EV cargo, environment modulated the RNA and protein composition and abundance of both groups, with remarkable differences between EVs and EV-producing cells, suggesting that EV selective cargo packing exists.

Taking into account the described HG003 EV RNA and protein cargo, we decided to further investigate their impact in the intra-species and inter-kingdom contexts. Regarding intra-species interactions, we showed that HG003 EVs had a media-dependent positive impact on bacterial growth. While growth of *S. aureus* remained unchanged after the addition of supplementary EVs in BHI medium, a dose-dependent increase was observed when EVs supplemented *S. aureus* grown in restrictive media NZM and RPMI + 10 % LB. This is the first study evidencing the positive impact of *S. aureus* EVs on bacterial population growth, paving the way for other studies in this area. In the context of host-pathogen interactions, we explored the impact of *S. aureus* HG003 and its derived EVs on three different host cell lines: pulmonary cells, keratinocytes, and osteoblasts. Although HG003 EVs were not cytotoxic to neither of the cell lines, they induced the expression of several pro-inflammatory cytokines. Interestingly, gene activation varied a lot between the cell lines tested. For instance, EVs and

bacteria induced the expression of more genes in the osteoblasts than in the two other cell lines. Some differences were also observed relative the source of stimulation, with less genes activated by EVs and with lower expression levels when compared to live bacteria. These data bring new insights in an area still largely unexplored, showing the role of *S. aureus* EVs in different contexts of infection, and how their exerted functions diverge from that of live bacteria.

Finally, in the last part of this project, we used a RNA-seq approach to provide a deeper characterization of the host response to *S. aureus* N305-derived EVs in the context of mastitis. This is also the first transcriptome of host cells exposed to *S. aureus* EVs in the literature. Bovine mammary epithelial cells (bMECs) were exposed to EVs during different times: 3 h, 4 h, 8 h and 24 h, while live bacteria were used as positive control after 3 h exposition. We found that the number of differentially expressed (DE) genes impacted by N305 EVs increased over time, some of which were modulated in all conditions. Moreover, remarkable genes modulated by both N305 EVs and live bacteria at 3 h, such as chemokines and pro-inflammatory cytokines, presented different expression levels depending on the source of stimulation. Interestingly, almost half of the genes modulated by N305 EVs after 24 h exposition is also modulated by *S. aureus* at 3 h, suggesting a late-EV modulatory effect. We also found that some genes modulated by N305 EVs at 3 h, are not modulated by bacteria in the same condition, a finding that is in accordance with gene enrichments analyses showing that several KEGG pathways were induced exclusively by N305 EVs (e.g. *Apoptosis*, *Necroptosis*). Finally, our study also uncovered new chemokines and cytokines modulated by *S. aureus* EVs that have not been previously described, bringing new knowledge and insights to this field.

7.2 Open questions and perspectives

7.2.1 What are the mechanisms involved in S. aureus *EV biogenesis?*

The mechanisms governing *S. aureus* EV biogenesis remain to be elucidated. In Gram-negative bacteria, turgor pressure and membrane curvature modifications have been proposed as triggers of OMV release (ELHENAWY et al., 2016; MCBROOM; KUEHN, 2007; ZHOU et al., 1998). In *S. aureus*, it has been shown that mutations in autolysins, PSMs and σ^B genes impacts EV production and concentration (LIU et al., 2018a; SCHLATTERER et al., 2018; WANG et al., 2018b), as well as different environmental conditions and stresses (ANDREONI et al., 2019; ASKARIAN et al., 2018; BRIAUD et al., 2021; KIM et al., 2020;

WANG et al., 2021b). In our study, EV biophysical properties remained unchanged after exposure to antibiotic, while EVs released at late-stationary growth phase were significantly larger than those released at early-stationary phase. This result could be explained by modifications in peptidoglycan cross-linking and cell-wall morphology at different *S. aureus* growth stages (ZHOU; CEGELSKI, 2012). However, EVs derived from the *S. aureus* C-29 strain at 6 h, 17 h and 24 h did not present differences in particle size and concentration (YAMANASHI et al., 2022). These data suggest that other intrinsic factors may also contribute to the observed differences. Indeed, Tartaglia et al. reported that EVs derived from five *S. aureus* strains from different host origins (human, bovine and ovine) presented significant differences in EV concentration and size (Tartaglia et al. 2020). This data raises the question if *S. aureus* EV biogenesis, although conserved across several strains, may occur by distinct pathways. In fact, Andreoni evidenced that antibiotic treatment may induce EVs release in both phage-dependent and -independent routes (ANDREONI et al., 2019).

Here, we identified at least 16 features from the HG003 genome that were present in both mRNA and protein forms in EVs. They include the autolysin (Atl), elongation factor FusA, enolase, and DnaK chaperone, being the last two proteins also part of the EV core proteome derived from five *S. aureus* strains, and in EVs derived from species of other Gram-positive genera, including *Bacillus*, *Clostridium*, *Listeria*, *Streptococcus*, and *Mycobacterium* (Tartaglia et al. 2020). These data lead us to hypothesize that they may play important role in EV biogenesis. Indeed, it has been shown that chaperones participate in selective protein packing of OMVs (ALTINDIS; FU; MEKALANOS, 2014; HUSSAIN; BERNSTEIN, 2018). Moreover, autolysins Atl and Sle1 have been shown to be involved in *S. aureus* EV release, since mutants for these genes release EVs with reduced size and concentration (WANG et al., 2018b). Much research is necessary in this field, and the characterization and identification of common EV elements across different bacterial species may provide insights on global biogenesis mechanisms. Experiments assessing EV release in mutants for proteins widely found in EVs, such as those involved in cell wall remodeling and protein adressing systems, may help elucidate EV biogenesis in *S. aureus* and other species.

7.2.2 What are the mechanisms involved in S. aureus *EV cargo selective packing?*

Our results showed that both RNA and protein cargo were relatively enriched in EVs when compared to bacteria, and were also environment-dependent. Nevertheless, enrichment results, mainly of RNAs, must be interpreted with caution, since it may reflect differences in

RNase activity, or due to the accumulation over time in EVs of RNAs with longer half-lives (LANGLETE; KRABBERØD; WINTHER-LARSEN, 2019; LÉCRIVAIN; BECKMANN, 2020). Either way, we found that the sRNA RNA RsaC involved in *S. aureus* response to oxidative stress identified in this study is also present in EVs derived from the *S. aureus* MSSA476 strain (JOSHI et al., 2021b), suggesting its common occurrence across different strains. Although packing mechanisms for RNA into EVs are still unknown, characteristics such as RNA size, location, and affinity for other molecules have been proposed as possible drivers (LANGLETE; KRABBERØD; WINTHER-LARSEN, 2019). Regarding the protein content, two studies conducted by our group have shown an association between proteins found in bacterial EVs and their biophysical properties. It was demonstrated that *S. aureus* EV proteins contain less hydrophobic, and more polar and charged residues than the whole cell proteome, suggesting that they are likely positively charged at a physiological pH (TARTAGLIA et al., 2020). Moreover, higher codon adaptation index (C.A.I.) and specific amino acid composition, such as overrepresented lysine, and underrepresented tryptophan and histidine, are protein properties found in EVs derived from both *S. aureus* (TARTAGLIA et al., 2020) and *Propionibacterium freudenreichii* species (unpublished data).

Here, we observed that approximately 60% of the HG003 EVs proteome is conserved across the four conditions tested. Additionally, proteomic analysis comparing EVs derived from five *S. aureus* strains of diverse host origins (human, bovine, and ovine) using the same approach also revealed a highly conserved proteome (TARTAGLIA et al., 2020). More interestingly, more than 80% of the EV core proteome identified in these five strains also overlap with HG003 EV proteome (this study) and EVs derived from the *S. aureus* RN4220 strain (HE et al., 2017), reinforcing that some EVs elements are present irrespective of specific strains and conditions. Several other studies also support the EV cargo selective packing in different bacterial species. In Gram-negative bacteria, *Serratia marcescens* and *Porphyromonas gingivalis* exclude proteins abundant in the outer membrane from OMVs, while they pack virulence factors and elements absent in this compartment (HAURAT et al., 2011; MCMAHON et al., 2012; VEITH et al., 2014). In Gram-positive bacteria, *Mycobacterium* and *Bacillus* species releases EVs enriched with lipoproteins and siderophore-binding proteins (BROWN et al., 2014; PRADOS-ROSALES et al., 2011, 2014b).

Note that most mentioned studies focus mainly on EV proteomic analysis, and thus, there is still a big gap in the characterization of other elements, such as DNA, metabolites, and lipids. Although the presence of DNA was evidenced in *S. aureus* EVs (ANDREONI et

al., 2019; BITTO et al., 2021a; RODRIGUEZ; KUEHN, 2020), none of these studies provided a detailed characterization, therefore, it is still unclear if DNA species may also be selectively packed into *S. aureus* EVs. To date, metabolite and lipid characterizations of *S. aureus* EVs are still lacking. In other bacterial species, it has been shown that OMVs derived from *Bacteroides thetaiotaomicron* are significantly enriched with metabolites linked to mouse metabolic networks (BRYANT et al., 2017). Studies with Gram-positive *Streptococcus*, *Propionibacterium*, and *Enterococcus* also revealed that EV lipid profile differ from that of bacteria (AFONINA et al., 2021; JEON et al., 2018; RESCH et al., 2016), suggesting selective packing. If this is a common featured of bacterial EVs, we could speculate that *S. aureus* EV may also present specific composition of lipids and metabolites. New studies are still necessary to further characterize EV components, and uncover mechanisms behind *S. aureus* EVs cargo packing.

7.2.3 What are the eventual advantages of S. aureus *EV cargo selective packing?*

At early stages of infection, *S. aureus* is expressing mainly elements linked to adhesion and invasion, while up-regulation of the expression of toxins necessary for host cell lysis and bacterial dissemination occurs latter. Accordingly, toxins known to be produced by *S. aureus* at high cell densities, such as Hld and PSMβ1 (JENUL; HORSWILL, 2019; LE; OTTO, 2015), were found in EVs only at late-stationary phase in our study. In this line, we could hypothesize that selective packing of toxins and other immunostimulatory elements into EVs could help bacteria exerted specific functions in host-pathogen interactions that may contribute to *S. aureus* survival and persistence. Indeed, in a report by Briaud et al., temperatures variations affected EV RNA and protein composition and abundance, directly reflecting in the EV cytotoxic properties towards host cells (BRIAUD et al., 2021). In fact, EVs derived from 34°C presented higher protein diversity, while EVs released at 40°C presented increased packaging of virulence factors (BRIAUD et al., 2021). As another example, *S. aureus* C-29 EVs recovered at 6 h, 17 h and 24 h cultures induced different inflammation-related genes in HaCaT keratinocytes (YAMANASHI et al., 2022).

It has been shown that various environmental stresses, such as antibiotic treatment, iron-depletion, ethanol, oxidative, and osmotic stresses may significantly alter EV size and concentration (ASKARIAN et al., 2018; KIM et al., 2020; WANG et al., 2021b), but characterization of EV cargo in most of these cases is still lacking, especially in lipid and metabolite composition. It has been demonstrated that short-chain fatty acids (SCFAs) produced by *P. freudenreichii* exert an anticancer effect by shifting host cell death mode from

apoptosis to necroptosis (JAN et al., 2002; LAN et al., 2007, 2008). In another study, administration of SCFA butyrate to ulcerative colitis (UC) patients was shown to inhibit NF-κB activation in macrophages, presenting an anti-inflammatory effect (LÜHRS et al., 2002). Moreover, *B. thetaiotaomicron* OMVs enriched with metabolites related to host metabolic networks also suggests their role in interactions between gut bacteria and mammals (BRYANT et al., 2017). In light of all these data, we could imagine that selective packing of proteins, lipids, metabolites, and nucleic acids into *S. aureus* EVs may provide advantage during infection development. The study of conditions mimicking the host environment are promising strategies. Indeed, additional experiments using DMEM and RPMI supplemented with fetal bovine serum (FBS) as bacterial culture media revealed hyper vesiculation in *S. aureus* (data not shown). The restrictive iron concentrations and host factors present in FBS may affect *S. aureus* physiology and consequently EV release and cargo. Future studies addressing the impact of other environmental conditions or stresses in EV selective cargo packing and roles may provide new information about *S. aureus* pathogenic processes.

7.2.4 What are S. aureus *EV roles in intra-species interactions?*

In this work, we provided the first evidence demonstrating that *S. aureus* EVs exert trophic functions. This phenomenon has already been demonstrated in marine and microbiome systems, where released OMVs support the growth of other species by serving as direct or indirect nutrient sources (BILLER et al., 2014; RAKOFF-NAHOUM; COYNE; COMSTOCK, 2014; SCHWECHHEIMER; KUEHN, 2015). Here, transcriptomic and proteomic characterization revealed that HG003 EVs contain several RNAs and proteins involved in metabolic processes and energy production, such as substrate transporters, glycolytic enzymes, pyruvate dehydrogenase, and cytochrome c oxidase complexes. The transfer of these elements via EVs could contribute to recipient cell functioning by boosting translation and nutrition, especially in a restringing context. We could also imagine that translation may occurs within EVs independent of the bacteria cell, which could further optimize these interactions. In fact, our analysis detected intact mRNA operons, suggesting that *S. aureus* EVs RNAs may be present as full-length transcripts. Moreover, ribosomal units, tRNAs and other elements essential to translation, such as elongation factors, are also present in EVs. During my thesis, experiments were conducted to try to validate this hypothesis (data not shown). Ribosomes were extracted and purified from both whole cell and EV samples for an *in vitro* protocol of translation. EVs samples were negative for translation whereas cell samples were positive. However, this result is inconclusive, since

experiments were performed with low EV-ribosome concentrations. EV isolation and purification by density sucrose gradient is a long and laborious work, and the quantity of EV samples acquired is limited. Strategies that optimize EV recovery, such as size exclusion chromatography, may be a way to obtain more material and further explore this hypothesis.

Previous studies have already demonstrated the transfer of *S. aureus* EV content intra-species and inter-species. For instance, biologically active BlaZ protein present in *S. aureus* EVs were able to provide transient resistance to ampicillin to both Gram-positive and Gram-negative susceptible bacteria, including *E. coli*, *Salmonella enterica*, *Staphylococcus epidermidis*, and *S. aureus* (LEE et al., 2013). In another study, Lee et al. reported that transfer of antibiotic resistance happened at long-term. *E. coli* treated with EVs derived from methicillin resistant *S. aureus* (MRSA) started producing OMVs with 12-fold increase of β-lactamase activity (LEE et al., 2022). This everlasting *E. coli* resistance profile raises the question of the role of *S. aureus* EVs on epigenetic regulation, as it has been already shown for OMVs derived from *E. coli*, *Vibro cholerae*, and *Pseudomonas aeruginosa* (KYUNG LEE et al., 2021; VDOVIKOVA et al., 2018). Despite these examples, it is still unclear how this transfer of EV content occurs, especially in Gram-positive bacteria presenting thick cell walls. Considering that degrading molecules, such as autolysins, are involved in *S. aureus* EV release, we could imagine that the presence of these elements in EVs may also favor material transfer in EV-bacteria interactions through peptidoglycan hydrolysis. This raises the question of what is the topography of EV proteins. Are they inside the EV lumen, embebbed in the EV membrane, or exposed on the EVs' surface? These questions and the mechanistic behind the delivery of *S. aureus* EV cargo content, as well as their role on inter-species interactions must be addressed in future studies.

7.2.5 Are S. aureus *EVs fine-tuning vehicles in host-pathogen interactions?*

Although *S. aureus* EVs were not cytotoxic against host cells (this study, Tartaglia et al., 2018), they induced the expression of several pro-inflammatory genes, as well as live bacteria. Moreover, they differently activated gene expression of three human cell lines, a result already reported with EVs derived from another S. aureus strain (KIM et al., 2012). This result is interesting because *S. aureus* may colonize several human niches and cause a wide range of diseases, and since EVs exert varied functions towards distinct host cells, infection outcomes may also be affected. Moreover, while comparing human cell stimulation by EV and live bacteria, we noted that less genes were activated by EVs, and those modulated by both groups presented higher expression levels after exposure to bacteria. In contrast,

RNA-seq analysis with bovine mammary epithelial cells (bMECs) indicated that some chemokines and cytokines were more strongly activated by EVs, compared to bacteria in the same conditions. These disparities are not surprising considering the different *S. aureus* strains and infection contexts. Indeed, proteomic analysis have shown that *S. aureus* EV proteome appears to reflect the severity of infections caused by different host isolates (human, bovine, ovine), with virulence factors less abundant in EVs derived from mild strains when compared to severe strains (TARTAGLIA et al., 2020). Moreover, in spite of examples showing the protective effect of EVs against *S. aureus* challenge (ASKARIAN et al., 2018; CHOI et al., 2015), *S. aureus* EVs may also induce hypersensitivity and mice death post-infection (ASANO et al., 2021).

Still regarding dynamics of bMECs exposed to *S. aureus* EVs in different conditions, we found that chemokines and cytokines, such as IL-8, IL-1β, and TNF-α, are induced by EVs through all conditions, suggesting that they promote unceasing inflammatory stimulation. Oppositely, many genes induced by *S. aureus* at early infection (3 h), were also induced by EVs after 24 h stimulation, suggesting that some host responses to EVs are delayed when compared to bacteria. Interestingly, a recent study by Zwack and collaborators have shown that infection with minor *S. aureus* CFUs produces a host muted transcriptional response that inhibits chemokine and cytokine signaling (ZWACK et al., 2022). In this line, we could hypothesize that specific EVs properties may trigger similar phenotypes in host cells. In summary, these data suggests that EVs evolved to adapt to specific contexts and to exert the necessary host responses, functioning as fine-tuned systems in host-pathogen interactions. Considering that EVs can travel long distances, their immunostimulatory properties could help construct a favorable environment for *S. aureus* survival during the course of infection. Experiments could be performed to investigate the impact of EV pre-stimulation followed by bacterial infection on host cells. Measurement of bacterial adhesion and internalization into host cells, as well as induction of host gene expression could bring new insights into the roles exerted by EVs in the context of pathogenesis.

7.2.6 Are EV RNAs protagonists in S. aureus *host-pathogen interactions?*

Several studies have shown the role of *S. aureus* EVs on host-pathogen interactions, such as cytotoxicity, cytokine release *in vitro* and *in vivo*, recruitment of defence cells, tissue inflammation and deterioration, infection exacerbation, and bacterial survival *ex vivo* and *in vivo* (ASANO et al., 2021; ASKARIAN et al., 2018; BITTO et al., 2021a; BRIAUD et al., 2021; CHOI et al., 2015; GURUNG et al., 2011; HONG et al., 2011, 2014; JUN et al., 2017;

KIM et al., 2012; KWON et al., 2018, 2019; STAUDENMAIER et al., 2022; TARTAGLIA et al., 2018; THAY; WAI; OSCARSSON, 2013; WANG et al., 2018b, 2021b; WANG; EAGEN; LEE, 2020; YAMANASHI et al., 2022). *S. aureus* EV proteins were linked to many of these functions, which were validated mainly through the study of mutant strains lacking important virulence factors, such as pore-forming toxins (e.g. Hla, PSMs, leukocidins) (BRIAUD et al., 2021; THAY; WAI; OSCARSSON, 2013), immunoglobulin-binding proteins (e.g. Spa protein) (WANG et al., 2018b), and global regulators (e.g. Agr, Sae) (WANG et al., 2021b; WANG; EAGEN; LEE, 2020). Nevertheless, the potential role of other components present in bacterial vesicles, such as RNAs, are still unknown.

This work provided the first extensive characterization of *S. aureus* EV RNAs, which comprises mRNAs, tRNAs, rRNAs and sRNAs classes. Interestingly, they corresponded to around 78 % of the annotated transcripts for this strain, which is in accordance with the 73 % of annotated transcripts identified in EVs derived from *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) (Malabirade et al., 2018). Most *S. aureus* EV RNAs presented low RNA gene coverage, a result also observed in Gram-negative bacteria (MALABIRADE et al., 2018). The presence of degraded RNAs in EVs could be attributed to the release of fragmented and unwanted material, and / or to the progressive degradation of transcripts in the case of transcription absence on this compartment. Although we focused mainly on highly covered EV RNAs, fragmented RNA corresponding to very small RNAs (vsRNAs) can also be modulators of host-pathogen interactions (DIALLO et al., 2022). Indeed, vsRNAs were found to be enriched in *E. coli* OMVs and to target several human mRNAs with diverse functions (DIALLO et al., 2022). One of these vsRNAs, the tRNA fragment Ile-tRF, was shown to be delivered to host cells and to compete with miRNA silencing machinery. This interaction promotes regulation of host mitogen-activated protein kinase pathway, resulting in the enhanced proliferation of colorectal carcinoma cells (Diallo et al., 2022, unpublished data). Interestingly, vsRNAs are highly abundant in the *S. aureus* strain HG001 (DIALLO et al., 2022), which is closely related to HG003 used in this study.

Finally, it is well known that sRNAs also play essential roles in pathogen-host interactions. For instance, it was shown that a sRNA carried by outer membrane vesicles (OMVs) from *P. aeruginosa* were transferred into human airway cells, provoking a reduction in IL-8 expression (KOEPPEN et al., 2016). In Gram-positive bacteria, Frantz et al. reported that the EV-associated rli32 sRNA of *Listeria monocytogenes* triggers the induction of the type I IFN response in host cells (FRANTZ et al., 2019). It is possible that secreted *S. aureus* EVs may also contain RNAs involved in inter-kingdom communication. Indeed, our analysis

identified 28 highly covered sRNAs. The specific characterization of *S. aureus* EV sRNAs and vsRNAs and the further investigation of their roles in host-pathogen interactions should be addressed in further studies.

7.3 Conclusion

This thesis's project provides the first work extensively characterizing the RNA and protein content of profile of *S. aureus* EVs and its producing cells. *S. aureus* HG003-derived EVs carry important RNAs and proteins, including key virulence factors, that differ from that of bacterial cells. The present study also demonstrates the impact of environmental conditions on EV content composition and abundance, suggesting that selective cargo packing exists. Furthermore, we also provide the first evidence that *S. aureus* EVs have a positive impact on bacterial growth. In the context of host-pathogen interactions, *S. aureus* EVs were shown to impact the host immunity in distinct magnitudes, time-points, and by different routes from that of living bacteria, suggesting their important and specific role in the infection process. This work is a new step towards a better understanding of how these released particles contribute to *S. aureus* physiology and pathogenesis. The further characterization of *S. aureus* EV elements, especially of nucleic acids, lipids, and metabolites, as well as the exploration of the mechanistic events involved in both EV release and EV cell-to-cell delivery is of great interest. Additional research on the *S. aureus* EV functions in bacterial physiology and in *in vivo* models should be conducted to validate previous data, to try elucidate the exact contribution of EVs in intra- and inter-kingdom interactions.

Thesis outputs

The work developed during this Ph.D. thesis resulted in the scientific products enumerated below:

1. Publications as first author

Published Article. da Luz BSR, Nicolas A, Chabelskaya S, Rodovalho VR, Le Loir Y, Azevedo VAC, Felden B, Guédon E. Environmental Plasticity of the RNA Content of *Staphylococcus aureus* Extracellular Vesicles. Front Microbiol. 2021 Mar 11;12:634226. doi: 10.3389/fmicb.2021.634226. eCollection 2021.PMID: 33776967.

Published Article. da Luz BSR, Nicolas A, Chabelskaya S, Rodovalho VR, Le Loir Y, Azevedo VAC, Guédon E. Impact of environmental conditions on the protein content of *Staphylococcus aureus* and its derived extracellular vesicles. Microorganisms 2022, Vol. 10, Page 1808. doi: 10.3390/microorganisms10091808.

Published Book Chapter. da Luz BSR, Azevedo, Vasco; Le-loir, Yves; Guedon, Eric. Extracellular Vesicles and Their Role in *Staphylococcus aureus* Resistance and Virulence. Book Chapter. In: Dr. Amjad Islam Aqib. (Org.). *Staphylococcus aureus*. 1ed:, IntechOpen 2021. doi: 10.5772/intechopen.96023.

Article in preparation. da Luz BSR, Nicolas A, Tartaglia N., Le Loir Y, Azevedo VAC, Guédon E. Remodeling of the bovine mammary epithelial cell transcriptome in response to *Staphylococcus aureus* extracellular vesicles (Chapter 6 of thesis).

2. Publications as co-autor

Published Article. Tartaglia NR, Nicolas A, Rodovalho VR, da Luz BSR, Briard-Bion V, Krupova Z, Thierry A, Coste F, Burel A, Martin P, Jardin J, Azevedo V, Le Loir Y, Guédon E. Extracellular vesicles produced by human and animal *Staphylococcus aureus* strains share a highly conserved core proteome. Sci Rep. 2020 May 21;10(1):8467. doi: 10.1038/s41598-020-64952-y. PMID: 32439871.

Published Article. Rodovalho VR, da Luz BSR, Rabah H, do Carmo FLR, Folador EL, Nicolas A, Jardin J, Briard-Bion V, Blottière H, Lapaque N, Jan G, Le Loir Y, de Carvalho

Azevedo VA, Guédon E. Extracellular Vesicles Produced by the Probiotic *Propionibacterium freudenreichii* CIRM-BIA 129 Mitigate Inflammation by Modulating the NF-κB Pathway. Front Microbiol. 2020 Jul 7;11:1544. doi: 10.3389/fmicb.2020.01544. eCollection 2020. PMID: 32733422.

Published Article. de Rezende Rodovalho V, da Luz BSR, Nicolas A, do Carmo FLR, Jardin J, Briard-Bion V, Jan G, Le Loir Y, de Carvalho Azevedo VA, Guedon E. Environmental conditions modulate the protein content and immunomodulatory activity of extracellular vesicles produced by the probiotic *Propionibacterium freudenreichii*. Appl Environ Microbiol. 2020 Dec 11;87(4):e02263-20. doi: 10.1128/AEM.02263-20. Online ahead of print.PMID: 33310709.

Article in Preparation. de Rezende Rodovalho V, da Luz BSR, do Carmo FLR, Nicolas A, Jardin J, Briard-Bion V, Folador EL, Santos AR, Jan G, Le Loir Y, de Carvalho Azevedo VA, Guedon E. The extracellular vesicles from *Propionibacterium freudenreichii* obtained by different purification methods and growth conditions share a distinctive core proteome.

3. Conference oral presentations

da Luz BSR, Nicolas A, Le Loir Y, Azevedo VAC, Felden B, Guédon E. Remodeling of the bovine mammary epithelial cell transcriptome in response to *Staphylococcus aureus* extracellular vesicles. FSEV - Focus on Young Investigators December 2021 (Video conference, France)

da Luz BSR, Nicolas A, Chabelskaya S, Rodovalho VR, Le Loir Y, Azevedo VAC, Felden B, Guédon E. Environmental Plasticity Of *Staphylococcus aureus* Extracellular Vesicles RNA Content. National Congres Société Française de Microbiologie – SFM, 22-24 September 2021 (Nantes, France).

da Luz BSR, VR, Le Loir Y, Azevedo VAC, Guédon E. Impact of *Staphylococcus aureus* N305 extracellular vesicles on the host immune response (Journée Scientifiques Université de Nantes - June, 2021, Video conference Nantes, France).

da Luz BSR, Nicolas A, Chabelskaya S, Rodovalho VR, Le Loir Y, Azevedo VAC, Felden B, Guédon E. Environmental Plasticity Of *Staphylococcus aureus* Extracellular Vesicles RNA Content. International Congres ISEV 2021, 18-21 Maio 2021 (Video Conference, France).

da Luz BSR, Nicolas A, Chabelskaya S, Rodovalho VR, Le Loir Y, Azevedo VAC, Felden B, Guédon E. Plasticidade do conteúdo de RNA das vesículas extracelulares de *Staphylococcus aureus* em diferentes condições ambientais. Session Prize Professor Cláudio Antônio Bonjardim. VII Microbiology Simposium UFMG – CONECTA SIM 2020 (Video conference, Brazil).

da Luz BSR, Rodovalho VR, Le Loir Y, Azevedo VAC, Guédon E. Bacterial EVs and health: positive and negative effects – EVs from *Staphylococcus aureus* and *Propionibacterium freudenreichii* (STLO OpenDays – 19-21 Mar 2019, France).

4. Conference poster presentations

da Luz BSR, VR, Le Loir Y, Azevedo VAC, Guédon E. Impact of extracellular vesicles of *Staphylococcus aureus* N305 on the on innate immune response of mice mammary gland (STLO OpenDays – 19-21 Mar 2019, France).

da Luz BSR, Nicolas A, Chabelskaya S, Rodovalho VR, Le Loir Y, Azevedo VAC, Felden B, Guédon E. Effect of vancomycin on the production of extracellular vesicles in *Staphylococcus aureus.* (FSEV Congress Oct 2019, Nantes, France).

da Luz BSR, VR, Le Loir Y, Azevedo VAC, Guédon E. Impact of extracellular vesicles derived from *Staphylococcus aureus* Newbould 305 on innate immune response of mice mammary gland. (FSEV Congress Oct 2019, France).

da Luz BSR, Nicolas A, Chabelskaya S, Rodovalho VR, Le Loir Y, Azevedo VAC, Felden B, Guédon E. Environmental plasticity of *Staphylococcus aureus* extracellular vesicles RNA content. Poster session RNA and Transcriptomics - X-meeting eXperience, 09-11 Nov 2020.

Résumé étendu (Extended abstract)

1. Introduction

Staphylococcus aureus est la bactérie la plus importante du genre *Staphylococcus* spp en raison de son impact sur la santé animale et humaine dans le monde entier, provoquant un large spectre de maladies allant d'infections mineures à graves chez plusieurs espèces de mammifères, en particulier l'homme et les bovins (Peton et Le Loir, 2014 ; Tong et al., 2015 ; Gnanamani et al., 2017). Le portage de *S. aureus* par des adultes en bonne santé est un phénomène courant associé à un risque plus élevé d'infections staphylococciques, entraînant des coûts de traitement, une morbidité et une mortalité élevés dans les établissements de santé (Eiff et al., 2001 ; Nouwen et al., 2006 ; Sivaraman et al., 2009 ; Bode et al., 2010 ; Salgado-Pabón et Schlievert, 2014). Les maladies humaines comprennent plusieurs infections aiguës et chroniques (p. ex. bactériémie, pneumonie, méningite) et syndromes induits par des toxines (Lowy, 1998 ; Silversides et al., 2010 ; Sunderkötter et Becker, 2015 ; Mishra et al., 2016 ; Fisher et al., 2018). En médecine vétérinaire, *S. aureus* provoque diverses infections affectant économiquement des animaux d'élevage importants, tels que les ruminants (Fitzgerald, 2012 ; Haag et al., 2019). Un exemple est la mammite, une inflammation de la glande mammaire qui affecte les troupeaux laitiers entraînant des pertes économiques importantes pour l'industrie laitière dans le monde entier (April et al., 2020).

La capacité de *S. aureus* à provoquer un large éventail d'infections chez différents hôtes et niches est principalement attribuée à l'expression d'un vaste arsenal de facteurs de virulence, impliqués dans l'adhésion, l'invasion, l'inflammation, la modulation de la réponse immunitaire de l'hôte, la survie et la persistance. Ils comprennent des protéines de surface, des exoenzymes, des cytotoxines et des superantigènes qui aident les bactéries à détourner et à échapper à la réponse immunitaire de l'hôte (Becker et al., 2014 ; Tong et al., 2015). Le type et la gravité des infections dépendent de facteurs de virulence spécifiques à la souche, principalement exprimés à partir d'éléments génétiques accessoires (Gill et al., 2011). Les facteurs de virulence de *S. aureus* sécrétés et exposés à la surface sont responsables de l'affaiblissement de la réponse immunitaire de l'hôte, de l'évasion immunitaire, des dommages aux tissus de l'hôte et de l'apparition de l'infection (Foster, 2005). De plus, des réseaux de régulation complexes contrôlent finement l'expression de ces facteurs de virulence, contribuant à l'adaptation bactérienne à diverses conditions environnementales. A

ce jour, aucun vaccin contre *S. aureus* n'est disponible et les infections sont principalement traitées avec des antibiotiques. Cependant, l'augmentation de la résistance à plusieurs souches antimicrobiennes fait du traitement de *S. aureus* une préoccupation mondiale. Une alternative potentielle de nouvelle génération pour le développement de vaccins contre *S. aureus* récemment proposée est l'utilisation de vésicules extracellulaires (VEs).

La libération de vésicules extracellulaires (VEs) est un phénomène connu de longue date et largement rapporté, notamment chez les eucaryotes (Guillaume et al., 2018 ; Doyle et Wang, 2019 ; Mathieu et al., 2019 ; Stahl et Raposo, 2019). Les archées et les bactéries libèrent également des VEs, faisant de leur présence une caractéristique conservée au cours de l'évolution dans les trois règnes (Gill et al., 2019). Ils peuvent être appelés vésicules membranaires, microvésicules, ectosomes, exosomes, corps apoptotiques, vésicules membranaires externes (OMVs) et autres, selon leur origine et leurs caractéristiques (Deatheragea et Cooksona, 2012 ; Gill et al., 2019). L'étude de ces particules est d'un grand intérêt, car elles sont considérées comme un mécanisme de communication intercellulaire et d'interactions trans-royaumes (Maas et al., 2017). Les VEs sont des bio-particules nanométriques (de 20 à 1000 nm), composées d'une bicouche lipidique, produites par la plupart des cellules et sécrétées dans le milieu environnant. Elles jouent un rôle essentiel dans la communication entre les cellules (intra et inter-espèce) et dans la vectorisation des propriétés biologiques des cellules, de par leur capacité à transporter des molécules bioactives (protéines, lipides, métabolites, acides nucléiques) d'une cellule productrice à une cellule receveuse et à déverser les molécules transportées dans les cellules receveuses pour y moduler leur métabolisme et physiologie (Brown et al., 2015). Récemment, l'étude des VEs bactériens a attiré l'attention car ces véhicules peuvent affecter les interactions pathogène-hôte et contribuer à la pathogenèse bactérienne.

La première étude concernant les VEs de bactéries remonte à 1966, lorsque des structures de type lipidique purifiées à partir de surnageants de culture d'*Escherichia coli* ont été observées au microscope électronique (Work et al., 1966). Chez les bactéries à Gram négatif, la vésiculation se produit à partir du bourgeonnement de la membrane externe (OM) qui capture les composants présents dans le périplasme. Ce processus forme des nanoparticules appelées vésicules membranaires externes (OMV), qui sont libérées dans le milieu extracellulaire (Schwechheimer et Kuehn, 2015). Les bactéries à Gram positif n'ont pas de membrane externe et ont une paroi cellulaire de peptidoglycane (PGN) plus épaisse, qui était considérée comme une barrière à la libération des VEs. Cela pourrait expliquer pourquoi les premières observations de libération des VEs chez des bactéries à Gram positif

ont été rapportées bien plus tard, en 2009, lorsque Lee et ses collaborateurs ont démontré la production des VEs par *S. aureus* (Lee et al., 2009). Depuis, d'autres études ont confirmé la libération des VEs par d'autres bactéries à Gram positif appartenant à divers genres tels que *Bacillus* sp, *Bifidobacterium* sp, *Cutibacterium* sp, *Clostridium* sp, *Enterococcus* sp, *Lactobacillus* sp, *Mycobacterium* sp, *Propionibacterium* sp et *Streptococcus* sp, entre autres. (Rivera et al., 2010 ; López et al., 2012 ; Brown et al., 2 014 ; Jiang et al., 2014 ; Choi et al., 2017 ; Jeon et al., 2017 ; Wagner et al., 2018 ; Dean et al., 2019 ; Nishiyama et al., 2020 ; Rodovalho et al., 2020).

Un domaine émergent de grand intérêt est l'implication des VEs dans les infections causées par *S. aureus*. Des études récentes ont montré que les VEs de *S. aureus* sont porteurs de protéines impliquées dans la survie et la virulence de la bactérie, tels que les β-lactamases, les superantigènes, les toxines, les coagulases et les protéines associées à l'adhérence bactérienne aux cellules hôtes (Lee et al., 2009, 2013 ; Gurung et 2009, 2013 ; Gurung et al. 2011 ; Hong et al., 2014 ; Jeon et al., 2016 ; Tartaglia et al., 2018, 2020). Dans certains cas, les VEs déclenchent la production de cytokines et favorisent l'inflammation des tissus (Hong et al., 2011 ; Kim et al., 2012, 2019 ; Jun et al., 2017). Les VEs sont également considérés comme des véhicules potentiels pour des applications biotechnologiques et cliniques, telles que le développement de vaccins (Davenport et al., 2008 ; Choi et al., 2015 ; Wang et al., 2018 ; Yuan et al., 2018). Ainsi, leut étude est un domaine attractif en microbiologie pour le développement futur de nouvelles stratégies contre les infections bactériennes.

2. Question de recherche

À ce jour, les VEs de *S. aureus* étaient principalement caractérisés par des approches protéomiques, laissant une lacune importante dans la connaissance des autres composants des VEs, tels que les acides nucléiques. Récemment, l'étude des ARN, y compris les petits ARN régulateurs (ARNs), a attiré l'attention en raison de leurs rôles intra et interspécifiques de régulation des gènes et d'immunomodulation. La caractérisation de la teneur en ARN des VEs a été rapportée chez certaines bactéries. Cependant, les données sur la cargaison d'ARN des VEs de *S. aureus* font toujours défaut. De plus, la plupart des rapports concernant les VEs staphylococciques se sont concentrés sur les souches cliniques, car les connaissances sur les VEs dérivés d'isolats animaux de *S. aureus* ont été négligées. Enfin, étant donné que les VEs bactériens semblent présenter une cargaison enrichie par rapport aux cellules productrices, les études récentes comparent rarement leur effet immunomodulateur avec des

bactéries vivantes. Par conséquent, il n'est toujours pas clair si les VEs libérés exercent les mêmes fonctions que les bactéries dans le contexte de l'infection.

Ici, nous avons émis l'hypothèse que les VEs dérivés de *S. aureus* portent non seulement des protéines, mais également des ARN (y compris des ARNs), qui peuvent être emballés de manière sélective pour exercer des fonctions spécifiques. Dans ce contexte, nous postulons que les VEs de *S. aureus*, y compris ceux provenant de souches animales, peuvent moduler la réponse immunitaire de l'hôte différemment de celle des bactéries vivantes.

3. Objectives

Ce travail vise à l'étude des VEs de *S. aureus*, y compris leur caractérisation biophysique et leur contenu (protéine et ARN), et à approfondir les mécanismes moléculaires impliqués dans les interactions VEs-bactéries et VEs-hôte (principalement de modèles animaux), y compris réponses cellulaires et immunitaires de l'hôte. Pour cela, une approche interdisciplinaire a été utilisée, comprenant des techniques de caractérisation des nanoparticules, des tests fonctionnels et des approches omiques telles que la transcriptomique et la protéomique pour évaluer les caractéristiques et les rôles biologiques des VEs dérivés de *S. aureus*.

Deux souches de *S. aureus* ont été utilisées dans cette étude. Le premier est la souche modèle de laboratoire *S. aureus* HG003, dérivant d'un isolat clinique et qui est largement utilisée dans les études de résistance aux antibiotiques. Ce travail fournit le premier travail exploratoire des VE dérivés de cette souche, fournissant la caractérisation de la composition en ARN et en protéines des VEs et l'étude de leur impact sur la réponse immunitaire de l'hôte. La deuxième souche, le modèle de mammite *S. aureus* Newbould 305 (N305), a été choisie pour effectuer une enquête plus approfondie sur leur rôle dans les interactions hôte-pathogène. La teneur en protéines et les premières informations sur l'impact des VEs dérivés de *S. aureus* N305 sur l'immunomodulation ont été publiées en 2018 (Tartaglia et al., 2018). Ici, nous élargissons ces connaissances.

Ce travail a été divisé en plusieurs étapes pour étudier l'hypothèse proposée. La figure (page 147) donne un aperçu général de la stratégie utilisée pour atteindre les objectifs spécifiques suivants :

1. Isolement et purification des VEs à partir de la souche *S. aureus* HG003 cultivée dans différentes conditions environnementales par gradient de densité de saccharose.

2. Description des caractéristiques biophysiques des VEs de *S. aureus* HG003 (taille, concentration, morphologie) dans différentes conditions environnementales.

3. Caractérisation de la composition en ARN des VEs de HG003 à partir de différentes conditions environnementales par ARN-Seq.

4. Caractérisation de la composition protéique des VEs HG003 dans différentes conditions environnementales.

5. Évaluation de l'effet intra-espèce des VEs dérivés de *S. aureus* HG003.

6. Étude de l'impact des VEs dérivés de *S. aureus* HG003 sur l'expression génique de différentes lignées cellulaires humaines.

7. Isolement et purification des VEs dérivés de la souche *S. aureus* N305 par gradient de densité de saccharose.

8. Évaluation du profil d'expression génique des cellules épithéliales mammaires bovines (bMEC) stimulées in vitro avec des VEs de N305 à différents temps d'exposition par ARN-seq.

Les objectifs 1 à 3 sont abordés dans le chapitre 3, qui présente un article de recherche original rapportant la caractérisation physique complète et la description du contenu en ARN des VEs dérivés de la souche *S. aureus* HG003 cultivée dans différentes conditions environnementales.

L'objectif 4 est abordé dans le chapitre 4, qui présente un article de recherche original décrivant la teneur en protéines de la souche *S. aureus* HG003 et de ses VE dérivés cultivés dans différentes conditions environnementales.

Les objectifs 5 et 6 sont abordés dans le chapitre 5, où des résultats supplémentaires montrent l'impact de l'ajout de VEs de HG003 sur la croissance de *S. aureus* et sur l'expression génique de trois lignées cellulaires humaines différentes.

Les objectifs 7 et 8 sont abordés dans le chapitre 6, où l'analyse de séquençage donne une compréhension approfondie de la réponse immunitaire eucaryote après exposition aux VEs de *S. aureus* N305, donnant de nouvelles informations sur leurs rôles dans la pathogenèse staphylococcique.

Les conclusions générales et les perspectives de ce travail sont présentées dans le chapitre 7.

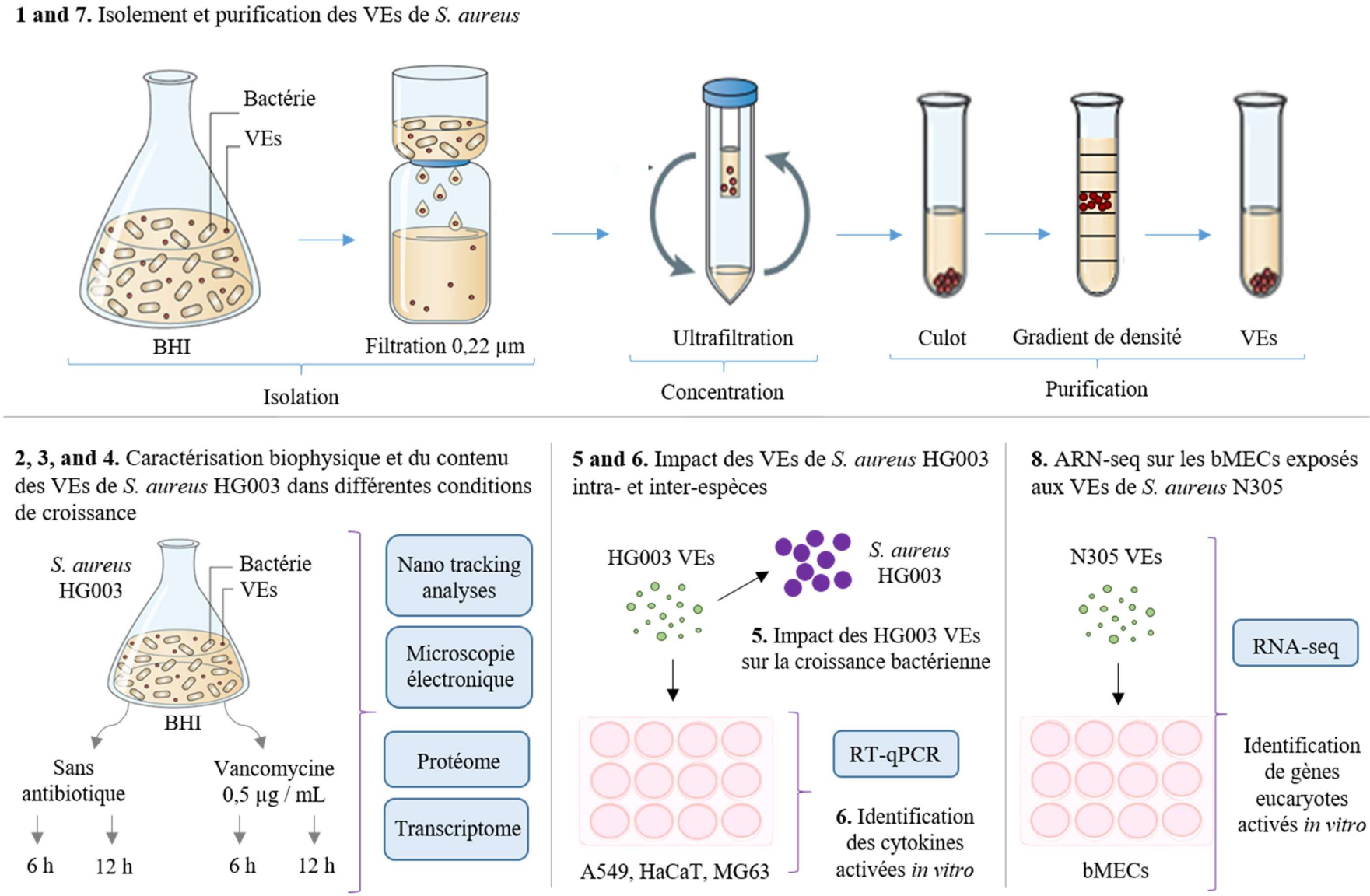

Figure. Stratégie expérimentale utilisée dans cette thèse de doctorat.

4. Résultats

4.1 Caractérisation des VEs de *S. aureus*

Dans la première partie de la thèse, nous avons fourni les premiers travaux exploratoires sur les VEs libérés par la souche *S. aureus* HG003. Au cours de l'infection, *S. aureus* est confronté à différentes conditions environnementales. Puisque les conditions de l'environnement peuvent influencer la libération, les propriétés et les fonctions des VE (Tashiro et al., 2010 ; Kim et al., 2016b ; Orench-Rivera et Kuehn, 2016 ; Askarian et al. 2018 ; Yun et al., 2018 ; Andreoni et al., 2019), dans ce travail, nous avons voulu caractériser l'impact de différentes conditions environnementales sur les propriétés des VEs de *S. aureus* HG003. Ainsi, au-delà des propriétés physiques des VEs, nous avons déterminé leur composition en ARN et en protéines et comparé ces compositions à celles des cellules productrices des VEs. Dans notre travail, nous avons testé deux conditions de croissance : les phases stationnaires précoces et tardives (respectivement 6 h et 12 h), et l'impact de l'absence et de la présence d'une concentration sublétale (0,5 µg/ml) de l'antibiotique vancomycine (V- et V+, respectivement). Nos données ont révélé que les VEs récupérés en phase stationnaire tardive sont plus grands que ceux des cultures en phase stationnaire précoce, alors qu'aucune différence significative n'a été observée en présence de vancomycine. L'analyse transcriptomique et protéomique a montré des variations dans la composition et l'abondance de l'ARN et des protéines des VEs, comme présenté ci-dessous.

Article 1

En ce qui concerne la cargaison en ARN, cette étude fournit la première caractérisation approfondie des ARN des VEs de *S. aureus*, et également la première chez les bactéries à Gram positif. Ici, les échantillons d'ARN extraits à partir des VEs purifiés et des cellules productrices des VEs ont été soumis à la déplétion de l'ARN ribosomal (ARNr), et au séquençage. Nous avons constaté que les VEs de HG003 portent des ARN de toutes les classes (y compris des ARNr résiduels), ce qui correspond à environ 78 % des transcrits annotés pour cette souche. Fait intéressant, la plupart des transcrits présents dans les VEs étaient fragmentés et seulement 5 % de tous les ARN identifiés étaient fortement couverts (couverture de séquençage > 90 %), donc probablement sous une forme intacte. Selon les conditions de croissance, 286 ARN uniques ont été détectés, dont 28 ARNs et 220 ARNm codant pour de nombreux facteurs de virulence (hld, agrBCD, psmβ1, sbi, spa et isaB), des protéines ribosomiques, des régulateurs transcriptionnels et des enzymes métaboliques. La présence de 22 ARN dans les VEs a été confirmée par une approche complémentaire de RT-

qPCR. Plusieurs de ces 286 ARN se sont révélés appartenir aux mêmes unités de transcription chez *S. aureus*. La nature et l'abondance des ARN des VEs ont été considérablement affectées en fonction de la phase de croissance et de la présence de vancomycine, tandis que beaucoup moins de variations ont été observées chez les cellules productrices. De plus, l'abondance d'ARN différait entre les VEs et les cellules productrices en fonction des conditions de croissance. Ces résultats montrent que l'environnement façonne la cargaison d'ARN des VEs, suggérant leur conditionnement sélectif. Cette étude met en lumière les rôles possibles des ARN présents dans les VEs de *S. aureus*, notamment dans les interactions hôte-pathogène.

Ces résultats ont été publiés comme un article de recherche original dans Frontiers in Microbiology (DOI : 10.3389/fmicb.2021.634226), intitulé "Environmental Plasticity of the RNA Content of the *Staphylococcus aureus* Extracellular Vesicles".

Article 2

En ce qui concerne la composition en protéines, l'analyse de la localisation subcellulaire a révélé que, proportionnellement, les VEs libérés contiennent moins de protéines du cytoplasme et plus de protéines membranaires, de surfaces exposées et de lipoprotéines que les bactéries. De plus, nous avons également constaté que 25 % (n = 144) des protéines identifiées dans les VEs étaient exclusives à ce groupe, bien que les mêmes conditions et paramètres aient été appliqués pour la détection des protéines dans les bactéries et les VEs. Curieusement, 45 de ces protéines exclusives appartenaient à des classes de protéines mal caractérisées et hypothétiques, suggérant que les VE présentent bien des caractéristiques et des fonctions spécifiques encore inconnues. Nous avons également constaté que l'abondance relative des protéines des VEs est modulée par l'environnement et diffère de celle de la cellule entière (WC). De plus, les conditions environnementales qui ont été testées ont eu un impact plus prononcé sur la composition en protéines des VEs par rapport au WC, soutenant l'existence de mécanismes pour l'emballage sélectif de la cargaison des VEs.

Des analyses comparatives entre le RNome des VEs de *S. aureus* HG003 précédemment publié et les données protéomiques ont montré que sur un total de 220 ARNm, plus de la moitié n'étaient pas trouvés sous la forme protéique correspondante dans les VEs de HG003. Fait intéressant, 16 éléments étaient présents dans toutes les conditions testées à la fois sous forme d'ARNm et de protéines. La présence ubiquitaire de ces protéines dans le protéome des VEs derivées de plusieurs souches de *S. aureus* et d'autres espèces, y compris des bactéries à Gram négatif, suggère leur rôle dans la biogenèse des VEs de bactéries. Cette

étude fournit la première image générale de l'impact de différentes conditions de croissance dans le protéome des VEs de *S. aureus* et de ses cellules productrices. Elle ouvre la voie à de futures études pour mieux comprendre la production, la composition et les rôles des VEs de *S. aureus*.

Ces résultats ont été publiés comme un article de recherche original dans la revue Microorganisms (DOI: 10.3390/microorganisms10091808), intitulé "Impact of Environmental Conditions on the Protein Content of *Staphylococcus aureus* and Its Derived Extracellular Vesicles".

4.2 Rôles et fonctions des VEs

Résultats supplémentaires - Partie 1

Compte tenu de la cargaison d'ARN et de protéines des VEs de la souche HG003 décrite, nous avons décidé d'étudier leurs rôles biologiques dans un contexte intra-espèce et inter-royaume. En ce qui concerne les interactions intra-espèces, nous avons montré que les VEs de HG003 avaient un impact positif sur la croissance bactérienne selon le milieu de croissance utilisé. Alors que la croissance de *S. aureus* reste inchangée après l'ajout de VEs lors d'une croissance en milieu BHI, une augmentation dose-dépendante a été observée lorsque les VEs ont été ajoutés à une culture de *S. aureus* cultivé dans les milieux restrictifs NZM et RPMI + 10% LB. Il s'agit de la première étude mettant en évidence l'impact positif des VEs de *S. aureus* sur la croissance de la population bactérienne, ouvrant la voie à d'autres études dans ce domaine. Dans le contexte des interactions hôte-pathogène, nous avons exploré l'impact de *S. aureus* HG003 et de ses VEs sur trois lignées cellulaires humaines : les cellules pulmonaires, les kératinocytes et les ostéoblastes. Bien que les VEs de HG003 ne soient pas cytotoxiques pour aucune des lignées testées, ils ont induit l'expression de plusieurs cytokines pro-inflammatoires. Fait intéressant, l'activation des gènes variait beaucoup entre les lignées cellulaires testées. Par exemple, les VEs et les bactéries ont induit l'expression de plus de gènes dans les ostéoblastes que dans les deux autres lignées cellulaires. Certaines différences ont également été observées par rapport à la source de stimulation, avec moins de gènes activés par les VEs et avec des niveaux d'expression plus faibles par rapport aux bactéries vivantes. Ces données apportent de nouvelles informations dans un domaine encore largement inexploré, montrant le rôle des VEs de *S. aureus* dans

différents contextes d'infection, et comment leurs fonctions exercées divergent de celles des bactéries vivantes.

Résultats supplémentaires - Partie 2

Enfin, dans la dernière partie de ce projet, nous avons utilisé l'approche ARN-seq pour fournir une caractérisation plus approfondie de la réponse de l'hôte aux VEs dérivés de *S. aureus* N305 dans un contexte mammite. Il s'agit également du premier transcriptome de cellules hôtes exposées aux VEs de *S. aureus* dans la littérature. Des cellules épithéliales mammaires bovines (bMEC) ont été exposées aux VEs pendant différentes durées : 3 h, 4 h, 8 h et 24 h, tandis que des bactéries vivantes ont été utilisées comme contrôle positif après 3 h d'exposition. Nous avons constaté que le nombre de gènes exprimés de manière différentielle (DE) touchés par les VEs de N305 augmentait avec le temps, dont certains étaient modulés dans toutes les conditions. De plus, des gènes remarquables modulés à la fois par les VEs de N305 et des bactéries vivantes à 3 h, tels que les chimiokines et les cytokines pro-inflammatoires, présentaient différents niveaux d'expression en fonction de la source de stimulation. Fait intéressant, près de la moitié des gènes modulés par les VEs de N305 après 24 h d'exposition sont également modulés par *S. aureus* à 3 h, suggérant un effet modulateur tardif des VEs. Notre étude a également découvert de nouvelles chimiokines et cytokines modulées par les VEs de *S. aureus* qui n'avaient pas été décrites auparavant. Enfin, nous avons trouvé que certains gènes modulés par les VEs de N305 à 3 h ne sont pas modulés par les bactéries dans le même état, une découverte qui est conforme aux analyses d'enrichissements génétiques montrant que plusieurs voies KEGG étaient induites exclusivement par les VEs de N305 (e.g. Apoptose, Nécroptose). Dans l'ensemble, nos données montrent que les VEs de N305 modulent la réponse de l'hôte avec différentes intensités, périodes d'exposition et par des voies différentes de celles des bactéries vivantes, apportant de nouvelles connaissances sur les rôles fonctionnels potentiels de *S. aureus* VEs dans le contexte des infections à staphylocoques.

5. Discussion générale et perspectives

5.1 Quels sont les mécanismes impliqués dans la biogenèse des VEs de S. aureus?

Les mécanismes régissant la biogenèse des VEs de *S. aureus* restent à élucider. Chez les bactéries à Gram négatif, des modifications de la pression de turgescence et de la courbure de la membrane ont été proposées comme déclencheurs de la libération des OMVs (Zhou et

al., 1998 ; McBroom et Kuehn, 2007 ; Elhenawy et al., 2016). Chez *S. aureus*, il a été montré que des mutations dans les autolysines, les PSMs et les gènes sigma σ^B impactent la production et la concentration des VEs (Liu et al., 2018a ; Schlatterer et al., 2018 ; Wang et al., 2018b), ainsi que différentes conditions et stress environnementaux (Askarian et al., 2018 ; Andreoni et al., 2019 ; Kim et al., 2020 ; Briaud et al., 2021 ; Wang et al., 2021b). Dans notre étude, les propriétés biophysiques des VEs sont restées inchangées après une exposition à la vancomycine, tandis que les VEs récoltés pendant la phase de croissance stationnaire tardive étaient significativement plus importants que ceux récoltés au moment de la phase stationnaire précoce. Ce résultat pourrait s'expliquer par des modifications de la réticulation du PGN et de la morphologie de la paroi cellulaire à différents stades de croissance de *S. aureus* (Zhou et Cegelski, 2012). Cependant, les VEs dérivés de la souche *S. aureus* C-29 à 6 h, 17 h et 24 h n'ont pas présenté de différences de taille et de concentration des particules (Yamanashi et al., 2022). Ces données suggèrent que d'autres facteurs intrinsèques peuvent également contribuer aux différences observées. En effet, Tartaglia et al. ont rapporté que les VEs dérivés de cinq souches de *S. aureus* provenant de différents hôtes (humain, bovin et ovin) présentaient des différences significatives dans la concentration et la taille des VEs (Tartaglia et al. 2020). Ces données suggérent qui la biogenèse des VEs de *S. aureus*, bien que conservée dans plusieurs souches, peut se produire par des voies distinctes. En fait, Andreoni et al. (2019) ont mis en évidence que le traitement antibiotique peut induire la libération des VEs via différentes voies, plus ou moins dépendantes de phages.

Ici, nous avons identifié au moins 16 élements du génome de HG003 qui étaient présentes à la fois sous une forme ARNm et protéique dans les VEs. Cela concerne l'autolysine (Atl), le facteur d'élongation FusA, l'énolase et le chaperon DnaK, ces deux derniers éléments faisant également partie du protéome partagé par les VEs dérivés de cinq souches de *S. aureus*, et d'autres espèces de bactéries à Gram positif, y compris *Bacillus*, *Clostridium*, *Listeria*, *Streptococcus* et *Mycobacterium* (Tartaglia et al. 2020). Ces données nous amènent à émettre l'hypothèse qu'ils pourraient jouer un rôle important dans la biogenèse des VEs. En effet, il a été montré que les chaperons participent au conditionnement sélectif des protéines des OMVs (Altindis et al., 2014 ; Hussain et Bernstein, 2018). De plus, il a été démontré que les autolysines Atl et Sle1 sont impliquées dans la libération des VEs de *S. aureus*, puisque les mutants de ces gènes libèrent des VEs avec une taille et une concentration réduites (Wang et al., 2018b). De nombreuses recherches sont nécessaires dans ce domaine, et la caractérisation et l'identification d'éléments communs aux VEs de différentes espèces bactériennes peuvent fournir des informations sur les mécanismes

généraux de leur biogenèse. Des expériences évaluant la libération des VEs chez des mutants pour des protéines largement présentes dans les VEs, telles que celles impliquées dans le remodelage de la paroi cellulaire et les systèmes d'adressage de protéines, peuvent aider à élucider les mécanismes de la biogenèse des VEs chez *S. aureus* et d'autres espèces.

2.2 Quels sont les mécanismes impliqués dans la seléction du cargo des VEs de S. aureus ?

Nos résultats ont montré des phénomènes d'enrichissement de certaines protéines et ARN dans les VEs par rapport aux bactéries, selon les conditions de l'environnement. Néanmoins, ces résultats d'enrichissement, notamment ceux relatifs aux ARN, doivent être interprétés avec prudence, car ils peuvent refléter des différences d'activité des RNase, ou résulter d'une accumulation, dans les VEs et au cours du temps, d'ARN à demi-vie plus longue (Langlete et al., 2019 ; Lécrivain et Beckman, 2020). Quoi qu'il en soit, nous avons constaté que l'ARNs RsaC, impliqué dans la réponse de *S. aureus* au stress oxydant, identifié dans les VEs de HG003 lors de cette étude, est également présent dans les VEs dérivés de la souche *S. aureus* MSSA476 (Joshi et al., 2021b), suggérant sa présence commune à travers différentes souches. Bien que les mécanismes d'emballage de l'ARN dans les VEs soient encore inconnus, des caractéristiques telles que la taille, l'emplacement et l'affinité de l'ARN pour d'autres molécules ont été proposées comme facteurs possibles (Langlete et al., 2019). Concernant la composition en protéines, deux études menées par notre groupe ont montré une association entre les protéines présentes dans les VEs bactériennes et leurs propriétés biophysiques. Il a été démontré que les protéines des VEs de *S. aureus* contiennent moins de résidus hydrophobes et plus de résidus polaires et chargés que l'ensemble des protéines du protéome cellulaire, ce qui suggère que les protéines adressées aux VEs sont des protéines probablement chargées positivement à un pH physiologique (Tartaglia et al., 2020). De plus, un indice d'adaptation des codons (C.A.I.) plus élevé et une composition spécifique en acides aminés, tels que la lysine surreprésentée et le tryptophane et l'histidine sous-représentés, sont des propriétés des protéines retrouvées dans les VEs dérivés à la fois de *S. aureus* (Tartaglia et al., 2020) et de l'espèce *Propionibacterium freudenreichii* (données non publiées).

Ici, nous avons observé qu'environ 60 % du protéome des VEs de HG003 est conservé dans les quatre conditions testées. De plus, l'analyse protéomique comparant les VEs dérivés de cinq souches de *S. aureus* d'origines d'hôtes diverses (humain, bovine, et ovin) utilisant la même approche a également révélé un protéome hautement conservé (Tartaglia et al., 2020). Plus intéressant, plus de 80% du protéome central des VEs identifié dans ces cinq souches chevauche également le protéome des VEs de HG003 (cette étude) et les VEs dérivés de la

souche *S. aureus* RN4220 (He et al., 2017), renforçant que certains éléments sont présents dans les VEs quelles que soient les souches et les conditions spécifiques. Plusieurs autres études soutiennent également l'emballage sélectif de la cargaison des VEs dans différentes espèces bactériennes. Chez les bactéries Gram négatifs, *Serratia marcescens* et *Porphyromonas gingivalis* excluent les protéines abondantes dans la membrane externe des OMVs, alors qu'elles emballent des facteurs de virulence et des éléments absents dans ce compartiment (Haurat et al., 2011 ; McMahon et al., 2012 ; Veith et al. 2014). Chez les bactéries à Gram positif, les espèces *Mycobacterium* et *Bacillus* libèrent des VEs enrichis en lipoprotéines et en protéines de liaison aux sidérophores (Prados-Rosales et al., 2011, 2014b; Brown et al., 2014).

Notons que la plupart des études mentionnées se concentrent principalement sur l'analyse protéomique des VEs, et donc, il y a encore un grand écart dans la caractérisation d'autres éléments, tels que l'ADN, les métabolites et les lipides. Bien que la présence d'ADN ait été mise en évidence dans les VEs de *S. aureus* (Andreoni et al., 2019 ; Rodriguez et Kuehn, 2020 ; Bitto et al., 2021a), aucune de ces études n'a fourni une caractérisation détaillée, par conséquent, il n'est toujours pas clair si les molécules d'ADN peuvent également être emballées de manière sélective dans les VEs de *S. aureus*. À ce jour, les caractérisations des métabolites et des lipides des VEs de *S. aureus* font toujours défaut. Chez d'autres espèces bactériennes, il a été montré que les OMVs de *Bacteroides thetaiotaomicron* sont significativement enrichies en métabolites liés aux réseaux métaboliques de la souris (Bryant et al., 2017). Des études sur les bactéries à Gram positif *Streptococcus* sp, *Propionibacterium* sp et *Enterococcus* sp ont également révélé que le profil lipidique des VEs diffère de celui des bactéries productrices (Resch et al., 2016 ; Jeon et al., 2018; Afonina et al., 2021), suggérant un emballage sélectif. S'il s'agit d'une caractéristique commune des VEs bactériens, nous pourrions supposer que les VEs de *S. aureus* peuveut également présenter une composition spécifique de lipides et de métabolites. De nouvelles études sont encore nécessaires pour caractériser les composants des VEs et les mécanismes derrière la seléction de la cargaison des VEs de *S. aureus*.

2.3 Quels sont les avantages éventuels de la sélection du cargo des VEs de S. aureus ?

Aux premiers stades de l'infection, *S. aureus* exprime principalement des éléments liés à l'adhésion et à l'invasion, tandis que la régulation de l'expression des toxines nécessaires à la lyse des cellules hôtes et à la dissémination bactérienne se produit plus tard. En conséquence, les toxines connues pour être produites par *S. aureus* à des densités cellulaires

élevées, telles que Hld et PSMβ1 (Le et Otto, 2015 ; Jenul et Horswill, 2019), n'ont été trouvées dans les VEs qu'à la phase stationnaire tardive de notre étude. Ainsi, nous pourrions émettre l'hypothèse que l'emballage sélectif de toxines et d'autres éléments immunomodulateurs dans les VEs pourrait aider les bactéries en exerçant des fonctions spécifiques dans les interactions hôte-pathogène susceptibles de contribuer à la survie et à la persistance de *S. aureus*. En effet, dans un rapport de Briaud et al., les variations de température ont affecté la composition et l'abondance des ARN et des protéines des VEs, se reflétant directement dans les propriétés cytotoxiques des VEs envers les cellules hôtes (Briaud et al., 2021). En fait, les VEs dérivés de 34°C présentaient une plus grande diversité protéique, tandis que les VEs libérés à 40°C présentaient un conditionnement accru des facteurs de virulence (Briaud et al., 2021). Comme autre exemple, les VEs de *S. aureus* C-29 récupérés à 6 h, 17 h et 24 h de cultures ont induit différents gènes liés à l'inflammation dans les kératinocytes HaCaT (Yamanashi et al., 2022).

Il a été démontré que divers stress environnementaux, tels que le traitement antibiotique, l'épuisement du fer, l'éthanol, les stress oxydatifs et osmotiques peuvent modifier de manière significative la taille et la concentration des VEs (Askarian et al., 2018 ; Kim et al., 2020 ; Wang et al, 2021b), mais la caractérisation de la cargaison des VEs dans la plupart de ces cas fait encore défaut, en particulier dans la composition des lipides et des métabolites. Il a été démontré que les acides gras à chaîne courte (AGCC) produits par *P. freudenreichii* exercent un effet anticancéreux en faisant passer le mode de mort de la cellule hôte de l'apoptose à la nécroptose (Jan et al., 2002 ; Lan et al., 2007, 2008). Dans une autre étude, il a été démontré que l'administration de butyrate à des patients atteints de colite ulcéreuse (CU) inhibe l'activation de NF-κB dans les macrophages, présentant un effet anti-inflammatoire (Lührs et al., 2002). De plus, les OMVs de *B. thetaiotaomicron* enrichis en métabolites liés aux réseaux métaboliques de l'hôte suggèrent également leur rôle dans les interactions entre les bactéries intestinales et les mammifères (Bryant et al., 2017). À la lumière de toutes ces données, nous pourrions imaginer que l'emballage sélectif de protéines, de lipides, de métabolites et d'acides nucléiques dans les VEs de *S. aureus* peut présenter un avantage lors du développement de l'infection. L'étude des conditions mimant l'hôte sont des stratégies prometteuses. En effet, des expériences supplémentaires utilisant du DMEM et du RPMI complétés par du sérum de veau fœtal (FBS) comme milieu de culture bactérienne ont révélé une hyper vésiculation sur *S. aureus* (données non présentées). Les concentrations de fer restrictives et les facteurs hôtes présents dans le FBS peuvent affecter la physiologie de *S. aureus* et, par conséquent, la libération et la cargaison des VEs. De futures études portant

sur l'impact d'autres conditions ou contraintes environnementales sur l'emballage et les rôles sélectifs des cargaisons des VEs pourraient fournir de nouvelles informations sur les processus pathogènes de *S. aureus*.

2.4 Quels sont les rôles des VEs de S. aureus dans les interactions intra-espèces ?

Dans ce travail, nous avons fourni la première preuve démontrant que les VEs de *S. aureus* exercent des fonctions trophiques. Ce phénomène a déjà été démontré dans les systèmes marins et du microbiome, où les OMVs libérés soutiennent la croissance d'autres espèces en servant de sources directes ou indirectes de nutriments (Biller et al., 2014 ; Rakoff-Nahoum et al., 2014 ; Schwechheimer et Kuehn, 2015). Ici, la caractérisation transcriptomique et protéomique a révélé que les VEs de HG003 contiennent plusieurs ARN et protéines impliqués dans les processus métaboliques et la production d'énergie, tels que les transporteurs de substrat, les enzymes glycolytiques, la pyruvate déshydrogénase et les complexes cytochrome c oxydase. Le transfert de ces éléments via les VEs pourrait contribuer au fonctionnement des cellules réceptrices en stimulant la traduction et la nutrition, en particulier dans un contexte de restriction. Nous pourrions également imaginer que la traduction puisse se produire au sein des VEs indépendamment de la cellule bactérienne, ce qui pourrait encore optimiser ces interactions. En fait, notre analyse a détecté des opérons d'ARNm intacts, ce qui suggère que les ARN des VEs de *S. aureus* peuvent être présents sous forme de transcrits de pleine longueur. De plus, des unités ribosomiques, des ARNt et d'autres éléments essentiels à la traduction, tels que des facteurs d'élongation, sont également présents dans les VEs. Au cours de ma thèse, des expériences ont été menées pour tenter de valider cette hypothèse (données non présentées). Les ribosomes ont été extraits et purifiés à partir d'échantillons de cellules entières et des VEs pour un protocole de traduction in vitro. Les échantillons des VEs étaient négatifs pour la traduction alors que les échantillons de cellules étaient positifs. Cependant, ce résultat n'est pas concluant, car les expériences ont été réalisées avec de faibles concentrations de ribosomes des VEs. L'isolement et la purification des VEs par gradient de densité de saccharose est un travail long et laborieux, et la quantité d'échantillon des VEs acquis est limitée. Les stratégies qui optimisent la récupération des VEs, telles que la chromatographie d'exclusion de taille, peuvent être un moyen d'obtenir plus de matériel et d'explorer plus avant cette hypothèse.

Des études antérieures ont déjà démontré le transfert intra-espèce et inter-espèce du contenu des VEs de *S. aureus*. Par exemple, la protéine BlaZ biologiquement active présente dans les VEs de *S. aureus* a pu conférer une résistance transitoire à l'ampicilline à des

bactéries à Gram positif et Gram négatif sensibles à l'antibiotique, notamment *E. coli*, *Salmonella enterica*, *Staphylococcus epidermidis* et *S. aureus* (Lee et al., 2013). Dans une autre étude, Lee et al. ont rapporté que un transfert à long terme de la résistanceà un antibiotique. *E. coli* traité avec des VEs dérivés de *S. aureus* résistant à la méticilline (MRSA) a commencé à produire des OMVs avec une augmentation de 12 fois de l'activité β-lactamase (Lee et al., 2022). Ce profil de résistance pérenne à *E. coli* pose la question du rôle des VEs de *S. aureus* sur la régulation épigénétique, comme cela a déjà été montré pour les OMVs dérivés de *E. coli*, *Vibro cholerae* et *Pseudomonas aeruginosa* (Vdovikova et al., 2018 ; Kyung Lee et al., 2021). Malgré ces exemples, on ne sait toujours pas comment ce transfert de contenu des VEs se produit, en particulier chez les bactéries à Gram positif présentant des parois cellulaires épaisses. Considérant que des molécules à activité de dégradation, telles que les autolysines, sont impliquées dans la libération des VEs de *S. aureus*, nous pourrions imaginer que la présence de ces éléments dans les VEs peut également favoriser le transfert de matière dans les interactions VEs-bactéries via l'hydrolyse du peptidoglycane. Cela soulève la question de savoir quelle est la topographie des protéines des VEs. Sont-elles à l'intérieur des VEs, intégrées dans la membrane ou exposées à la surface des VEs ? Ces questions et les mécanismes sous-jacents de la livraison de leur contenu, ainsi que leur rôle dans les interactions inter-espèces devraient être abordés dans des études futures.

2.5 Les VEs de S. aureus ajustent-ils avec précision les interactions hôte-pathogène ?

Bien que les VEs de *S. aureus* ne soient pas cytotoxiques contre les cellules hôtes (cette étude, Tartaglia et al., 2018), ils induisent, comme les bactéries vivantes, l'expression de plusieurs gènes pro-inflammatoires. De plus, les VEs peuvent moduler de manière différente l'expression de gènes selon les lignées cellulaires humaines étudiées, un résultat déjà rapporté avec des VEs dérivés d'une autre souche de *S. aureus* (Kim et al., 2012). Ce résultat est intéressant car *S. aureus* peut coloniser plusieurs niches humaines et provoquer un large éventail de maladies. Puisque les VEs exercent des fonctions variées envers des cellules hôtes distinctes, les résultats de l'infection peuvent également être affectés. De plus, en comparant la stimulation des cellules humaines par les VEs et les bactéries vivantes, nous avons noté que moins de gènes étaient activés par les VEs et que ceux modulés par les deux groupes présentaient des niveaux d'expression plus élevés après exposition aux bactéries. En revanche, l'analyse ARN-seq avec des cellules épithéliales mammaires bovines (bMECs) a indiqué que certaines chimiokines et cytokines étaient plus fortement activés par les VEs, par rapport aux bactéries dans les mêmes conditions. Ces disparités ne sont pas surprenantes compte tenu des différentes souches de *S. aureus* et des contextes d'infection. En effet,

l'analyse protéomique a montré que le protéome des VEs *S. aureus* semble refléter la sévérité des infections causées par différents isolats hôtes (humain, bovin, ovin), avec des facteurs de virulence moins abondants dans les VEs dérivés de souches légères par rapport aux souches sévères (Tartaglia et al., 2020). De plus, malgré des exemples montrant l'effet protecteur des VEs face à une exposition à *S. aureus* (Choi et al., 2015; Askarian et al., 2018), les VEs de *S. aureus* peuvent également induire une hypersensibilité et la mort de souris après l'infection (Asano et al., 2021).

Toujours en ce qui concerne la dynamique des bMECs exposées aux VEs de *S. aureus* dans différentes conditions, nous avons constaté que les chimiokines et les cytokines, telles que l'IL-8, l'IL-1β et le TNF-α, sont induites par les VEs dans toutes les conditions, ce qui suggère qu'ils favorisent incessamment stimulation inflammatoire. À l'opposé, de nombreux gènes induits par *S. aureus* au début de l'infection (3 h) ont également été induits par les VEs après une stimulation de 24 h, ce qui suggère que certaines réponses de l'hôte aux VEs sont retardées par rapport aux bactéries. Fait intéressant, une étude récente de Zwack et de ses collaborateurs a montré que l'infection par des CFU (des unités formant des colonies) mineures de *S. aureus* produit une réponse transcriptionnelle atténuée de l'hôte qui inhibe la signalisation des chimiokines et des cytokines (Zwack et al., 2022). Nous pourrions ainsi émettre l'hypothèse que des propriétés spécifiques des VEs pourraient déclencher des phénotypes similaires dans les cellules hôtes. En résumé, ces données suggèrent que les VEs ont évolué pour s'adapter à des contextes spécifiques et pour exercer les réponses nécessaires de l'hôte, fonctionnant comme des systèmes affinés dans les interactions hôte-pathogène. Considérant que les VEs peuvent parcourir de longues distances, leurs propriétés immunomodulatrices pourraient aider à construire un environnement favorable à la survie de *S. aureus* au cours de l'infection. Des expériences pourraient être réalisées pour étudier l'impact de la pré-stimulation des VEs suivie d'une infection bactérienne sur les cellules hôtes. La mesure de l'adhésion et de l'internalisation bactériennes dans les cellules hôtes, ainsi que l'induction de l'expression des gènes hôtes pourraient apporter de nouvelles informations sur les rôles exercés par les VEs dans le contexte de la pathogenèse.

2.6 Les ARNs des VEs sont-ils des protagonistes des interactions hôte-pathogène ?

Plusieurs études ont montré le rôle des VEs de *S. aureus* sur les interactions hôte-pathogène, telles que la cytotoxicité, la libération de cytokines in vitro et in vivo, le recrutement de cellules de défense, l'inflammation et la détérioration des tissus, l'exacerbation de l'infection et la survie bactérienne ex vivo et in vivo. (Gurung et al., 2011 ;

Hong et al., 2011, 2014 ; Kim et al., 2012 ; Thay et al., 2013 ; Choi et al., 2015 ; Jun et al., 2017 ; Askarian et al., 2018; Kwon et al., 2018, 2019; Tartaglia et al., 2018; Wang et al., 2018b, 2020, 2021b; Asano et al., 2021; Bitto et al., 2021a; Briaud et al., 2021; Staudenmaier et al., 2022 ; Yamanashi et al., 2022). Les protéines des VEs de *S. aureus* étaient liées à plusieurs de ces fonctions, qui ont été validées principalement par l'étude de souches mutantes dépourvues de facteurs de virulence importants, tels que les toxines porogènes (par exemple Hla, PSMs, leucocidines) (Thay et al., 2013 ; Briaud et al., 2021), les protéines de liaison aux immunoglobulines (par exemple, la protéine Spa) (Wang et al., 2018b) et les régulateurs mondiaux (par exemple, Agr, Sae) (Wang et al., 2020, 2021b). Néanmoins, le rôle potentiel d'autres composants présents dans les vésicules bactériennes, tels que les ARN, est encore inconnu.

Ce travail a fourni la première caractérisation approfondie d'ARN des VEs de *S. aureus*, qui comprend les classes d'ARNm, d'ARNt, d'ARNr et d'ARNs. Fait intéressant, ils correspondaient à environ 78 % des transcrits annotés pour cette souche, ce qui est conforme aux 73 % de transcrits annotés identifiés dans les VEs dérivés de *Salmonella enterica* sérovar Typhimurium (*S.* Typhimurium) (Malabirade et al., 2018). La plupart des ARN des VEs de *S. aureus* présentaient une faible couverture de séquençage, un résultat également observé chez les bactéries à Gram négatif (Malabirade et al., 2018). La présence d'ARN dégradés dans les VEs pourrait être attribuée à la libération de matériel fragmenté et indésirable, et/ou à la dégradation progressive des transcrits en cas d'absence de transcription au sein des VEs. Bien que nous nous soyons concentrés principalement sur les ARN très couverts des VEs, les ARN fragmentés correspondant à de très petits ARN (ARNvs) peuvent également être des modulateurs des interactions hôte-pathogène (Diallo et al., 2022). En effet, les ARNvs se sont révélés être enrichis en OMVs d'*E. coli* et cibler plusieurs ARNm humains aux fonctions diverses (Diallo et al., 2022). L'un de ces ARNvs, le fragment d'ARNt Ile-tRF, s'est révélé être délivré aux cellules hôtes et entrer en compétition avec la machinerie de « silencing » des miARN. Cette interaction favorise la régulation de la voie de la protéine kinase activée par le mitogène de l'hôte, entraînant une prolifération accrue des cellules de carcinome colorectal (Diallo et al., 2022, données non publiées). Fait intéressant, les ARNvs sont très abondants dans la souche HG001 de *S. aureus* (Diallo et al., 2022), qui est étroitement liée à HG003 utilisée dans cette étude. Enfin, il est bien connu que les ARNs jouent également un rôle essentiel dans les interactions pathogène-hôte. Par exemple, il a été montré qu'un ARNs porté par des vésicules de la membrane externe (OMVs) de *P. aeruginosa* était transféré dans des cellules des voies respiratoires humaines, provoquant une réduction de l'expression de

l'IL-8 (Koeppen et al., 2016). Chez les bactéries à Gram positif, Frantz et al. ont rapporté que l'ARNs rli32 associé aux VEs de *Listeria monocytogenes* déclenche l'induction de la réponse IFN de type I dans les cellules hôtes (Frantz et al., 2019). Il est possible que les VEs sécrétés par *S. aureus* contiennent également des ARNs impliqués dans la communication avec les cellules de mammifères. En effet, notre analyse a identifié 28 ARNs hautement couverts. La caractérisation spécifique des ARNs et des ARNvs des VEs de *S. aureus* et l'étude plus approfondie de leurs rôles dans les interactions hôte-pathogène devraient être abordées dans d'autres études.

6. Conclusion

Le projet de cette thèse fournit un premier travail de caractérisation approfondie de la composition en ARN et en protéines des VEs de *S. aureus* HG003 et de ses cellules productrices. Les VEs dérivés de la souche HG003 portent des ARN et des protéines importants, y compris des facteurs de virulence clés, qui diffèrent de ceux des cellules bactériennes. La présente étude démontre également l'impact des conditions environnementales sur la composition et l'abondance du contenu des VEs, suggérant qu'il existe un emballage sélectif de la cargaison des VEs. De plus, nous fournissons également la première preuve que les VEs de *S. aureus* ont un impact positif sur la croissance bactérienne. Dans le contexte des interactions hôte-pathogène, il a été démontré que les VEs de *S. aureus* affectent l'immunité de l'hôte dans des ampleurs, des moments et des voies différentes de celles des bactéries vivantes, ce qui suggère leur rôle important et spécifique dans le processus d'infection. Dans l'ensemble, cette étude a apporté de nouvelles informations sur le contenu des VEs de *S. aureus* et leurs rôles potentiels. Il s'agit d'une nouvelle étape vers une meilleure compréhension de la façon dont ces particules libérées contribuent à la physiologie et à la pathogenèse de *S. aureus*. La caractérisation plus poussée des éléments des VEs de *S. aureus*, en particulier des acides nucléiques, des lipides et des métabolites, ainsi que l'exploration des événements mécanistes impliqués à la fois dans la libération des VEs et dans la livraison de cellule à cellule des VEs est d'un grand intérêt. Des recherches supplémentaires sur les fonctions des VEs de *S. aureus* dans la physiologie bactérienne et dans des modèles in vivo devraient être menées pour valider les données précédentes afin d'essayer d'élucider la contribution exacte des VEs dans les interactions intra- et inter-royaumes.

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Annexes

Supplementary Material – Chapter 6

Supplementary Tables S1-10 from Chapter 6 are available at:
https://docs.google.com/spreadsheets/d/11r4NtsTDeRGtiYy0S-y1zI3y5q1IfQk6/edit?usp=sharing&ouid=100366295432850026370&rtpof=true&sd=true

Supplementary Figure 1

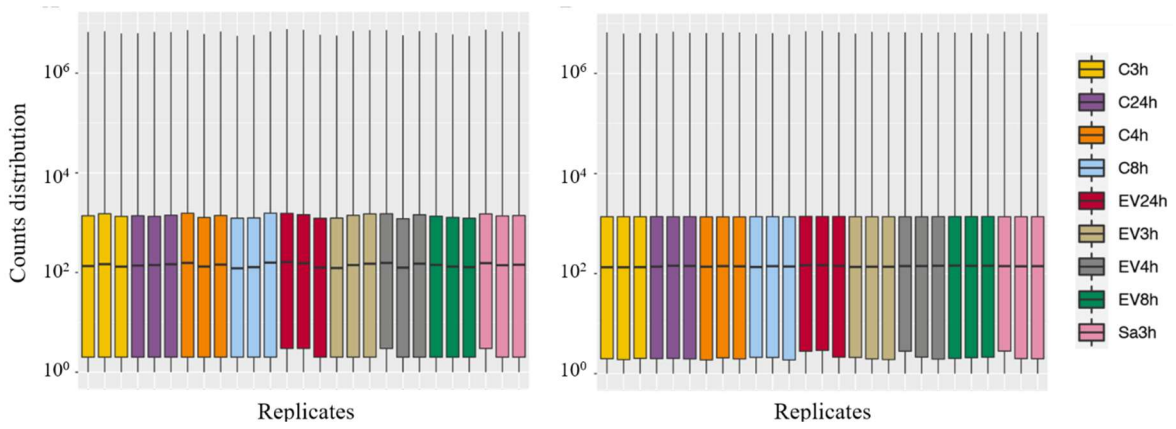

Figure S1. DESeq2 data normalization was performed in order to make the number of read counts comparable among samples. Boxplots of raw (left) and normalized (right) read count distribution.