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The role of wall teichoic acids in the host immune response to *Staphylococcus epidermidis*

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Dissertation for the Degree of Master in Bioengineering

Integrated Masters in Bioengineering, Molecular Biotechnology

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November 1, 2020

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This study was supported by the Portuguese Foundation for Science and Technology (FCT) by the funded project PTDC/BIA-MOL/29553/2017, under the scope of COMPETE2020 (POCI-01-0145-FEDER-029553)

Resumo

Staphylococcus epidermidis é um organismo comensal da microbiota humana. No entanto, esta bactéria é uma das principais causas de infeções nosocomiais devido à sua capacidade de formar biofilmes na superfície de dispositivos médicos. Estas infeções são geralmente persistentes e difíceis de tratar. Os ácidos teicóicos da parede (WTAs) são glicopolímeros importantes da parede celular *de S. epidermidis,* associados à virulência bacteriana. No entanto, a sua interação com o sistema imunológico do hospedeiro é pouco conhecida.

Este trabalho teve por objetivo contribuir para o conhecimento sobre a forma como os WTAs influenciam a resposta imunitária do hospedeiro a *S. epidermidis.* Foi então estudada uma estirpe de *S. epidermidis* deficiente em WTAs, construída através da deleção do gene *tagO,* e a sua interação com células do sistema imunitário.

A estirpe mutante evidenciou alterações no tamanho das células bacterianas, uma provável reestruturação da parede celular e defeitos no septo, afetando o crescimento da bactéria. Também mostrou uma capacidade reduzida de formar biofilmes. Comparativamente à respetiva estirpe selvagem, o mutante deficiente em WTAs foi menos fagocitado e induziu uma menor secreção de citocinas pró- e anti-inflamatórias por macrófagos de murganho e humanos. No entanto, não foram detetadas diferenças significativas entre as duas estirpes bacterianas na indução da produção de citocinas por células dendríticas (DCs) humanas nem na indução da expressão de marcadores indicativos de maturação na superfície das DCs de murganho e humanas. Por outro lado, foram produzidos níveis mais elevados de citocinas pró- e anti-inflamatórias por DCs de murganho, após infeção com a estirpe mutante.

Estes resultados indicaram que os WTAs são um constituinte importante da parede celular de *S. epidermidis,* com impacto na sua estrutura, e na morfologia e proliferação desta bactéria, afetando ainda a sua capacidade de formar biofilmes. Os WTAs parecem estar também envolvidos na modulação da fagocitose por macrófagos e da secreção de citocinas por DCs de murganho. Parecem ter também impacto na produção de citocinas por macrófagos humanos.

Embora preliminares, estes resultados sugerem um papel dos WTAs na resposta imunitária do hospedeiro a *S. epidermidis,* assim como um papel estrutural nesta bactéria, tornando pertinente a continuação deste trabalho, de forma a melhor esclarecer os mecanismos subjacentes aos papéis dos WTAs aqui sugeridos.

Abstract

Staphylococcus epidermidis is a commensal organism of the human microbiota. However, this bacterium is one of the main causes of nosocomial infections due to its capacity to form biofilms on the surface of indwelling medical devices. These infections are usually persistent and difficult to treat. Wall teichoic acids (WTAs) are important glycopolymers of *S. epidermidis* cell wall, associated with bacterial virulence. Yet, their interaction with the host immune system remains to be characterized.

The aim of this work was to determine whether WTAs could modulate the host immune response to *S. epidermidis*. Herein, a WTA-deficient *S. epidermidis* strain, built through the deletion of the *tagO* gene, was studied and its interaction with the host immune system was evaluated.

The mutant strain showed alterations in bacterial cell size, a probable restructuring of the cell wall and septum defects, with impairment of bacterial growth. It also showed impaired ability to form biofilms. Comparatively to the wild type (WT) strain, the WTA-deficient mutant was less phagocytosed and induced a lower production of pro- and anti-inflammatory cytokines by mouse and human macrophages. However, no significant differences were detected between the mutant and WT strains in the induced cytokine production by human dendritic cells (DCs) and expression of maturation markers on the surface of human and murine DCs. In contrast, higher levels of pro- and anti-inflammatory cytokines were produced by mouse DCs, after infection with *S. epidermidis* mutant strain.

Altogether these results indicated that WTAs are an important constituent of *S. epidermidis* cell wall, with relevant functions in its structure and in bacterial cell morphology and proliferation, as well as in its ability to form biofilms. Wall teichoic acids appear to be involved in modulation of phagocytosis by mouse macrophages and cytokine secretion by mouse DCs. In addition, they seem to have an impact in cytokine production by human macrophages.

Although preliminary, these results provided insights concerning the role of WTAs in the host immune response to *S. epidermidis*, as well as its structural part in this bacterium. This work highlights the relevance of further studies that could contribute for a better understanding on the mechanisms underlying the roles of WTAs in *S. epidermidis* physiology and interaction with the host immune system.

Acknowledgments

From mentors, coworkers, friends, and family, all these people were extremely important to me and crucial for me to achieve all I have been able to. Therefore, I would like to thank:

Primarily to Professor Manuel Vilanova for his mentoring, for always believing in me, and for his constant motivation towards the project, even when things seemed to go wrong. I also want to thank him for everything that he taught me, not only when it comes to immunology and infection, but also about problem-solving, perseverance, and determination. But above all, I want to thank him for welcoming me in his group, with open arms, both times I asked him. Thanks to him, I had the opportunity to learn throughout my academic path and to successfully finish it.

Next, to Ângela França and Alexandra Correia for their guidance and for the care they demonstrated every time a doubt or problem arose. Thank you for always being ready to help me, in any way possible.

To Tânia Lima, for all the guidance and support over these months, always allied with a good mood. Thank you for teaching me everything you have taught me. Without a doubt, I was able to learn a lot from you. Thank you for all the patience you had and for always being available to help in whatever I needed. Undoubtedly, you were indispensable for the progress of this project and for the conclusion of my dissertation.

To Carina Duarte for being a part of this project with me. I entered the project after her, so her help in the beginning was crucial. Working alongside her was great and, eventually, I gained a friend.

To the rest of the members of the Immunobiology Group at i3s, particularly Anna Clara, Elena Pérez, Margarida Silva, Mariana Resende, Rita Bastos and Rita Pedro. Thank you for being so friendly and for your willingness to help, in any way.

To all my friends, the ones I know since I was a kid and the ones I met in college. All play an important part in my life and have contributed, in some way, for me to get here. A special thank you to Beatriz Moura, Francisca Cardoso, and Joana Sousa for supporting me and for always being there, regardless. They made this journey so fun and worthwhile and I couldn't have asked for better people to have by my side.

And last but not least, to all my family members, for being supportive, believing in me, and trusting that I would succeed, not only on my dissertation but in everything throughout the years. Particularly, I would like to thank my parents, Laurinda Soares and Carlos Soares, for all those reasons, for never doubting my capacities, even when I do, for raising me, and for giving me everything I could ever ask for. To finish, I would like to thank my sister, Sara Soares, again for the reasons already mentioned and for being kind of a role model to me, for never giving up pursuing what she believes and loves to do.

Andreia Silva Soares

"We keep moving forward, opening new doors, and doing new things, because we're curious and curiosity keeps leading us down new paths"

Walt Disney

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Abbreviations

ABS	ATP-binding cassette
AIDS	Acquired immunodeficiency syndrome
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
APC	Antigen presenting cell
BMDC	Bone marrow derived dendritic cells
BSA	Bovine serum albumin
CFU	Colony forming unit
Cm	Chloramphenicol
CNS	Central nervous system
CoNS	Coagulase-negative *Staphylococci*
CoPS	Coagulase-positive *Staphylococci*
CWA	Cell-wall anchored
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRI	Device-related infection
EDTA	Ethylenadiaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting (or flow cytometry)
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate

GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBSS	Hanks' Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMD	Implanted medical device
KO	Knockout
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M-CSF	Macrophage colony-stimulating factor
MDM	Monocyte-derived macrophages
MDDC	Monocyte-derived dendritic cells
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
OD	Optic density
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PG	Peptidoglycan
PGA	Poly-γ-glutamic acid
PI	Propidium iodide
PIA	Polysaccharide intercellular adhesin (also named PNAG)
PNAG	Poly-N-acetyl-glucosamine (also named PIA)
PRR	Pattern recognition receptor

PSM	Phenol-soluble modulin
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SD	Standard deviation
TA	Teichoic acid
TB	Trypan blue
TCR	T-cell receptor
TEM	Transmission electron microscopy
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TSA	Tryptic soy agar
TSB	Tryptic soy broth
WT	Wild type
WTA	Wall teichoic acid

Chapter 1

Introduction

1. *Staphylococci* and related infections: characterization and clinical significance

Staphylococci belong to the vast and heterogeneous group of bacteria, which are unicellular and prokaryotic microorganisms. The designation *Staphylococcus* was first presented in 1882 by W. Ogston, but the species belonging to this genus were only labelled as virulent and pathogenic in 1884 by Rosenbach. The genus *Staphylococcus* is included in the *Staphylococcaceae* family, in the order *Bacillales* and, up to 2014, 47 species and 23 subspecies had already been portrayed as members of this genus (Becker, Heilmann, & Peters, 2014).

Staphylococci are Gram-positive bacteria, characterized by spheric (cocci), nonmotile and non-spore forming cells arranged in grape-like clusters. These bacteria present a diameter comprehended between 0.5 μm and 1.5 μm, produce catalase and are facultatively anaerobic (França & Cerca, 2012). *Staphylococci* have been identified in the air, dust, soil, food and water. Moreover, they are ubiquitous commensals that reside the skin and mucosa of humans and other animals, in which some have shown a preference to inhabit particular niches (Huebner & Goldmann, 1999; Widerström, Wiström, Sjöstedt, & Monsen, 2012).

In 1940, R. W. Fairbrother divided *Staphylococcus* spp. in two main groups, coagulase-negative *Staphylococci* (CoNS) and coagulase-positive *Staphylococci* (CoPS), according to their ability to produce coagulase, a blood-clotting enzyme (Becker et al., 2014; França & Cerca, 2012). *Staphylococcus aureus* is the most representative species of CoPS. The CoNS comprise distinct species and subspecies, whose recognition and characterization can be demanding. Until 2014, of the 47 *Staphylococcus* species identified, 38 fit in the CoNS group (Becker et al., 2014), although only some were found in humans (Widerström et al., 2012), where *S. epidermidis* is the most commonly isolated species (Becker et al., 2014). Species of CoNS present differing phenotypes, morphology, biochemistry and susceptibility (Rupp & Archer, 1994).

Coagulase-negative *Staphylococci* are commensal microorganisms normally present in the human microbiota since an early phase in life. Initially, CoNS were considered apathogenic, however, this changed throughout the years (Huebner & Goldmann, 1999). In the late 1950s CoNS pathogenicity was primarily demonstrated, as the cause of endocarditis and septicemia. Between the 1960s and the 1970s their clinical relevance was evidenced, once they were shown to originate bacteremia, osteomyelitis, several medical device-associated infections, among others (Widerström et al., 2012). Coagulase-negative *Staphylococci* greatly affect immunocompromised patients and premature newborns. These opportunistic bacteria are capable of infecting, both locally and systemically, patients that have received an indwelling medical device, thus representing a major cause of nosocomial infections related with implanted medical devices (França & Cerca, 2012).

Firstly, CoNS infections were presumed to emerge from commensal species; nevertheless, it has been proposed that most originate from nosocomial strains. Since the discrimination between

commensal and pathogenic CoNS can be challenging, the acknowledgment of infection may be too. The pathogenicity of CoNS and clinical manifestations raised by these bacteria vary among species and strains, according to the virulence factors and protection mechanisms they carry. Unlike *S. aureus,* which has a broad and defined set of virulence factors, CoNS species do not, making these bacteria usually responsible for the development of either subacute or chronic infections (Becker et al., 2014; von Eiff, Peters, & Heilmann, 2002). Furthermore, in device-related infections (DRI), the clinical significance diverges depending on the type of medical device used, the place of implantation and the state of health of the patient (von Eiff et al., 2002). In this sort of infections, CoNS have displayed their propensity to adhere to the medical device and further form a biofilm, which enhances their protection from the host immune response and antibiotics. Following this, they have also proved their capacity to spread throughout the human body and colonize other native tissues. These features highly contribute to the virulence and pathogenicity of CoNS and hamper infection treatment, causing a negative impact on social, economic and medical fields (Becker et al., 2014; Sabaté Brescó et al., 2017).

2. *Staphylococcus epidermidis* species

S. epidermidis was discovered in 1884 by F. J. Rosenbach and was firstly denominated as *Staphylococcus albus*, until later when it was classified with the currently known name *S. epidermidis* (Rupp & Archer, 1994).

As a *Staphylococcus* species, *S. epidermidis* is a non-motile and non-sporulating Gram-positive coccus that forms grape-like clusters. This bacterium originates colonies characterized by a round, smooth, opaque, shiny and elevated morphology, with a white color and a variable diameter, when growing on tryptic soy agar. In terms of metabolism, it is facultative anaerobe, hence displaying aerobic respiration or fermentation. This organism is capable of producing catalase but not coagulase, thus belonging to the CoNS group (França & Cerca, 2012).

S. epidermidis is a ubiquitous commensal microorganism, typically encountered in the normal human skin microbiota. In fact, *S. epidermidis* is the CoNS species most frequently isolated from humans (França & Cerca, 2012), covering from 65% to 90% of the isolated *Staphylococci*. It was estimated that up to 24 strains of this bacterium are able to reside in healthy patients (Huebner & Goldmann, 1999).

Yet, *S. epidermidis* is an opportunistic pathogen that may cause infections in humans and is the most prevalent isolated species in hospital-acquired infections. *S. epidermidis* has been often linked with nosocomial infections originating from indwelling medical devices utilization, which mainly result from the establishment of biofilms in such devices, given that *S. epidermidis* is a biofilm-forming pathogen (McCann, Gilmore, & Gorman, 2008).

The infections caused by this pathogen are usually defined by a subacute or chronic character and may be relapsing. In addition, they can be quite difficult to prevent or treat (Otto, 2009; Sabaté Brescó et al., 2017). These intrinsic characteristics of *S. epidermidis* infections are the result of multiple features and mechanisms identified in this bacterium that will be addressed below.

2.1. *S. epidermidis* as a commensal organism

S. epidermidis is a common commensal organism, normally inhabiting the human skin and mucous membranes. This bacterium can be found *in utero*, in the amniotic fluid and later in the first feces and in the newborn skin. It afterwards becomes a resident organism of the human skin, largely found

in moist areas such as nares, groin, fossae, toe webs and armpits; sebaceous areas, as for instance the facial skin; and mucosal tissues, for example the conjunctiva and the gastrointestinal and lower reproductive tracts (Nguyen, Park, & Otto, 2017; Sabaté Brescó et al., 2017). *S. epidermidis* gathers various mechanisms that enable this organism to reside and survive in these sites. It has surface adhesins to adhere to the host tissue, detects signaling molecules and host antimicrobial peptides (AMPs), acting against the latter, and circumvents desiccation and osmotic stress (Sabaté Brescó et al., 2017).

This bacterium has the ability to maintain an innocuous or even beneficial relationship with the human host (Nguyen et al., 2017). As an important member of the commensal microbiota, this bacterium is able to contribute to the preservation of healthy conditions (Otto, 2012). In this regard, *S. epidermidis* supports nutrition, instructs the immune system and outcompetes potentially harmful microorganisms, such as *S. aureus*. The latest may be achieved by interfering with the viability or colonization of these microorganisms through i) production of phenol-soluble modulins (PSMs), which are amphipathic, α-helical, multifunctional peptides that may present proinflammatory or cytolytic functions; ii) generation of bacteriocins, which are antimicrobial peptides; iii) production of metabolic products; and iv) degradation of other microorganisms biofilms (Otto, 2009; Sabaté Brescó et al., 2017).

2.2. *S. epidermidis* as a pathogen

Despite being beneficial for the normal human microbiota, *S. epidermidis* can switch from a commensal to an opportunistic lifestyle and cause serious infections. As a matter of fact, *S. epidermidis* is considered one of the most important causative agents of nosocomial infections (Otto, 2009; Vuong & Otto, 2002). In order to succeed, it requires a predisposed host, due to, for instance, a compromised epithelial barrier (Nguyen et al., 2017; Vuong & Otto, 2002). As a pathogen, the factors used to live as a commensal, also contribute to the development of infection (Otto, 2009).

S. epidermidis predominantly leads to infections on immunocompromised individuals, such as premature newborns, or patients under immunosuppressive therapy (e.g. cancer or HIV/AIDS treatment (Nguyen et al., 2017)) and immunodeficient patients (França & Cerca, 2012).

Generally, *S. epidermidis*, unlike *S. aureus*, do no causes pyogenic infections in non-compromised patients. This feature is associated with the limited number of tissue-damaging exoenzymes and toxins produced by *S. epidermidis*. These features also explain the subacute or chronic character of *S. epidermidis* infections (Vuong & Otto, 2002).

In healthy patients, this bacterium usually leads to infection through the penetration of epithelial barriers of the human body following, for instance, a trauma or medical device implantation (França & Cerca, 2012). Indeed, this bacterium has emerged as a major cause of nosocomial infections associated with implantation of indwelling medical devices (O'GARA & Humphreys, 2001).

2.3. *S. epidermidis* medical device-associated infections

Biomaterials are widely used in biomedical and clinical applications. These materials must be biocompatible, which translates in the ability to carry out their functions without leading to a damaging host response. Nonetheless, the implantation of biomaterials in patients may result in material rejection and emergence of inflammatory responses and infections (Arciola, Campoccia, Speziale, Montanaro, & Costerton, 2012; Oliveira et al., 2018).

The implantation of medical devices has been increasing over the years. Since medical devices are made up of biomaterials, in order to be successfully implanted, these devices have to tackle the adversities previously enumerated, besides performing its desirable role in the patient. The major

challenge that has been associated with indwelling medical devices is their colonization by bacteria, which may originate infections (Arciola et al., 2012). The infecting pathogens colonizing the device can be already present in the medical device before implantation, due to airborne contamination, or can be transported from the tissue of the implantation site, or from the skin of the medical staff. Multiple aspects, such as health condition, type and duration of the surgery, extended hospitalization, other surgeries or infections and increased utilization of medical devices, predispose to the occurrence of infections related to implanted medical devices (IMDs) (McCann et al., 2008). The clinical manifestations of different infections vary according to the type of medical device and where it is implanted (Longauerova, 2006). The originated infections can even evolve into a chronic state and impair the efficiency of many given treatments (Arciola et al., 2012). Additionally, device dysfunction commonly occurs, implicating its removal and reinsertion (McCann et al., 2008; Scherr, Heim, Morrison, & Kielian, 2014), which is unfeasible for patients in which the medical device is required for their survival (Arciola et al., 2012).

As mentioned above, *S. epidermidis* is frequently responsible for the establishment of nosocomial infections in patients with IMDs. Examples of medical device-associated infections caused by this bacterium comprise: i) prosthetic valve endocarditis after prosthetic valve insertion; ii) eye keratitis and endophthalmitis due to contact lenses implantation; iii) bacteriuria upon urinary catheters inclusion; iv) prosthetic joint-, intravascular catheter- or graft-related infections; v) surgical site infections; and vi) CNS shunt-associated infections (McCann et al., 2008; Otto, 2009).

S. epidermidis is a recognized biofilm-forming pathogen. Biofilms are constituted by multiple and heterogeneous bacterial populations embedded in a self-produced extracellular matrix, with a variable composition of, predominantly, extracellular DNA (eDNA), proteins, and polysaccharides (Scherr et al., 2014). Infections associated with the implantation of medical devices were the first to be recognized as originating due to biofilm development, representing approximately 60% to 70% of nosocomial infections reported in the United States of America (França & Cerca, 2012).

As *S. epidermidis* is a prevalent colonizer of human tissue and has the capacity to rapidly and efficiently adhere to the surface of IMDs, this enables biofilm formation on their surface, promoting infection and its progression (McCann et al., 2008). Following the establishment of the infection, *S. epidermidis* may disseminate into the bloodstream (bacteremia), making this bacterium a leading agent causative of nosocomial sepsis (Nguyen et al., 2017). The cells released from the biofilm can spread from the original site of infection to other niches (McCann et al., 2008). Furthermore, this bacterium has the capacity to form biofilms on native surfaces of the host (Scherr et al., 2014).

The biofilms established by *S. epidermidis* are characterized by different cell mechanisms, when it comes to gene expression and growth rates, comparatively to planktonic cells (Scherr et al., 2014). Biofilm cells exhibit decreased cell division, and cell wall biosynthesis and fermentation processes are favored rather than aerobic ones. Additionally, the extracellular matrix of biofilms represents a mechanical barrier, which is able to diminish the penetration and action of immune cells and molecules, such as cytokines, chemokines, antimicrobial peptides and antibiotics, leading to a decrease in inflammation (McCann et al., 2008; Otto, 2012). To worsen the scenario, not only does the host immune response fail to eliminate the biofilm, but also might harm the surrounding tissues in the process (O'GARA & Humphreys, 2001). All the alterations verified in biofilm cells lead to a less hostile state that more easily evades the host immune system and the action of antibiotics, protecting the bacteria, which has the ability to persist and lead to a chronic infection (McCann et al., 2008; Scherr et al., 2014). In addition, *S. epidermidis* biofilm-released cells were shown to be more resistant to antibiotics than biofilm or planktonic cells, being less efficiently eliminated and then able to create new sites of infection (França et al., 2016).

Apart from biofilm formation, *S. epidermidis* displays multidrug resistance, withstanding the action of multiple antibiotics. Moreover, it has demonstrated an inherent genetic variability, which can result in improved and resistant characteristics, facilitating the adjustment to the circumstances to which they are submitted (McCann et al., 2008).

As emphasized above, biofilm related characteristics and the intrinsic traits of *S. epidermidis* contribute to the endurance and success of this pathogen, hampering the eradication of the infections it originates by colonizing IMDs. Regardless of the fact that these infections hardly lead to life-threatening diseases, they are frequent and difficult to treat, resulting in the establishment of chronic and persistent infections with a recurrent character. Thus, *S. epidermidis* infections pose not only substantial medical and economic burdens but also serious implications on patients' quality of life. Also, these infections have a significant impact on morbidity and, at a less extent, on mortality rates among infected patients (O'GARA & Humphreys, 2001; Otto, 2009), making this pathogen currently regarded a leading cause of morbidity in Europe.

3. Biofilm formation: the major virulence factor of *S. epidermidis*

As mentioned above, the key virulence factor of *S. epidermidis* is this bacterium ability to form biofilms. This process encompasses: i) initial planktonic cell adhesion to a surface, which may occur on either abiotic or biotic surfaces; ii) the subsequent phase of accumulation and maturation that includes intercellular adhesion; and, at last, iii) cell detachment from the biofilm, promoting the dissemination of infection (Figure 1) (França & Cerca, 2012).

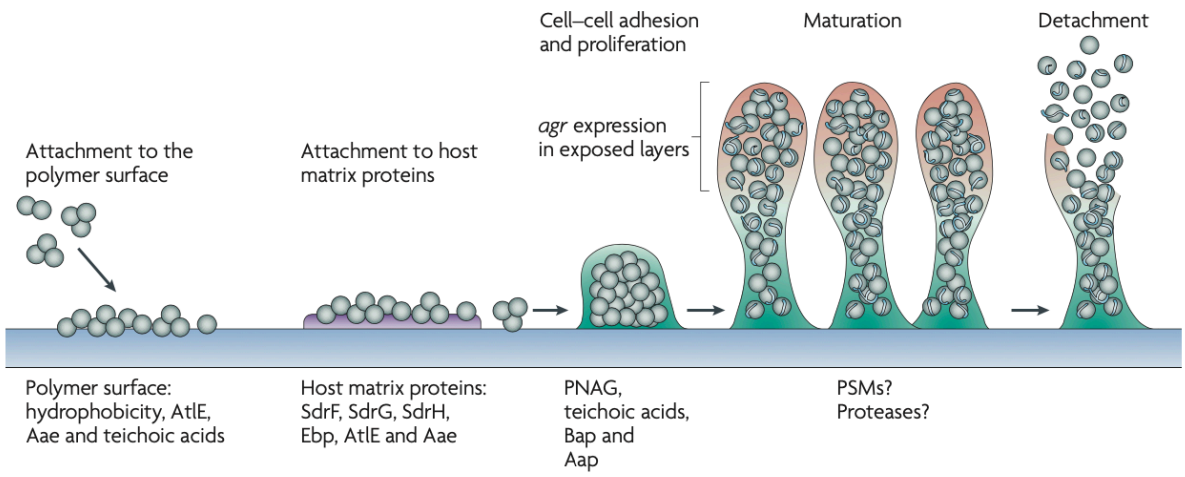


Figure 1 – Stages of biofilm development by *S. epidermidis*. Biofilm formation encompasses the attachment of *S. epidermidis* to an abiotic or biotic surface and subsequent intercellular adhesion and biofilm maturation. Lastly, biofilm cells can detach from the biofilm and spread (Otto, 2009).

Primary attachment

Initial attachment is the first phase that takes place in biofilm formation and is influenced by several factors related to *S. epidermidis* virulence, the host and the characteristics of the implanted device. This adhesion may be carried out on abiotic surfaces, when the bacteria binds directly to the unmodified polymer surface of the implanted devices; or on biotic surfaces, when bacteria adheres to host extracellular matrix proteins coating the surface of IMDs (McCann et al., 2008).

Attachment to abiotic surfaces

The adhesion of planktonic bacterial cells to the uncovered surface of the indwelling medical devices depends on nonspecific physicochemical properties of the bacterial cell surface and the material of which the implanted device is made of (França & Cerca, 2012). These involve electrostatic, hydrophobic and van der Waals interactions, polarity, surface tension, steric effects and temperature (McCann et al., 2008).

In addition, certain *S. epidermidis* proteins have been implicated in the adhesion process. One example are the staphylococcal surface proteins 1 and 2 (SSP-1 and SSP-2) (McCann et al., 2008). Also, the autolysin-adhesin AtlE, a peptidoglycan hydrolase that besides its hydrophobic interactions, is involved in bacterial cells division and lysis. This then contributes to the release of DNA (eDNA), which also appears to be associated with adhesion. Finally, the biofilm associated homolog protein (Bhp) grants an hydrophobic character to the bacterial cell surface (França & Cerca, 2012).

Attachment to biotic surfaces

The adhesion to biotic surfaces might portray a more relevant mechanism (Vuong & Otto, 2002) because, when a medical device is implanted, its surface is immediately coated with extracellular matrix, plasma proteins and coagulation products from the host. These modify the physicochemical properties of the surface and the molecules that will attach to it (França & Cerca, 2012).

In *S. epidermidis*, specific cell-wall anchored (CWA) proteins are involved in binding the bacteria to these molecules, supporting primary attachment to coated indwelling devices. These CWA adhesive proteins can be microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Examples include the serine-aspartate repeat protein G (SdrG/Fbe) that binds to fibrinogen; the extracellular matrix-binding protein (Embp) that binds to fibronectin; the serine-aspartate repeat protein F (SdrF/GehD), which binds to collagen; AtlE and Aae (autolysin-adhesin) that bind to vitronectin; and elastin-binding protein (EbpS) that binds to elastin. Furthermore, WTAs are able to bind to absorbed fibronectin and fibrin clots, promoting adhesion to coated indwelling devices (Sabaté Brescó et al., 2017).

Intercellular adhesion and maturation

The phase of biofilm accumulation and maturation comprises intercellular adhesion and three-dimensional structuring of the biofilm (França & Cerca, 2012). At this stage, bacterial cells multiply and accumulate in multilayered cell clusters, through intercellular adhesion, which is promoted by multiple molecules. In parallel occurs the production of a slime glycocalyx that enfolds bacterial cells in a matrix, which contributes to the establishment of a steady architecture. The cell aggregates are usually separated by fluid-filled channels (McCann et al., 2008), created due to disruptive forces (Otto, 2009) and PSMs (Sabaté Brescó et al., 2017), that are essential for nutrient and oxygen supply and elimination of metabolic products (McCann et al., 2008). The combination of these mechanisms, together with the existence of structuring forces, assists in the development of a stable and mature three-dimensional biofilm (França & Cerca, 2012).

Adhesive forces are accountable for cell-cell adhesion, which is achieved through several molecules, essentially polymeric substances, including matrix adhesion proteins and polysaccharides, mostly responsible for extracellular matrix production. Extracellular DNA and teichoic acids (TAs) are also engaged, as a result of their polyanionic character. This characteristic facilitates aggregation and binding to components of the matrix, which supports biofilm maturation (França & Cerca, 2012; Otto, 2009).

Poly-N-acetyl-glucosamine (PNAG), also designated as polysaccharide intercellular adhesin (PIA), is a major constituent of *S. epidermidis* extracellular matrix slime implicated in intercellular adhesion and biofilm accumulation (Namvar et al., 2014; Otto, 2012). This is eased by PNAG unbranched structure, which enhances distant interactions between adjacent polysaccharides strands and the cell wall or lectins (McCann et al., 2008). In addition, PNAG takes part in the hemagglutination of erythrocytes induced by *S. epidermidis*, which contributes to biofilm formation as well (Vuong & Otto, 2002).

Synthesis of PNAG is carried out by the gene products encoded in the *ica* locus. This locus comprises the *icaADBC* operon, which is composed by the open reading frames *icaA*, *icaD*, *icaB* and *icaC*, and the *icaR* gene, a negative regulator of the *icaADBC* (McCann et al., 2008). Nevertheless, it was demonstrated that *S. epidermidis* strains that lack *ica* genes are able to develop PNAG-independent biofilms, even though they were shown to be less robust than the PNAG-dependent ones. Instead, they can form biofilms through protein-mediated mechanisms, involving the accumulation associated protein (Aap), the Bhp, Embp, or *S. epidermidis* surface protein (Ses)C and SesE (Sabaté Brescó et al., 2017).

Biofilm detachment and dissemination

Eventually, cell detachment from the biofilm takes place, which consists on the controlled release of individual cells or groups of cells to the surrounding environment. This allows control of biofilm expansion and the regulation of bacterial dissemination. This step is crucial for biofilm persistence and for spread of bacteria, which will be able to colonize other niches. Subsequently, this aids in the dissemination and success of the infection (França & Cerca, 2012; McCann et al., 2008).

The release of bacterial cells may be triggered by several factors, such as mechanical strengths, enzymatic degradation of exopolymers, and disruption of non-covalent interactions due to surfactant molecules. Regarding the last mechanism, in *S. epidermidis*, surfactant molecules may be involved in the breakdown of the bonds between PNAG and surface polymers; or between hydrophobic regions of the bacterial surface. One example of molecules with surfactant properties are PSMs (França & Cerca, 2012), peptides that are present at the biofilm interface (McCann et al., 2008), and assist in biofilm structuring and dissemination (França & Cerca, 2012). The PSMδ, also named δ-toxin, inhibits hydrophobic interactions among bacterial cell surfaces, thus reducing the surface tension at the biofilm interface (McCann et al., 2008).

4. Additional virulence factors of *S. epidermidis*

As mentioned above, the biofilm mode of growth is the major virulence factor of *S. epidermidis*. Ergo, all the molecules and regulatory factors, presented above as components implicated in the mechanisms of biofilm formation, contribute to the virulence and pathogenicity of this bacterium. Beyond this, other mechanisms may contribute to *S. epidermidis* virulence, as the ones introduced below.

Multiple Drug Resistance

S. epidermidis typically exhibits an intrinsic genetic resistance against several antibiotics, a characteristic that can be spread throughout the bacterial population. This feature can arise from the presence of efflux pumps, modifying enzymes, target mutations or staphylococcal cassette chromosome *mec* (SCC*mec*) islands (Uckay et al., 2009). Those antibiotics include methicillin or other β-lactam antibiotics, erythromycin, clindamycin, tetracycline, chloramphenicol (Cm),

trimethoprim/sulfamethoxazole, aminoglycosides, glycopeptide antibiotics and quinolones. As a result of this virulence factor, treatment of *S. epidermidis* infections is impaired (McCann et al., 2008; Rupp & Archer, 1994).

Genotypic and phenotypic variability

The genome of *S. epidermidis* contains insertion sequence (IS) elements, which are short fragments of mobile and autonomous DNA. These IS elements are responsible for recombination mechanisms. Furthermore, they can be transposed and upregulate or downregulate gene expression. Both of these roles increase genetic and phenotypic variability, which can be spread among bacteria and lead to the emergence of evolved organisms that may be more resistant to the variable conditions they encounter (Ziebuhr et al., 2006).

Small colony variants

S. epidermidis has shown to form colonies characterized by a different morphology and biochemistry, along with a decrease in both growth and virulence gene expression. Additionally, these colonies have demonstrated to create an anti-inflammatory state and persist intracellularly. As a consequence, they present a higher tolerance to antibiotics and host immune responses. These colonies are designated small colony variants (SCVs) and constitute a virulence factor of *S. epidermidis* that contributes to the development of chronic and relapsing infections (Sabaté Brescó et al., 2017; Uckay et al., 2009).

Internalization and intracellular persistence

It has been suggested that *S. epidermidis,* through the mediation of AtlE and SdrG, is able to invade cells and persist therein. This enhances the ability of this pathogen to lead to chronic and recurrent infections in the host (Sabaté Brescó et al., 2017).

Exoenzymes and toxins

It has been evidenced that *S. epidermidis* produces exoenzymes, such as a metalloprotease and a cysteine protease, both with elastase properties. In addition, a serine protease has been recognized as well. These molecules appear to have a negative impact on the host, by damaging some constituents of the extracellular matrix, tissues or molecules involved in the immune response (Vuong & Otto, 2002). Moreover, *S. epidermidis* produces lipases, crucial in skin colonization (Vuong & Otto, 2002), and fatty acid modifying enzymes (FAME), which detoxify bactericidal fatty acids (Otto, 2012). *S. epidermidis* also produces δ-toxin (Vuong & Otto, 2002), that apart from its role in the detachment phase of biofilms (McCann et al., 2008), also lyses erythrocytes and neutrophils (Otto, 2012).

Lantibiotics

Gram-positive bacteria, as *S. epidermidis*, can generate lantibiotics, which are antimicrobial peptides. Some examples produced by these bacteria include epidermin, Pep5, epilancin K7 and epicidin 280. Lantibiotics have revealed to be essential for a successful colonization of *S. epidermidis* as they outcompete potential pathogens (von Eiff et al., 2002).

Iron uptake

S. epidermidis, as all bacteria, must be able to acquire iron, in order to proliferate and develop an infection in the host. Since a large amount of iron is captured by glycoproteins with binding affinity to this element, for instance transferrin, the quantity of iron left to be used by these pathogens is

limited. *S. epidermidis* seems to produce the siderophores staphyloferrin A and B, which are iron chelators that have the ability to snatch the iron bound to transferrin, when its released levels are low. Apart from this, iron uptake can be achieved through interaction between transferrin and receptors of *S. epidermidis* cell surface, such as glyceraldehyde-3-phosphate dehydrogenase, present on the cell wall, and a membrane-related lipoprotein (von Eiff et al., 2002).

5. *S. epidermidis* cell surface

The cell surface of bacteria is structurally variable among species, having multiple and essential functions. Gram-positive bacteria, unlike Gram-negative ones, do not have an outer membrane or a distinct periplasm (Swoboda et al., 2010). Although the structure of the bacterial surface can differ amongst bacteria, peptidoglycan (PG) layers are a constituent of all bacterial surfaces (Brown, Santa Maria Jr, & Walker, 2013). In Gram-positive bacteria, these layers are thicker than the ones present in the Gram-negative (Swoboda et al., 2010). Peptidoglycan mesh-like layers are constituted by linear chains of glycans and peptides that are cross-linked between peptides of different chains. These layers are vital for bacteria survival, membrane stabilization and anchoring of several molecules (Brown et al., 2013). The PG of Gram-positive bacteria is greatly functionalized with anionic glycopolymers, which promote membrane integrity, being the TAs the main group. Teichoic acids may be divided in lipoteichoic acids (LTAs) and WTAs as evidenced in Figure 2 (Swoboda et al., 2010). Furthermore, Gram-positive bacteria commonly contain lipoproteins, which, in *S. epidermidis* cell surface, are anchored to the membrane via a fatty acid anchor (Otto, 2009).

In addition, *S. epidermidis* cell surface contains various CWA proteins (Figure 2), which are essential for bacterial survival, virulence and pathogenesis In addition, they allow nutrient acquisition, microenvironment sensing, evasion of host immune response, cell-cell adhesion and binding to host tissue constituents, which enhances bacterial attachment (Sousa, Henriques, Teixeira, & Oliveira, 2009). These functions are extremely important for successful biofilm development in this bacterium. These CWA proteins generally have a common structure, encompassing from the N-terminal to the C-terminal: i) a secretion signal sequence (S); ii) a non-repeat A domain, which is typically ligand-binding; iii) a B repeats domain; iv) several other regions, comprising a proline-rich wall-spanning site (W), a LPXTG motif, a hydrophobic transmembrane domain and a cationic cytoplasmic tail, involved in sorting and anchoring to the cell wall (Bowden et al., 2005).

In *S. epidermidis*, CWA proteins include the MSCRAMMs, adhesive proteins that play a critical part in primary attachment of *S. epidermidis* to surfaces (Sabaté Brescó et al., 2017). The MSCRAMMs can be non-covalently bound to the cell surface, possibly by interacting with polymers like TAs, or covalently anchored to the PG, due to a sortase-mediated mechanism that cleaves the LPXTG motif. The serine-aspartate repeat (Sdr) family is one example of MSCRAMMs in *S. epidermidis*. The SdrG is the most well characterized protein of this family, however, SdrF and SdrH have also been identified. The SdrG is covalently bound to the PG and binds to fibrinogen. As for SdrF, it is also covalently attached to the PG but it binds to collagen (Otto, 2009). On the other hand, the SdrH is non-covalently anchored to the surface (Vuong & Otto, 2002). The AtlE and the Aae, which are autolysins-adhesins non-covalently linked to the surface, are another example of MSCRAMMs (Otto, 2012). These molecules are able to bind to vitronectin, besides their role in cell-wall turnover. Other MSCRAMMs are the elastin-binding protein EbpS (Sabaté Brescó et al., 2017) and the Embp, which is covalently anchored to the PG and binds to fibronectin, facilitating intercellular adhesion (Otto, 2012).

Another example of a CWA protein is the Bhp, which contributes to initial adhesion to abiotic surfaces and cell-cell aggregation during biofilm development (Bowden et al., 2005).

At last, the accumulation-associated protein (Aap) is also an CWA protein that is covalently bound to the PG (Otto, 2009). This molecule may also be found in the extracellular fluid, due to its secretion by biofilm cells (McCann et al., 2008). It has the capacity to facilitate cell-cell adhesion contributing to biofilm formation. Additionally, it is able to attach PNAG, a cationic molecule that it is thought to interact with LTAs, WTAs and poly-γ-glutamic acid (PGA), and that is crucial in biofilm-related intercellular aggregation (Otto, 2009).

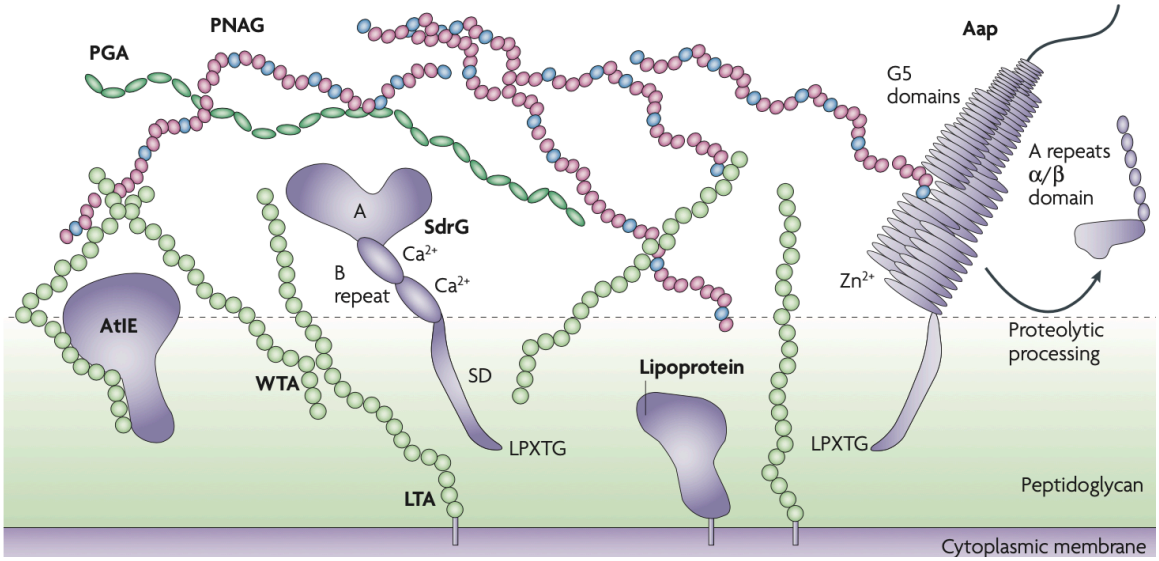


Figure 2 - Cell wall of *Staphylococcus epidermidis*. *S. epidermidis*, as a Gram-positive bacterium, is a characterized by the presence of thick peptidoglycan layers on its cell wall. Multiple molecules, such as lipoteichoic acids, wall teichoic acids and cell-wall anchored proteins are important constituents of *S. epidermidis* cell surface (Otto, 2009).

5.1. *S. epidermidis* teichoic acids

Teichoic acids comprehend a diverse class of anionic glycopolymers, mainly composed of repeating units of phosphate and polyol ribitol or glycerol, linked by phosphodiester bonds. These compounds are encountered within the cell wall of most Gram-positive bacteria, as *S. epidermidis*, in which they modify the PG layers. Teichoic acids encompass LTAs, which are anchored to the cell membrane through a glycolipid and prolong until the PG layer; and WTAs, which are covalently bound to the PG of the cell wall, which they surpass (Brown et al., 2013; Swoboda et al., 2010).

Teichoic acids are structural components of Gram-positive bacteria with essential functions. These constituents are involved in multiple mechanisms crucial for physiology and pathogenesis of these bacteria. Notwithstanding, the role of TAs is not entirely understood (Otto, 2012).

Some functions of TAs appear to be mutual to both WTAs and LTAs. As a consequence, these two types of TAs may offset each other. In parallel, both WTAs and LTAs comprise phosphate and polyol-linked repeating units that can be modified through D-alanylation, the attachment of cationic D-alanine esters, which suggests that both share some biosynthetic mechanisms. Therefore, the similar structure between these two TAs can be accountable for their similar functions (Swoboda et al., 2010).

5.1.1. *S. epidermidis* wall teichoic acids

Wall teichoic acids are the subject of this dissertation and, therefore, a major focus is given to these molecules, with a more detailed explanation of its structure and functions. It has been shown that WTAs may have a role in *S. epidermidis* virulence, which emphasise their importance in the pathogenesis of infections caused by this bacterium (Holland, Conlon, & O'Gara, 2011). Accordingly, WTAs pose a potential target to overtake *S. epidermidis* associated infections.

In *Staphylococci*, such as *S. aureus*, WTAs are predominantly constituted by ribitol and phosphate (Holland et al., 2011). Nevertheless, the polymer structure of WTAs varies amongst different bacterial species and even within the same species, which may result from adjustments to distinct environments (Brown et al., 2013; Holland et al., 2011). For instance, the WTAs of the biofilm-forming *S. epidermidis* strain RP62A are made up of repeating units of glycerol and phosphate (Gro-P), along with substitutions in the glycerol residues (Holland et al., 2011).

As illustrated in Figure 3, the poly (glycerol-phosphate) WTAs are commonly constituted by a highly conserved disaccharide linkage unit, which includes a ManNAc (β1→4) GlcNAc-1P disaccharide, along with glycerol phosphate units linked to the C4 hydroxyl of ManNAc. The GlcNAc-1P end of the linkage unit is the component that allows the covalent attachment to the PG, through a phosphodiester bond to the C6 hydroxyl of the MurNAc unit present in the PG. Furthermore, the final glycerol phosphate unit of the linkage element is followed by a main chain, which contains the repeating units of glycerol and phosphate, linked by phosphodiester bonds (Brown et al., 2013; Swoboda et al., 2010).

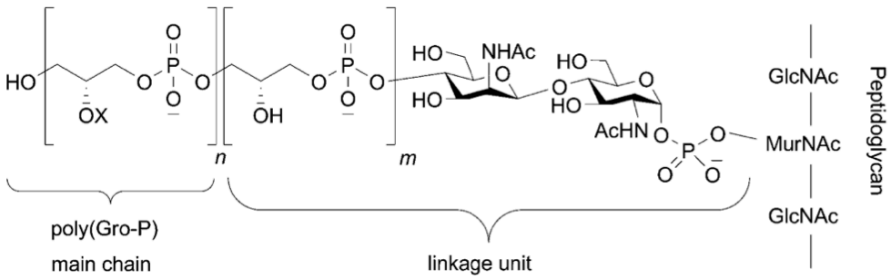


Figure 3 - Structure of poly(Gro-P) wall teichoic acids on Gram-positive bacteria. Poly(Gro-P) WTAs are constituted by a main chain of Gro-P repeating units and a disaccharide linkage unit, whose end is covalently attached to the peptidoglycan of the cell wall (Adapted from (Swoboda, Campbell, Meredith, & Walker, 2010).

The biosynthesis of poly (glycerol-phosphate) WTAs takes place in the cytoplasm and encompasses multiple steps. These are carried out by specific enzymes, the Tag (teichoic acid glycerol) enzymes, which are encoded by the *tag* genes. These genes, along with the process of synthesis of WTAs, were primarily identified in *B. subtilis* 168 (Ward, 1981). The first step of WTA biosynthesis comprises the linkage of GlcNAc-1P to an undecaprenyl phosphate, a lipid carrier attached to the membrane, through the TagO enzyme. This step, along with the binding of ManNAc to the C4 hydroxyl of the GlcNAc by the TagA and the connection of a glycerol-phosphate unit to the C4 hydroxyl of ManNAc by the TagB, complete the formation of the WTA linkage unit. Next, the TagF links several glycerol-phosphate units to the glycerol added by the TagB. Thereafter, the built polymer is glycosylated through addition of sugars to the poly (glycerol-phosphate) chain, by the TagE. After, it is transferred to the outer surface of the bacterial cell by the TagGH, an ATP-binding cassette (ABC) transporter. Then, the GlcNAc-1P of the formed WTA is covalently bound, by a phosphodiester linkage, to the C6

hydroxyl of the MurNAc unit of the PG, possibly through the action of TagTUV. At last, WTAs can be D-alanylated by four enzymes encoded by the operon *dltABCD* (Brown et al., 2013; Swoboda et al., 2010).

Wall teichoic acids are essential components of Gram-positive bacteria cell wall, due to their crucial functions in bacterial physiology and pathogenesis. These compounds have shown to guarantee cell morphology and division; cation binding and homeostasis; molecular interactions, through scaffold or receptor mimicking; anchoring of cell surface proteins; autolytic enzymes regulation; and resistance to host defense and antibiotics (Brown et al., 2013; Swoboda et al., 2010). Moreover, they are engaged in colonization and biofilm development, enhancing bacterial virulence and pathogenesis. Regarding this, WTAs promote primary attachment of *S. epidermidis* to a surface, through binding to absorbed fibronectin and fibrin clots. They also facilitate intercellular adhesion, on account of their anionic charge (Sabaté Brescó et al., 2017). Furthermore, WTAs are crucial constituents of *S. epidermidis* biofilms, in which they assure structuring. Even though WTAs are involved in inflammation, when it comes to immune response stimulation or *S. epidermidis* evasion from the host immune response, although highly probable, the influence of WTAs has not been entirely proven (Brown et al., 2013; Swoboda et al., 2010). Regardless, the modification of WTAs, via D-alanylation, diminishes the anionic character of *S. epidermidis* surface, which may affect the action of cationic AMPs (Otto, 2009).

6. Host immune response to commensal *S. epidermidis*

S. epidermidis is a crucial colonizer of the skin microbiota that provides multiple benefits to the host. This important role of *S. epidermidis,* as a commensal microorganism, shows that this bacterium has the ability to modulate the host immune response. Accordingly, *S. epidermidis* may be able to influence both innate and adaptive host immune responses, since an early life stage. The latter can be achieved through antigen presenting cells (APCs), such as DCs. These are responsible for presenting antigens to T cells, which can produce memory T and B cells, along with antibodies (Sabaté Brescó et al., 2017).

When it comes to the innate response, it was demonstrated in mice that *S. epidermidis* LTAs lead to a decrease in skin inflammation, via a TLR (toll-like receptor)-2 pathway (Lai et al., 2009). Moreover, this bacterium also triggers TLR-2 and generate AMPs, targeted at pathogenic organisms (Lai et al., 2010).

As for the adaptive response, two studies in mice evidenced divergent results. One of them exhibited that commensal *S. epidermidis* colonization in neonatal skin leads to a local and systemic immune response, mediated by $CD4^+$ regulatory T (T_{reg}) cells (Scharschmidt et al., 2015). In contrast, the other study revealed, in adult mice, that commensal *S. epidermidis* colonization, due to $CD103^+$ skin-resident DCs, gave rise to a skin-specific immune response, mediated by $IL\text{-}17A^+$ $CD8^+$ T cells (Naik et al., 2015).

These results were not demonstrated in humans, in which this knowledge is limited. However, *in vitro* studies have shown that, after stimulation of human monocytes, monocyte-derived DCs (moDCs) and T cells, with commensal *S. epidermidis,* all these cells, despite producing IL-6 and TNF-α, generated a large amount of IL-10, which promoted the establishment of a more anti-inflammatory environment (Laborel-Préneron et al., 2015).

7. Host immune response during *S. epidermidis* infection

Once *S. epidermidis* penetrates the epithelial barrier, it encounters several factors with the purpose of fighting this bacterium and preventing infection development. Therefore, many innate and adaptive host immune responses can be generated (Otto, 2009). Specifically, in the case of biofilm-relation infections, this defense response can be inefficient in elimination of infection, which can develop into a chronic state (Nguyen et al., 2017).

7.1. Innate immunity

The innate immune system generates early protective and non-specific responses against pathogens. These responses are induced by the recognition of pathogen-associated molecular patterns (PAMPs), through pattern-recognition receptors (PRRs) present on immune or tissue cells. The TLR-2 is one of the PRRs that mediate recognition of *S. epidermidis* surface constituents, such as PG, LTA and lipoproteins (Akira, Uematsu, & Takeuchi, 2006; Fournier, 2013). In addition, PSMs proved to be capable of triggering TLR-2/TLR6 heterodimers (Hajjar et al., 2001). *S. epidermidis* is also recognized by intracellular NOD-like receptors, which sense PG structures (Natsuka, Uehara, Yang, Echigo, & Takada, 2008); CD14 of monocytes and macrophages, that acts as a co-receptor of TLR-2 (Hatakeyama et al., 2003); and formyl peptide receptor 2 (FPR2/ALX) of neutrophils that detects PSMs (Kretschmer, Nikola, Dürr, Otto, & Peschel, 2012; Kretschmer, Rautenberg, Linke, & Peschel, 2015). Following *S. epidermidis* recognition by these PRRs, signaling and effector pathways are activated (Figure 4).

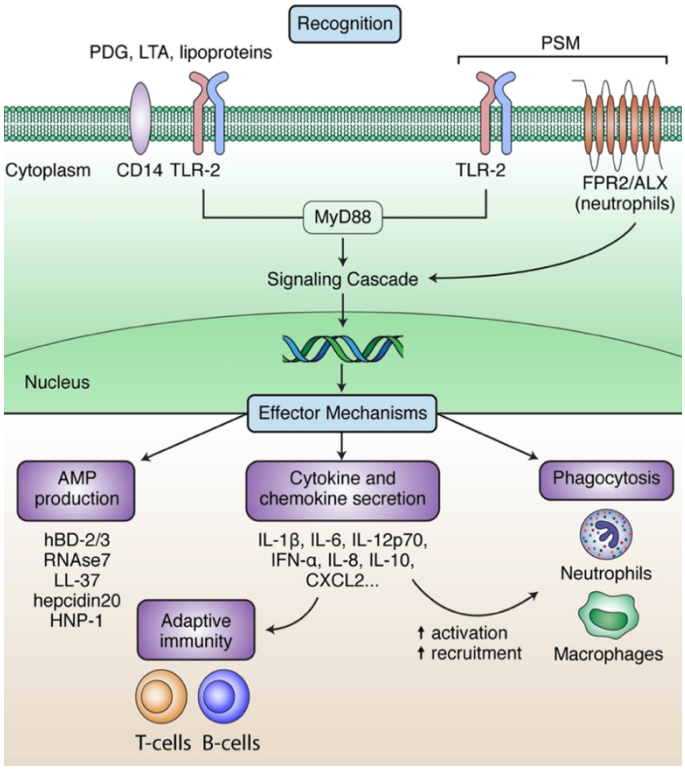


Figure 4 – Immune response to *Staphylococcus epidermidis*. Following recognition of *S. epidermidis* through specific PRRs, signaling and effector mechanisms are activated. These induced pathways comprise AMP production, the secretion of cytokines and chemokines, phagocytosis by macrophages and neutrophils and stimulation of adaptive response (Sabaté Brescó et al., 2017).

The AMPs encountered in the human body comprise a class of heterogeneous cationic peptides with diverse functions that promote a protective response against *S. epidermidis* and its related infections. These functions comprehend direct killing and immune system regulation, through production of chemokines and recruitment of cells (Sabaté Brescó et al., 2017). It was discovered, *in vitro*, that *S. epidermidis* stimulates AMPs expression, such as human β-defensin-2 (hBD-2), hBD-3, RNase7 and cathelicidin LL-37 (Figure 4) (Burgey, Kern, Römer, & Rieg, 2016; Lai et al., 2010; Li et al., 2013; Ommori et al., 2013; Park, Ommori, Imoto, & Asada, 2014; Percoco et al., 2013). In a *S. epidermidis* DRI model it was shown that neutrophils and monocytes are induced to express AMPs, which are able to kill this bacterium within phagolysosomes (Sabaté Brescó et al., 2017). Moreover, it was proved, *in vitro*, that hBD-3, LL-37 and hepcidin 20 (Figure 4) inhibit adhesion and biofilm development by *S. epidermidis* (Brancatisano et al., 2014; Hell, Giske, Nelson, Römling, & Marchini, 2010; Zhu et al., 2013).

Through the detection of signaling molecules or constituents of *S. epidermidis*, neutrophils have the capacity to migrate to the infection site, where they phagocytose and kill this pathogen, by means of reactive oxygen species (ROS), proteases and AMPs (Sabaté Brescó et al., 2017). Additionally, *in vitro* neutrophil-mediated killing of *S. epidermidis* biofilms may occur through NETosis or by the production of AMP-containing neutrophil extracellular traps (NETs), through DNA release (Dapunt, Gaida, Meyle, Prior, & Hänsch, 2016; Meyle, Brenner-Weiss, Obst, Prior, & Hänsch, 2012). It was observed, both *in vitro* and *in vivo*, that macrophages are triggered by IFN-γ to phagocytose *S. epidermidis* and present its antigens to T lymphocytes (Boelens, van der Poll, Dankert, & Zaat, 2000; Magryś et al., 2015). Contradictory results concerning biofilm cells phagocytosis have been published. While some studies have demonstrated that *S. epidermidis* biofilms promote phagocytosis by neutrophils and macrophages, others have shown that these biofilms impair both neutrophil and macrophage mediated phagocytosis and killing, in comparison with planktonic cells (Nguyen et al., 2017). Likewise, PNAG-producing biofilm cells have shown to impair killing by macrophages and neutrophils (N. Cerca, Jefferson, Oliveira, Pier, & Azeredo, 2006; Kristian et al., 2008; Spiliopoulou et al., 2012; Vuong et al., 2004), but have evidenced, in mice, to improve neutrophil recruitment due to PNAG expression (Ferreirinha et al., 2016). Still in mice, *S. epidermidis* biofilm-released cells have demonstrated to upregulate the expression of genes associated with innate immunity, with roles in activation and neutrophil recruitment (França et al., 2016).

Distinct cytokines secreted by cells of the innate system have been implicated in the immune response to *S. epidermidis* (Sabaté Brescó et al., 2017). The production of IL-6, TNF-α and IL-1β , triggered by *S. epidermidis*, has been observed *in vivo* (Bi et al., 2015; Ferreirinha et al., 2016; Simojoki, Salomäki, Taponen, Iivanainen, & Pyörälä, 2011), such as in DRI models (Boelens, van der Poll, Zaat, et al., 2000; Svensson et al., 2015). Furthermore, *in vitro* and *in vivo* experiments have shown IL-8 release in response to *S. epidermidis* (Boelens, van der Poll, Zaat, et al., 2000; Simojoki et al., 2011; Svensson et al., 2015). Also, the production of anti-inflammatory cytokines in response to *S. epidermidis*, such as IL-10, is rather high. This supports the sub-acute or chronic character of *S. epidermidis* infections, as it allows the pathogen to persist in the host (Ferreirinha et al., 2016; Sabaté Brescó et al., 2017). In comparison with planktonic cells, *S. epidermidis* biofilm cells induce a lower production of pro-inflammatory cytokines, namely IL-1β, IFN-γ and IL-12, and a higher production of IL-13 (Spiliopoulou et al., 2012); while biofilm-released cells lead to a greater induction of pro-inflammatory cytokines and a lower production of IL-10 (França et al., 2016). Moreover, *S. epidermidis* enhances the production of several chemokines, such as CXCL-1 and CXCL-2 (Bi et al., 2015; Ferreirinha et al., 2016).

7.2. Adaptive immunity

Adaptive immune responses elicited by *S. epidermidis* infections are poorly understood (Nguyen et al., 2017). Regardless, both cellular and humoral adaptive responses appear to be relevant in anti-*S. epidermidis* immunity (Sabaté Brescó et al., 2017). This was demonstrated in mice deficient in T or both T and B lymphocytes, as these mice were more susceptible to *S. epidermidis* DRIs (Vuong, Kocianova, Yu, Kadurugamuwa, & Otto, 2008).

S. epidermidis has shown, both *in vitro* and *in vivo*, to activate DCs, as assessed by the upregulation of antigen-presenting major histocompatibility complex (MHC) class II and the costimulatory CD86 and CD80 molecules (F. Cerca et al., 2014; França et al., 2016; Laborel-Préneron et al., 2015). However, cytokine production by DCs, following *S. epidermidis* stimulation, is not well understood, since some studies evidenced that, depending on the DC origin and the stimuli, they can produce low or elevated levels of IL-10 (F. Cerca et al., 2014; Laborel-Préneron et al., 2015). The PSMs also proved this stimuli dependency, as cytotoxic T lymphocytes were only activated if APCs were involved and after recognition by specific TLRs (Durantez et al., 2010).

Concerning humoral immunity, numerous components of *S. epidermidis* have displayed their immunogenic role and propensity to increase the production of specific antibodies, crucial in infection clearance (Sabaté Brescó et al., 2017). Some examples include Na+/H+ antiporter, Acetyl-CoA C-acetyltransferase, lipoate ligase, cysteine synthase, alanine dehydrogenase, AtlE, Staphylococcal conserved antigen B (ScaB), GehD lipase, and staphylococcal Major amidase (Atl- AM) (Nair et al., 2015; Pourmand, Clarke, Schuman, Mond, & Foster, 2006; Sellman, Howell, Kelly-Boyd, & Baker, 2005). In addition, Aap and antibodies targeted at constituents of *S. epidermidis* surface have shown to affect the development of *S. epidermidis* biofilms (Yan, Zhang, Ma, Chiu, & Bryers, 2014). This was also achieved using antibodies against PNAG or phosphonate ABC transporter substrate binding protein (PhnD) (França, Vilanova, Cerca, & Pier, 2013; Lam, Kesselly, Stegalkina, Kleanthous, & Yethon, 2014). Also, an SdrG-specific antibody proved to impair *S. epidermidis* infection (Vernachio et al., 2006).

As for cellular immunity, triggering of Th1 and Th2 responses via Atl-AM (Nair et al., 2015), the activation of Th17-mediated responses following opsonization by IgG (Den Dunnen et al., 2012), the stimulation of Th1 by IFN-γ (Boelens, van der Poll, Dankert, et al., 2000), and the induction of Th1 and Th17 after the characteristic release of IL-6, IFN-γ, and IL-12, may be important in adaptive defense against *S. epidermidis*. This indicates an essential role for these T cell subtypes in this response (Sabaté Brescó et al., 2017). A study in mice confirmed that biofilm cells of a PNAG-producing *S. epidermidis* strain induced IFN-γ- and IL-17A-producing $CD4^+$ T lymphocytes, together with regulatory T cells and IL-10, reinforcing the capacity of *S. epidermidis* to survive and persist in the host (Ferreirinha et al., 2016). Ultimately, it was observed in mice that *S. epidermidis* biofilm-released cells upregulate the expression of genes involved in the adaptive responses, such as the ones involved in APCs prompting of T cell activation (França et al., 2016).

8. Evasion of host immune response by *S. epidermidis*

S. epidermidis has numerous mechanisms and constituents that contribute to evasion of host immune responses. Many of them probably play a role in the commensal lifestyle of this bacterium, since *S. epidermidis* infections are generally regarded as accidental (Otto, 2009). Those factors lead to impairment of the immune system, through diverse paths, by decreasing inflammation and favoring intracellular survival. Therefore, since the immune system is not capable of efficiently eliminating the infection, it can develop into a chronic state (Sabaté Brescó et al., 2017).

The ability of *S. epidermidis* to form biofilms is one of the mechanisms that allow this bacterium to escape from the host immune system. Firstly, the metabolism of biofilm cells can alter the pH and the oxygen rates of the microenvironment, which, may modify the response induced by the host immune system (Scherr et al., 2014). In addition, biofilms pose a mechanical barrier that hampers the penetration and activity of immune cells and numerous molecules. For instance, the action of AMPs is impaired, since the extracellular biofilm matrix prevents them from reaching the cytoplasmic membrane (Otto, 2012). Lastly, it was demonstrated that biofilms produced by *Staphylococci* establish a more anti-inflammatory profile, due to induction of arginase-1 (Arg1)-expressing M2-type macrophages (Scherr et al., 2014).

Beyond biofilm development, *S. epidermidis* produces various molecules, crucial for evasion from the host immune system. One major class are the exopolymers, such as PNAG and PGA. The PNAG molecules can protect the bacterium from phagocytosis and killing by neutrophils, complement and Ig deposition, and from both cationic and anionic AMPs (Otto, 2009). Comparatively to planktonic cells, *S. epidermidis* biofilms produce a greater amount of PNAG, which may inhibit deposition on the bacteria cell surface of opsonic antibodies targeted at this polysaccharide. Subsequently, despite their ability to penetrate into the biofilm, phagocytic killing mediated by these antibodies is greatly impaired (N. Cerca et al., 2006). The PGA is an anionic polymer, constituted by glutamic acid residues (Otto, 2012), with a role in circumventing the immune system. It has shown to affect innate immunity by suppressing phagocytosis, neutrophil-mediated killing and AMPs activity (Otto, 2009). Moreover, peptides such as PSMs, which represent the narrowed set of toxins in *S. epidermidis* (Otto, 2009), are also important in defense against host immune response. Specifically, PSMδ has evidenced a high cytolytic activity, leading to neutrophil and erythrocyte lysis (Otto, 2012). Another group of molecules are the exoenzymes secreted by *S. epidermidis*. For example, SepA is engaged in AMP degradation (Otto, 2012). *S. epidermidis* also expresses SdgA and SdgB, glycocyltransferases that alter Sdr-proteins with the intent of avoiding breakdown via the neutrophil protein cathepsin G (Hazenbos et al., 2013).

Ultimately, *S. epidermidis* has an AMP-sensing system, the *aps* system, which detects AMPs and initiates protective mechanisms against these peptides, such as TA D-alanylation and MprF-mediated phospholipid lysylation. Both mechanisms are able to inhibit cationic AMPs affinity by lowering the surface anionic character (Otto, 2009).

9. Wall teichoic acids in the host immune response to *S. epidermidis*: The use of WTA-deficient *S. epidermidis* strains

A better understanding of the host immune response to *S. epidermidis*, combined with the comprehension of its evasion mechanisms, are of major importance. The attainment of this knowledge would facilitate the development of novel and improved approaches targeted at *S. epidermidis* biofilm-based infections.

Wall teichoic acids can be implicated in *S. epidermidis* virulence, contributing to its pathogenicity. Hence, as they may represent an important target to treat these infections, it would be relevant to comprehend how WTAs are involved in the host immune response (Otto, 2012). However, the role played by WTAs in this response is poorly understood. In fact, it is not known if WTAs have some influence in this response, and if so, how do they modulate it. Likewise, it has not been proven that WTAs are directly connected with immune evasion by *S. epidermidis*. This knowledge is mostly limited to the recognition that *S. epidermidis* has the capacity to modify WTAs, via D-alanylation, which may prevent the action of AMPs, as identified in *S. aureus* (Peschel et al., 1999; Sabaté Brescó et al., 2017).

The genes involved in the biosynthesis of poly (glycerol-phosphate) WTAs are the *tag* genes and the *tagO* gene, which encodes the enzyme TagO, catalyzes the initial step of WTA biosynthesis. Therefore, the utilization of *tagO* deletion mutants, which do not have the *tagO* gene and, consequently, are WTA-deficient, can be highly useful to understand several potential roles of WTAs (Holland et al., 2011).

Actually, this was already done in a study that proved the role of WTAs in *S. epidermidis* biofilm development. The *tagO* mutant exhibited a deficient biofilm formation, related to reduced bacterial adhesion. This study was able to prove the important role that WTAs have in biofilm development. It provided details concerning the mechanisms in which WTAs are directly engaged (Holland et al., 2011).

Since the implication of WTAs on the host immune response to *S. epidermidis* infections is poorly acknowledged, the characterization of the host immune response to a WTA-deficient *S. epidermidis* strain, could be the way to finally comprehend the role of WTAs in the immune response to *S. epidermidis*. This information would certainly help the development of novel immune-mediated approaches, with the purpose of preventing or treating medical device-associated *S. epidermidis* infections.

Chapter 2

Objectives

S. epidermidis is one of the major causes of nosocomial infections due to its capacity to form biofilms on the surface of indwelling medical devices. These infections are usually persistent and difficult to treat, thus contributing to the increase of morbidity in Europe. Wall teichoic acids are important constituents of *S. epidermidis* cell wall, implicated in bacterial virulence. Nonetheless, the interaction of WTAs with the host immune system remains to be characterized. With this in view, the main objective of this dissertation is to understand the influence of WTAs in the host immune response to *S. epidermidis*, to better comprehend these infections and discover how to prevent/treat them.

To achieve this main goal, a WTA-deficient strain of *S. epidermidis* was used on this project. Accordingly, the dissertation focuses on:

1) the optimization of *S. epidermidis* growth protocols for further normalization of cell number between strains;

2) the characterization and differentiation between the wild type and the mutant strains of *S. epidermidis* in terms of morphology and proliferation;

3) the assessment of the interaction of planktonic cells of these strains with both mouse and human cells of the innate immune system and, finally

4) the evaluation/comparison of biofilm production by each of *S. epidermidis* strains.

Dissertation Structure

This thesis encompasses the theoretical introduction on Chapter 1; the main objectives of this dissertation and the overall structure of the document on the present Chapter 2; a brief explanation of the materials and methods employed throughout this project on Chapter 3; the achieved results along with their discussion and validated interpretation are presented on Chapter 4; and the main conclusions coupled with the future perspectives are outlined on Chapter 5.

Chapter 3

Materials and Methods

1. Characterization of *S. epidermidis* strains

1.1. Bacterial strains of *S. epidermidis*, cultures and setup conditions

S. epidermidis strain 1457 (Mack, Siemssen, & Laufs, 1992) was used in this dissertation, in particular: the wild type (WT), a mutant for the gene *tagO* (Δ*tagO*, or simply ΔT) and the complemented (Δ*tagO*::*tagO*, or ΔT::T for short) strains, that have been produced by A. França. As explained above, the WT contains the *tagO* gene, involved in the biosynthesis of the WTAs, whereas the ΔT was constructed from the deletion of that gene in a WT strain. As for the ΔT::T, this strain was constructed using the ΔT strain, in which the plasmid pRB473 carrying the *tagO* gene and promotor sequences was transformed. This ΔT::T serves as a control to ensure that the phenotype observed in the mutant strain is the result of the gene *tagO* deletion, ergo, this and the WT strain are excepted to display a similar behavior.

All of them were preserved with 30% glycerol, as frozen stocks at -80°C. Bacterial cells were cultured and grown on tryptic soy agar (TSA) plates at 37°C, during approximately 24h. The ΔT::T strain was cultured in plates of TSA with 10μg/mL Cm, in order to maintain the expression plasmid. To obtain liquid cultures, a single colony from TSA plates was transferred to tryptic soy broth (TSB) supplemented with human AB serum (10%) (Figure 6) and incubated at 37°C and 180 rpm, for approximately 14h to 15h (stationary phase) (Figure 5). Once again, the liquid culture in which the ΔT::T strain grew was also supplemented with 10μg/mL Cm (TSB_{Cm}). The grown cultures were then centrifuged at 4000 rpm, for 10 minutes. Thereafter, the supernatants were discarded, the cells were resuspended in sterile PBS buffer, supplemented with human serum (2%) (Figure 6), and filtrated using a cell strainer. The optical density (OD) of each strain was measured in a spectrophotometer at 640nm and the inoculum, with the desired OD_{640nm} for each strain (WT-0.25; ΔT-0.50 and ΔT::T-0.50) (Figure 7) was prepared in order to attain an equal number of cells among all strains ($2x10^8$ CFU/mL). Following this, the OD_{640nm} of each strain was confirmed in the spectrophotometer and the suspension was plated on TSA or TSA+Cm plates, according to the strain, and maintained at 37°C for up to 24h. Finally, the number of CFU/mL in each suspension was counted to confirm the normalization performed between the strains. All these steps were employed in the beginning of all the experiments that will be presented below on the sections 2 and 3 of the present chapter.

1.2. Growth curves

A growth curve for each of the *S. epidermidis* strains was determined. For that, a liquid culture of each strain was prepared and allowed to grow in TSB or TSB_{Cm}, non-supplemented with human serum, at 37ºC and 180 rpm for up to 24h. At several time points 0, 1, 2, 3, 4, 5, 6, 7, 8 and 24h, an aliquot of each strain was collected their OD_{640nm} measured.

1.3. Optimization test: Growth conditions

Liquid cultures of the strains were grown overnight at 37ºC and 180 rpm, up to their stationary phase. In the next day, overnight cultures were centrifuged, resuspended and filtrated. Then, the OD_{640nm} of each inoculated strain was measured and adjusted to different OD_{640nm} values: (WT- 0.06; 0.12; 0.25 or 0.50); (ΔT- 0.12; 0.25; 0.50 or 1.00) and (ΔT::T- 0.12; 0.25; 0.50 or 1.00). CFUs of all samples were counted. The key factor for this optimization was the use of three different conditions: i) growth in TSB + 10% human serum and subsequent resuspension in PBS + 2% human serum; ii) growth in TSB and resuspension in PBS; iii) growth in TSB + 10% BSA and resuspension in PBS + 2% BSA.

1.4. Optimization test: CFU count

For this optimization test, the inoculum, grown with TSB + 10% human serum and resuspended in PBS + 2% human serum, as explained on 1.3, was considered. Following the filtration step, the OD_{640nm} of each *S. epidermidis* strain was adjusted to different values: (WT- 0.06; 0.12; 0.25 or 0.50); (ΔT- 0.12; 0.25; 0.50 or 1.00) and (ΔT::T- 0.12; 0.25; 0.50 or 1.00) and all the prepared suspensions were plated on TSA/TSA_{Cm} and incubated at 37ºC for up to 24h. CFUs were then counted and a specific OD_{640nm} value was chosen for each strain, taking into account that the number of cells should be equal among the strains. This optimization test was done in duplicate, both times using the growth condition with human serum and replicating all the other parameters.

1.5. Confirmation test of optimization: Cell count by FACS

A flow cytometry assay, using BD Accuri™ C6 Plus, was employed for cell count. The strain cultures were grown overnight (37ºC, 180 rpm) in TSB + 10% human serum. Then, they were centrifuged, resuspended in PBS + 2% human serum and filtrated. Their OD_{640nm} was measured and adjusted to 0.25 (WT) or to 0.50 (ΔT and ΔT::T). These suspensions were then diluted to $5x10^5$ CFU/mL in order to be in the range of values that the cytometer performs accurate measures. Next, the cells were stained with a LIVE/DEAD staining: SYTO 9, at 1:5000, and propidium iodide (PI), at 5μg/mL. Controls for each strain were used, which included an unstained sample, one stained with only SYTO (single SYTO) and one with just PI (single PI). Finally, all samples were analyzed in the cytometer and cell counts for the three strains were obtained.

1.6. Morphology analysis by TEM

All three strains of *S. epidermidis* were grown overnight as liquid cultures (TSB+10% human plasma, and Cm in the case of ΔT::T) during 12 hours. With the purpose of obtaining bacterial cells in their exponential phase, the grown cultures were diluted to $3×10^7$ CFU/mL and grown for 6h (Figure 5), at 37ºC and 250 rpm. Upon this growth, cultures were centrifuged (5000*g*, 10 min and 4ºC), washed three times with metal-free ultrapure water to eliminate salts, fixed with 2.5% (w/v) glutaraldehyde/ 2% (w/v) paraformaldehyde in cacodylate buffer 0.1 M (pH 7.4) and left overnight. Next, they were washed with 0.1M sodium cacodylate buffer and fixed overnight in 2% (w/v) osmium tetroxide in the 0.1M sodium cacodylate buffer. One last time, they were fixed overnight with 1% (w/v) uranyl acetate. After, gradient dehydration was applied to the samples, using ethanol and propylene oxide, and then they were embedded in EPON™ resin, in a silicon mold, through 1h absorptions in rising ratios of propylene oxide:EPON™, up to 0:1. Subsequently, samples were sectioned into 60 nm-thick sections with a diamond knife attached to an RMC Ultramicrotome (PowerTome, USA), collected to 200 mesh Formvar Ni-grids. Finally, samples were stained with 2% (w/v) uranyl acetate and saturated

lead citrate solution.

For morphology characterization purposes, the prepared samples were further analyzed by transmission electron microscopy (TEM), using a JEM 1400 microscope (JEOL, Japan), operating at 80kV. The TEM procedure was done by a skilled worker at the i3s Institute, Porto, Portugal, and a CCD digital camera Orious 1100 W (Tokyo, Japan) was utilized to digitalize the images.

2. Innate immune response promoted by planktonic cells of *S. epidermidis* strains

2.1. Interaction with murine macrophage-like cell line

2.1.1. Culture of RAW 264.7 macrophages

Frozen aliquots of RAW 264.7, a murine monocyte/macrophage-like cell line, were thawed, resuspended in RPMI and centrifuged at 1300 rpm for 5-10 min, to remove the DMSO present. Subsequently, the supernatant was discarded and RPMI, supplemented with 10% fetal bovine serum (FBS), 2% L-glutamine, 1% HEPES, 50μM β-mercaptoethanol and 1% penicillin-streptomycin, was added to suspend the cells. The cell culture was then transferred into a T25 flask and maintained at 37ºC with 5% CO_2. Passages of the cell culture to a new T75 flask were conducted every 3 to 4 days. Once the cells were confluent and adherent to the flask, they were ready to be used in phagocytosis assays.

2.1.2. Phagocytosis assay

The RAW 264.7 cells were recovered from the culture flask and counted using the trypan blue exclusion method. Subsequently, they were resuspended in RPMI, supplemented with 10% FBS, 1% HEPES, 2% L-glutamine and 50μM β-mercaptoethanol. Then, they were seeded in a 96-well plate, with a flat bottom to enhance macrophage adhesion, at a concentration of $1x10^6$ cells/mL and were allowed to attach for approximately 1 hour at 37ºC and 5% CO_2. During this time, the three strains, which have grown overnight, were prepared. Completed the time of macrophage attachment, these were infected with the three *S. epidermidis* strains, which were plated at a concentration of $1x10^7$ cells/mL, to attain a MOI of 1 macrophage to 10 bacteria (1M:10B). The plate was centrifuged at 300*g* during 2 min, to enable a synced phagocytosis. Then, it was incubated at 37ºC with 5% CO_2 and phagocytosis was evaluated 30, 60 and 180 min after the infection. At each of the indicated time points, supernatants were discarded and warm RPMI, supplemented with 2% L-glutamine, 1% HEPES, 50μM β-mercaptoethanol and 50 μg/mL gentamicin, was added for 1h to remove non internalized bacteria. Next, supernatants were discarded once again, cells were washed with warm PBS 1x and incubated for 15 min, in the cold, with PBS 1x supplemented with 0,1% saponin to lyse the cells. Finished this time, the lysate was sonicated by ultrasounds for 5 min to try to break aggregates of bacteria. At last, serial dilution were prepared with PBS + 2% human serum and plated on TSA/TSA_{Cm} plates for CFU counting. Plates were incubated for approximately 24h.

2.2. Interaction with mice bone marrow derived dendritic cells (BMDCs)

2.2.1. Mice

BALB/c WT mice were bred at the Animal Facility of the Instituto de Investigação e Inovação em Saúde (i3s) Porto, Portugal, under specific-pathogen-free conditions. All the underlying practices were conducted in agreement with the European Convention for the Protection of Vertebrate Animals

Used for Experimental and Other Scientific Purposes (ETS 123), the 2010/63/EU directive of the European Parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes, and Portuguese rules (DL 113/2013). All experimental procedures were permitted by the institutional board responsible for animal welfare (ORBEA) at i3s and their approval was emitted by the competent national authority (Direção Geral de Alimentação e Veterinária), reference number 014036/2019-07-24.

2.2.2. BMDCs differentiation

BALB/c mice were anaesthetized with CO_2, euthanized through cervical dislocation and briefly immersed in 70% ethanol. Thereafter, their femurs and tibias were isolated, and their tips were cropped. 27G and 25G needles pierced the tibias and the femurs, respectively, allowing HBSS 1x to cross the bones, and bone marrow cells to be recovered. The cell suspension was then filtered with a cell strainer, centrifuged at 300*g* for 10 min and resuspended in RPMI, supplemented with 10% FBS, 2% L-glutamine, 1% HEPES, 50μM β-mercaptoethanol and 1% penicillin-streptomycin. Upon being counted with trypan blue, cells were seeded in 6-well culture plates, at a concentration of $1x10^6$ cells/mL. In order to differentiate the obtained cells into BMDCs, 20ng/mL of GM-CSF was added to the cells in culture. Cells were incubated at 37ºC with 5% CO_2 for 7 days. Every two days, all of the cell culture medium was exchanged.

2.2.3. Infection of BMDCs with *S. epidermidis* strains

The BMDCs were allowed to differentiate for 7 days and, afterwards, they were lifted, through a technique based on up and down. Next, they were collected from the 6-well culture plates, resuspended in RPMI (supplemented with 10% FBS, 2% L-glutamine, 1% HEPES and 50μM β-mercaptoethanol) and counted with trypan blue. Then, they were seeded in a 96-well plate with round bottom, where they were co-incubated with all *S. epidermidis* strains. The DCs were infected with each of the bacterial strains at a MOI of 1 DC to 4 bacteria (1DC:4B) and (1DC:10B). Some cells were left unstimulated (negative control) or stimulated with LPS (positive control) and were all incubated at 37ºC and 5% CO_2. After 2h, 50 μg/mL gentamicin was added to each well to stop the reaction by killing the bacteria. The plate was again incubated at 37ºC and 5% CO_2.

Upon an incubation of 24h, the 96-well plate was centrifuged at 300*g* for 10 min, the supernatants were collected and stored at -80ºC for upcoming cytokine quantification by ELISA and the cells were stained and prepared for activation assays by flow cytometry studies.

2.2.4. Cytokine release quantification through sandwich ELISA

Sandwich ELISA was performed to quantify the secretion of cytokines by the BMDCs infected with *S. epidermidis* strains. The presence of secreted IL-10, TNF-α, IL-1β, IL-6, and IL-12p70 in the supernatants was assessed. The ELISA technique was carried out following the manufacturer's protocol (Mouse IL-10 DuoSet® ELISA Development System from R&D systems and Mouse TNF-α Uncoated ELISA Kit, Mouse IL-1β Uncoated ELISA Kit, Mouse IL-6 Uncoated ELISA Kit, Mouse IL-12p70 Uncoated ELISA Kit from Invitrogen by Thermo Fisher Scientific).

2.2.5. Assessment of BMDCs activation by FACS

Flow cytometry studies, using BD FACSCanto™ II, were held to evaluate the presence of activation markers at the cell surface. Following the 24h incubation of the BMDCs, infected with the strains of

S. epidermidis, these cells were recovered, incubated with Fixable Viability Dye (FVD) and antibodies against cell surface markers [anti-mouse CD11c-FITC conjugated (clone N418), anti-mouse F4/80-APC/Cy7 conjugated (clone BM8), anti-mouse Ly6G-AF647 conjugated (clone 1A8), anti-mouse MHC class II-PerCP conjugated (clone M5/114.15.2), anti-mouse CD80-PE conjugated (clone 16-10A1) and anti-mouse CD86-PECy7 conjugated (clone GL1)], fixed with paraformaldehyde (PFA) and resuspended in FACS buffer (PBS + 2% FCS + 10mM sodium azide).

2.3. Interaction with human monocyte-derived macrophages (MDMs) and dendritic cells (MDDCs)

2.3.1. Ethics Statement

Buffy coats from healthy blood donors were acquired at the Immunohemotherapy Department of Centro Hospitalar São João (CHSJ) from Porto, Portugal. All donors gave informed written consent for the use of their blood collection for scientific purposes (Protocol reference 260/11), under the ethical approval of the service. Procedures employed in obtention of peripheral blood mononuclear cells (PBMCs) from buffy coats were in agreement with the principles of the Declaration of Helsinki.

2.3.2. MDDCs and MDMs differentiation

Initially, PBMCs were isolated from the human blood through density gradient centrifugation, following the septmate™ procedure protocol, and through the use of histopaque-1077 as the density gradient medium. In order to selectively collect monocytes ($CD14^+$ cells), the methodology of magnetic labeling and separation of cells was employed, and CD14 microbeads were used. The recovered monocytes were then resuspended in RPMI, supplemented with 10% FBS, 2% L-glutamine, 1% HEPES, 50μM β-mercaptoethanol and 1% penicillin-streptomycin, counted with trypan blue and seeded in 6-well culture plates at a concentration of $1x10^6$ cells/mL. In order to differentiate the monocytes into MDMs and MDDCs, specific cytokines were added to the cells in culture. To obtain MDDCs, GM-CSF and IL-4 were added and to have MDMs, either GM-CSF or M-CSF was added as a means to originate M1 or M2 macrophages, respectively. All cell cultures were incubated at 37ºC with 5% CO_2 for 7 days. Every three days, half of the cell culture medium was changed.

2.3.3. Infection of MDDCs and MDMs with *S. epidermidis* strains

The MDDCs and both types of MDMs were lifted through up and down or with 5 mM EDTA, respectively. Then, cells were recovered from the 6-well plates, resuspended in RPMI (supplemented with 10% FBS, 2% L-glutamine, 1% HEPES and 50μM β-mercaptoethanol) and counted with trypan blue. Afterwards, the MDDCs were seeded in 96-well plates with round bottom; while the MDMs were seeded in flat bottom ones. Both types of human cells were infected with each of the strains at a MOI of 1DC:10B or 1M:10B. Some cells were not stimulated (negative control) and others were stimulated with LPS (positive control). Next, they were incubated during at 37ºC and 5% CO_2. After 2h, 50 μg/mL gentamicin was added to each well to stop the reaction, by killing the bacteria, and the plate was again incubated at 37ºC and 5% CO_2. Finished a 24h incubation period, the plates were centrifuged (300*g*, 10 min) and the supernatants were collected and stored at -80ºC until measurement of cytokine secretion by ELISA. Cells were prepared for activation assays by flow cytometry.

2.3.4. Estimation of cytokine release through sandwich ELISA

The secretion of TNF-α, IL-6, IL-8, IL-1β, IL-10 and IL-12p70, by the MDMs and the MDDCs infected with the strains of *S. epidermidis*, was quantified by sandwich ELISA. The methodology used was in accordance with the manufacturer's protocol (Human TNF-α DuoSet® ELISA Development System, Human IL-6 DuoSet® ELISA Development System, Human IL-8 DuoSet® ELISA Development System, Human IL-1β DuoSet® ELISA Development System, Human IL-10 DuoSet® ELISA Development System and Human IL-12p70 DuoSet® ELISA Development System from R&D systems).

2.3.5. Evaluation of MDDCs activation by FACS

To detect cell surface activation markers, MDDCs were analyzed by flow cytometry, using a BD FACSCanto™ II. The human cells infected with the *S. epidermidis* strains were collected, following the 24h incubation, stained with FVD and antibodies against the activation markers [anti-human CD11c-APC conjugated (clone BU15), anti-human HLA-PECy7 conjugated (clone L243), anti-human CD83-FITC conjugated (clone HB15e), anti-human CD14-PE conjugated (clone 61D3), anti-human CD80-BV510 conjugated (clone 2D10) and anti-human CD86-PECy5 conjugated (clone IT2.2)], fixed with PFA and resuspended in FACS buffer.

3. Biofilm production by *S. epidermidis* strains

3.1. Biofilm development

For the formation of biofilms, on day 1, the strains were allowed to grow overnight as liquid cultures according to the conditions referred on 1.1. On day 2, overnight cultures were prepared as detailed on 1.1. Accordingly, they were centrifuged (4000 rpm, 10 min), resuspended in PBS 2% human serum, filtrated and, at last, their OD_{640nm} was adjusted (WT-0.25; ΔT-0.50; ΔT::T-0.50). CFUs of each strain were prepared, to confirm, on the next day, if the cell number between strains was identical. Completed the preparation steps of the bacterial strains, 1,5μL/well of each bacterial suspension was seeded in a 96-well plate, with 150μL/well of TSB supplemented with 0,4% glucose and 10% human serum. The medium of the ΔT::T was also supplemented with 10μg/mL Cm. Next, they were incubated at 37ºC and 80 rpm for 24h. On day 3, finished the 24h incubation period, spent media were discarded, biofilms were washed twice with PBS1x, and fresh medium was added to the biofilms. Negative controls, with only TSB+0.4% glucose+10% human serum, were made at both 0h and 24h of biofilm formation. The plate was incubated again at 37ºC and 80 rpm, during 24h more, as a way of obtaining a biofilm grown for 48h. On the last day of biofilm formation, day 4, the suspensions of the biofilms were discarded, since those suspensions hold the cells released from the biofilms. Biofilms were washed twice with PBS1x, resuspended in this buffer, and removed from the bottom of the wells with a 1 mL syringe plunger. Subsequently, each biofilm suspension was recovered to an eppendorf and further sonicated (10s, mode 3), to disaggregate the biofilm cells.

3.2. Comparative assay between strains: biomass quantification and biofilm cell count

Two biofilms of each *S. epidermidis* strain were developed, following the methodology described in 3.1. After following all the protocol up to the sonication step, the ODs_{640nm} of biofilms formed by

each strain were measured. Finally, CFUs of those samples were prepared and grew at 37°C for approximately 24h, time upon which they were counted.

4. Statistical Analysis

For result interpretation, gathered data were analyzed in GraphPad Prism (version 8.0.2, Windows 10) and represented as mean ± standard deviation (SD). The one-way ANOVA with Tukey test was used for statistical analysis. Divergence between different groups was regarded as statistically significant for: *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Chapter 4

Results and Discussion

1. Characterization of *S. epidermidis* strains

1.1. Growth curve

Bacterial cells can be grown in liquid cultures in which cell proliferation can vary among different bacterial species or within strains of the same species. Growth curves were determined for all three *S. epidermidis* strains (WT, ΔT and ΔT::T) used herein, under the same growth conditions. Bacteria were allowed to grow, and a sample of each strain was recovered at different time points (0h, 1h, 2h, 3h, 4h, 5h, 6h, 7h, 8h and 24h) for OD_{640nm} measurements (Figure 5).

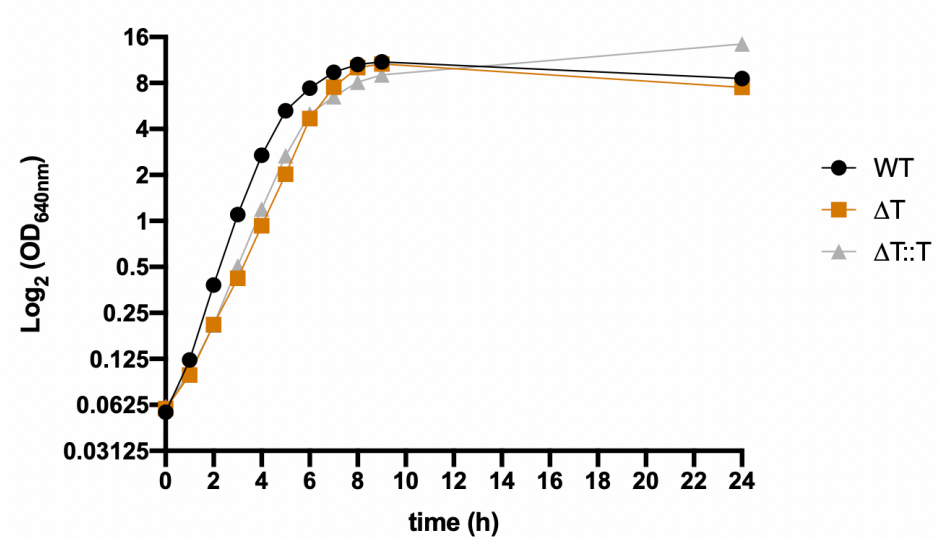


Figure 5 – Growth curve representative of each *S. epidermidis* strain. Each strain (WT, ΔT and ΔT::T) was allowed to grow in culture medium at 37ºC and 180 rpm. The growing process occurred up to 24h and, at the indicated time points, an aliquot of each strain was collected and their OD_{640nm} was measured. The results displayed are representative of two experiments.

All *S. epidermidis* strains show an exponential growth, approximately until 8 hours (exponential phase). During this time, the WT strain was the one presenting a higher growth rate, whereas the ΔT and ΔT::T strains showed similar growth rates. After the 8h time point, the curves reached the early stationary phase of growth. At the 24 hour time-point, the ΔT::T strain appears to have a multiplication rate higher than the death rate, while the opposite seems to happen with the WT and the ΔT strains.

1.2. Optimization of growth conditions

The conditions to which bacteria are subjected influence their multiplication and cell count. Hence, both the conditions that they are under, while growing as liquid cultures, and after, when their cultures are being prepared for experimental purposes, affect the bacterial proliferation and their biomass concentration. Furthermore, *S. epidermidis* bacteria tend to aggregate, an intrinsic feature of this microorganism. Hence, minimizing its aggregation would help having more precise quantifications of biomass quantification and CFU or cell count, necessary to establish accurate inoculum doses.

In order to determine the best growth conditions for the *S. epidermidis* strains under study, to be employed for all the experiments, the three strains were grown overnight and further prepared for an optimization test. As presented in Figure 6, the three conditions studied were: i) growth in TSB and resuspension in PBS, both without supplementation, or ii) TSB + 10% BSA and PBS + 2% BSA, or iii) TSB + 10% human serum and PBS + 2% human serum. Although TSB and PBS buffer did not avoid aggregation, it was prevented using either BSA or human serum in the growth medium. For each strain, several samples were prepared with distinct OD_{640nm} values and the number of CFUs assessed.

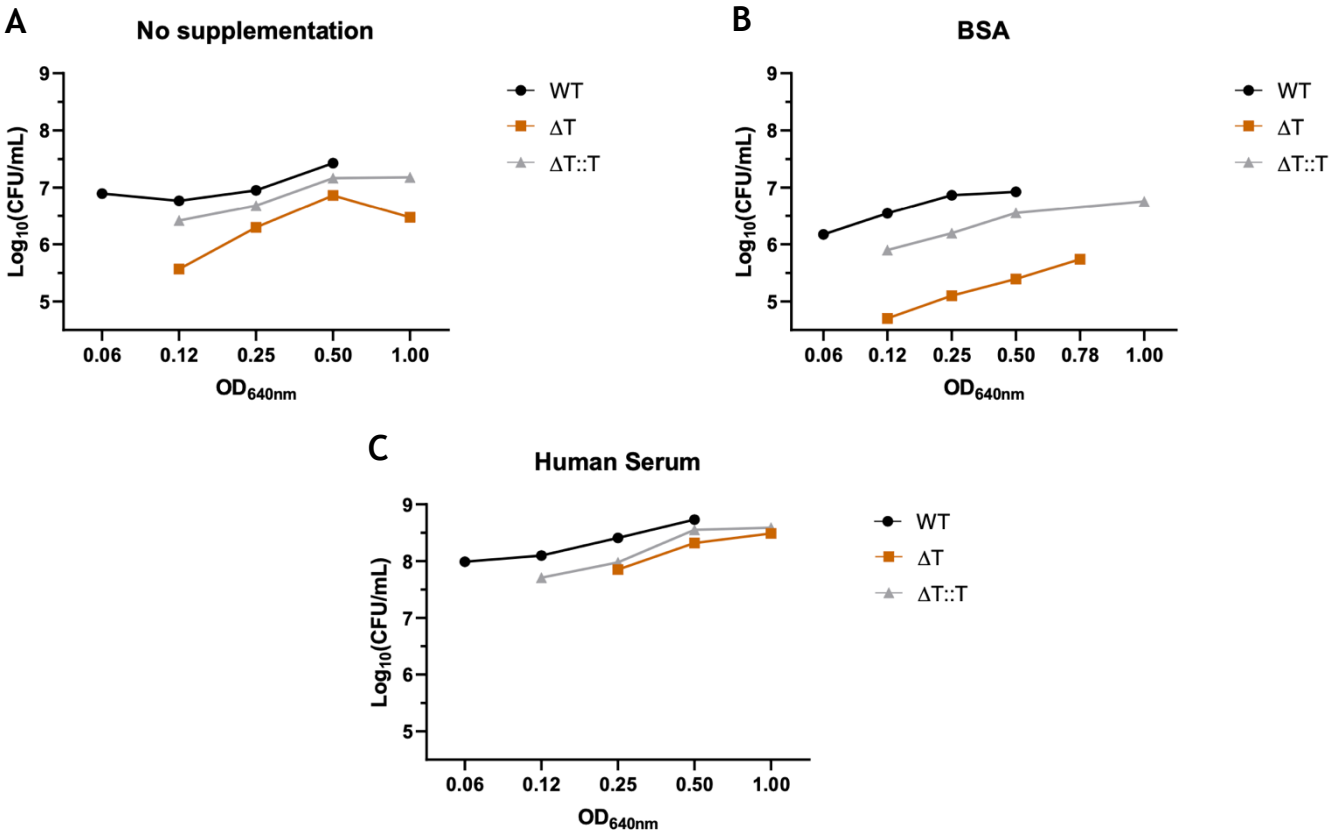


Figure 6 - Evaluation of *S. epidermidis* proliferation while under three distinct growth conditions: No supplementation and supplementation with BSA or human serum. The three strains were grown in liquid cultures at 37ºC and 180rpm until reaching their stationary phase. They were resuspended in a specific solution and the OD_{640nm} of each strain was measured. Three conditions were tested: growth and resuspension, respectively, (A) with TSB and in PBS (no supplementation), or (B) with TSB + 10% BSA and in PBS + 2% BSA, or (C) with TSB + 10% human serum and in PBS + 2% human serum. The OD_{640nm} of each strain was adjusted to multiple values: (WT- 0.06; 0.12; 0.25 or 0.50); (ΔT- 0.12; 0.25; 0.50 or 1.00) and (ΔT::T- 0.12; 0.25; 0.50 or 1.00). CFUs of all samples were counted after 24h.

From the three tested conditions, the one that used human serum yielded the highest log_{10}(CFU/mL) values, which means that these were able to have an enhanced growth. When comparing the non-supplemented and BSA conditions, the first one seems to have values of log_{10}(CFU/mL) slightly superior in all strains.

Specifically, TSB medium and PBS buffer, with no supplementation, were the least effective in preventing bacterial aggregation. Apart from this, they did not grant a consistent growth, as shown by the results presented in Figure 6 A. In that regard, the log_{10}(CFU/mL) values of the ΔT::T strain increased as the OD_{640nm} values rose, even with a low difference between the ODs_{640nm} of 0.50 and 1.00. In contrast, the WT and the ΔT strains showed both increases and drops of log_{10}(CFU/mL) while the OD_{640nm} increased. This is not an accurate result, once the log_{10}(CFU/mL) was expected to rise as the OD_{640nm} increased. The possible explanation for these variations may have to do with the bacterial aggregation that could then translate in erroneous quantifications of the biomass concentration and, subsequently, misleading CFU counts.

In the condition with BSA (Figure 6 B), the mutant strain showed a highly impaired growth as compared to the other strains. In fact, the highest log_{10}(CFU/mL) value, corresponding to the highest OD_{640nm} (0.78), that this strain was able to reach, was still lower than the lowest values of log_{10}(CFU/mL) attained by the WT and the ΔT strains. As a matter of fact, the growth of the ΔT was so reduced that it was not possible to study a sample of ΔT with an OD_{640nm} of 1.00 and one with an OD_{640nm} of 0.78 had to be used instead. This would then constitute a problem, since it would affect the choice of an OD_{640nm} value for each strain that could permit a common cell count among the three used.

Finally, concerning the condition in which human serum was used for supplementation (Figure 6 C), besides hindering the formation of *S. epidermidis* aggregates, it presents the highest log_{10}(CFU/mL) values, thus potentiating the growth of the three strains. It led to accurate results that demonstrate an increase of log_{10}(CFU/mL) as the values of ODs_{640nm} rose. Moreover, the growth among the three strains, although not equivalent, showed small divergences. For all these reasons, the condition with the human serum revealed to be the best one for the growth of *S. epidermidis* strains.

1.3. Optimization: CFU count for normalization between strains

Bacterial proliferation and, therefore, their biomass quantity and cell count, are variable among bacteria, as they are between strains, such as the three strains used here (WT, ΔT and ΔT::T).

In the previous experiment, each *S. epidermidis* strain suspension was adjusted to specific OD_{640nm} values [(WT- 0.06; 0.12; 0.25 or 0.50); (ΔT- 0.12; 0.25; 0.50 or 1.00) and (ΔT::T- 0.12; 0.25; 0.50 or 1.00)] and CFUs in each sample were determined. Hence, in parallel to the optimization previously presented, that assay was carried out with the intent of identifying the OD_{640nm} value that each strain should be adjusted to, in order to attain an equivalent cell count between distinct strains. This would subsequently enable the normalization of cell count and of experimental results, by preventing the problem of strain cell count variation. Once the human serum condition proved to be the optimal choice for *S. epidermidis* growth, only those results were considered herein. A duplicate experiment using solely human serum, which replicated the parameters used in the human serum condition of the first test, was performed to confirm the results obtained in the first experiment. The means of the results obtained for each strain are displayed in Figure 7.

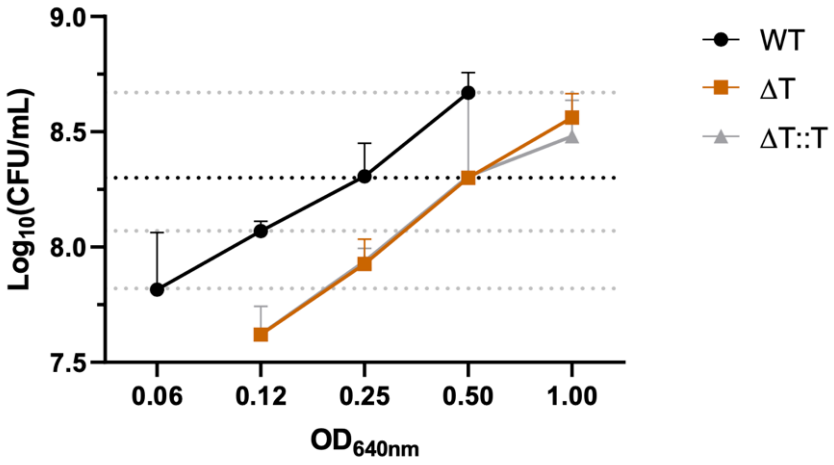


Figure 7 – Identification of the strains OD_{640nm} value for CFU count normalization. The three strains were grown in TSB + 10% human serum at 37ºC and 180rpm until reaching their stationary phase. They were resuspended in PBS + 2% human serum and the OD_{640nm} of each strain was measured and adjusted to several values: (WT- 0.06; 0.12; 0.25 or 0.50); (ΔT- 0.12; 0.25; 0.50 or 1.00) and (ΔT::T- 0.12; 0.25; 0.50 or 1.00). CFUs of all samples were counted after 24h. Grey dotted lines show the log_{10}(CFU/mL) values of the WT, to which the other strains do not show equivalent values. The black dotted line represents the log_{10}(CFU/mL) value that is common among the three strains. The experiment was conducted twice (n=2). Mean ± SD values are represented.

The grey dotted lines (Figure 7) represented on the graphic show that for those established log_{10}(CFU/mL) values of the WT, neither the ΔT nor the ΔT::T achieved an equal value. Then, a high log_{10}(CFU/mL) difference is identified between strains. In contrast, the black dotted line (Figure 7) evidences that the samples with ODs_{640nm} of 0.25, 0.50 and 0.50, respectively for the WT, ΔT and ΔT::T, show practically no divergence between their log_{10}(CFU/mL) values. Therefore, this set of ODs, which leads to a log_{10}(CFU/mL) of approximately 8.30 and, thereby, a CFU/mL of around $2x10^8$, appears to be the optimal choice to obtain the same concentration of cells in all strains.

1.4. Confirmation of CFU optimization: bacterial cell count using flow cytometry

The optimization test using CFU counts, described above, was validated by an alternative approach, using flow cytometry.

The protocol used for bacterial cell count by flow cytometry required several optimizations. Once the procedure was fully optimized, the strains were grown until stationary phase, in TSB medium supplemented with human serum. Then, their ODs_{640nm} were adjusted as follows: WT-0.25; ΔT-0.50; ΔT::T-0.50 to further confirm that these conditions lead to an equal cell count between strains, as determined by the optimization with CFUs. Upon staining of samples with SYTO 9 and PI, they were analyzed in the flow cytometer and their cell concentration was determined. Controls (unstained, single SYTO and single PI) were used for each strain to subsequently apply color compensation in the analysis of results. The results obtained by flow cytometry are depicted in Figure 8, as well as the ones from the optimization test with CFUs, for comparison purposes.

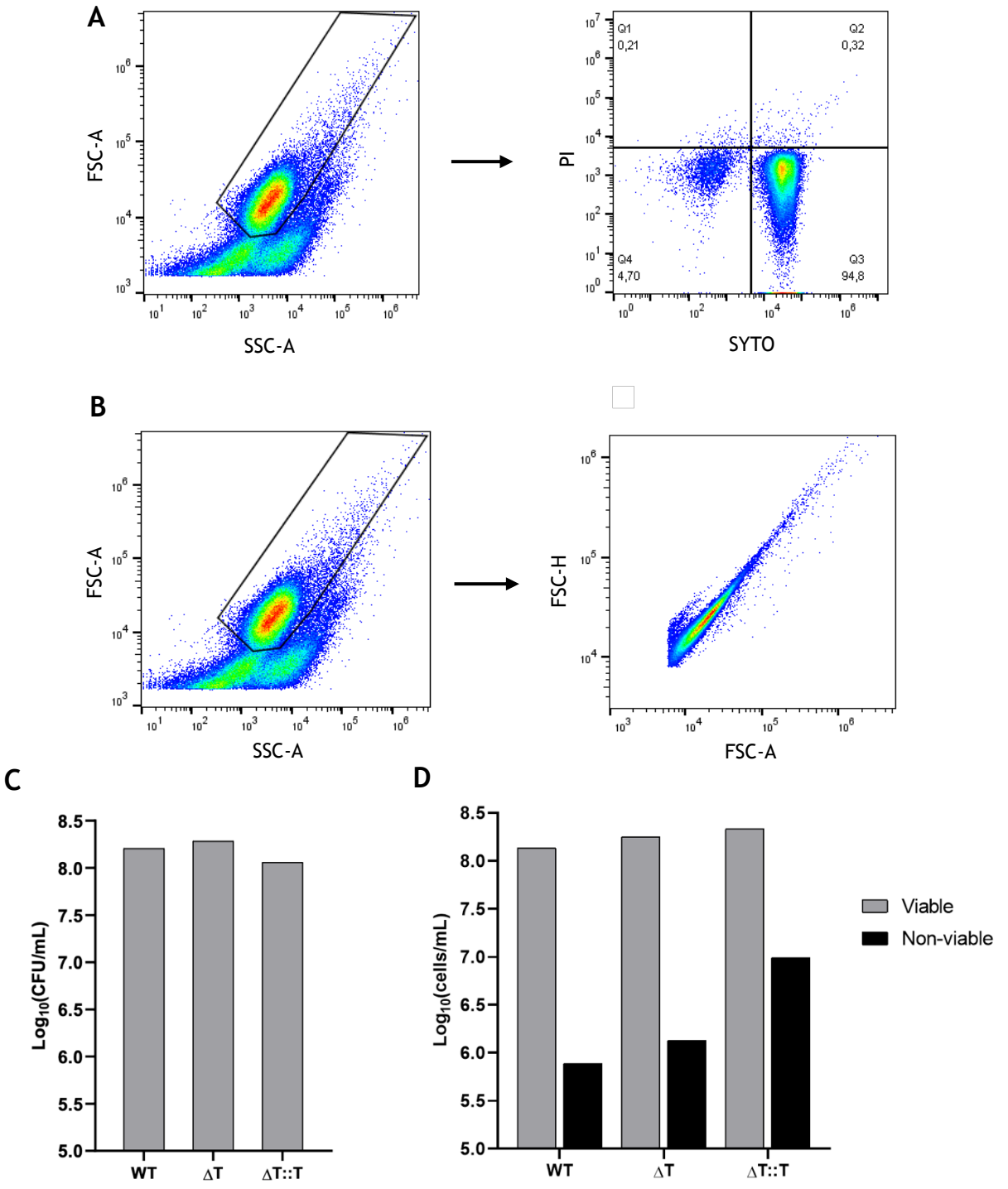


Figure 8 – *S. epidermidis* cell count by flow cytometry. All three strains were grown with TSB + 10% human serum at 37ºC and 180rpm until reaching their stationary phase. They were resuspended in PBS + 2% human serum and the OD_{640nm} of each strain was adjusted to: WT- 0.25; ΔT- 0.50 and ΔT::T-0.50. Samples were labelled with SYTO 9 and PI (LIVE/DEAD staining). Controls (unstained, single SYTO, single PI) were used for color compensation. Samples were analyzed and counted in the flow cytometer. A) Representative gating strategy. After gating the bacteria population (left), the viable cells ($SYTO^+/PI^-$) were selected (right). B) Illustration of single cells and bacterial aggregates. Next to selecting the bacteria population (left), single cells and few aggregates were identified (right). C) Log_{10}(CFU/mL) values obtained in each strain, after OD_{640nm} adjustment to either 0.25 or 0.50, in the CFU optimization test. D) Values of log_{10}(cells/mL) attained for each strain, with the adjusted ODs_{640nm}, in the flow cytometry assay, with representation of viable ($SYTO^+/PI^-$) and non-viable cells ($SYTO^-/PI^+$ and $SYTO^+/PI^+$).

In Figure 8 B is evident that mostly single cells and almost no bacterial aggregates were present in the samples, which can prove that the use of human serum may in fact be preventing the formation of *S. epidermidis* aggregates.

The cell number counted by flow cytometry appeared to be very similar between all three strains, despite some minor variations (Figure 8 D).

The log_{10}(CFU/mL) values obtained for each strain in the optimization test with CFU counts were equivalent to the values of log_{10}(cells/mL) attained by the viable cells ($SYTO^+/PI^-$) of each strain in the flow cytometry assay (Figure 8 C and D), especially for the WT and the ΔT strains. As for the ΔT::T, a small variance was noticed in its count.

Therefore, not only did the strains achieved similar counts in each optimization assay, but also showed identical counts when comparing both tests. Then, this strongly confirms that the set of ODs_{640nm} WT-0.25; ΔT-0.50; ΔT::T-0.50 is the ideal one to ensure the normalization of cell number among the three strains.

1.5. Morphological characterization

Bacteria include a wide and heterogeneous group of microorganisms, which can be very distinctive in terms of morphological characteristics, such as shape, size and structure. As a consequence, these differences modulate the behavior of bacteria, for instance how they interact with the surrounding environment. Thereby, morphological assays are always important when studying bacteria and/or different strains. This can be crucial for the interpretation of further experimental results, for instance related to the interaction with the host immune system, with which these microorganisms may act divergently.

In accordance, morphological studies were performed by TEM, using samples containing bacteria that were grown until attaining the exponential phase. As it is noticeable in Figure 9, bacterial cells were observed in a stage of cellular division, being even possible to identify many cell division phases.

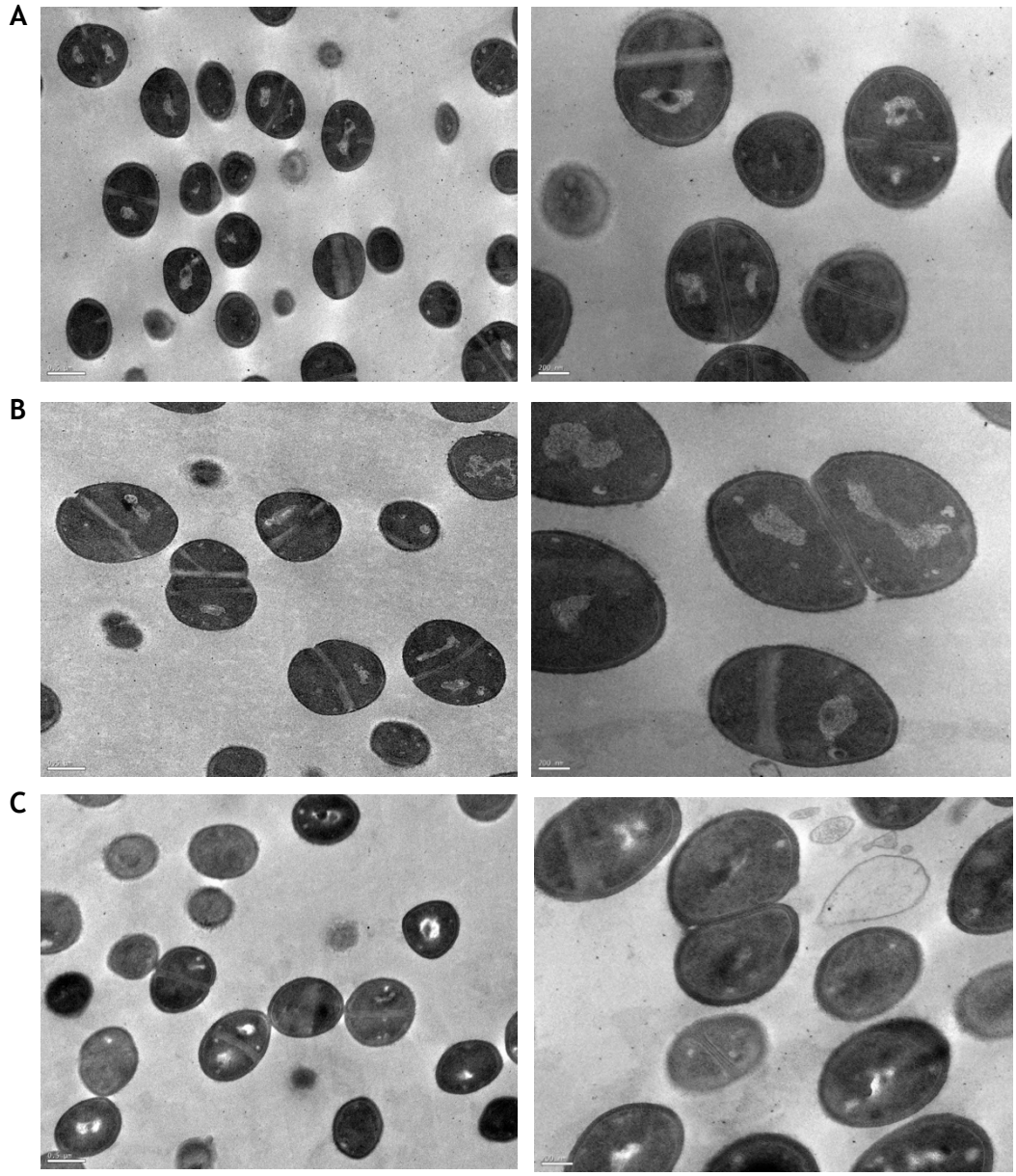


Figure 9 – Morphological analysis of *S. epidermidis* strains, WT, ΔT and ΔT::T. Each strain was grown until attaining the exponential phase. Images of each strain were obtained through TEM. (A) WT, Mag. 25000X (left), Mag. 50000X (right); (B) ΔT, Mag. 25000X (left), Mag. 50000X (right); (C) ΔT::T, Mag. 25000X (left), Mag. 50000X (right).

The TEM results confirm the spherical or ovoid shape of the three *S. epidermidis* strains, representative of coccus bacteria. Likewise, cells of the different strains display variable arrangements according to their division stage, which is a feature of cocci as well.

In relation to bacterial size, the WT and the ΔT::T strains appear to have a similar size. Nonetheless, the WTA-deficient strain appeared to be larger (Figure 9 B), than both the WT and the ΔT::T. The differences in the cell wall, when comparing the mutant with the other two strains, may explain the size variations observed. The fact that the ΔT lacks the *tagO* gene would translate in the absence of WTAs in its cell wall. This deficiency in WTAs, affects the rigidity of the cell wall, since WTAs make up a large percentage of the cell wall, which shapes the cell. Then, the cell shape can be

dramatically changed and hence the change in size. Accordingly, the lack of WTAs may lead to a reorganization of the cell wall, which may result in an entirely different structure. Consequently, its components might be interacting differently, and some may even be more exposed in comparison with a cell wall rich in WTAs.

Another difference between the ΔT and the other two strains is that the mutant strain shows successive division planes frequently placed at nonorthogonal angles and duplicated septa (Figure 9 B). These distinct features recognized in the mutant may result, as well, from the lack of WTAs in its cell wall that can lead to its restructuring. Since the septum is the cell wall generated between two daughter cells during cellular division, variances in the septum may cause changes in the process of cell division. Consequently, alterations of cell division may directly affect the proliferation of bacteria and their growth. Accordingly, the defective septum of the mutant may explain the slower/lower growth of this strain, in comparison with the WT, as recognized on the results of section 1.1 of the current chapter.

In fact, it has been recognized in *B. subtilis* that interference in WTA biosynthesis increases the thickness of the bacterial cell wall and, consequently, the size of bacterial cells. In addition, it has shown to provoke septum defects, by altering their position or number, and then affect bacterial growth (Bhavsar, Beveridge, & Brown, 2001; Boylan, Mendelson, Brooks, & Young, 1972). These implications have also been reported in *S. aureus*, when the expression of WTA was blocked with tunicamycin (Santa Maria et al., 2014).

2. Innate immune response elicited by planktonic cells of *S. epidermidis* strains

2.1. Assessment of *S. epidermidis* phagocytosis by a murine macrophage-like cell line

Phagocytosis is a fundamental mechanism of the innate immune response generated in the host, following recognition of an infectious microorganism. This process generally consists in the internalization of the pathogen and its subsequent killing through oxidative or non-oxidative mechanisms inherent to the phagocytic cells. Infections developed by pathogenic *S. epidermidis* are no exception, once they have already proven to promote the phagocytic activity by host macrophages and neutrophils.

With the intent of determining whether the *tagO* mutation could affect phagocytosis of the bacterial strains WT, ΔT and ΔT::T, a murine macrophage cell line (RAW 264.7) was used. The macrophages were incubated with each one of the strains, at a MOI of (1M:10B), and phagocytosis was allowed to occur during three different time periods: 30, 60 and 180 minutes. Subsequently, intracellular CFUs were determined, enabling the quantification of the internalization of each strain by RAW 264.7 (Figure 10).

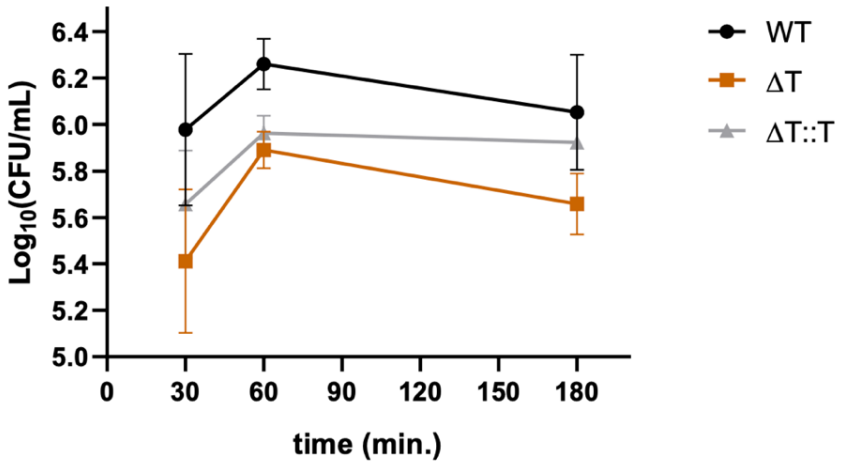


Figure 10 - Phagocytosis of WT, *Δ*T and *Δ*T::T strains of *S. epidermidis.* The internalization of the strains by RAW 264.7 macrophages was quantified through intracellular CFU counting. Macrophages were co-incubated with the strains at a MOI of (1M:10B) and the phagocytic process was allowed to occur for three different time periods: 30, 60 and 180 minutes. The assay was carried out in triplicate (n=3) and, in each experiment, each sample was analyzed in duplicate. Mean ± SD values are represented.

Pursuant to the results, the three strains demonstrate the same tendency. All of them reveal an increase of their $\log_{10}$(CFU/mL) counts from 30 min to the 60 min, and a decrease of the same parameter from 60 min to 180 min. This means that the number of internalized bacteria rises, up to 60 min of phagocytosis, but at a certain point, it begins to drop, indicating intracellular control inside the macrophages.

When comparing the phagocytic process for each strain, it is clear that they present different predispositions to be internalized. The WT strain appears to be the most susceptible one, followed by the ΔT::T strain. As for the ΔT strain, of all three strains, it is the less internalized one, thus evidencing the lowest $\log_{10}$(CFU/mL) values in all assessed time-points.

The percentage of phagocytosed cells was calculated through the division of the internalized bacteria with the concentration of bacteria seeded with the RAW 264.7 macrophages. It was estimated for each strain and at each time point (30 min, 60 min and 180 min). The estimated results are represented in Figure 11.

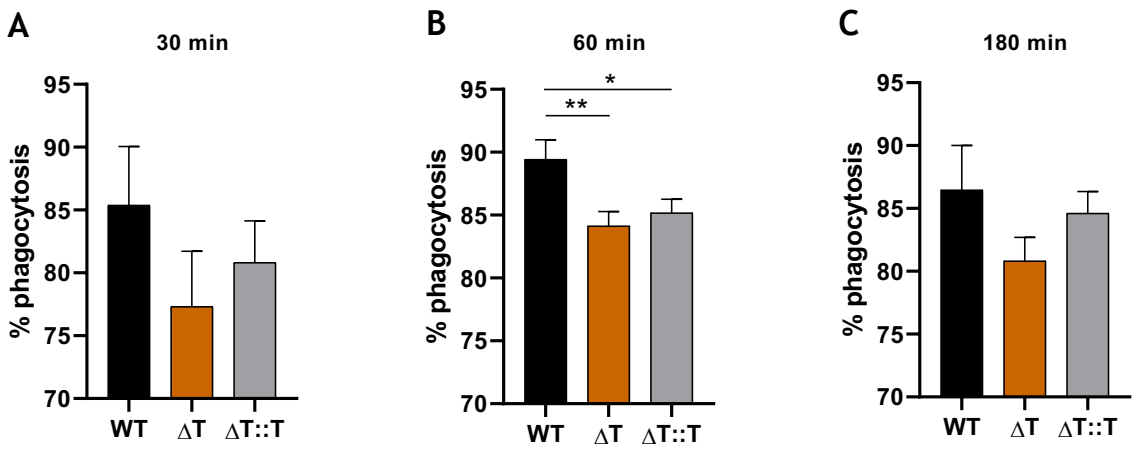


Figure 11 – Percentage of phagocytosed cells of each *S. epidermidis* strain. The internalization of each strain by RAW 264.7 macrophages was estimated through intracellular CFU counting. Macrophages were incubated with the strains at a MOI of (1M:10B) and phagocytosis was evaluated for a duration of (A) 30 min, (B) 60 min and (C) 180 min. The experiment was carried out in triplicate and, in each one, each condition was examined in duplicate. Percentage of phagocytosed cells was calculated through the division of the internalized bacteria at each time point with the concentration of bacteria seeded with the RAW 264.7 macrophages. Mean ± SD values are represented. For statistical analysis, the one-way ANOVA with Tukey test was used. (*P<0.05 and **P<0.01).

Once again, the mutant strain can be easily recognized as the least internalized by the RAW 264.7 macrophages, due to its lowest phagocytosis percentage at all three time points. In fact, the differences in internalization between the ΔT and the WT strains were shown to be statistically significant after 60 min of phagocytosis (Figure 11 B).

When comparing the percentages of phagocytosis for each strain over the three time periods, it is evident that in all strains the percentage increases from the 30 min to the 60 min time-point and diminishes from the latter to the 180 min one (Figure 11), corroborating the tendency already seen in Figure 10.

The result showing the mutant as the less phagocytosed strain proves that it is less susceptible to internalization by macrophages. This may result from an impaired recognition of this particular strain by specific PRRs. A possible role of WTAs in recognition of *S. epidermidis* and/or stimulation of the subsequent phagocytosis mechanisms can be hypothesized. Then, because the mutant strain does not possess WTAs in its cell wall, its recognition and phagocytosis would be impaired, while the WT is more easily recognized and internalized by the phagocytic cells.

On the other hand, the lack of WTAs may lead to a reorganization of the cell wall, as suggested by TEM results. This could result in the exposure of some constituents that were previously concealed, or the other way around. This could explain the results shown by the mutant, because, if a constituent, with the capacity of inhibiting recognition or phagocytosis, was more exposed in the mutant, its internalization would be diminished.

In contrast, if a component responsible for its recognition became hidden, as a consequence, the internalization of this microorganism would be impaired. For instance, the PG, LTAs and lipoproteins, all constituents of the cell wall of *S. epidermidis*, have shown to be important for the recognition of this bacterium by specific PRRs, such as TLR-2 (Fournier, 2013). Hence, if one of these components were to be less approachable in the ΔT, its recognition would be disturbed, as would be the activation of phagocytosis mechanisms downstream.

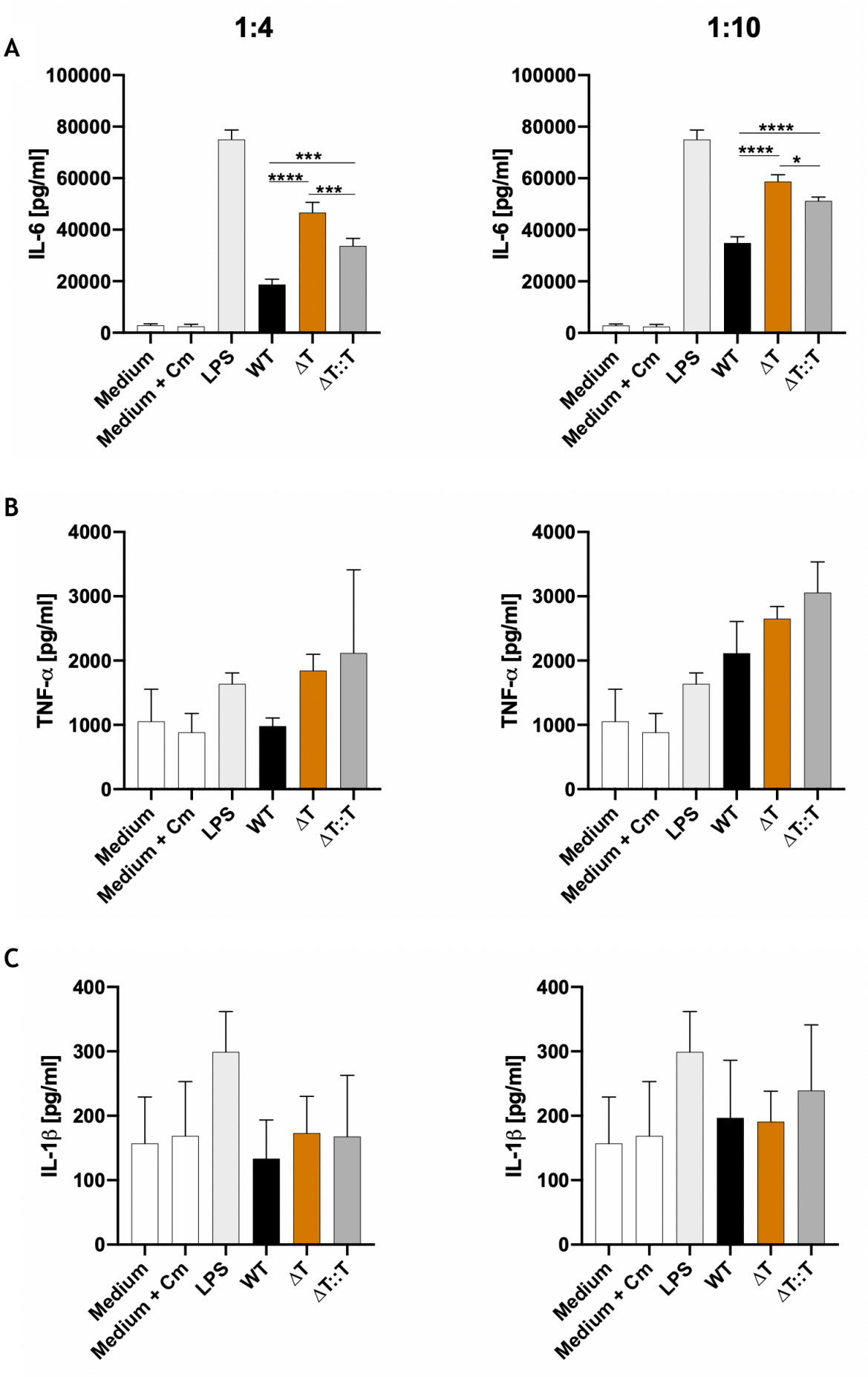
As for the reduction of internalized bacteria, identified on all three strains from the 60 to the 180 minutes of phagocytosis, it certainly results from the death of the bacteria. Several killing mechanisms, intrinsic to the phagocytic cells, may have occurred. Indeed, macrophages have proven to be capable of internalizing and killing *S. epidermidis* through similar mechanisms to the ones used by neutrophils, which include production of ROS, AMPs and proteases (Riool et al., 2014; Sabaté Brescó et al., 2017).

2.2. Analysis of mouse BMDCs following infection with strains

2.2.1. Measurement of cytokine release by sandwich ELISA

Once a pathogen is recognized by PRRs, signaling and effector mechanisms are triggered in the host immune cells. The production of cytokines by a broad range of cells is included in those mechanisms and significantly modulates the emerging innate and adaptive responses. Following infections by *S. epidermidis*, cytokine secretion by immune cells has been reported and some cytokines may actually be crucial in the defense against these infections.

Fittingly, to assess cytokine production, studies with infected DCs were performed. After the co-incubation of BMDCs from BALB/c mice with the strains of *S. epidermidis* (WT, ΔT, ΔT::T), at a MOI of (1DC:4B) and (1DC:10B), culture supernatants were recovered after 24h, and cytokine secretion by the DCs was estimated by ELISA. In this assay, the production of the pro-inflammatory cytokines IL-6, TNF-α, IL-1β, IL-12p70 and anti-inflammatory IL-10 was assessed. Negative controls, with BMDCs incubated with either medium alone, or medium supplemented with Cm, and a positive control, with cells incubated with LPS, were used. The levels of cytokines produced by the BMDCs at both MOI are displayed in Figure 12.


A
1:4
1:10
IL-6 [pg/ml]
100000
80000
60000
40000
20000
0
***
****
***
****
****
*
Medium
Medium + Cm
LPS
WT
ΔT
ΔT::T
B
TNF-α [pg/ml]
4000
3000
2000
1000
0
C
IL-1β [pg/ml]
400
300
200
100
0

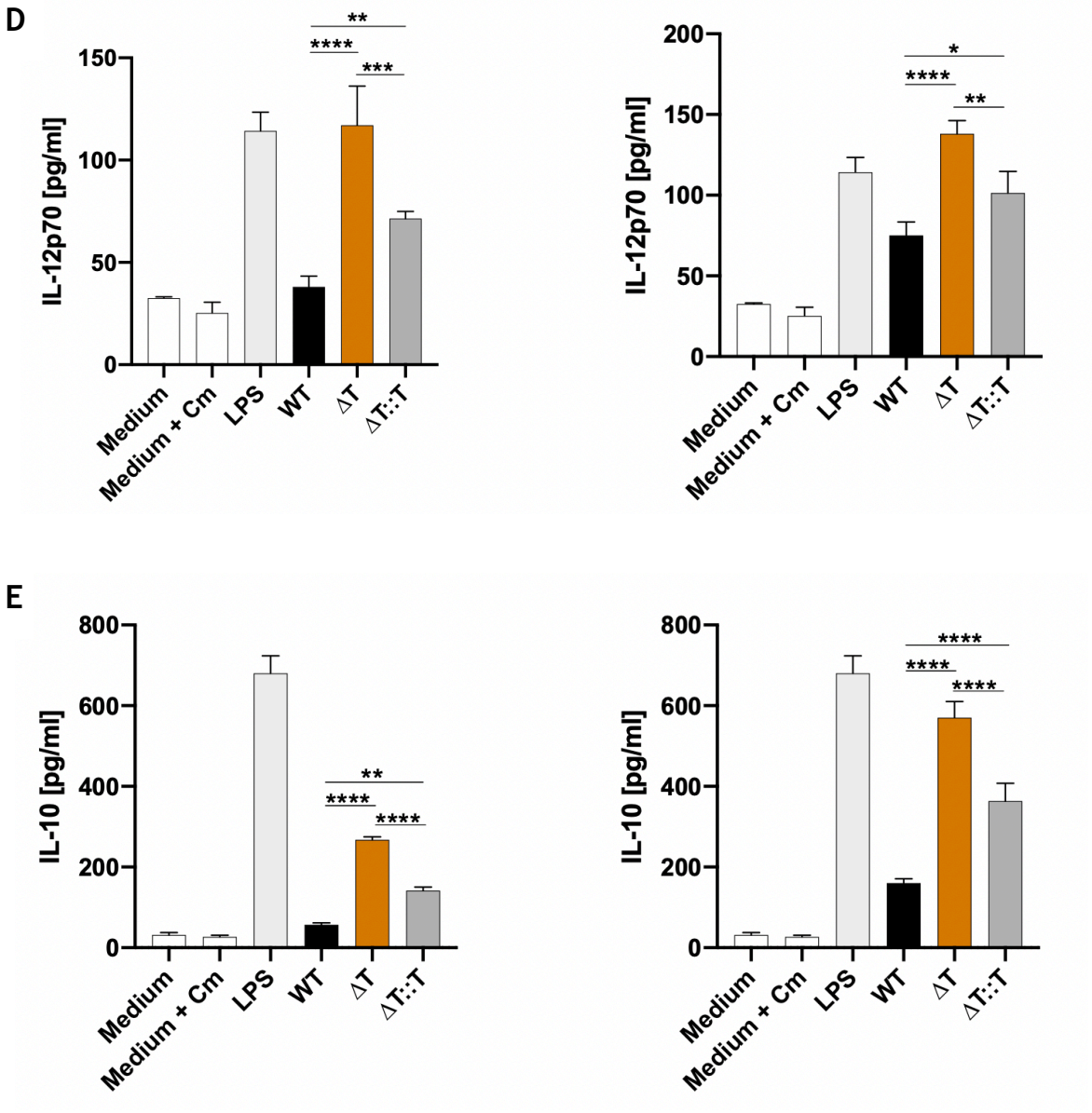


Figure 12 – Assessment of cytokine production by BMDCs from BALB/c mice. BMDCs from BALB/c mice were infected with the three strains at a MOI of (1DC:4B) and (1DC:10B). Samples with DCs incubated with medium or medium plus Cm (negative controls) and with LPS (positive control) were made. After 24h, the culture supernatants were recovered. The levels of (A) IL-6, (B) TNF-α, (C) IL-12p70, (D) IL-1β and (E) IL-10 in the supernatants were quantified by ELISA. The results presented are representative of three experiments (n=3), in which each sample was done in triplicate. Mean $\pm$ SD values are represented. For statistical analysis, the one-way ANOVA with Tukey test was used. (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001). To simplify, only statistical significance between strains was illustrated.

In general, the WT strain was the one inducing the lowest levels of all cytokines, both pro- and anti-inflammatory (Figure 12). The ΔT strain was the one that led to the highest levels of the pro-inflammatory cytokines IL-6 and IL-12p70 at both MOI. The same was found for the production of the anti-inflammatory cytokine IL-10. Notably, the difference in the production of these three cytokines between the WT and the ΔT strains is statistically significant. (Figure 12 A, D and E) As for TNF-α, the ΔT led to a slightly increased, but not significant, secretion of this pro-inflammatory cytokine, at both

MOI (Figure 12 B). Lastly, no significant differences were observed in the induction of IL-1β (Figure 12 C). In fact, the WT and ΔT strains led to equivalent levels of this pro-inflammatory cytokine.

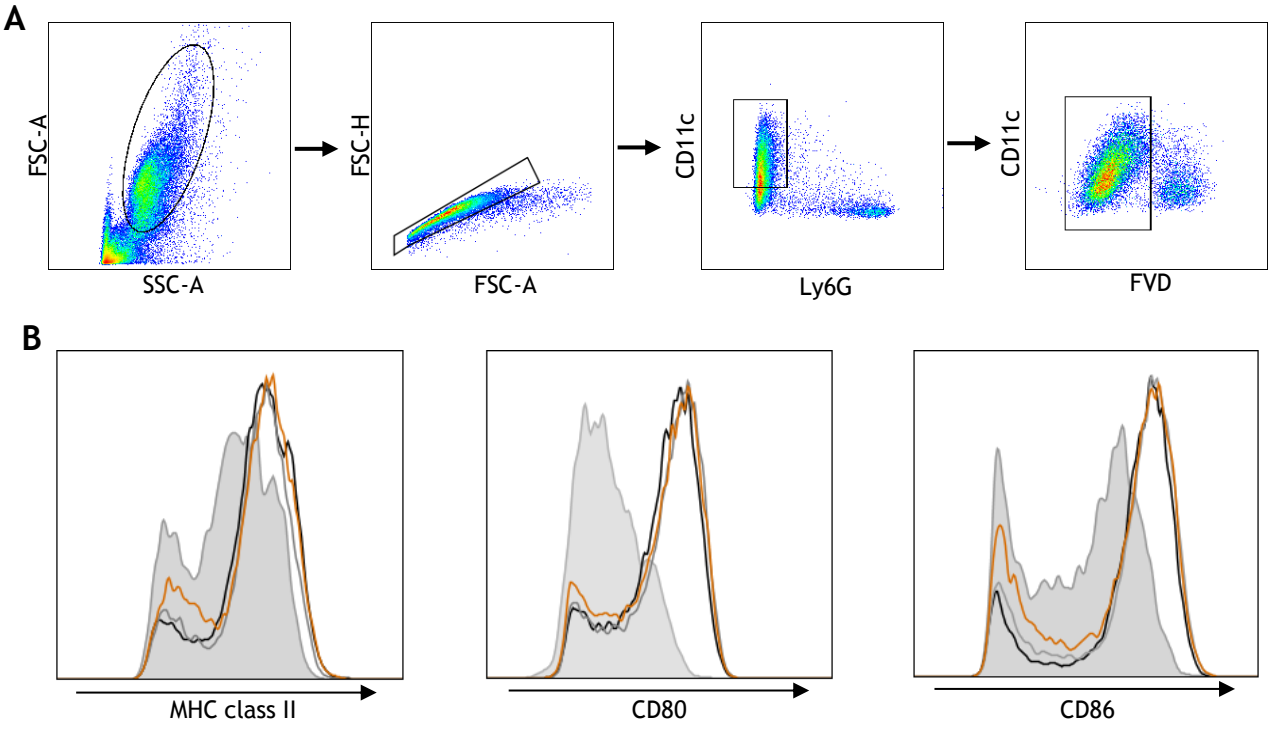
The inflammatory profile of the mutant strain, characterized by induction of higher levels of pro- and anti-inflammatory cytokines than the WT, may indicate an indirect influence of WTAs in cytokine production by mouse DCs. The lack of WTAs in the ΔT may impact its cell wall and cause its restructuring. This may lead to the exposure of more inflammatory components. These constituents may then promote recognition of the mutant by specific PRRs and/or provoke an increased cytokine production by DCs.

Another possible explanation would be that WTAs may be involved in evasion to recognition and/or cytokine production by mouse DCs. This would explain why the WT strain leads to lower levels of secreted cytokines, in comparison with the mutant. Indeed, in *S. aureus*, WTAs have already been recognized in immune response evasion mechanisms. This bacterium was shown to alter WTAs, via D-alanylation, to prevent the action of AMPs (Peschel et al., 1999), which is a mechanism already recognized in *S. epidermidis* (Otto, 2009). Therefore, it would be reasonable to assume that WTAs could be involved in other evasion mechanisms.

2.2.2. Evaluation of BMDC activation by flow cytometry

Dendritic cells play a crucial and acknowledged part in translating innate to adaptive immune responses against pathogens. After the recognition of the infectious microorganism through PRRs, DCs demonstrate an upregulated expression of MHC and co-stimulatory molecules, such as CD80 and CD86, markers that indicate activation and maturation of these cells. Afterwards, DCs are able to activate T cells, through presentation of the pathogen antigens, and further induce antibody-specific responses.

With the purpose of assessing the activation of BALB/c mice BMDCs, challenged with the *S. epidermidis* strains at a MOI of (1DC:4B) and (1DC:10B), after 24h, the DCs were stained with FVD and monoclonal antibodies specific for mouse DC activation markers, and were analyzed by flow cytometry. Regarding this, the expression of the activation markers MHC class II, CD80 and CD86, at the surface of mouse DCs, was evaluated, as shown in Figure 13.



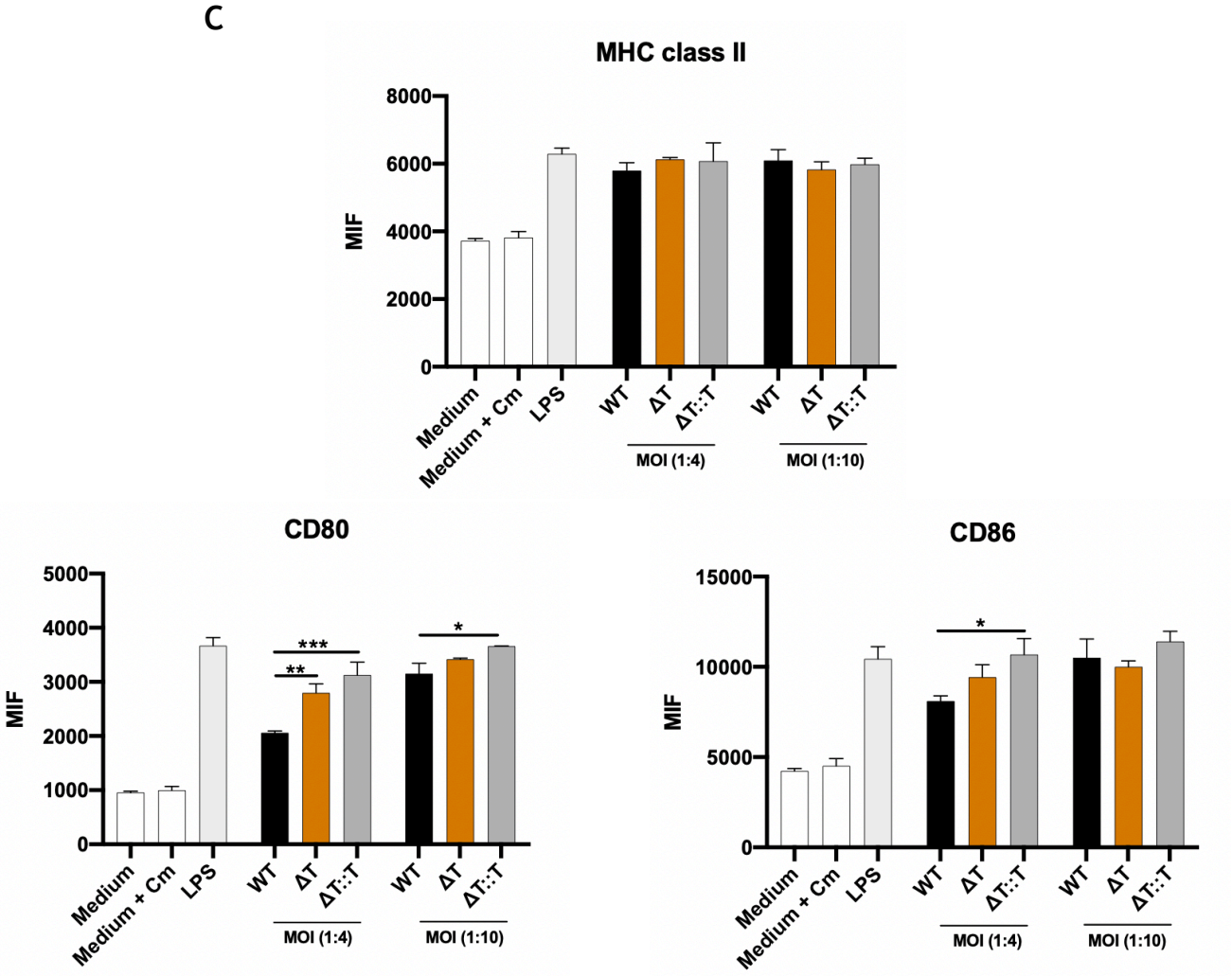


Figure 13 – Activation/maturation of BALB/c mice BMDCs. BALB/c mice BMDCs were infected with the strains at a MOI of (1DC:4B) and (1DC:10B). Negative controls, with DCs co-incubated with medium or medium+Cm, and a positive control, with DCs incubated with LPS, were used. After 24h, the cells were labelled with FVD, anti-CD11c-FITC conjugated, anti-F4/80-APC/Cy7 conjugated, anti-Ly6G-AF647 conjugated, anti-MHC class II-PerCP conjugated, anti-CD80-PE conjugated and anti-CD86-PECy7 conjugated. Cells were analyzed by flow cytometry. A) Representative gating strategy. After gating the population of interest (first), single cells were selected (second), in which dendritic cells ($CD11c^{+}Ly6G^{-}$) were chosen (third) and live cells (FVD^{-}) were considered (fourth). B) Representative histogram overlays of each activation marker under study, with illustration of non-stimulated BMDCs (grey witch filling) and BMDCs infected with the WT (black line) ΔT (orange line) and ΔT::T (grey line) strains at MOI (1DC:10B). C) Quantification of the expression of mouse BMDC activation markers. The results presented are representative of three experiments (n=3), in which each sample was done at least in duplicate. Mean ± SD are represented. For statistical analysis, the one-way ANOVA with Tukey test was used. (*P<0.05, **P<0.01 and ***P<0.001). To simplify, only statistical significance between strains was illustrated.

According to Figure 13 B, some BMDCs were already expressing MHC class II and CD86, prior to infection with the strains. This may have happened due to the process of cell detachment from the culture plates, which may have caused stress and subsequent DC activation.

Nevertheless, BMDCs infected with *S. epidermidis*, regardless of the strain, led to an increased expression of the activation markers, MHC class II, CD80 and CD86, at both MOI, in comparison with

non-stimulated BMDCs (Figure 13 B and C), which were only incubated with medium. Therefore, all strains were able to successfully activate and induce maturation of BMDCs.

In Figure 13 C, when comparing the WT and ΔT strains, no significant differences are evident in their expression of either MHC class II or CD86. When it comes to MHC class II, its expression is identical between these strains at both MOI. As for the CD86, a small difference can be noticed at MOI (1:4), in which the ΔT strain demonstrates a slightly increased expression of CD86, when comparing with the WT. Lastly, in terms of CD80, although at MOI (1:10) the ΔT shows an insignificant increase of its expression, at MOI (1:4) it has a significantly higher expression of CD80 than the WT.

With these results, it was shown that all three strains of *S. epidermidis* can induce the expression of MHC class II and the co-stimulatory molecules CD80 and CD86 at the surface of mouse DCs, thus activating these cells. As a matter of fact, DC activation, with expression of these activation markers, after infection with *S. epidermidis*, has already been proven in *in vitro* and *in vivo* studies (F. Cerca et al., 2014; Sabaté Brescó et al., 2017).

WTAs probable do not display an essential role in mouse DC activation, since no significant divergences in the expression of both MHC class II and CD86 were demonstrated between the WT and ΔT strains. Other constituents of *S. epidermidis* may be involved in this mechanism and be the key for the generation of this type of immune response.

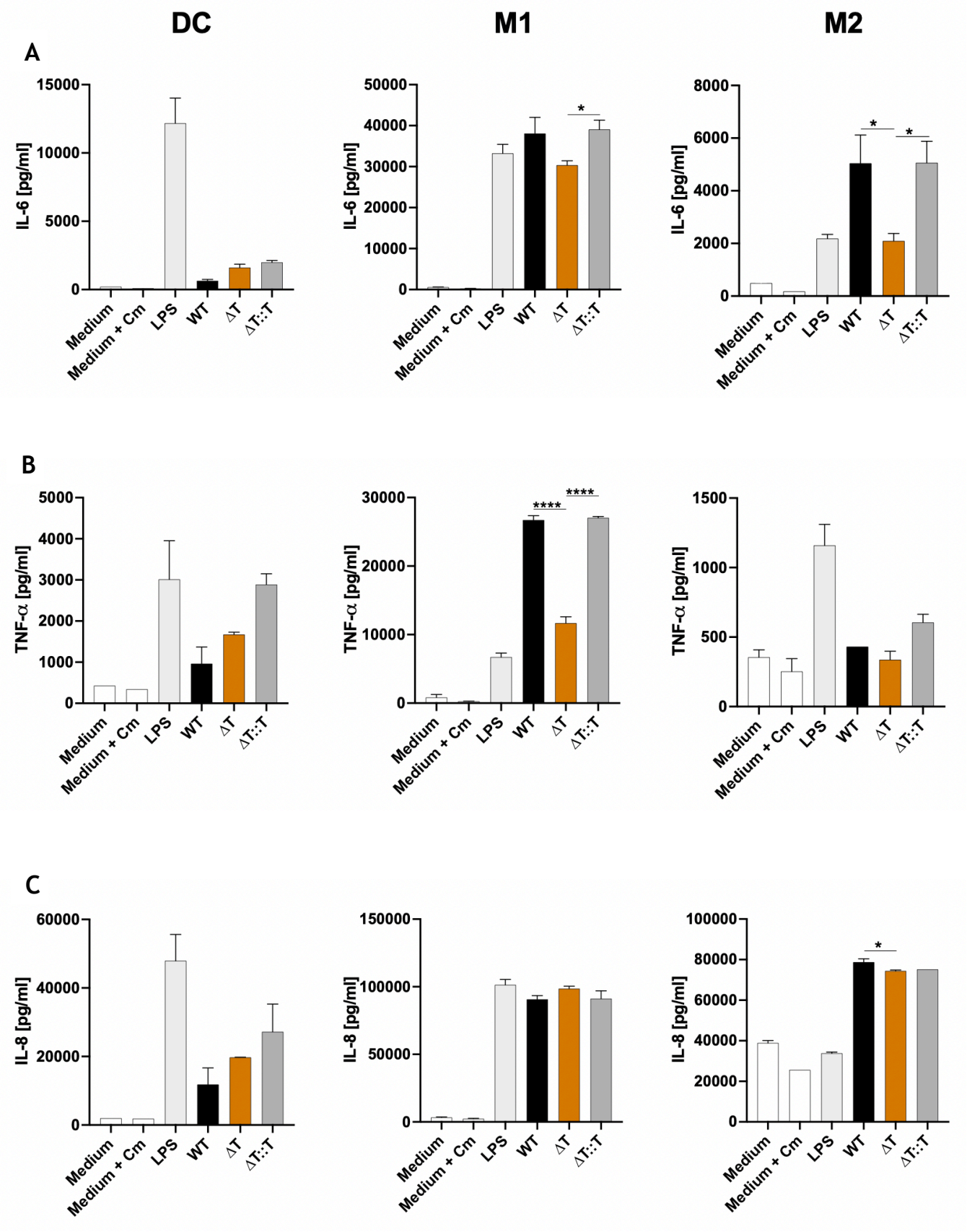
Nonetheless, a significant increase in the expression of CD80 was detected in the ΔT, in comparison with the WT. Thus, the mutant appears to lead to a greater stimulation of DCs, as observed in the ELISA assay. A possible explanation for this difference in significance between markers could be a late detection of DC activation. Perhaps the activation of mouse BMDCs should have been evaluated at a sooner time point, instead of 24h later. After this time, DCs might have already decreased their expression of MHC class II and CD86. Thus, an optimization of the protocol or a kinetic study should be performed to truly confirm if the presence/absence of WTAs has an impact or not in mouse DC activation. This approach was employed in a study of BMDC activation, following stimulation with *S. epidermidis*. A sooner time point of 6h was used and it allowed a better detection of differences in the expression of these markers between strains (F. Cerca et al., 2014).

2.3. Study of human MDDCs and MDMs after infection with strains

2.3.1. Quantification of cytokine secretion by sandwich ELISA

Following the study in murine cells, the secretion of cytokines in response to *S. epidermidis* was evaluated with human cells. Assays using mouse cells are usually employed before the ones with human cells and can provide crucial and accurate insights, since mice and humans share multiple similarities in terms of genetics, anatomy and physiology. Nonetheless, the disparities they evidence cannot be ignored, because they may result in different immune responses after infection. Thus, the results obtained with mouse cells always need to be corroborated in human cells.

Consequently, human MDDCs and MDMs were infected with the three *S. epidermidis* strains, at a MOI of (1DC:10B) or (1M:10B). Then, the secretion of the pro-inflammatory cytokines IL-6, TNF-α, IL-8, IL-1β, IL-12p70 and the anti-inflammatory cytokine IL-10 by these cells was quantified by ELISA, in culture supernatants collected 24h after the co-incubation. Cells incubated with medium or medium with Cm were used as negative controls, while cells incubated with LPS served as a positive control. The levels of secreted cytokines by DCs and both M1- and M2- type macrophages are presented in Figure 14.


DC
M1
M2
A
IL-6 [pg/ml]
15000
10000
5000
0
50000
40000
30000
20000
8000
6000
4000
2000
*
Medium
Medium + Cm
LPS
WT
ΔT
ΔT::T
B
TNF-α [pg/ml]
5000
4000
3000
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150000
100000
50000
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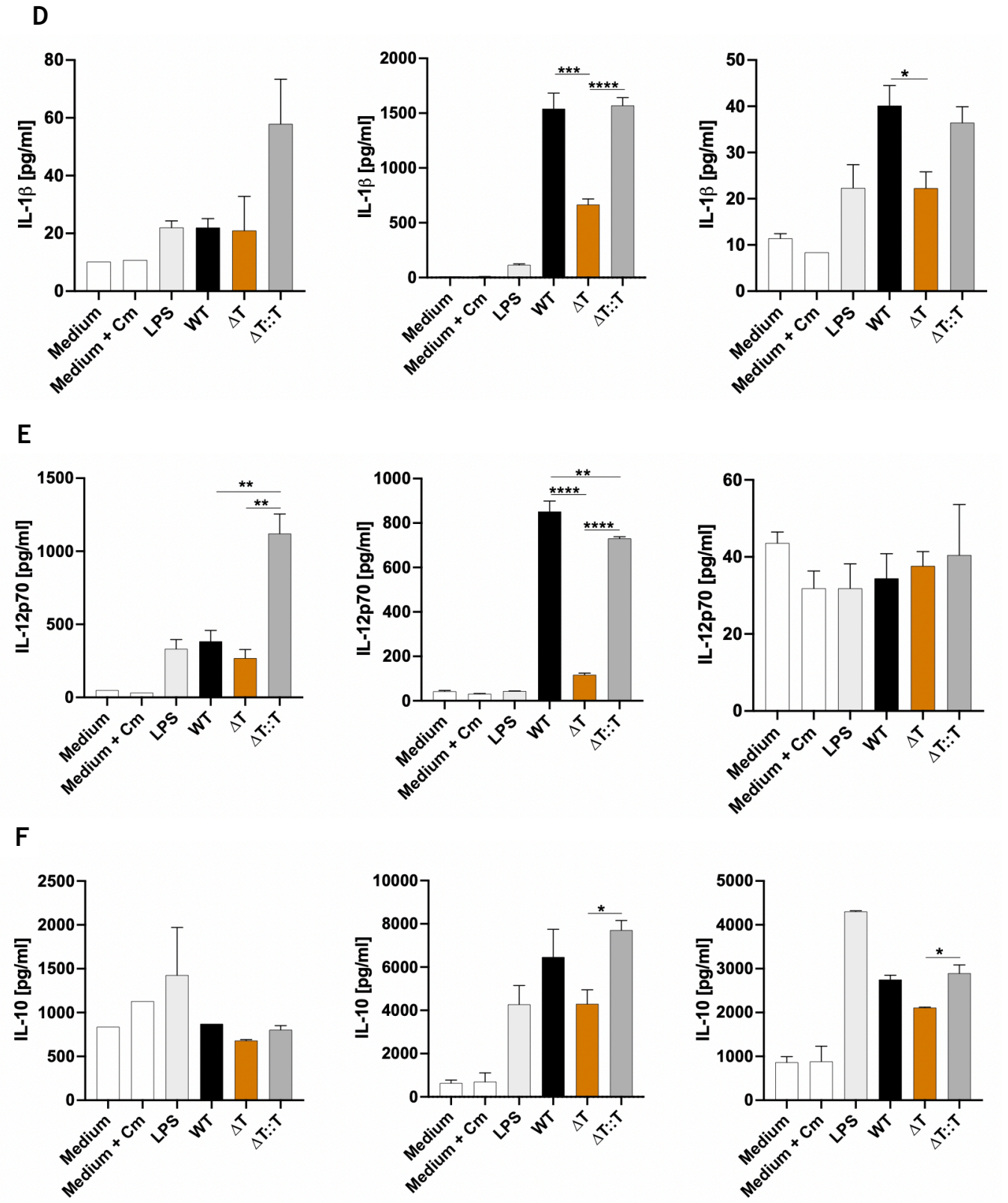


Figure 14 - Quantification of secreted cytokines by human monocyte-derived dendritic cells and M1- and M2-type macrophages. Human MDDCs and MDMs, both M1- and M2-type, were infected with the three strains at a MOI of (1DC:10B) or (1M:10B). DCs incubated with medium or medium plus Cm (negative controls) and with LPS (positive control) were used. After 24h, the culture supernatants were collected. The levels of (A) IL-6, (B) TNF-α, (C) IL-8, (D) IL-12p70, (E) IL-1β and (F) IL-10 in the supernatants were measured by ELISA. The results presented are representative of three experiments (n=3), in which each condition was done in duplicate. Mean $\pm$ SD are represented. For statistical analysis, the one-way ANOVA with Tukey test was used. (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001). To simplify, only statistical significance between strains was illustrated.

In DCs no statistically significant differences were found between the WT and the ΔT strains in the secretion of none of the pro-inflammatory cytokines.

As for the M1 macrophages, the WT strain led to a considerably higher production of the pro-inflammatory cytokines TNF-α, IL-1β and IL-12p70 than the ΔT (Figure 14 B, D and E).

The M2 macrophages infected with the WT strain generated significantly increased levels of the pro-inflammatory interleukins IL-6, IL-8 and IL-1β, in comparison with M2 macrophages co-incubated with the ΔT strain (Figure 14 A, C and D).

With regard to the induction of the anti-inflammatory IL-10, no meaningful variances were noticed when comparing the WT and the ΔT, regardless of the cell type (DC, M1 or M2) (Figure 14 F). However, the stimulation of IL-10 secretion seemed to be inhibited in the mutant.

With human DCs, no significant differences were encountered when comparing the levels of secreted cytokines, induced by the WT or the ΔT. Regarding this, WTAs do not seem to possess an important role in the recognition of *S. epidermidis* and in the stimulation of cytokine production by human DCs. Ways to optimize the protocol and possibly enable a better detection would be: i) to perform a kinetics assay, with later time-points such as 48h; and ii) to replicate the experiments, to gather stronger results.

The results gathered with human DCs do not corroborate what was evidenced with mouse DCs. Although in mouse DCs the presence/absence of WTAs appears to influence cytokine secretion, in human DCs WTAs may be insignificant for generation of that type of response. The disparities confronted with mouse and human cells may be explained by the fact that these cells may possess distinct recognition mechanisms of *S. epidermidis*, which lead to different signaling pathways. For instance, the PAMPs recognized by the PRRs of human DCs may be constituents of *S. epidermidis* that were not affected from the absence of WTAs, being common to the WT and the ΔT strains. This result is legitimate because the cells under study are from two different species and present differences in both innate and adaptive immunity (Mestas & Hughes, 2004). In fact, several research studies have shown that the results obtained with mouse and human cells can be variable. An example includes a study that reports differences in the interaction of mouse and human DCs with *Candida albicans* (Newman & Holly, 2001).

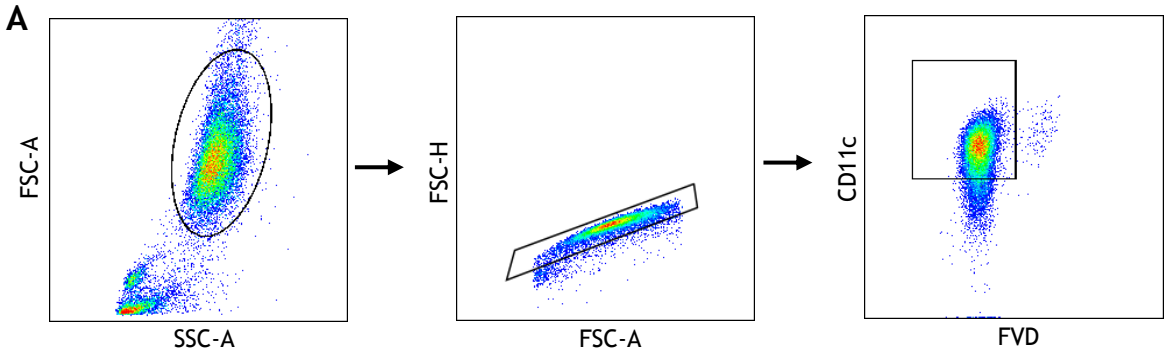
Concerning macrophages, when comparing the WT and ΔT strains, the first one leads to a significantly increased production of pro-inflammatory cytokines in both M1- and M2-type macrophages. This can imply a direct role of WTAs in cytokine secretion by macrophages. These compounds may be involved in recognition of *S. epidermidis* by PRRs on macrophages, which would justify not only these results, but also the results obtained in the phagocytosis assay. On the other hand, if not directly implicated in recognition, WTAs may be capable of enhancing cytokine secretion by human macrophages.

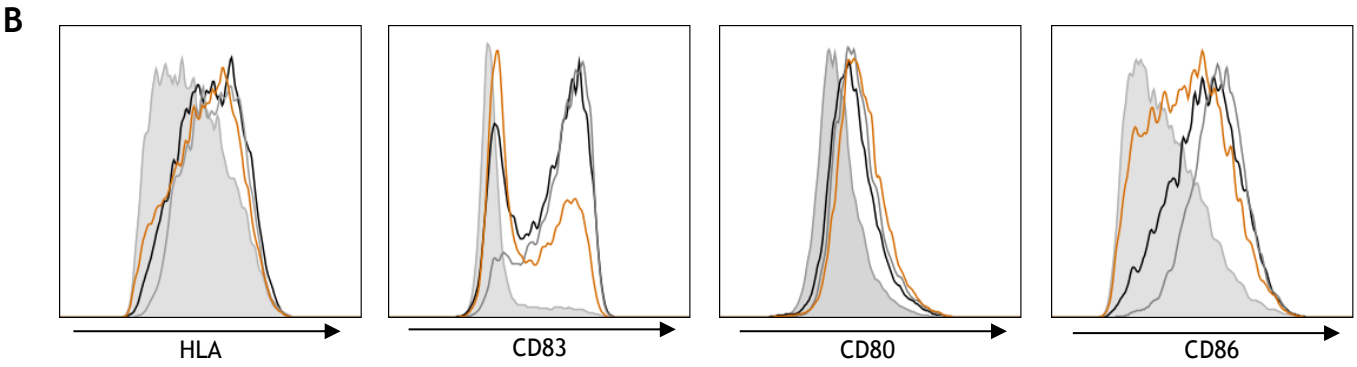
These results show different phenotypes concerning the immune response to *S. epidermidis* between human DCs and macrophages. This suggests that, in these cells, distinct PRRs may be involved in the recognition mechanisms of this bacterium. In fact, these two cell types show variable expressions of the same receptors, along with different signal transductions. Subsequently, this can lead to divergent responses against various stimulus, such as bacterial pathogens (Zanoni & Granucci, 2009).

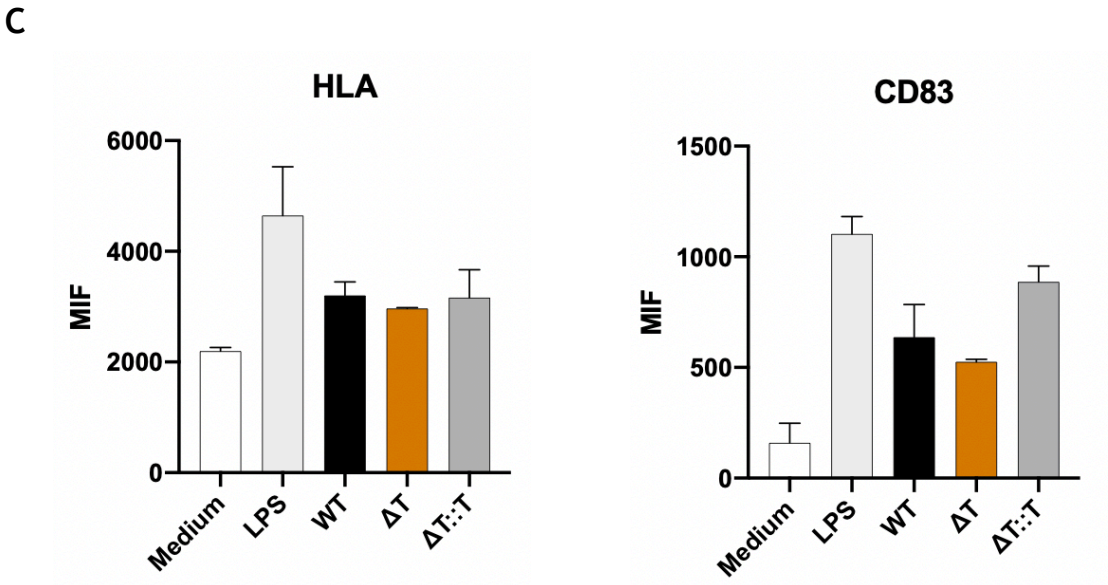
2.3.2. Evaluation of MDDC activation by FACS

Once more, the results acquired with mouse cells should be validated using human cells. Accordingly, the activation/maturation of human DCs ought to be assessed.

With this in view, human MDDCs were co-incubated with the WT, ΔT and ΔT::T strains at a MOI of (1DC:10B). After 24h, the DCs were labelled with FVD and antibodies specific for the activation markers of human DCs. Subsequently, the expression of the activation markers HLA, CD83, CD80 and CD86 of human DCs was identified through flow cytometry, to determine if these cells had been activated. The results acquired are illustrated in Figure 15.







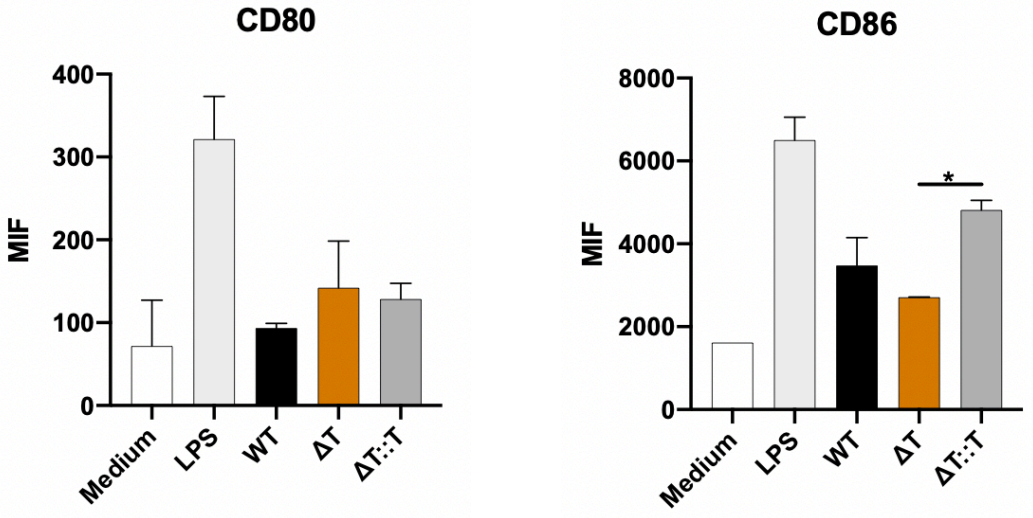


Figure 15 - Activation/maturation of human MDDCs. Human MDDCs were infected with the strains at a MOI of (1DC:10B). A negative and a positive control were used, with DCs incubated with medium or LPS, respectively. After 24h, the cells were stained with FVD, anti-CD11c-APC conjugated, anti-HLA-PECy7 conjugated, anti-CD83-FITC conjugated, anti-CD14-PE conjugated, anti-CD80-BV510 conjugated and anti-CD86-PECy5 conjugated. Cells were analyzed by flow cytometry. A) Representative strategy of gating. After selecting the population of interest (first), single cells were chosen (second), and live dendritic cells ($CD11c^{+}FVD^{-}$) were gated (third). B) Representative histogram overlays of each activation marker analyzed, with illustration of non-stimulated MDDCs (grey with filling) and MDDCs infected with the WT (black line) ΔT (orange line) and ΔT::T (grey line) strains. C) Quantification of the expression of human MDDC activation markers. The results presented are representative of two experiments (n=2), in which each sample was done in duplicate. Mean ± SD are represented. For statistical analysis, the one-way ANOVA with Tukey test was used. (*$P<0.05$). To simplify, only statistical significance between strains was illustrated.

The results in Figure 15 B and C reveal that the three strains were able to induce the activation/maturation of MDDCs, once DCs infected with the strains show an increased expression of the activation markers HLA, CD83, CD80 and CD86, in comparison with the MDDCs incubated with medium (non-stimulated), which were immature before infection.

As for differences in expression of the activation markers between the different strains, no significance was found between the WT and the ΔT strains (Figure 15 C). A minor decrease in the expression of HLA, CD83 and CD86 can be detected in the ΔT strain, while the WT led to a slightly upregulated expression (Figure 15 B and C). Specifically, for the CD83, it is clear that the ΔT strain was not capable of activating its expression in a great number of MDDCs, since the increased expression of CD83 was identified in the lowest peak (Figure 15 B). Conversely, the ΔT appears to have induced an expression of CD80 a bit superior to the WT (Figure 15 B and C).

The results obtained with human DCs corroborate what was observed in mouse DC activation. Herein, the three *S. epidermidis* strains were able to successfully activate DCs, inducing the expression of its activation markers (HLA, CD83, CD80 and CD86). In fact, activation of human MDDCs by *S. epidermidis* has already been reported (Laborel-Préneron et al., 2015).

Although the mutant strain appears to have a decreased expression of three markers, in comparison to the WT, only differences with no significance were revealed between the two. Thus, a role for the WTAs in human DC maturation and further eliciting of adaptive responses is unlikely. This is in concordance of what was observed in ELISA results, in which the WTAs did not appear to have an important role in stimulating human DCs for cytokine secretion.

3. Biofilm development by *S. epidermidis* strains

3.1. Comparative analysis of biofilm production among strains

S. epidermidis is a biofilm producer species. This feature is the major virulence factor evidenced by this bacterium, which can contribute to its pathogenicity. Consequently, it facilitates the development of infections in a host. As such, biofilm formation capacity was evaluated in each *S. epidermidis* strain (WT, ΔT, ΔT::T), as it may differ between them.

Hence, to quantify and subsequently compare biofilm production by the three strains, biofilms were grown for 48h in TSB supplemented with 0.4% glucose and 10% human serum. Then, biofilms were disrupted, the cells were collected, and the OD_{640nm} of each sample was measured. Afterwards, the number of CFUs in each sample was determined. The biofilms established are presented in Figure 16.

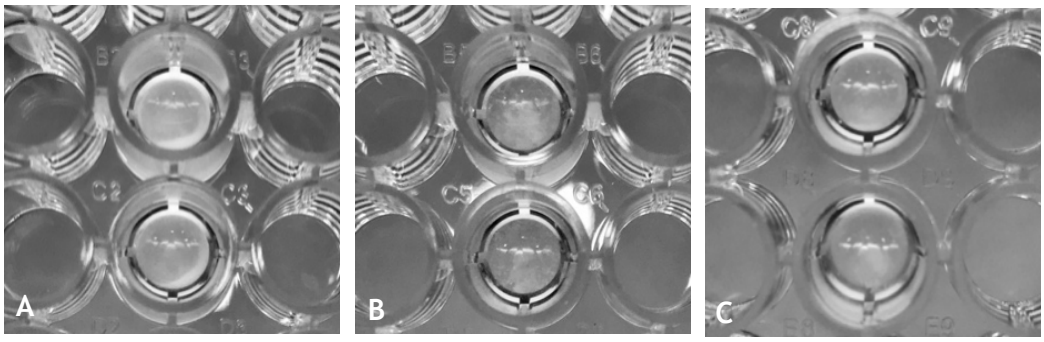


Figure 16 – Biofilm formation by *S. epidermidis* strains. Biofilms of the (A) WT, (B) ΔT and (C) ΔT::T strains were developed during 48h in a 96-well plate in TSB+0.4% glucose+10% human serum at 37ºC and 80 rpm. After 24h of growth, spent media was removed and replaced by fresh media. Two biofilms of each strain were formed and analyzed.

As observable in Figure 16 A, B and C, every strain was capable of developing biofilms, once all the wells correspondent to each strain were covered by biomass. Afterwards, the two biofilms produced by each strain were evaluated through OD_{640nm} measurements and CFUs counting. The OD_{640nm} quantification for each strain is exhibited in Figure 17.

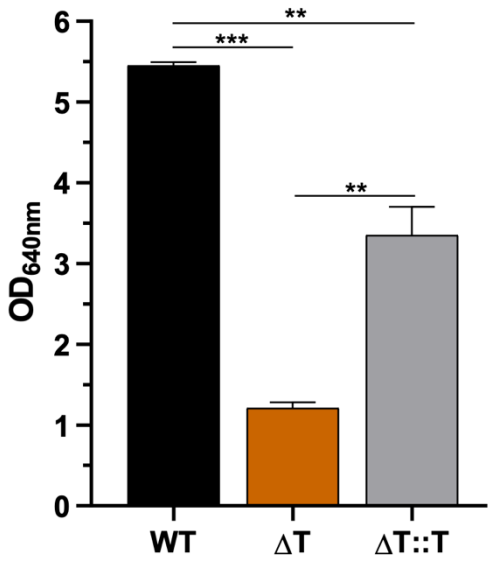


Figure 17 – Estimation of biofilm production through OD_{640nm} measurements. Biofilms of the three strains were developed during 48h. Biofilm cells of each strain were recovered and their OD_{640nm} was measured. The results illustrated are respective to the OD_{640nm} quantification of the two biofilm samples developed by each strain. Mean ± SD are represented. For statistical analysis, the one-way ANOVA with Tukey test was used. (**P<0.01, ***P<0.001).

According to the OD_{640nm} measurements, the ΔT shows significantly less biomass than the WT and the ΔT::T, since it revealed the lowest OD_{640nm} value.

The measurement of OD_{640nm} allows indirect quantification of biomass concentration, which encompasses several other factors besides the number of bacterial cells. Consequently, to quantify the number of biofilm cells, the number of CFUs of these same samples was determined. The CFU results are displayed in Figure 18.

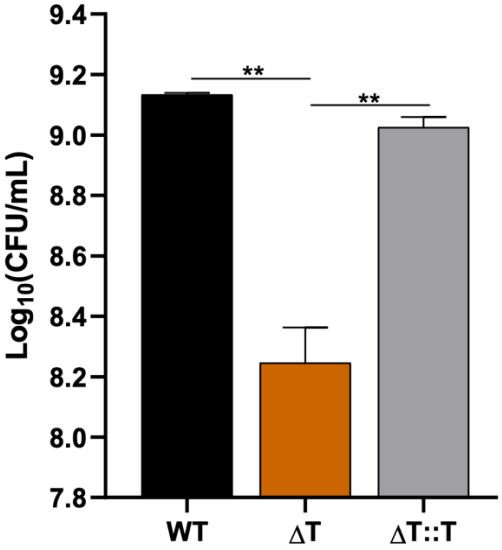


Figure 18 – Quantification of biofilm cells through CFU count. Biofilms of the three strains were developed during 48h. Biofilm cells of each strain were recovered, their OD_{640nm} was measured and CFUs were determined. The results displayed are respective to the CFU counts of the two biofilm samples developed by each strain. Mean ± SD values are represented. For statistical analysis, the one-way ANOVA with Tukey test was used. (**P<0.01).

Once more, it was proven that the ΔT strain presents a significantly lower amount of biofilm cells. Therefore, the results obtained through OD_{640nm} quantification were confirmed by CFUs.

Conversely to the results found when using the method of OD_{640nm} quantification, here, the number of biofilm cells was identical between the WT and the ΔT::T. The reason for this might be linked to the fact, already mentioned above, that the measurement of OD quantifies biomass, which includes multiple components other than cells. In conformity with this, the WT biofilms, when comparing to the ΔT::T, may be richer in other factors. Actually, it makes more sense for CFU counts results to be the most viable for that very reason, because that approach is quantifying, exclusively, the number of cells in the biofilm.

The impaired biofilm formation exhibited by the mutant strain suggests that the absence of WTAs affects this mechanism. Thereby, WTAs may play an important part in biofilm formation and their removal could be the way to prevent this virulence mechanism. As a matter of fact, it was demonstrated that WTAs are structural components of *S. epidermidis* biofilms. They were shown to be involved in *S. epidermidis* cells primary attachment to a coated surface, through binding of absorbed fibronectin and fibrin clots. In addition, they are also important in the phase of intercellular adhesion, which they support due to their anionic charge (Sabaté Brescó et al., 2017). The importance of WTAs in biofilm formation was even evidenced using a WTA-deficient mutant of *S. epidermidis*, which showed an impaired biofilm development (Holland et al., 2011).

Chapter 5

Conclusions and Future Perspectives

S. epidermidis is a bacterium that presents the typical growth profile of microorganisms in closed systems. This bacterium is characterized by an exponential growth phase (from 0h to 8h) and a succeeding stationary growth phase, which starts at 8 hours of multiplication.

As a microorganism that easily forms bacterial aggregates, its growth and handling conditions should minimize this phenomenon. The use of human serum appeared to be the optimal choice for these conditions. Besides inhibiting the development of *S. epidermidis* aggregates, as observed by flow cytometry analysis, it provided an identical growth among the WT, ΔT and ΔT::T strains. Furthermore, the supplementation with human serum highly potentiated the proliferation of the strains.

An equivalent cell count between strains had to be achieved in order to normalize experimental conditions and allow comparing results. The way to attain this was to adjust the OD_{640nm} to 0.25 or 0.50, respectively for the WT, and for the ΔT and the ΔT::T strains. This set of OD_{640nm} proved to be the ideal one in both optimization assays, through CFU counts and viable cell count by flow cytometry.

The morphology studies using TEM revealed differences between the mutant strain and the other two, in terms of size and septum. Both differences may be related to the lack of WTAs in the cell wall of the mutant. This deficiency can alter the cell shape, leading to variations in cell size and cell wall restructuring. These may explain the appearance of duplicated septa and successive division planes at nonorthogonal angles. The defects shown in the ΔT septum may impact its cell division and, consequently, impair the multiplication mechanisms. This could then clarify the slower/inferior growth rate identified in the mutant strain, when comparing with the WT, in the assay of growth curve assessment.

Wall teichoic acids appear to be directly involved in the recognition of *S. epidermidis* and downstream activation of phagocytosis and cytokine production by macrophages. This is suggested by the inhibited phagocytosis rate of the mutant by mouse RAW 264.7 macrophages, and the lower secretion of pro- and anti-inflammatory cytokines by human M1- and M2-type macrophages infected with the mutant strain. On the other hand, instead of directly involved in the initial recognition of *S. epidermidis*, WTAs may act as a molecule capable of eliciting phagocytosis or cytokine production by macrophages. In regard to phagocytosis by mouse macrophages, other hypotheses may justify the reduced internalization of the ΔT by these cells. These include: i) the exposure of molecules that could inhibit phagocytosis; or ii) the concealing of PAMPs, crucial for the recognition by specific PRRs and further internalization of the bacterium by macrophages. Both of these possibilities could be caused by the restructuring of the mutant cell wall, due to WTA deficiency, as suggested by TEM results.

The increased levels of pro- and anti- inflammatory cytokines, produced by mouse DCs infected with the mutant strain, may suggest, as well, that the absence of WTAs in the cell wall leads to its reorganization. This could then cause the exposure of molecules that may facilitate recognition

and/or stimulate cytokine production. Alternatively, WTAs may be involved in immune evasion mechanisms, hence the lower cytokine levels identified in the WT. The results obtained with human DCs did not indicate the same. Wall teichoic acids do not seem crucial for recognition and subsequent cytokine secretion by human DCs. These disparities imply that recognition of *S. epidermidis* by mouse and human DCs may be carried out by distinct recognition mechanisms, leading to divergent signaling pathways.

Accordingly, a distinct phenotype was found between human DCs and macrophages. Although WTAs appear to have a role in recognition of *S. epidermidis* and further cytokine secretion by macrophages, in DCs they do not. The recognition mechanisms of this bacterium probably vary from one cell type to the other.

S. epidermidis strains have proven to induce the maturation of both mouse and human DCs, with a high expression of specific activation markers at their surface. Nonetheless, WTAs do not come out as an important molecule for the recognition of *S. epidermidis* and DC activation for further generation of adaptive responses.

Finally, deficiency in WTAs translates into an impaired capacity to form biofilms. Accordingly, WTAs confirmed to be essential for the development of biofilms by *S. epidermidis* and their removal could be the approach to inhibit this virulence mechanism.

Throughout the discussion of the experimental results, no focus was given to the results obtained by the ΔT::T strain. This was due to the fact that this strain should show an identical behavior to the WT, which was not verified at all times. At one point this divergence was noticed and it was further discovered that the expression levels of the *tagO* gene were variable between these two strains. For this reason, a major focus was given to the comparison between the WT and ΔT strains, which was the main objective of this project.

In the future, in-depth characterization studies of the cell wall of each *S. epidermidis* strain should be performed. These would confirm whether the *tagO* gene mutation actually prevented the production of WTA in the mutant. Additionally, they would show if there was in fact a restructuring of the cell wall and if some constituents became more exposed or concealed. Subsequently, these studies would facilitate the comprehension of the results here presented.

The DC activation assays should be reproduced following an optimized protocol and, preferably, with evaluation of the activation kinetics of DCs.

Afterwards, it would be pertinent to repeat the assays that studied the interaction of *S. epidermidis* planktonic cells with cells of the innate immune response. Yet, specific receptors of the innate cells, which could be involved in the recognition of *S. epidermidis*, should be blocked, in order to understand which receptor is more important in which cell. This would help elucidate the differences in recognition, suggested by the results obtained here, between macrophages and DCs and between mouse and human cells. Another approach would be the use of detection antibodies that would bind to a specific receptor and emit a signal if that receptor was exposed/accessible for binding.

To complement the phagocytosis assays here performed, which only considered the internalization process, killing mechanisms of the different strains within the phagocytes should be analyzed. For instance, the quantification of ROS production by the phagocytic cells could be assessed.

Completed the study of *S. epidermidis* planktonic cells, assays with biofilms cells should be initiated. To eliminate the dissimilarities in biofilm formation among the three strains, an optimization test should be carried out. This should identify the OD value for the biofilms cells of each strain that would permit the same CFU/mL for all. This would then allow the normalization of

results of further experiments, namely the study of the interaction between biofilms produced by the three *S. epidermidis* strains with cells of the innate system. Obviously, these studies should replicate the assays carried out with planktonic cells, for comparison purposes. Then, the results obtained should be compared with the ones of planktonic cells. This would facilitate the study of the innate immune response elicited in the host by these two cell types. Furthermore, it would be possible to recognize the similarities and/or differences between the innate responses induced by these cell types.

Besides investigating the innate immune response, the host adaptive response to planktonic and biofilms cells of the three strains could also be evaluated *in vitro*.

Upon *in vitro* testing, *in vivo* assays should be conducted. In these assays, mouse infection models should be used, for instance T-cell receptor (TCR) transgenic mouse systems and knockout (KO) mice, given that the last could lack a relevant component, such as a recognition receptor. Once again, the response triggered by the infection with both planktonic and biofilm cells of *S. epidermidis* strains should be assessed. These models would contribute to the understanding of the recognition of WTAs, the polarization of T $CD4^+$ lymphocytes and how the generated response would be modulated.

All this future work would help to understand how the presence/absence of WTAs impacts *S. epidermidis* cell wall and the immune response induced in the host. Moreover, it would elucidate the recognition and downstream pathways preferably activated by *S. epidermidis* planktonic and biofilm cells, in different immune cells from distinct hosts. The understanding of these mechanisms would then facilitate the development of novel immune-mediated approaches to prevent or treat infections caused by *S. epidermidis*.

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