Book Chapter

Staphylococcal Extracellular Vesicles (EVS): Function, Pathogenesis, and Therapy

Fernando Gómez-Chávez[1], Sandra Martínez-Rodríguez[2], Mario E Cancino-Diaz[2] and Juan C Cancino-Diaz[3]*

[1]Laboratorio de Enfermedades Osteoarticulares e Inmunológicas, Sección de Estudios de Posgrado e Investigación, Escuela Nacional de Medicina y Homeopatía, Instituto Politécnico Nacional, Mexico
[2]Laboratorio de Inmunidad Innata, Departamento Inmunología, Escuela Nacional de Ciencias Biológicas-Instituto Politécnico Nacional, Mexico
[3]Laboratorio de Inmunomicrobiología, Departamento Microbiología, Escuela Nacional de Ciencias Biológicas-Instituto Politécnico Nacional, Mexico

***Corresponding Author:** Juan C Cancino-Diaz, Laboratorio de Inmunomicrobiología, Departamento Microbiología, Escuela Nacional de Ciencias Biológicas-Instituto Politécnico Nacional, 11340 Mexico City, Mexico

Published **March 15, 2022**

This Book Chapter is an excerpt of an article published by Juan C Cancino-Diaz, et al. at International Journal of Molecular Sciences in December 2021. (Gómez-Chávez, F.; Cedillo-Peláez, C.; Zapi-Colín, L.A.; Gutiérrez-González, G.; Martínez-Torres, I.; Peralta, H.; Chavez-Galan, L.; Avila-Calderón, E.D.; Contreras-Rodríguez, A.; Bartolo-Aguilar, Y.; Rodríguez-Martínez, S.; Cancino-Diaz, M.E.; Cancino-Diaz, J.C. The Extracellular Vesicles from the Commensal Staphylococcus Epidermidis ATCC12228 Strain Regulate Skin Inflammation in the Imiquimod-Induced Psoriasis Murine Model. Int. J. Mol. Sci. 2021, 22, 13029. https://doi.org/10.3390/ijms222313029)

How to cite this book chapter: Fernando Gómez-Chávez, Sandra Martínez-Rodríguez, Mario E Cancino-Diaz, Juan C Cancino-Diaz. Staphylococcal Extracellular Vesicles (EVS): Function, Pathogenesis, and Therapy. In: Saeed Tarighi, editor. Prime Archives in Molecular Sciences: 3rd Edition. Hyderabad, India: Vide Leaf. 2022.

Funding: This research was funded by The "Secretaria de Investigación y Posgrado (SIP)" of Instituto Politécnico Nacional, grant number 20221352.

Abstract

Communication between bacteria and host cells is relevant to understanding the bacterial infection process. Bacteria use the production and release of extracellular vesicles (EVs) to communicate. EVs are formed by a double-membrane of phospholipids and membrane proteins containing metabolites, proteins, lipids, nucleic acids, phages, enzymes, and toxins. EVs can deliver their contents to cells by membrane fusion. Depending on the pathogenicity of the bacterial species, the content of the EVs will differentially affect the host cells. For example, pathogenic bacterium EVs cause an inflammatory response, while commensal bacteria EVs modulate the immune response. Gram-negative bacteria EVs have been studied in greater depth than those of Gram-positive bacteria. This chapter focuses on updating the role of the EVs from Gram-positive bacteria and specifically Staphylococci. The EVs of *Staphylococcus aureus* have several functions, emphasizing their ability to transport molecules that participate in the pathogenesis of this bacterium, such as antibiotic resistance, protein A, and the stimulation of an inflammatory immune response in the skin. In contrast, the EVs of *S. epidermidis* have been poorly studied, showing regulatory functions in inflammatory diseases such as

psoriasis. Also, in this chapter, the staphylococci EVs proteomics and proposals for the use of EVs as therapeutics are addressed.

Introduction

The communication between bacteria and human organ tissue cells is highly relevant since knowing this communication mechanism can help understand bacterial infection. This communication is mainly through the exchange of bacterial molecules towards the host cells [1]. Bacteria have generated several pathogenic molecules secretion systems, such as the secretomes, which are protein complexes inserted in the bacterial cytoplasmic membrane that transport specific molecules from the cytoplasm to the external environment. Another system is the external membrane vesicles or extracellular vesicles (EVs). Bacterial EVs release a large number of molecules, mainly those found in the cytoplasmic membrane or the cytoplasm [2]. Bacterial EVs are produced from the outermost layer of the bacteria, and this process occurs by evagination from the cytoplasmic membrane of Gram-positive bacteria or the outer membrane of Gram-negative bacteria. EVs are double-membrane vesicles with an interior content from the periplasmic space for Gram-negative bacteria or the cytoplasmic space for Gram-positive bacteria. Evs' content can be metabolites, proteins, lipids, nucleic acids, phages, enzymes, and toxins [3–5].

A variety of functions of EVs have been described. Those participating in bacterial physiology and ecology stand out since EVs can exchange different molecules involved in the bacteria-host interaction, promoting survival, infection, invasion, immune evasion, and immune modulation [6]. Regarding the EVs of Gram-positive bacteria, it has been shown that they participate in cellular defense [7], cell communication [8], DNA transfer [9], pathogenesis through the delivery of virulence factors [10], and the inactivation of antimicrobials by enzymatic degradation [11].

The EVs can also participate in protection against pathogenic microbial agents; for example, the vaginal microbiota protects

women from sexually transmitted infections, particularly HIV-1. This protection depends on lactobacillus in the vaginal microbiota, and the protection mechanism against HIV-1 is due to the EVs released by Lactobacillus [12]. However, this vaginal protection against HIV-1 also occurs from EVs released by other Gram-positive bacteria such as *Staphylococcus aureus*, *Gardnerella vaginalis*, *Enterococcus faecium*, and *Enterococcus faecalis*, present also in the vagina of healthy women [13]. On the other hand, the EVs of *Streptococcus pneumoniae* are internalized by macrophages activating NF-kB for the polarization to an M2 macrophage phenotype, this activation leads the macrophages to increase the uptake of *S. pneumoniae*, and the macrophage in these conditions acts as a reservoir for bacteria increasing their survival inside this cell [14].

This chapter will review the most recent research on the EVs of Gram-positive bacteria and specifically on the EVs produced by staphylococci.

EVs Isolation

Bacterial EVs are isolated from different *in vitro* and *in vivo* sources [15]. Generally, the bacteria are grown in conventional media, and it has not been determined if there is a specific condition for EVs production. After bacterial growth, the cells are separated by centrifugation, and the culture supernatant is filtered through 0.22 µm filters to remove bacterial cells. Then, EVs are purified from cell debris by ultracentrifugation using a sucrose or iodixanol gradient [16]. Finally, transmission electron microscopy is used to observe the EVs' characteristic structure.

Structure, Composition, and Formation of the EVs

In general, bacterial EVs are composed of a lipid membrane bilayer where proteins and glycoproteins are incorporated; different molecules such as enzymes, toxins, or nucleic acids may also exist within the vesicles.

The composition and structure of EVs are still unclear, and research continues using various techniques such as electron

microscopy, mass spectroscopy, proteomic analysis, among others, to know its composition and possible bacterial EVs functions. However, the approach to determining the Evs' function is based on their bioactivity in vitro or in vivo models.

The mechanism of biogenesis of EVs from Gram-positive bacteria is still not fully established. However, some lines of research suggest that the release of EVs through the cell wall may be by different mechanisms; among the most notable are: 1) a turgor pressure on the cell wall caused by the accumulation of EVs, which causes the release of EVs by the cytoplasmic membrane; 2) degradation of the cell wall by the presence of enzymes that modify the cell wall; 3) EVs can deform into smaller diameters that can pass through cell wall pores [17].

Bacteria-Bacteria Communication by EVs

In a microbial community, bacteria communicate with each other through complex interactions, and one of these interactions may be the EVs that play an essential role in the processes of cooperation and competition [18]; including but not limited to antibiotic resistance [19], biofilm formation [20], survival [21], virulence factors [22], and quorum sensing [23].

In antibiotic resistance, it has been reported that bacterial EVs can mediate their resistance through different mechanisms, such as horizontal gene transfer, EV entrapment of extra- and intracellular antimicrobials, and the presence of enzymes in EVs that degrade antibiotics. For example, the latter occurs with *Staphylococcus aureus* EVs containing the beta-lactamase enzyme within them. These enzyme lactamase-carrying EVs are released into the medium to be taken up by other bacteria such as *Salmonella enterica*, *Escherichia coli*, and *Staphylococcus epidermidis* [19] to confer them ampicillin resistance. This mechanism of antibiotic resistance transferred by EVs is widespread among various Gram-negative and Gram-positive bacteria. In addition, the enzymes that produce antibiotic resistance inside the EVs are protected from degradation or inactivation [24], as is the case of *Moraxella catarrhalis* EVs, which protect the beta-lactamase from neutralization by the

binding of the immunoglobulin IgG [25]. Regarding horizontal gene transfer, it has been observed that carbapenem-resistant *Acinetobacter baumannii* can transfer the carbapenemase gene via EVs to other carbapenem-sensitive *A. baumanii* strains [26]. On the other hand, bacterial EVs can contain quorum-sensing molecules that support communication between bacteria, such as quinolines or lactones [23].

EVs-Host Communication

The interaction mechanism between bacterial EVs and human host cells include the interaction of these EVs with host receptors, delivery of the internal cargo of the EVs into the host cell, and complete incorporation of the EVs into the host cell cytoplasm [27]. The molecular mechanism of EVs uptake is unknown, but three routes of uptake have been proposed: 1) Endocytosis, 2) EV internalization by lipid rafts, and 3) direct membrane fusion.

Toll-like receptors (TLRs) were reported to be involved in the interaction of bacterial EVs with host cells. EVs from *M. catarrhalis* are internalized into human epithelial cells via interaction with TLR2 [28]. *Bifidobacterium* and *Lactobacillus* EVs potentiate the TLR2/1 and TLR4 response in dendritic cells [29]. The interaction of *Mycobacterium* EVs with mouse macrophages stimulates the release of cytokines and chemokines in a TLR2-dependent manner [30].

The effect of EVs components on host cells after its delivery can alter the activation of signaling cascades related to the immune response [31]; for example, *Clostridium perfringens'* EVs upregulate the expression of tumor necrosis factor-alpha (TNF-α), IL-6, and the granulocyte colony-stimulating factor (GM-CSF) in vitro experiments [32]. However, contrary to this, the EVs of *Akkermansia muciniphila*, a gut bacterium, reduce IL-6 production from the colonic epithelial cells during colitis [33].

EVs from pathogenic bacteria affect host immunity. For example, EVs isolated from *Bacillus anthracis*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, and *Neisseria*

meningitidis can trigger an immune response that can protect against disease development opening a new potential use for Bacterial EVs as vaccines [34]. On the other hand, the gut microbiota has essential functions, but EVs have been shown to have protective effects. For example, the administration of EVs isolated from the commensal bacteria *Bacteroides fragilis* can mimic immune tolerance to prevent inflammatory bowel disease caused by administering the bacterium itself [35]. Furthermore, EVs from probiotic bacteria have anti-inflammatory effects and promote immune tolerance, as is the case with EVs from *Bifidobacterium longum* that alleviates food allergy mediated by mast cell suppression [36], or the case of EVs from probiotic *E. coli* Nissle strain, which trigger the production of IL-10 [37], as well as the EVs from *lactobacillus* [38]. This data depicts that bacterial EVs can be considered an alternative for cases where the intestinal barrier is damaged, or the use of live bacteria in the host could be dangerous, such as immunocompromised individuals.

EVs of Staphylococci

Coagulase-positive staphylococci such as *Staphylococcus aureus* and coagulase-negative staphylococci such as *Staphylococcus epidermidis* have had a significant impact on health due to their ability to cause infectious diseases. Therefore, these two species of staphylococci have been studied to identify the biological potential of their EVs. In this context, *S. aureus* EVs are the most studied, and we now have the most information about them. In contrast, there is little information about EVs from *S. epidermidis*.

Staphylococcus aureus EVs

EVs from Gram-positive bacteria are poorly understood, especially in the bacterial community and particularly EVs from *S. aureus*. Under antibiotic stress conditions, *S. aureus* releases EVs containing the BlaZ protein, a beta-lactamase protein [4,39]. EVs shuttle the BlaZ protein to other Gram-negative and Gram-positive bacteria helping them to survive in the presence of ampicillin but not tetracycline, chloramphenicol, or kanamycin.

The EVs of *S. aureus* transport the BlaZ protein but not the blaz gene. In addition, the EVs protect the BlaZ protein from digestion by extracellular proteases [39], indicating that the EVs are a highly efficient molecular transfer mechanism, showing the importance of *S. aureus* EVs in antibiotic resistance which allows the polymicrobial community to continue to grow against antibiotics.

The EVs of *S. aureus* can induce cytotoxicity and neutrophil activation. The exogenous administration of EVs increases the resistance of *S. aureus* to death in the presence of whole blood or ex vivo purified human neutrophils, and this also occurs in the in vivo model. The use of *S. aureus* EVs as antigens to immunize mice produces IgM, total IgG, IgG1, IgG2a, and IgG2b; these EVs immunization generate protection against systemic and subcutaneous *S. aureus* infection [40], suggesting that *S. aureus* EVs may influence the bacteria-host interaction during systemic infection and provide protective immunity in murine models of infection.

The SPA protein of *S. aureus* is considered a virulence factor; this protein has been found present in keratinocytes and the intercellular space of the epidermis of atopic dermatitis lesions colonized with *S. aureus*. SPA is a component of the EVs of *S. aureus*. The EVs of *S. aureus* deliver their components to the keratinocytes, and these keratinocytes induce the expression of pro-inflammatory cytokine genes (such as IL-8) in vitro, and this induction by EVs is by TLR2 or by NOD2 (nucleotide-binding oligomerization domain containing 2). Topical application of *S. aureus* EVs to atopic dermatitis-like skin lesions in a mouse model induces massive infiltration of inflammatory cells generating eczematous dermatitis; the inflammatory reaction is associated with Th1/Th2 cell immune response and enhanced expression of chemokine genes in atopic dermatitis-like skin lesions [41]. In this way, it is shown that the EVs of *S. aureus* are powerful mediators to worsen atopic dermatitis and that it could be a therapeutic target for the management of this disease. Furthermore, this is the opposite of the above information that EVs can be vaccine antigens against *S. aureus* infection [40].

In order to understand what happens to the cells that receive components of the *S. aureus* EVs, four strains of *S. aureus* (two wild type strains and two clinical isolates) produced EVs in vitro, and these EVs interact with the plasma membrane of the host cells via a cholesterol-rich membrane microdomain for the delivery of their EVs components to cells, one of them being protein A. This delivery of EVs to HEp-2 cells induces apoptosis in a dose-dependent manner, while lysed EVs do not produce this cell death process. In addition, in a mouse pneumonia model by infection with a clinical isolate of *S. aureus*, EVs can be recovered from the infected mouse [42]. In another study very similar to the previous one, it was shown that the cholesterol-dependent fusion of *S. aureus* EVs with the plasma membrane of the host cell represents a route for the entry of key virulence factors of this bacterium, such as α-toxin (α-hemolysin; Hla) to human cells. Most *S. aureus* strains produce this α-toxin, and it can lyse a wide range of human cells and induce apoptosis in T-lymphocytes. In agreement with this, the α-toxin present in EVs contributes to the cytotoxicity of the EVs for HeLa cells and for the lysis of erythrocytes [43]. These studies demonstrate that *S. aureus* EVs contribute to host cell cytotoxicity.

Another important factor used by *S. aureus* infection is biofilm formation as a defense mechanism against the host's immune system and antibiotic treatment. In a challenging in vitro model system with *S. aureus* in biofilm with human neutrophils, bacteria EVs were identified in the supernatant, indicating that the challenge with immune cells induces the production of EVs from the biofilm. Furthermore, these EVs treated with phospholipase C were wholly destroyed, while the addition of proteinase K caused EVs partial structural disorganization; however, the treatment with DNAse did not affect the structure of the EVs. These observations allow us to conclude that phospholipids and proteins play a role in the Evs' shape-structure formation. In addition, EVs were shown to have anti-biofilm activities with other staphylococci species since *S. aureus* EVs inhibit *S. epidermidis* (strain 178M and strain 328/5, respectively) biofilm formation. However, this anti-biofilm effect does not occur for *S. aureus* biofilms (strains 5983/2, 5663, and 18A) [44], indicating that EVs are selective to inhibit

biofilm formation since with a different species of staphylococci it has an anti-biofilm. Until now, the mechanism of anti-biofilm action of *S. aureus* EVs is unknown.

On the other hand, the management of infectious osteomyelitis relies on identifying the causative microorganism for appropriate antibiotic therapy. A systemic bacterial infection rapidly accumulates EVs in the circulation; moreover, in a rat osteomyelitis model infected by *S. aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*, after three days, EVs are isolated in the serum. The size and the number of EVs in serum from infected rats are significantly increased; in addition, bacterial aggregation assays show that *S. aureus* and *E. coli* form large aggregates in response to stimulation of EVs from rats serum infected with *S. aureus* or *E. coli*. Interestingly, these same experiments using serum from 28 osteomyelitis patients infected with *S. aureus* also showed that the size and number of EVs are larger and induce strong bacterial aggregations of *S. aureus* [45]. These findings indicate that the size and number of serum EVs may help diagnose potential infections and that EVs-induced bacterial aggregation may be a rapid test for identifying osteomyelitis-causing microorganisms.

Another *S. aureus* infection is the bovine mammary gland infection, generating mastitis. A bovine mastitis isolate, the *S. aureus* Newbould 305 (N305), can generate EVs with high protein content in vitro. EVs secreted from *S. aureus* N305 are non-cytotoxic when tested in vitro with bovine mammary epithelial cells; however, EVs induce inflammatory cytokine gene expression at the same level as that induced by live bacteria. The in vivo immune response to *S. aureus* N305 EVs in a mouse model of mastitis showed a dose-dependent induction of neutrophil recruitment and the production of pro-inflammatory mediators such as IL-17 [46], meaning that EVs may be involved in the development of bovine mastitis due to *S. aureus.*

When studying the role of EVs derived from *S. aureus* with the pathogenesis of atopic dermatitis, in a tape-stripped mouse skin model, EVs were applied three times a week, showing an increase in the production of pro-inflammatory mediators such as

IL -6, thymic stromal lymphopoietin, macrophage inflammatory protein-1α, and eotaxin; as well as epidermal thickening with infiltration of the dermis by mast cells and eosinophils. Furthermore, these changes were associated with enhanced cutaneous production of IL-4, IL-5, IFN-gamma, and IL-17, and a significant increase in specific IgE antibodies, in the serum of patients with atopic dermatitis, against EVs of *S. aureus* compared to healthy individuals [47]. These results indicate that EVs of *S. aureus* induce atopic dermatitis-like inflammation in the skin and that EVs can be used as a new diagnostic and therapeutic target for controlling atopic dermatitis. Something similar occurs with the case of *Propionibacterium acnes* EVs since they can induce acne-like phenotypes in human epidermal keratinocytes and a model of reconstituted human skin. Furthermore, *P. acne* EVs induce inflammatory cytokines such as IL-8 and GM-CSF and deregulation of epidermal differentiation by increased proliferation of keratinocytes and decreased levels of epidermal keratin and desmocollin 1 [48]. All these data suggest that the presence of EVs from skin pathogenic bacteria can induce inflammation and skin disease phenotypes as the bacteria themselves do, indicating that EVs secrete molecules that can trigger skin inflammation.

Staphylococcus epidermidis EVs

Like *S. aureus*, *S. epidermidis* can cause osteomyelitis. Osteomyelitis models in rats infected by *S. epidermidis* show the presence of EVs of this bacterium at three days after infection, with a diameter size of 137.13 nm and a significantly high number of EVs in the serum, around $7.03x10^9$ particles/mL. Neutrophils infected with *S. epidermidis* induce the production of *S. epidermidis* EVs, in addition, treatment of *S. epidermidis* bacteria with their own EVs induce a high bacterial aggregation of this bacterium [45], indicating that the EVs of *S. epidermidis* are produced in an infection model of osteomyelitis; however their EV role in the pathology is unknown.

S. epidermidis causes infections associated with orthopedic implants due to its ability to establish biofilms causing infections to become chronic and difficult to treat. The role of *S.*

epidermidis EVs in biofilm formation and survival is entirely unknown. A study related to osteomyelitis showed that a gentamicin-susceptible *S. epidermidis* strain grown at subinhibitory concentrations of the antibiotic gentamicin produced many EVs with high protein content. The gentamicin-susceptible *S. epidermidis* strain grown under gentamicin pressure and treated with its EVs promotes different antimicrobial tolerance mechanisms depending on the concentrations of EVs and gentamicin. Regarding the biofilm, cell adhesion to polystyrene plates decreases in the presence of gentamicin and EVs, having a synergistic effect. In the case of EVs obtained from a strain of *S. epidermidis* resistant to gentamicin, they increased cell division during the exponential growth phase, faster maximum growth rate, shorter generation time, and dramatic inhibition of adhesion cell [49]. These findings suggest that the formation of EVs in *S. epidermidis* is related to the survival response towards subinhibitory concentrations of gentamicin. This survival by EVs is due to the modulation of growth rate and affecting cell adhesion towards polystyrene, which reduces biofilm.

Isolates of *S. epidermidis* from patients with osteomyelitis associated with septic loss of orthopedic prostheses can produce EVs under in vitro growth conditions. Then, these EVs of *S. epidermidis* are internalized by THP-1 cells and cause an upregulation of the TLR3 gene expression, activation of NF-kB, and promotion of expression and secretion of IL-8, the monocyte chemoattractant protein (MCP)-1, the matrix metallopeptidase (MMP)-9, and the IL-10. In addition, these EVs also upregulated the expression of the proapoptotic DNA damage-inducible transcript 4 (DDIT4) gene; in contrast, they downregulated the antiapoptotic B-cell lymphoma 2 (Bcl-2) gene [50]. In conclusion, staphylococcal EVs possess potent cytolytic and immunomodulatory properties.

Finally, our research group obtained EVs of *S. epidermidis* from the ATCC12228 strain and the 983 clinical strain. Characteristically, the EVs from *S. epidermidis*, ATCC12228 (ATCC12228EVs), sized on average 66.9 nm (from 27.9 to 135 nm). In both strains, EVs showed a spherical shape and a

bilayer-lipidic membrane. The amount of protein was similar in both strains, 1,421 mg/mL for ATCC12228EVs and 1377 mg/mL for 983EVs [51].

Proteomics in Staphylococcal EVs

Proteomic studies have been carried out on the staphylococci EVs to find the component proteins of the EVs and deduce those proteins that participate in the different reported functions of the EVs.

Regarding *S. aureus*, mass spectrometry has been done on the EVs, and a total of 90 protein components have been identified, of which enrichment of extracellular proteins are present, suggesting that a specific sorting mechanism for vesicular proteins exists. Proteins that facilitate the transfer of proteins to other bacteria have also been detected and proteins involved in the elimination of competition organisms, antibiotic resistance, pathological functions in systemic infections, and proteins of the biogenesis of EVs [4]. Another proteomic study found that EVs are mostly of cytoplasmic proteins, followed by proteins of unknown localization, cytoplasmic membrane proteins, extracellular or secretable proteins, and cell wall-associated proteins. These proteins' molecular function is described as potential DNA/RNA/ATP binding or ligase, endopeptidase activity, or proteins that participate in crucial metabolic or virulence processes. Proteins involved in carbohydrate synthesis or host cell cytolysis, protein translation, and the formate acetyltransferase and delta-hemolysin precursor are identified as the most abundant proteins in the *S. aureus* EVs [40]. A total of 143 proteins were identified in the EVs of *S. aureus* 06ST1048, with a putative pyruvate dehydrogenase E1 component beta subunit (D1GS80) detected in the highest abundance, followed by dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (D1GS81). In addition, β-lactamases and protein A were identified in EVs from *S. aureus* 06ST1048 isolate. The identified proteins were classified into five groups based on their location in the bacterium: cytoplasm, cytoplasmic membrane, cell wall, extracellular region, and unknown

localization [42]. This location suggests that many biologically active bacterial proteins are packaged in the EVs of *S. aureus*.

Very little has been studied regarding the proteins present in the EVs of *S. epidermidis*. A single study published by us demonstrates the proteins present in *S. epidermidis* EVs of the type strain ATCC12228 and of a clinical strain 983. The LC-MS/MS analysis identified 105 proteins in the 983EVs and ATCC12228EVs. From these proteins, 22 were exclusive for 983EVs, 89 for the ATCC12228EVs, and 16 proteins were shared between them. When the Clusters of Orthologous Groups of proteins (COG) functions were compared between both EVs origins, the 983EVs had more abundant proteins with unknown function (COG S) than in the ATCC12228EVs. Proteins with different functions were more abundant in the ATCC1228EVs, such as translation (COG J), transcription (COG K), replication and recombination (COG L), energy obtainment (COG C), carbohydrate metabolism (COG G), amino acid biosynthesis (COG E), coenzyme biosynthesis (COG H), inorganic ion transport/metabolism (COG P), and general activities (COG R). In the 983EVs, the proteins found were related to nucleotide synthesis (COG F) and intracellular trafficking and secretion (COG U). The ATCC12228EVs had proteins with exclusive biological processes, including metabolic process, catabolic process, tricarboxylic acid cycle, acetyl-CoA catabolic process, macromolecule biosynthetic, and coenzyme catabolic process. The 983EVs had exclusive biological processes such as pathogenesis, cellular process, gene expression, multi-organism process, primary metabolic process, and nucleobase metabolic process. From all these data and the analysis of the metabolic processes, we can conclude that the proteins in each vesicle could participate in essential functions of the cell, with ATCC12228EVs having a higher number of proteins. Some proteins present in ATCC12228EVs could suggest an exciting role, such as HTH-type transcriptional regulator rot, universal stress protein UspA, penicillin-binding protein 2, glycosyltransferase, 60 kDa chaperonin, ornithine carbamoyltransferase, arginine deiminase, glutamate dehydrogenase, carbamate kinase, catalase, superoxide dismutase, soluble phenol modulin β1/β2, and poly (glycerol-

phosphate) α-glucosyltransferase). In the case of 983EVs, the proteins found are antiholin-like protein LrgA, N-acetylmuramoyl-L-alanine amidase, antibacterial protein 2, and siphovirus Gp157 [51].

To our knowledge, this is the first report about proteomics in *S. epidermis* EVs. Surprisingly, EVs composition analysis reveals that some proteins detected in *S. epidermidis* EVs have also been reported in *S. aureus* EVs, highlighting the idea that some proteins are shared in the EVs of these two staphylococcal species. In 983EVs, we detected proteins such as the N-acetylmuramoyl-L-alanine amidase protein [4,52], ATP synthase subunit β [4,52], pyruvate dehydrogenase E1 component subunit α [4, 42], dihydro-lipoyl lysine- residue acetyltransferase component of pyruvate dehydrogenase complex, 30S ribosomal protein S3, and 30S ribosomal protein S4 [4]. In ATCCC12228EVs, we found proteins such as the penicillin-binding protein 2 [4,42,52], ATP synthase subunit β [4,52], glutamine synthetase [52], pyruvate kinase, DNA-directed RNA polymerase sub-unit β [52], 30S ribosomal protein S7, 50S ribosomal protein L5, 50S ribosomal protein L1 [4], catalase (560), enolase [52], phenol-soluble modulin β 1, superoxide dismutase [52], HTH-type transcriptional regulator rot [40], glycosyltransferase, 60 kDa chaperonin, and glutamate dehydrogenase [52].

Bacterial EVs as Therapeutics

Therapy with bacterial EVs can be an alternative because of its low cost, easy isolation and handling, and provides immunomodulatory properties. In addition, bacterial EVs can be concentrated in large quantities and stored for long periods.

One of the therapeutic approaches for EVs is vaccination, as they have multiple antigens simultaneously in a native state to generate an effective immune response. For example, EVs from *S. aureus*, *Streptococcus pneumoniae*, *Clostridium perfringens*, and *Bacillus anthracis* have been successfully used as vaccines or as vaccine adjuvants [53-55].

Immunization with EVs from *S. aureus* shows the induction of specific antibodies and a T-cell response, which confers protection against *S. aureus* infection in mice [56]. It has also been reported that EVs from *S. aureus* coated with indocyanine green-labeled mesoporous silica nanoparticles protect against drug-resistant *S. aureus* infection, which is an effective strategy against this infection [57].

Another possible application of EVs is as drug delivery vehicles, as EVs can protect their interior contents from degradation making delivery functional and suitable for target cells. During biogenesis of the EVs, compounds of interest can be encapsulated in the cells treated with the compound of interest, or the purified EVs can be loaded in vitro with the compound through electroporation.

Another use of bacterial EVs can be for the diagnosis. For example, EVs isolated from urine can be sequenced the 16S rRNA gene to characterize the bacteria present and identify them. Because EVs can travel to different parts of the body, they are important as an accessible diagnostic tool on body fluids.

In a study published by us, we demonstrated that the EVs of *S. epidermidis* ATCC12228 applied to psoriasis can improve this disease. In contrast to the pathogenic *S. aureus*, *S. epidermidis* is an active inhabitant of the skin microbiome [58] with critical roles as a regulator of the skin immune response [59], as it can inhibit the establishment of pathogenic bacteria in the skin [60]. On the injured skin of subjects with psoriasis, *S. epidermidis* is under-represented, indicating that its absence may have a role in the development of psoriasis [61]; however, studies on the role of *S. epidermidis* in psoriasis are scarce. Psoriasis is the most common chronic inflammatory skin disease, characterized by epidermal hyperplasia (acanthosis) due to hyperproliferation and impaired differentiation of keratinocytes, scaling, and erythematous plaque formation, eventually resulting in loss of the protective skin barrier [62]. Psoriasis pathogeny depends on the inflammatory environment produced by the innate/adaptive cells and the skin inflammatory resident cells. In psoriasis, activated resident immune cells and keratinocytes can produce

cytokines that initiate inflammatory processes such as IL-36, IL-23, and IL-22. Later, the environment induces the production of IL-17, TNF-α, IL-6, IL-8, and VEGF-A. These cytokines can exacerbate inflammation by recruiting T cells and neutrophils into the dermis and the epidermis [63]. As *S. epidermidis* participates in regulating the immune response of healthy skin, we hypothesized that *S. epidermis* EVs may regulate the immunological environment of psoriasis. The results found that in the murine psoriasis model, the topical imiquimod (IMQ) treatment in the ears in-duce redness, skin thickness, and scaling with similar signs as occurring in the human psoriasis phenotype. EVs from both origins decreased the characteristic psoriatic phenotype observed in mice treated only with IMQ, with reduced cellular infiltrate and epidermal thickness. However, the treatment with ATCC12228EVs showed a higher amelioration, reducing the degree of redness, epidermal thickness, and scaling than in the skin-like-psoriasis treated with 983EVs. The recruitment of neutrophils as Gr1+ cells was evaluated in the ears of mice as an indication of the inflammation progress, as typically seen in psoriatic lesions. The IMQ-induced psoriasis ears of mice treated with ATCC12228EVs showed a significant decrease in the percentage of neutrophils, such as that found in healthy skin, an observation not found in those mice treated with 983EVs [51].

On the other hand, the ATCC12228EVs treatment decreased the mRNAs skin expression of VEGF-A, IL-6, KC, IL-17F, IL-23, and the IL-36 family members IL-36γ and IL-36R, compared to the IMQ-induced psoriasis skin mice with no EV treatment. In addition, IL-36α mRNA was expressed in the IMQ control group, and IL-36β was induced. In contrast, 983EVs treatment only significantly decreased IL-6, IL-17F, IL-36γ, and IL-36R, while the cytokines IL-36α and IL-36β were overexpressed compared to the only IMQ-induced psoriasis skin. Furthermore, ATCC12228EVs did not significantly reduce the Foxp3 regulator expression but increased the IL-36 antagonist, the IL-36Ra, compared to the IMQ control group. In opposition, the 983EVs significantly inhibited Foxp3. In general, ATCC12228EVs reduced the IL-23, VEGF-A, KC, IL-17F, IL-

6, and non-significantly stimulated IL-36-α, -γ, and -R expression compared to 983EV-treated mice [51].

Conclusions

Staphylococcal EVs are important players in bacterial virulence, host immunomodulation, communication with other cells, survival, and biofilm. However, more information is still required about the biogenesis, composition, and effect of the EVs of staphylococci to be able to manipulate and use them as vehicles for the treatment of infections by this group of bacteria. Furthermore, more studies are needed on the other species of staphylococci since only *S. aureus*, and *S. epidermidis* have been documented.

References

1. Ulhuq FR, Gomes MC, Duggan GM, Guo M, Mendonca C, et al. A membrane-depolarizing toxin substrate of the Staphylococcus aureus type VII secretion system mediates intraspecies competition. Proc. Natl. Acad. Sci. USA. 2020; 117: 20836–20847.
2. Briaud P, Carroll RK. Extracellular vesicle biogenesis and functions in gram-positive bacteria. Infect. Immun. 2020; 88: 12.
3. Mashburn-Warren LM, Whiteley M. Special delivery: Vesicle trafficking in prokaryotes. Mol. Microbiol. 2006; 61: 839–846.
4. Lee EY, Choi DY, Kim DK, Kim JW, Park JO, et al. Gram-positive bacteria produce membrane vesicles: Proteomics-based characterization of Staphylococcus aureus-derived membrane vesicles. Proteomics. 2009; 9: 5425–5436.
5. Hong SW, Kim MR, Lee EY, Kim JH, Kim YS, et al. Extracellular vesicles derived from Staphylococcus aureus induce atopic dermatitis-like skin inflammation. Allergy. 2011; 66: 351–359.
6. Schooling SR, Hubley A, Beveridge TJ. Interactions of DNA with biofilm-derived membrane vesicles. J. Bacteriol. 2009; 191: 4097–4102.

7. Manning AJ, Kuehn MJ. Contribution of bacterial outer membrane vesicles to innate bacterial defense. BMC Microbiol. 2011; 11: 258.
8. Mashburn LM, Whiteley M. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. Nat. Cell Biol. 2005; 437: 422–425.
9. Perez-Cruz C, Carrion O, Delgado L, Martinez G, Lopez-Iglesias C, et al. New type of outer membrane vesicle produced by the Gram-negative bacterium Shewanella vesiculosa M7T: Implications for DNA content. Appl. Environ. Microbiol. 2013; 79: 1874–1881.
10. Rivera J, Cordero RJ, Nakouzi AS, Frases S, Nicola A, et al. Bacillus anthracis produces membrane-derived vesicles containing biologically active toxins. Proc. Natl. Acad. Sci. USA. 2010; 107: 19002–19007.
11. Ciofu O, Beveridge TJ, Kadurugamuwa J, Walther-Rasmussen J, Hoiby N. Chromosomal beta-lactamase is packaged into membrane vesicles and secreted from Pseudomonas aeruginosa. J. Antimicrob. Chemother. 2000; 45: 9–13.
12. Costantini PE, Vanpouille C, Firrincieli A, Cappelletti M, Margolis L, et al. Extracellular Vesicles Generated by Gram-Positive Bacteria Protect Human Tissues Ex Vivo From HIV-1 Infection. Front. Cell Infect. Microbiol. 2022; 11: 822882.
13. Yerneni SS, Werner S, Azambuja JH, Ludwig N, Eutsey R, et al. Pneumococcal extracellula vesicles modulate host immunity. mBio. 2021; 12: e0165721.
14. Liu Y, Defourny KAY, Smid EJ, Abee T. Gram-positive bacterial extracellular vesicles and their impact on health and disease. Front. Microbiol. 2018; 9: 1502.
15. Lee EY, Bang JY, Park GW, Choi DS, Kang JS, et al. Global proteomic profiling of native outer membrane vesicles derived from Escherichia coli. Proteomics. 2007; 7: 3143–3153.
16. Briaud P, Carroll RK. Extracellular Vesicle Biogenesis and Functions in Gram-Positive Bacteria. Infect. Immun. 2020; 88: 12-25.

17. Stubbendieck RM, Vargas-Bautista C, Straight PD. Bacterial Communities: Interactions to Scale. Front. Microbiol. 2016; 7: 1234.
18. Lee J, Lee EY, Kim SH, Kim DK, Park KS, et al. Staphylococcus aureus extracellular vesicles carry biologically active beta-lactamase. Antimicrob. Agents Chemother. 2013; 57: 2589–2595.
19. Schooling SR, Beveridge TJ. Membrane vesicles: an overlooked component of the matrices of biofilms. J. Bacteriol. 2006; 188: 5945–5957.
20. Ciofu O, Beveridge TJ, Kadurugamuwa J, Walther-Rasmussen J, Hoiby N. Chromosomal beta-lactamase is packaged into membrane vesicles and secreted from Pseudomonas aeruginosa. J. Antimicrob. Chemother. 2000; 45: 9–13.
21. Bomberger JM, Maceachran DP, Coutermarsh BA, Ye S, O'Toole GA, et al. Long-distance delivery of bacterial virulence factors by Pseudomona aeruginosa outer membrane vesicles. PLoS Pathog. 2009; 5: e1000382.
22. Mashburn LM, Whiteley M. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. Nature. 2005; 437: 422–425.
23. Alves NJ, Turner KB, Medintz IL, Walper SA. Protecting enzymatic function through directed packaging into bacterial outer membrane vesicles. Sci. Rep. 2016; 6: 24866.
24. Schaar V, Paulsson M, Morgelin M, Riesbeck K. Outer membrane vesicles shield Moraxella catarrhalis beta-lactamase from neutralization by serum IgG. J. Antimicrob. Chemother. 2013; 68: 593–600.
25. Rumbo C, Fernandez-Moreira E, Merino M, Poza M, Mendez JA, et al. Horizontal transfer of the OXA-24 carbapenemase gene via outer membrane vesicles: a new mechanism of dissemination of carbapenem resistance genes in Acinetobacter baumannii. Antimicrob. Agents. Chemother. 2011; 55: 3084–3090.
26. Brown L, Kessler A, Cabezas-Sanchez P, Luque-Garcia JL, Casadevall A. Extracellular vesicles produced by the Gram-positive bacterium Bacillus subtilis are disrupted by the lipopeptide surfactin. Mol. Microbiol. 2014; 93: 183–198.

27. Schaar V, de Vries SP, Perez Vidakovics ML, Bootsma HJ, Larsson L, et al. Multicomponent Moraxella catarrhalis outer membrane vesicles induce an inflammatory response and are internalized by human epithelial cells. Cell Microbiol. 2011; 13: 432–449.
28. van Bergenhenegouwen J, Kraneveld AD, Rutten L, Kettelarij N, Garssen J, et al. Extracellular vesicles modulate host-microbe responses by altering TLR2 activity and phagocytosis. PLoS ONE. 2014; 9: e89121.
29. Prados-Rosales R, Baena A, Martinez LR, Luque-Garcia J, Kalscheuer R, et al. Mycobacteria release active membrane vesicles that modulate immune responses in a TLR2-dependent manner in mice. J. Clin. Invest. 2011; 121: 1471–1483.
30. Kunsmann L, Ruter C, Bauwens A, Greune L, Gluder M, et al. Virulence from vesicles: Novel mechanisms of host cell injury by Escherichia coli O104:H4 outbreak strain. Sci. Rep. 2015; 5: 13252.
31. Jiang Y, Kong Q, Roland KL, Curtiss R. Membrane vesicles of Clostridium perfringens type A strains induce innate and adaptive immunity. Int. J. Med. Microbiol. 2014; 304: 431–443.
32. Kang CS, Ban M, Choi EJ, Moon HG, Jeon JS, et al. Extracellular vesicles derived from gut microbiota, especially Akkermansia muciniphila, protect the progression of dextran sulfate sodium-induced colitis. PLoS ONE. 2013; 8: e76520.
33. Acevedo R, Fernandez S, Zayas C, Acosta A, Sarmiento ME, et al. Bacterial outer membrane vesicles and vaccine applications. Front. Immunol. 2014; 5: 121.
34. Shen Y, Giardino Torchia ML, Lawson GW, Karp CL, Ashwell JD, et al. Outer membrane vesicles of a human commensal mediate immune regulation and disease protection. Cell Host Microbe. 2012; 12: 509–220.
35. Kim JH, Jeun EJ, Hong CP, Kim SH, Jang MS, et al. Extracellular vesicle-derived protein from Bifidobacterium longum alleviates food allergy through mast cell suppression. J. Allergy Clin. Immunol. 2016; 137: 507–516.
36. Fabrega MJ, Aguilera L, Gimenez R, Varela E, Alexandra Canas M, et al. Activation of immune and defense responses

in the intestinal mucosa by outer membrane vesicles of commensal and probiotic Escherichia coli strains. Front. Microbiol. 2016; 7: 705.
37. Behzadi E, Mahmoodzadeh Hosseini H, Imani Fooladi AA. The inhibitory impacts of Lactobacillus rhamnosus GG-derived extracellular vesicles on the growth of hepatic cancer cells. Microb. Pathog. 2017; 110: 1–6.
38. Lee J, Lee EY, Kim SH, Kim DK, Park KS, et al. Staphylococcus aureus extra-cellular vesicles carry biologically active beta-lactamase. Antimicrob. Agents Chemother. 2013; 57: 2589–2595.
39. Askarian F, Lapek JD Jr, Dongre M, Tsai CM, Kumaraswamy M, et al. Staphylococcus aureus membrane-derived vesicles promote bacterial virulence and confer protective immunity in murine infection models. Front. Microbiol. 2018; 9: 262.
40. Jun SH, Lee JH, Kim SI, Choi CW, Park TI, et al. Staphylococcus aureus-derived membrane vesicles exacerbate skin inflammation in atopic dermatitis. Clin. Exp. Allergy. 2017; 47: 85–96.
41. Gurung M, Moon DC, Choi CW, Lee JH, Bae YC, et al. Staphylococcus aureus produces membrane-derived vesicles that induce host cell death. PLoS ONE. 2011; 6: e27958.
42. Thay B, Wai SN, Oscarsson J. Staphylococcus aureus alpha-toxin-dependent induction of host cell death by mem-brane-derived vesicles. PLoS ONE. 2013; 8: e54661.
43. Chebotar I, Konchakova ED, Maianskii AN. Vesicle formation as a result of interaction between polymorphonuclear neutrophils and Staphylococcus aureus biofilm. J. Med. Microbiol. 2013; 62: 1153–1159.
44. Deng S, Wang Y, Liu S, Chen T, Hu Y, et al. Extracellular vesicles: A potential biomarker for quick identification of infectious osteomyelitis. Front. Cell. Infect. Microbiol. 2020; 10: 323.
45. Tartaglia NR, Breyne K, Meyer E, Cauty C, Jardin J, et al. Staphylococcus aureus extracellular vesicles elicit an immunostimulatory response in vivo on the murine mammary gland. Front. Cell Infect. Microbiol. 2018; 8: 277.
46. Hong SW, Kim MR, Lee EY, Kim JH, Kim YS, Jeon SG, et al. Extracellular vesicles derived from Staphylococcus

aureus induce atopic dermatitis-like skin inflammation. Allergy. 2011; 66: 351–359.
47. Choi EJ, Lee HG, Bae IH, Kim W, Park J, et al. Propionibacterium acnes-derived extracellular vesicles promote acne-like phenotypes in human epidermis. J. Invest. Dermatol. 2018; 138: 1371–1379.
48. Zaborowska M, Flores CT, Vazirisani F, Shah FA, Thomsen P, et al. Extracellular vesicles influence the growth and adhesion of staphylococcus epidermidis under antimicrobial selective pressure. Front. Microbiol. 2020; 11: 1132.
49. Zaborowska M, Vazirisani F, Shah FA, Firdaus R, Omar O, et al. Immunomodulatory effects exerted by extracellular vesicles from Staphylococcus epidermidis and Staphylococcus aureus isolated from bone-anchored prostheses. Biomaterials. 2021; 278: 121158.
50. Gómez-Chávez F, Cedillo-Peláez C, Zapi-Colín LA, Gutiérrez-González G, Martínez-Torres I, et al. The extracellular vesicles from the commensal Staphylococcus epidermidis ATCC12228 strain regulate skin inflammation in the imiquimod-induced psoriasis murine model. Int. J. Mol. Sci. 2021; 22: 13029.
51. Schlatterer K, Beck C, Hanzelmann D, Lebtig M, Fehrenbacher B, et al. The mechanism behind bacterial lipoprotein release: Phenol-soluble modulins mediate toll-like receptor 2 activation via extracellular vesicle release from Staphylococcus aureus. mBio. 2018; 9: e01851-18.
52. Fernandez S, Fajardo EM, Mandiarote A, Ano G, Padron MA, et al. A proteoliposome formulation derived from Bordetella pertussis induces protection in two murine challenge models. BMC Immunol. 2013; 14: S8.
53. Holst J, Martin D, Arnold R, Huergo CC, Oster P, et al. Properties and clinical performance of vaccines containing outer membrane vesicles from Neisseria meningitidis. Vaccine. 2009; 27: B3–12.
54. Martinon-Torres F, Safadi MAP, Martinez AC, Marquez PI, Torres JCT, et al. Reduced schedules of 4CMenB vaccine in infants and catch-up series in children: Immunogenicity and safety results from a randomised open-label phase 3b trial. Vaccine. 2017; 35: 3548–3557.

55. Choi SJ, Kim MH, Jeon J, Kim OY, Choi Y, et al. Active immunization with extracellular vesicles derived from Staphylococcus aureus effectively protects against Staphylococcal lung infections, mainly via Th1 cell-mediated immunity. PLoS ONE. 2015; 10: e0136021.
56. Chen G, Bai Y, Li Z, Wang F, Fan X, et al. Bacterial extracellular vesicle-coated multi-antigenic nanovaccines protect against drug-resistant Staphylococcus aureus infection by modulating antigen processing and presentation pathways. Theranostics. 2020; 10: 7131–7149.
57. Wang Y, Kuo S, Shu M, Yu J, Huang S, et al. Staphylococcus epidermidis in the human skin microbiome mediates fermentation to inhibit the growth of Propionibacterium acnes: Implications of probiotics in acne vulgaris. Appl. Microbiol. Biotechnol. 2013; 98: 411–424.
58. Leech JM, Dhariwala MO, Lowe MM, Chu K, Merana GR, et al. Toxin-triggered interleukin-1 receptor signaling enables early-life discrimination of pathogenic versus commensal skin bacteria. Cell Host Microbe. 2019; 26: 795–809.e5.
59. Xia X, Li Z, Liu K, Wu Y, Jiang D, et al. Staphylococcal LTA-induced miR-143 inhibits Propionibacterium acnes-mediated inflammatory response in skin. J. Investig. Dermatol. 2016; 136: 621–630.
60. Chang HW, Yan D, Singh R, Liu J, Lu X, et al. Alteration of the cutaneous microbiome in psoriasis and potential role in Th17 polarization. Microbiome. 2018; 6: 1–27.
61. Lowes MA, Bowcock AM, Krueger JG. Pathogenesis and therapy of psoriasis. Nat. Cell Biol. 2007; 445: 866–873.
62. Nadeem A, Ahmad SF, Al-Harbi NO, El-Sherbeeny AM, Alasmari AF, et al. Bruton's tyrosine kinase inhibitor suppresses imiquimod-induced psoriasis-like in-flammation in mice through regulation of IL-23/IL-17A in innate immune cells. Int. Immunopharmacol. 2020; 80: 106215.