



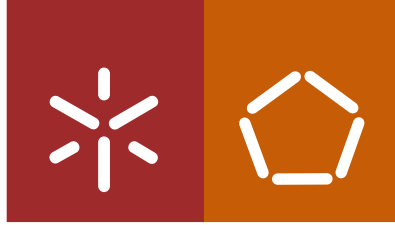
Universidade do Minho
Escola de Engenharia

S. epidermidis putative toxin-antitoxin module *mazEF*: the
role in VBNC formation and impact on the immune system

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mazEF: the role in VBNC formation and impact
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***S. epidermidis* putative toxin-antitoxin module
mazEF: the role in VBNC formation and impact
on the immune system**

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Trabalho realizado sob a orientação do
Doutor Nuno Cerca
da
Doutora Ângela França
e do
Prof. Doutor Manuel Vilanova

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mazEF, um potencial sistema toxina-antitoxina em *S. epidermidis* : papel na formação de células viáveis mas não-cultiváveis e impacto no sistema imunitário

RESUMO

Staphylococcus epidermidis é um microrganismo comensal que habita na pele e membranas mucosas dos seres humanos e outros mamíferos, mas que pode atuar como um agente patogénico oportunista, especialmente em pacientes imunocomprometidos e em bebés prematuros. Os principais fatores de virulência de *S. epidermidis* são a sua reduzida suscetibilidade a antibióticos e a sua capacidade de formação de biofilmes e de evasão à resposta do sistema imunitário. Os biofilmes estão também associados à recorrência de infeções, o que pode estar relacionado com a presença de células viáveis mas não-cultiváveis (CVNC) na sua composição. Um anterior estudo identificou o operão *mazEF*, descrito como um potencial sistema toxina-antitoxina (TA), como um possível regulador da ocorrência de CVNC em biofilmes de *S. epidermidis*. De forma a determinar o papel do *mazEF* na modulação do estado CVNC e o seu impacto noutros aspetos de virulência, construímos e caracterizamos duas estirpes mutantes sem o operão *mazEF*. Um modelo *in vitro* previamente desenvolvido para modular a indução do estado CVNC em biofilmes foi validado com as nossas estirpes e conseguimos também induzir a formação de células CVNC em culturas planctónicas, sendo este fenómeno de alguma forma regulado pela densidade celular. Surpreendentemente, ao compararmos a estirpe mutante de *mazEF* com a estirpe selvagem, não foram encontradas diferenças na indução do estado CVNC quer em biofilmes, quer em culturas planctónicas. Uma vez que homólogos do operão *mazEF* foram identificados como sistemas TA, os mutantes foram complementados com outro plasmídeo onde a expressão do *mazEF* foi regulada por um promotor induzível, permitindo assim determinar se a indução da toxina levaria à morte celular. Contudo, sob as condições experimentais utilizadas, a expressão destes genes não levou à morte celular, sugerindo, assim, que o homólogo de *mazEF* não funciona como um sistema TA nas estirpes testadas. Estudámos também o impacto do *mazEF* noutras características de virulência, incluindo a sobrevivência a antibióticos ou à ação bactericida do sangue e plasma humanos e a resposta de células dendríticas e macrófagos a infeções com as estirpes de *S. epidermidis* 1457. Dos fatores testados, apenas uma menor suscetibilidade à rifampicina e uma atividade metabólica distinta após tratamento com tetraciclina foram observados no mutante, levando-nos a concluir que o homólogo de *mazEF* tem um impacto pouco significativo no potencial de virulência das estirpes de *S. epidermidis* aqui usadas.

Palavras-chave: Biofilmes, CVNC, fatores de virulência, manipulação genética, *mazEF*.

***S. epidermidis* putative toxin-antitoxin module *mazEF*: role in VBNC formation and impact on the immune system**

ABSTRACT

Staphylococcus epidermidis is a commensal microorganism that inhabits the skin and mucous membranes of humans and several mammals, but acts as an opportunistic pathogen, particularly in immunocompromised patients and neonates. The main virulent properties of *S. epidermidis* are its reduced antimicrobial susceptibility profile, associated with biofilm formation ability, which contribute to the evasion from the host immune response. Biofilms have also been associated with recurrent infections, and this might be due to the high proportion of viable but non-culturable (VBNC) cells within them. A previous RNA-Seq study identified the *mazEF* putative toxin-antitoxin (TA) system as a possible key player in the regulation of the occurrence of VBNC cells in *S. epidermidis* biofilms. In order to determine the role of *mazEF* in the modulation of the VBNC state and its impact on other virulence aspects of biofilms, we constructed and characterized two mutant strains lacking the *mazEF* operon. The previously characterized *in vitro* model used to modulate VBNC induction in biofilm cultures was further validated and we demonstrated that VBNC induction can also occur in planktonic cultures, being this phenomenon somewhat related to cellular densities. Surprisingly, when comparing the *mazEF* operon mutant to the wild-type strain, no significant differences were observed in VBNC induction in either biofilms or planktonic cultures. As *mazEF* homologues have also been identified as TA systems, the mutants were complemented with a different plasmid, wherein the *mazEF* expression was regulated by an inducible promoter, in order to determine if the induction of the toxin module would lead to bacterial death. However, under our experimental conditions, neither gene expression led to bacterial death, suggesting that the *mazEF* homologue was not acting as a TA system in our strains. We also assessed the impact of *mazEF* in other relevant virulence characteristics, including the ability to survive after antibiotic challenges or in human blood and plasma, and the response of dendritic cells and macrophages to infections with *S. epidermidis* 1457 strains. Of the tested traits, only a lower susceptibility to rifampicin and a distinct metabolic activity upon treatment with tetracycline was observed in the mutant strain, leading us to conclude that the *mazEF* homologue operon had a minor impact on virulence of the *S. epidermidis* strains considered herein.

Keywords: Biofilms, gene manipulation, *mazEF*, VBNC, virulence factors.

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List of abbreviations

<i>agr</i>	Accessory gene regulator
AMPs	Antimicrobial peptides
APCs	Antigen-presenting cells
ATC	Anhydrotetracycline
ATP	Adenosine triphosphate
B	Bacteria
<i>bap</i>	Biofilm-associated protein
bp	Base pair
Brc	Biofilm-released cells
cDNA	Complementary DNA
CFU	Colony-forming unit
CLSM	Confocal laser scanning microscopy
CM	Chloramphenicol
CoNS	Coagulase-negative staphylococci
cRPMI	RPMI supplemented with 10% FBS
CT	Control
DAPI	4',6-diamidine-2'phenylindole dihydrochloride
DCFDA	2',7' –dichlorofluorescein diacetate
DNA	Deoxyribonucleic acid
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances

FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
gDNA	Genomic DNA
Glu	Glucose
GM-CSF	Granulocyte-macrophage colony-stimulating factor
<i>ica</i>	Intercellular adhesion operon
IFN- γ	Interferon gamma
IL	Interleukin
IND	Induced VBNC state
LPS	Lipopolysaccharide
LTA	Lipoteichoic acids
M-CSF	Macrophage colony-stimulating factor
MACS	Magnetic-activated cell sorting
MCS	Multiple-cloning site
MDDCs	Human monocyte-derived dendritic cells
MDMs	Human monocyte-derived macrophages
MFI	Mean fluorescence intensity
Mg ²⁺	Magnesium
MgCl ₂	Magnesium chloride
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid

NaCl	Sodium chloride
NETs	Neutrophil extracellular traps
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PI	Propidium iodide
PIA/PNAG	Polysaccharide intercellular adhesin / poly-N-acetylglucosamine
PMNs	Polymorphonuclear leukocytes
PMS	Phenazine methosulfate
PRE	Prevented VBNC state
PRRs	Pattern recognition receptors
PSC	Peak serum concentration
PSMs	Phenol-soluble modulins
qPCR	Quantitative PCR
R	Resistant
RBS	Ribosomal binding site
RFU	Relative fluorescence units
RIF	Rifampicin
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing

RPKM	Reads Per Kilobase of transcript per Million reads mapped
ROS	Reactive oxygen species
S	Susceptible
SEM	Scanning electron microscopy
sRPMI	RPMI supplemented with 10% FBS, 2% L-glutamine, 1% HEPES and 50 μ M β -mercaptoethanol
TA	Toxin-antitoxin
TET	Tetracycline
TGF- β	Transforming growth factor-beta
TLRs	Toll-like receptors
TNF- α	Tumour necrosis factor-alpha
TSA	Tryptic soy agar
TSA _{ATC}	TSA supplemented with 1 μ g/mL anhydrotetracycline hydrochloride
TSA _{CM10}	TSA supplemented with 10 μ g/mL chloramphenicol
TSB	Tryptic soy broth
TSB _{CM10}	TSB supplemented with 10 μ g/mL chloramphenicol
UV	Ultra-violet
VAN	Vancomycin
VBNC	Viable but non-culturable
WGA	Wheat germ agglutinin
WT	Wild-type
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2 <i>H</i> -Tetrazolium-5-Carboxanilide

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Scientific outputs

Part of the work described in this thesis has been published in international peer-reviewed journals and presented in international scientific conferences.

Articles published in peer-reviewed journals

Gaio, V., Lopes, N., Cerca, N., França, A. *codY* and *pdhA* expression is induced in *Staphylococcus epidermidis* biofilm and planktonic populations with higher proportions of viable but non-culturable cells. *Frontiers in Cellular and Infection Microbiology*. 2021;11:771666.

Gaio, V., Lima, T., Vilanova, M., Cerca, N., França, A. *mazEF* homologue has a minor role in *Staphylococcus epidermidis* 1457 virulence potential". *Frontiers in Cellular and Infection Microbiology*. 2022, 11:803134.

Poster communications at international scientific conferences

Gaio, V., Cerca, N., França, A. "Preliminary studies on the role of *mazEF* in *S. epidermidis* biofilm dormancy" in Microbiotec 2019 – Congress of microbiology and biotechnology, Coimbra, Portugal, December 5-7, 2019.

Gaio, V., Cerca, N., França, A. "The putative toxin-antitoxin *mazEF* gene cluster is not involved in cell death in *S. epidermidis*" in Microbiotec 2021 – Congress of microbiology and biotechnology, Online congress, November 23-26, 2021.

CHAPTER 1

Introduction

SUMMARY

This chapter presents the initial introduction to the thesis, research questions aimed to be answered and the main scope and aims of this work. Moreover, the thesis outline is herein presented.

1.1. Background

Staphylococcus epidermidis is a common colonizer of humans due to its ubiquitous presence in the skin and mucous membranes [1], with a great ability to form biofilms [2]. Despite its general commensal lifestyle and benign relationship with the host [1], *S. epidermidis* can cause severe complications, such as bloodstream infections [3, 4], which are often hard to treat due to the decreased antimicrobial susceptibility of bacteria [5, 6]. Since bacteraemia is associated with increased morbidity and mortality among hospitalized patients [7], this species has become an important object of study in the past years [4]. Importantly, this species presents a high ability to form biofilms on the surface of indwelling medical devices, being acknowledged as one of the most frequent causes of nosocomial infections in the United States of America [8, 9]. Moreover, according to the 2014 Annual Epidemiological Report on Antimicrobial Resistance and Healthcare [10], *S. epidermidis*, together with other coagulase-negative staphylococci (CoNS), is one of the most frequent pathogens isolated from intensive care units-acquired bloodstream infections in European countries [10]. Interestingly, the last Annual Epidemiological Report published by the European Centre for Disease Prevention and Control (ECDC), in 2019, still outlines CoNS as prevalent microorganisms in surgical site infections [11]. In fact, also in Portugal, CoNS have been found to be among the main pathogens involved in bloodstream nosocomial infections and in infections associated with central venous catheters [12], being *S. epidermidis* one of the most frequent pathogens associated with hospital-acquired infections [13]. Several studies have shown that *S. epidermidis* biofilms are more tolerant to antimicrobial therapies [14] and to the host immune defences [15], which results in relapsing and recurrent infections [1]. Moreover, it was found that these bacteria can enter a special state of dormancy, where cells are viable but non-culturable (VBNC) during the biofilm lifecycle, turning them even more tolerant to antibiotics [16, 17] and to the immune response [18]. As a result, the treatment of such infections becomes more difficult and, consequently, affects the quality of life of thousands of persons every year [19, 20]. It was recently found that *mazE* was only expressed in *S. epidermidis* biofilms where the VBNC state was induced [17]. The *mazE* gene encodes a protein of the *mazEF* complex that has been described as a toxin-antitoxin (TA) system in several species, such as *Escherichia coli* [21], *Pseudomonas aeruginosa* [22], *Streptococcus mutans* [23] and *Staphylococcus aureus* [24] and mentioned as a putative TA system in *S. epidermidis* as well, nonetheless this association was purely based in protein homology [25–27].

The possible involvement of *mazEF* on *S. epidermidis* bacterial stress response [28], decreased susceptibility to antimicrobials [29] and putative action as a TA system [26] raised interest in the study of the role of the *mazEF* complex in *S. epidermidis* biofilms.

1.2. Research questions

This thesis aimed to address the following questions:

1. Does *mazEF* act as a TA system in *S. epidermidis*?
2. Does *mazEF* have an influence on VBNC modulation on *S. epidermidis* biofilms?
3. Is the susceptibility to antimicrobials affected by the deletion of the *mazEF* complex?
4. Can innate cell immunity be more effective against *mazEF* mutants than to the wild-type strain?

It is expected that the answer to these questions will help elucidate the role of *mazEF* in *S. epidermidis* and, hopefully, provide significant outcomes for the treatment and control of *S. epidermidis* biofilm-associated infections.

1.3. Scope and aims

Due to the impact of bacterial dormancy on infections and, conversely, the lack of a detailed understanding of the mechanisms involved in this process, herein we propose to study the molecular determinants behind the generation of VBNC cells in *S. epidermidis*. The main goal of this thesis was to assess the role of *mazEF* complex, a putative TA system in *S. epidermidis*, in biofilm dormancy and host immune response. In particular, we aimed to construct *S. epidermidis mazEF* mutants to assess the role of *mazEF* system in biofilm and planktonic VBNC modulation, as well as in their susceptibility to antibiotics. Moreover, the role of this complex on the adaptation to the host response was addressed and complemented with a study focused on the survival in human blood.

In order to achieve our aims, the following objectives were pursued:

1. Construction of *S. epidermidis* mutants and characterization of *mazEF* putative TA function;
2. *In vitro* evaluation of the role of *mazEF* in VBNC state modulation;
3. Assessment of the role of *mazEF* in bacterial survival in human blood;
4. Analysis of the interactions of *S. epidermidis* mutants with different host immune effectors.

Overall, it is expected that the information gathered at the conclusion of all tasks proposed above will provide important insights related to the role of *mazEF* in *S. epidermidis* 1457, with emphasis on the effect of this operon on the modulation of the VBNC state, as well as the contribution of the *mazEF* complex in *S. epidermidis* evasion from the host response and susceptibility to antimicrobials. It is anticipated that relevant information regarding *S. epidermidis* infections will result from these outcomes, which can help conceive new and more effective strategies to increase the success in treating biofilm-associated infections caused by *S. epidermidis*.

1.4. Thesis outline

The present chapter (**Chapter 1**) presents a brief introduction to this thesis and the motivations behind this study, featuring the main questions and aims to be answered to contribute to a deeper knowledge on the role of *mazEF* in *S. epidermidis*.

A literature review is presented in **Chapter 2**, providing essential information on the aetiology of *S. epidermidis* as an opportunistic pathogen in humans. This chapter summarizes what has been described, so far, in the literature regarding *S. epidermidis* biofilm infections, with emphasis on the generation and modulation of VBNC cells and on the decreased susceptibility of biofilms to antimicrobials and to host immune defences. Moreover, a review of the role of *mazEF* in *S. epidermidis* and, especially, as a TA system in other species is also presented. Finally, a brief review of what is known so far regarding the response of the immune system to *S. epidermidis* infections is presented herein.

Chapters 3 to 6 address the experimental results obtained in this work and include a summary of the chapter and a brief introduction on the motivation of each experiment, followed by the description of the materials and methods, results, discussion and main conclusions.

More specifically, in **Chapter 3** an in-depth characterization of the previously established *in vitro* VBNC state induction model is presented and focuses on its application to modulate *S. epidermidis* biofilms and planktonic cells entrance into a VBNC state.

Then, the construction of *S. epidermidis mazEF* mutant and complemented strains and respective characterization is presented in **Chapter 4**. Herein, the study of *mazEF* as a putative TA system is described and a bioinformatics analysis of the *mazE* and *mazF* genes homology with other strains was performed. Moreover, the susceptibility studies of the target strains (WT, mutant and

complemented strains) upon incubation with antimicrobials and survival in human blood and plasma were also assessed.

The influence of *mazEF* deletion on the host immune response was addressed in **Chapter 5**, where the activation of the innate immune response upon infection of human-derived macrophages and dendritic cells with the different strains was assessed.

The last experimental chapter (**Chapter 6**) presents the study of the role of *mazEF* in the modulation of the VBNC phenotype, focusing on the ability of biofilm and planktonic populations of all strains to enter the VBNC state. Additionally, the structure of biofilms formed by the wild-type (WT) and mutant strains is presented. Finally, this chapter also focuses on the study of the transcription of some important virulence genes when the proportion of VBNC cells is modulated and on the influence of *mazEF* deletion on the expression of these genes.

The final chapter (**Chapter 7**) compiles the main conclusion of this thesis, aiming to respond to the initial questions, and presents the major findings and limitations of the studies performed. Moreover, this chapter is concluded with suggestions for future work that could be performed to increase the knowledge on the main topics covered throughout this work and possibly answer questions that remained unanswered or emerged in the present study.

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CHAPTER 2

Literature review

SUMMARY

This chapter compiles a review of the literature regarding *S. epidermidis* main virulence factors and the role of *mazEF* as a potential toxin-antitoxin system.

2.1. State of the art

Indwelling medical devices have been increasingly used in modern medicine and have saved millions of lives worldwide. Unfortunately, they can also be a source of very serious infections [1]. *Staphylococcus epidermidis*, a commensal bacterium that colonizes healthy human skin and mucosa, has emerged as one of the most common causes of medical device-associated infections [2]. This is due to its ability to adhere to the surface of medical devices and form biofilms, complex tridimensional communities of microorganisms surrounded by an extracellular polymeric matrix, which are highly tolerant to antibiotics [3] and to the host immune system response [4]. Hence, *S. epidermidis* biofilms represent a common source of recurrent and relapsing infections that, due to the lack of effective treatments, are often only resolved via surgical removal of the colonized device, resulting in increased patients' morbidity and an extra burden to healthcare systems [5].

The ability to form biofilms on medical devices is the most important virulence factor of *S. epidermidis* [5]. Moreover, the quantity of dormant bacteria in biofilms influences their ability to cause persistent and recurrent infections. It was previously shown that high amounts of dormant cells can turn biofilms less susceptible to both the host immune response [6] and antibiotic therapy [7]. Furthermore, cells in the dormant state have the particularity of not growing on standard culture media, and, consequently, are not detected in common infection's detection tests [8]. This can result in misleading interpretations of the actual status of the infection, e.g., the bacterial load of a biofilm with dormant cells can be significantly higher than the one detected in usual culture tests [8], or might even lead to false-negative results [9].

The molecular mechanisms of biofilm formation in *S. epidermidis* have been extensively studied, however, despite their clinical relevance, little is known regarding the mechanisms involved in *S. epidermidis* dormancy. A selection of genes potentially involved in the dormancy state was made by performing RNA-sequencing (RNA-Seq) analysis of biofilms in conditions of prevented or induced dormancy [10]. Some of the genes that were differentially expressed in biofilms with induced or prevented dormancy were ranked based on homology analysis with other bacterial species, and it was found that *mazE*, an homolog gene putatively encoding the antitoxin of the *mazEF* complex, was only detected in biofilms with induced dormancy [10]. The role of this gene in *S. epidermidis* biofilms raised interest due to its involvement in bacterial stress response [11] and decreased susceptibility to antimicrobials [12]. So far, to our knowledge, no studies have been performed to

understand the role of *mazEF* in *S. epidermidis* and its effect on biofilm dormancy and pathogenicity.

2.2. Microbial biofilms

Biofilms can be defined as an aggregation of microorganisms and their extracellular products, forming a well-structured population, generally attached to a surface [13], as represented in **Figure 2.1**. Human health can be deeply affected by the development of biofilms, not only because of the low susceptibility towards antimicrobial therapies, but also because biofilms can serve as a continuous reservoir of several opportunistic bacteria that are able to colonize different surfaces [14–16].

Formation of bacterial biofilms is accepted as a survival strategy, being accounted as responsible for several chronic and acute infections [15, 17], from which can be highlighted chronic bacterial wound infections [18], endocarditis [19] and respiratory tract infections [20]. The existence of a polymeric matrix surrounding bacteria has some benefits in protecting bacteria towards environmental changes [21, 22] and also protecting them from being removed from the surface where they are attached [23, 24]. Besides the contribution to the survival of the biofilm under diverse adverse conditions, such as the lack of nutrients, the biofilm matrix is also fundamental for the maintenance of the tridimensional structure of the biofilm [1, 25].

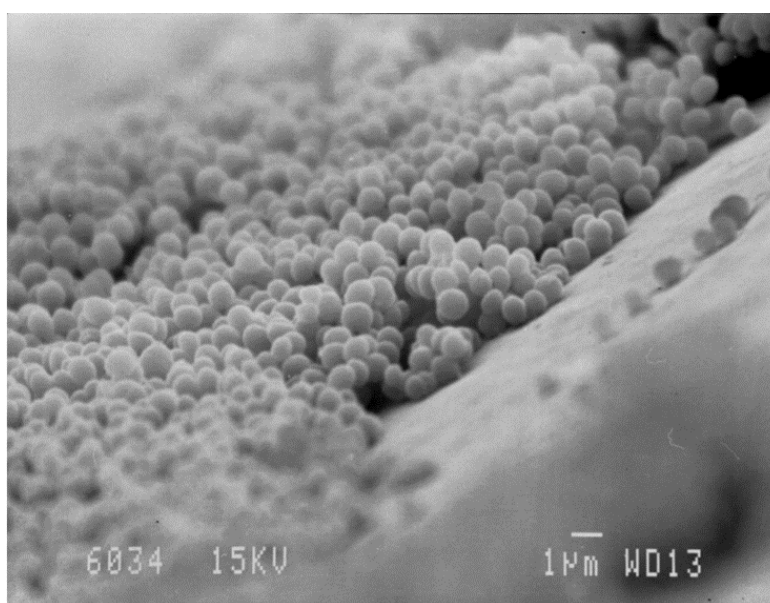


Figure 2.1– Scanning electron microscopy (SEM) of a *S. epidermidis* biofilm in early stages. Adapted from [26].

2.2.1. *S. epidermidis* biofilms and infections

Staphylococci are important pathogens of several animals and humans, and are responsible for a wide spectrum of infections, including a variety of life-threatening systemic diseases [24]. Skin and urinary tract infections, as well as infections of the soft tissues and bones, are common examples of injuries caused by several staphylococci, including by some opportunistic staphylococcal species [5, 27].

Colonization by *S. epidermidis* is frequent and can be very harmful in immunocompromised persons and neonates [5, 28]. On the other hand, this species is known to perform an important role in the maintenance of a healthy skin flora by competing with similar microorganisms which can be considerably more harmful, such as *Staphylococcus aureus* [28, 29]. Being a common inhabitant of the skin, *S. epidermidis* can easily invade this physical barrier through wounds and follicles. This happens mainly when the skin barrier is compromised, for example, due to medical practices as the insertion and removal of catheters and other medical devices, or upon fissures on the skin resulting from surgical procedures [30].

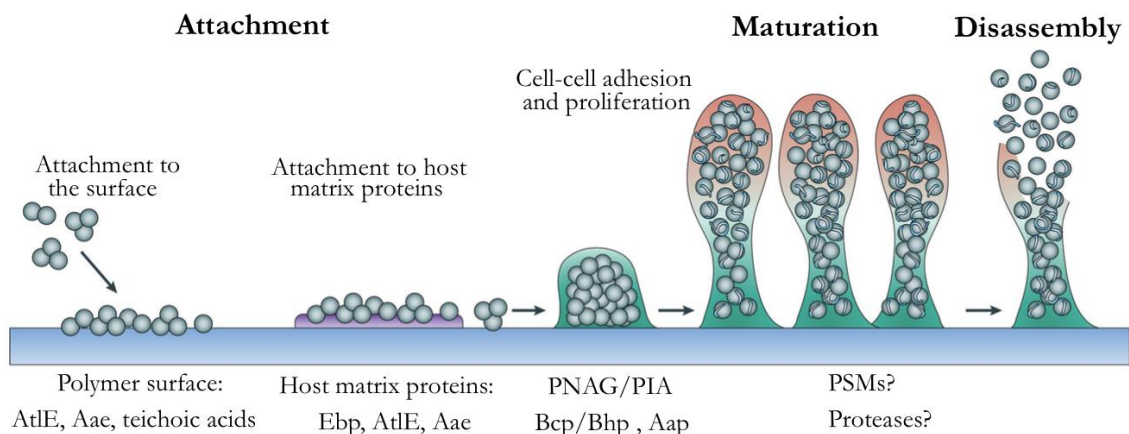


Figure 2.2 – Representation of *S. epidermidis* biofilm lifecycle and some of the molecules involved in the different phases of biofilm formation and disassembly. The process begins with initial attachment to the surface, followed by the adhesion of cells to each other, forming clusters. Maturation of the biofilm is achieved by the growth of bacterial clusters and the production of polymeric matrix by those aggregates, which will accumulate and surround bacteria. Lastly, a mature biofilm is obtained, and bacteria can detach and disperse from this biofilm and colonize other surfaces. Adapted from [5].

S. epidermidis biofilm formation (**Figure 2.2**) is regulated by a system of cell-to-cell communication known as staphylococcal accessory gene regulator (*agr*) [31]. The first phase of biofilm development is generally termed initial adhesion or attachment and comprises bacterial adhesion to surfaces [28]. Non-specific interactions, as hydrophobic and electrostatic interactions, generally mediate this primary attachment to inert surfaces, in which bacteria can adhere directly to the surface of the implanted medical devices [32]. Conversely, specific interactions between the cell surface and host proteins can occur when the medical device is coated with host-derived matrix molecules [32–35]. The AtlE (major autolysin of *S. epidermidis*) is among the bacterial proteins that mediate primary attachment, facilitating the adhesion of bacteria to surfaces or to previously attached host matrix proteins [32, 33]. Similarly, biofilm-associated protein (*bap*) may also be involved in the first stage of biofilm formation, by increasing the hydrophobicity of the cell surface to facilitate the initial adhesion process [36].

Some of the bacteria will detach from the surface, while only a part of those will enter the next phase and be able to form the biofilm, mainly due to intercellular adhesins and autolysins [37, 38]. As the biofilm grows and enters the maturation stage, specific interactions allow the growth of bacteria into clusters [32, 34] and the formation of the hydrated polymeric matrix that surrounds cells in biofilms [5, 34, 39, 40]. Moreover, some molecules, for instance adhesive and exopolysaccharide macromolecules, are produced to enhance cell-to-cell communication and aggregation [5, 34].

The *icaADBC* operon was the first and better studied molecular mechanism associated with biofilm formation by *S. epidermidis*. It is often present in invasive strains of *S. epidermidis* and accomplishes an important function in biofilm formation [40, 41]. Proteins from this operon produce a polymer of N-acetyl glucosamine (PNAG) [40], involved in aggregation of bacteria upon communication between adjacent polymers on the surface of bacteria [42]. As the first known function of PNAG was the intercellular adhesion, this polymer is also commonly known as a polysaccharide intercellular adhesin (PIA) [40, 43].

Furthermore, PNAG has a great influence on the structure of the biofilm [44] and has been shown to increase the survival rate of bacteria in the host [22]. On the other hand, it was also shown that PNAG promoted exacerbated liver inflammatory pathology in mice repeatedly challenged with *S. epidermidis* [45], and the secretion of PNAG acts as a decoy for the immune system [42].

The development of the biofilm continues with the maturation of the cell agglomerates and the production of extracellular polymeric substances (EPS) that will be located between cells [24, 46], as illustrated in **Figure 2.3**. The major components of these polymeric substances are polysaccharides, proteins and nucleic acids that result from cellular metabolism and/or cell death processes, however, the composition of the matrix varies among different biofilms [38, 47].

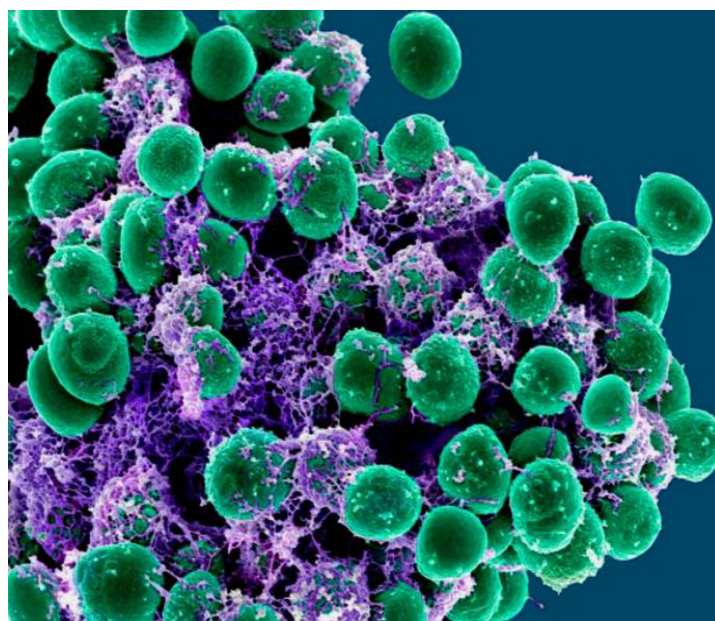


Figure 2.3 – SEM image of a grape-like cluster of *S. epidermidis*, with evidence of biofilm matrix (purple). Adapted from [48].

The biofilm maturation stage proceeds with the formation of channels, which allow the flow of molecules within the biofilm, and the increasing number of bacteria and production of the polymeric matrix allow the expansion of the biofilm thickness [15, 49]. This phase depends on adhesive and disruptive forces and facilitates the communication of cells with the exterior, enabling the circulation of nutrients and oxygen into deeper layers of the biofilm [24, 50].

Later, the mature biofilm reaches a state that no longer tolerates significant growth, the stationary phase, where the division of bacteria occurs at a similar rate to bacterial death due to nutritional and physicochemical limitations [51].

Finally, *S. epidermidis* biofilms are characterized by entering a disassembly process that occurs in order to regulate the cell density of the biofilm [52, 53], being influenced by several environmental conditions [52, 54–56]. The availability of nutrients [57, 58] and oxygen [57, 59–61] and

environmental parameters, such as the pH [62, 63], temperature [64, 65] and nature of the surfaces to which bacteria are attached [49, 52], cause an active release of biofilm cells, known as dispersion, allowing the regulation of the biofilm cell density. Moreover, a passive release process may also occur when the biofilm is affected by shear forces that cause the detachment of cells from the biofilm structure [66, 67].

It is important to have into consideration that smaller active and passive events of cell release may also occur before the final disassembly phase. Although less significant than disassembly, the release of smaller amounts of cells should not be disregarded, as some clusters formed during biofilm maturation may detach and enter blood circulation and, ultimately, cause thromboembolisms [68]. The cells disassembled from the biofilm may be designated as biofilm-released cells (Brc) [53] and have the ability to colonize other sites, contributing to the spreading of infections within the host and to the occurrence of inflammation processes [69, 70].

2.2.2. Antimicrobial susceptibility

An important issue associated with biofilm-related infections is the decrease in the susceptibility to antibiotics [33, 71]. This decreased susceptibility often leads to situations where it is unsuitable to treat infections with common antibiotic therapies, since the concentration of antibiotic needed to kill bacteria within biofilms is higher than the peak serum concentration (PSC) [3, 72, 73], which is the maximum concentration of an antibiotic that the human body can endure after administration. Importantly, the antimicrobial susceptibility of biofilms is mainly dependent on the species and antibiotics used [74, 75]. Additionally, biofilms are thought to present a lower susceptibility to antibiotics by a diversity of factors [76–79], as represented in **Figure 2.4**. Among those limitations, the diffusional barrier to antibiotics [80–82] and a slow growth rate of cells within the biofilm [83–85] are often highlighted as major causes of altered antimicrobial susceptibility.

Moreover, the existence of a phenotype that is more tolerant to antimicrobials [78, 86, 87], such as dormant cells, including both viable but non-culturable (VBNC) and persisters cells, can also partially explain the inefficacy of antibiotic treatments in biofilms [10, 88–91]. Notwithstanding, it is known that neither of the above-mentioned hypotheses *per se* can fully explain the diminished susceptibility of biofilms towards antimicrobial treatment [74, 92]. For instance, some antibiotics can easily diffuse through the biofilm matrix of some species, as was already shown for rifampicin

in *S. epidermidis* biofilms [93, 94] and for amikacin and ciprofloxacin in *S. epidermidis* and *S. aureus* [95], among others.

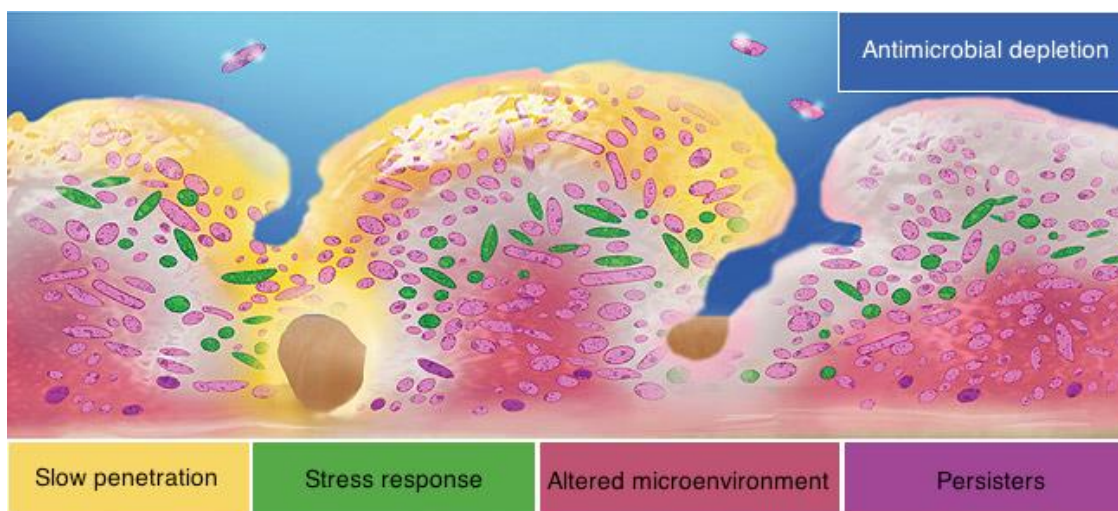


Figure 2.4 – Some hypotheses that attempt to explain the decreased susceptibility of biofilm cells to antibiotics. Adapted from [96].

The structure of the extracellular polymeric matrix can, occasionally, act as a physical diffusional barrier reducing and/or delaying the penetration of certain antibiotics into the biofilm, whereby some antibiotics can no longer reach a great number of bacterial cells [1, 73]. Furthermore, the negatively charged polymeric matrix may also behave as a chemical barrier to the positively charged antimicrobial agents, since these agents tend to bind to the matrix and, thus, the amount of antimicrobial drugs that successfully reach biofilm cells is limited [15, 97]. Moreover, some of the polysaccharides and proteins that constitute the matrix can specifically inactivate some antibiotics [1, 88, 98]. Among the modifications that bacteria experience upon growing as a biofilm, phenotypic changes are one of the most important, considering they may influence the susceptibility to antibiotics within the biofilm environment [1, 73]. It is now accepted that bacteria residing within the biofilm are phenotypically different from free-floating bacteria, whereby some bacteria may experience a differentiation process that leads to a particular dormant phenotype, which generally presents a lower susceptibility against antimicrobials [69–73]. Slow growing bacterial cells can be found in altered environment zones, since in the deeper layers of the biofilm the concentration of oxygen and nutrients is reduced, leading to distinct growth conditions [78, 88, 99]. The reduced bacterial growth rate, as well as the alteration of metabolic processes and reduced metabolic activity, present a limitation to the action of some classes of antibiotics in biofilm cells [78, 88, 99].

The heterogeneity of cells within biofilms [100] also contributes to the decreased susceptibility to antimicrobials [73, 88]. Dormant cells are characterized by being in a physiological state that does not allow growth and present a decreased metabolic activity when compared with other cells [91, 101]. This population generally emerges upon stressful conditions, and presents an altered susceptibility to antibiotics, contributing to recalcitrant infections [102–104], as will be explored in the next section.

As a consequence of the reduced susceptibility of biofilms to some common antibiotics, it is occasionally necessary to use a combination of different antibiotics to be able to treat biofilm infections [1, 99, 105]. When the difficulty is related to the diffusional barrier, the use of multiple antibiotics and other substances as adjuvants to promote diffusion through the matrix that envelops bacteria may be a solution. For instance, it was found previously that rifampicin used combined with vancomycin in *S. epidermidis* would provide a better result, as rifampicin improved the diffusion of vancomycin through the matrix [93]. In severe cases of antimicrobial therapy failure to treat medical device-related infections [104, 106], the removal of the colonized medical devices is often required, resulting in high costs and great inconvenience to patients [107, 108].

2.2.3. Cell dormancy within biofilms

There has long been some controversy regarding the definition and distinction between the terms: dormancy, persistence and VBNC [91, 109], but recent reviews have tried to clarify and standardize such definitions [89, 101, 110–112].

Nowadays, the concept of dormancy is widely applied to describe populations of cells that experienced a significant reduction in their metabolic activity, but not a complete loss [91], and, despite being alive, do not grow on laboratory cultures [89–91, 109, 110]. Dormant cells exist in a reversible non-replicative physiological state, i.e., these cells are in a temporary state where their reduced metabolic processes do not allow their replication, yet this state can be reverted with the appropriate growth conditions [103, 104]. In other words, dormancy may be viewed as a condition of extremely slow growth, which presents a decreased antimicrobial susceptibility [111, 113], but where cells maintain an intact membrane and membrane potential, contrasting with dead cells [114].

Several human pathogens have been shown to exist in a dormant state [115], which may impair the detection of the infections since traditional culture methods are unable to detect unculturable

cells [116]. On the other hand, most of these pathogens also present an ability to revert the unculturable physiological state of dormancy when in the presence of favourable microenvironmental conditions [91, 117], as happens in multiple staphylococcal species, including *S. epidermidis*, when grown in media without antibiotics and suitable concentrations of nutrients and oxygen [118–121].

Dormancy was previously defined as a state that occurs in response to unfavourable environmental conditions and did not contemplate the emergence of persisters, caused by stress related to antimicrobial therapies [122]. However, it is nowadays accepted that dormancy includes cells originated in both conditions, including VBNC and persister cells [89, 101, 110]. Altogether, the VBNC state is caused by environmental stresses [91, 116], such as nutritional starvation [123], oxygen limitations [124], oxidative stress [125] and substantial changes in the temperature, salinity and pH of the environment [65, 126, 127]. On the other hand, persister cells generally refer to a state in which cells can survive antimicrobial treatments [103, 128], entering the dormant state due to the interaction with antibiotics [129] or environmental stresses [89,91].

Persistence was first described in the early 40's in *Staphylococcus pyogenes*, when it was observed that a small population of cells (named persisters) survived after exposure to penicillin, a mechanism found to be different from traditional mechanisms of antibiotics resistance [130, 131]. Persisters were, back then, defined as cells within a genetically homogeneous population with the ability to survive different types of stress, being considered phenotypic variations of the wild-type [132]. Importantly, chemical inhibition of protein synthesis may lead to the development of persister-like cells. Therefore, antibiotics acting on the protein synthesis machinery can trigger the formation of persisters and, consequently, decrease their susceptibility to antimicrobials [106].

Thus, persisters are typically a sub-population of cells that entered a dormant state in which they do not proliferate and present a reduced susceptibility to antibiotics [133]. A particularity of persisters is that they are able to resist upon exposure and treatment with high doses of antibiotics, being able to revert the transient dormant phenotype when the antibiotic stimulus ceases [73, 87], which leads to the problem of relapsing infections [86, 103, 134].

As both VBNC and persisters cannot be detected by means of classical laboratory detection methods, standard tests may lead to a misleading interpretation of the status of infections [8]. To overcome the detectability problem of non-culturable cells by counting colony-forming units (CFUs), it is necessary to use methods and/or techniques able to detect viable bacteria, such as the use

of LIVE/DEAD staining together with microscopy or flow cytometry techniques [6], which are often not used for this purpose in healthcare units.

2.2.3.1. Induction of the VBNC state in *S. epidermidis* biofilms

Previous *in vitro* studies confirmed that biofilm dormant cells present a reduced susceptibility towards antimicrobials [7], as well as an increased ability to escape the host immune system response [71, 83], rendering biofilms more difficult to be treated. Moreover, using *in vitro* and *in vivo* models, it was possible to confirm that dormancy may impact the virulence of *S. epidermidis* biofilms under certain conditions [6, 79]. For those reasons, the interest in studying dormancy has increased over the last years, in an attempt to better understand the mechanisms and implications of dormancy in biofilm-associated infections.

An *in vitro* model to study the effect of dormancy in *S. epidermidis* biofilms was previously developed [6]. This model was developed to induce or prevent dormancy in biofilms with the main aim of analysing how dormancy influenced biofilm physiology [6]. In this model, dormancy was assessed by the quantification of VBNC cells and induced by growing biofilms in a glucose-enriched medium and prevented by adding divalent ions such as Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) [6]. Induced dormancy was achieved by incubating biofilms in TSB supplemented with 1% glucose, that, due to the glucose metabolism, leads to the acidification of media [6, 135], resulting in the inhibition of bacterial growth [136].

On the other hand, high levels of extracellular divalent calcium and magnesium ions (Ca^{2+} and Mg^{2+}) [137–141] were used to reduce the number of bacteria entering the dormant state, being this a pH-independent phenomenon, as no changes in the pH were detected with or without calcium and magnesium supplementation [6].

Importantly, it was later found that supplementation with magnesium alone was able to prevent dormancy induced by glucose, without using calcium [79], and this model was used herein. While the exact mechanism behind the prevention of VBNC cells formation by Mg^{2+} was not elucidated [6, 140], recent studies showed that bacteria can control the membrane potential to withstand the effect of antibiotics targeting ribosomes or against starvation-induced persisters, and this ion flux modulation can be achieved with magnesium [142, 143].

Additionally, it was demonstrated that deprivation of magnesium in the extracellular media would lead to a reduction in the cytoplasmic ATP [143]. Since ATP levels and the formation of persister

cells have been correlated [144–147], it is likely that magnesium prevented the induction of dormancy via an ATP-mediated mechanism.

Nevertheless, this dormancy model presents some limitations that should be considered when interpreting results. One of the major constraints is the fact that the model is not able to generate populations with either 0% or 100% dormant bacteria, which results in a mixture of VBNC and metabolic active cells in both conditions (prevented and induced dormancy). This results in the proportion of dormant cells relatively to the number of total culturable cells being significantly higher in biofilms with induced dormancy [6]. Another potential drawback of this model was shown when testing different clinical and commensal *S. epidermidis* isolates, as some were not able to produce VBNC cells under the conditions of the model used with the control strain (*S. epidermidis* 9142) [148]. Although this model may be suitable to study *in vitro* biofilms with induced dormancy and its implications, the correlation with *in vivo* biofilms may be questionable.

Previous studies using this model confirmed a decreased susceptibility of *S. epidermidis* VBNC cells to vancomycin or penicillin, that was maintained even after allowing the cells to grow in the planktonic state and under regular culture conditions (no induction or prevention of dormancy) [79]. Importantly, it was also shown that dormancy interfered with the host immune response to biofilms [6, 149], since, as assessed by the production of pro-inflammatory cytokines, *S. epidermidis* biofilms with higher proportions of dormant bacteria were shown to be less inflammatory [6].

Moreover, the transcriptomic and proteomic profiles of *S. epidermidis* biofilms with induced or prevented dormancy have been assessed [10, 150, 151]. Although the differences in the expression of the genes detected by RNA-Seq were not substantially high, with up to 3 fold of up- or downregulation, genes with potential involvement in biofilm dormancy were highlighted [10]. From all the genes assessed (RPKM >1), 22 were found to be expressed only in conditions of induced dormancy, 29 were only detected when dormancy was prevented, and, from the 2047 detected in both conditions, 28 genes were upregulated and 110 were downregulated, relatively to the prevented dormancy condition [10]. Genes that presented a different expression when dormancy was induced or prevented were mainly involved in metabolic processes as oxidation-reduction and acetyl-coA, as well as in ribosome activity [10].

Besides the evident alterations in genetic expression, the proteomic profile of biofilms with induced dormancy also presented differences, with most of the overexpressed proteins being associated

with the ribosome synthesis pathway [150]. Conversely, proteins associated with ion binding and catalytic activity were underexpressed when dormancy was stimulated, which correlates with the decreased metabolic activity of VBNC cells [150].

2.3. Pathogenicity and immune response activation

Being a common inhabitant of the skin and mucosa of humans and other mammals, *S. epidermidis* generally presents a benign interaction with the host. However, it can behave as an opportunistic pathogen when the host defences are compromised [5, 152]. In fact, previous studies designated this species as an “accidental” pathogen [5], however, it is still not clear if *S. epidermidis* infections are accidental or if this microorganism represents an unknown pathogenic profile with the ability to overcome the host defences and take advantage of immunocompromised patients and neonates [28, 153, 154].

Based on the previous, a concern was raised regarding the effect of the host immune response against a bacterium that presents a great proportion of the healthy human skin microbiome [154]. Concretely, former studies have confirmed that *S. epidermidis* presents the ability to surpass the host defences to cause infections [4, 152]. Conversely, it has also been shown that the immune response is triggered by sensing *S. epidermidis* infections as pathogenic invasions [152] and recent studies have found that, in the skin, this species can boost the innate immune response by activating $\gamma\delta$ T cells [155].

The immune system protects the host against microbial infections through innate and adaptive responses [156]. Of note, the immune response has to be controlled, as it is supposed to eliminate pathogens and toxic substances but has to avoid killing commensal microorganisms or even cause excessive damage to the host tissues [157]. Innate immunity consists of mechanisms that exist prior to the infection or are promptly triggered, and have broad specificity, i.e., an unspecific and immediate response that is usually activated upon contact with pathogen-associated molecular patterns (PAMPs). Adaptive immunity refers to a later response, characterized by high specificity for pathogen antigens, which may be recalled after each encounter with the same antigen [156, 157].

Importantly, both innate and acquired immune mechanisms can cooperate through cellular and molecular factors. Innate immunity stimulates the adaptive response, while the adaptive response stimulates innate immune cells to more effectively eliminate invading pathogens [157].

Nonetheless, despite the response of the immune system to *S. epidermidis* invasions being insufficiently studied, it is currently accepted that the response of adaptive immune system does not play an important role [152, 158], and, thus, the main focus will be put on the study of innate immunity response.

2.3.1. Innate immunity

Overall, innate immunity is composed of physical and chemical barriers, including the skin, antimicrobial substances produced by epithelia, phagocytic cells and soluble proteins, such as the complement system, that interact and communicate in a very complex and unique way [159]. When the primary line of immune defence is breached, for instance, upon wounds in the skin or mucosa epithelia, the innate immune effectors are required to act immediately through rapid identification of invading pathogens and recognition of PAMPs [160].

A first step in the innate immune response to pathogenic agents is their recognition by phagocytic cells, which include neutrophils, macrophages and dendritic cells [159]. Phagocytes are cells with the ability to engulf bacteria and other small cells and particles and eliminate them by oxidative and non-oxidative (e.g. enzymatic) mechanisms [161]. These cells may recognize pathogens through binding to conserved molecules on their surface (PAMPs), using pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). Binding to PRRs initiates downstream signalling events that ultimately lead to the production of cytokines, some of which may promote the recruitment of phagocytic cells to the site of infection [162, 163].

Previous findings in *S. epidermidis* showed that the immune response triggered by this bacterium involves recognition by Toll-like receptor 2 (TLR2), which usually binds to TLR1 and TLR6 forming heterodimers that recognize proteins associated with cell-wall, such as lipoproteins, lipoteichoic acids (LTA) and peptidoglycan [164–166]. Moreover, former studies have also reported that TLR2 can be stimulated by phenol-soluble modulins (PSMs) secreted by *S. epidermidis* or by PIA/PNAG mainly present in this species' biofilms [164, 167, 168].

After recognizing the pathogen, phagocytic cells will start the phagocytosis process aiming to ingest and destroy the threat, by going through an initial internalization involving the formation of phagosomes. Phagosomes mature into phagolysosomes, which have an acidic pH, necessary for full microbicidal activity [169, 170].

Phagocytes have the ability to produce reactive oxygen species (ROS), that can kill pathogens by causing oxidative stress and damage, or induce pathogen killing by non-oxidative mechanisms [171, 172]. The latter include the production of antimicrobial peptides (AMPs), lytic enzymes, or the release of nucleic acids, forming the so-called neutrophil extracellular traps (NETs), which can entrap bacteria and promote their killing [172–174].

Besides their phagocytic activity, macrophages, as well as dendritic cells, are also professional antigen-presenting cells (APCs), meaning that they are able to present pathogen antigens to T-cells, through class I and class II major histocompatibility complex (MHC) molecules. Macrophages and dendritic cells provide distinct signals that are needed for T cell effector function, such as the presentation of the antigen on MHC molecules, the expression of co-stimulatory molecules (such as CD80 and CD86) that bind to CD28 on the surface of T cells, and cytokine production, that, altogether, trigger their activation and differentiation [175, 176].

2.3.2. Cytokine production

Macrophages are key modulators and effectors in the host immune response. These cells can differentiate into distinct states. M1-type macrophages are mainly involved in pro-inflammatory processes with a phagocytic profile, while alternatively activated M2-type macrophages are more related to anti-inflammatory processes and wound repair [176, 177]. Despite some differences, both macrophage types are involved in the innate immune response and produce cytokines in response to their activation by bacterial constituents, triggering the activation of further immune cells.

Cytokines are cell signalling molecules that act as intermediates in cell-to-cell communication during the immune response process. Cytokines generally bind to specific receptors located on the surface of the immune cells, triggering a cascade of events that will lead to their activation and, often, to the production of more cytokines. Overall, these proteins have the ability to induce the production and migration of immune cells towards the site of infection and have a vital function in the regulation of inflammation, either with pro-inflammatory or anti-inflammatory roles [178, 179].

Cytokines are the general group of communication proteins and include lymphokines (produced by lymphocytes), monokines (produced by monocytes), chemokines (cytokines presenting chemotactic activity) and interleukins (cytokines produced by specific leukocytes that will produce an action on other leukocytes) [178]. Most of the cytokines are produced by distinct cells and can

either act on distinct effectors or on the producer cell itself, while also being able to act as positive or negative regulators, as schematized in **Figure 2.5**.

Due to their distinctive role in the mediation of inflammation, cytokines can be divided into pro- and anti-inflammatory, although both types are involved in the recruitment, differentiation and activation of immune cells. Among the more well-known cytokines, IL-1, IL-6, IL-12, IL-17, TNF- α and IFN- γ are usually referred to as pro-inflammatory, while IL-10, TGF- β or IL-35 are known as anti-inflammatory cytokines [180].

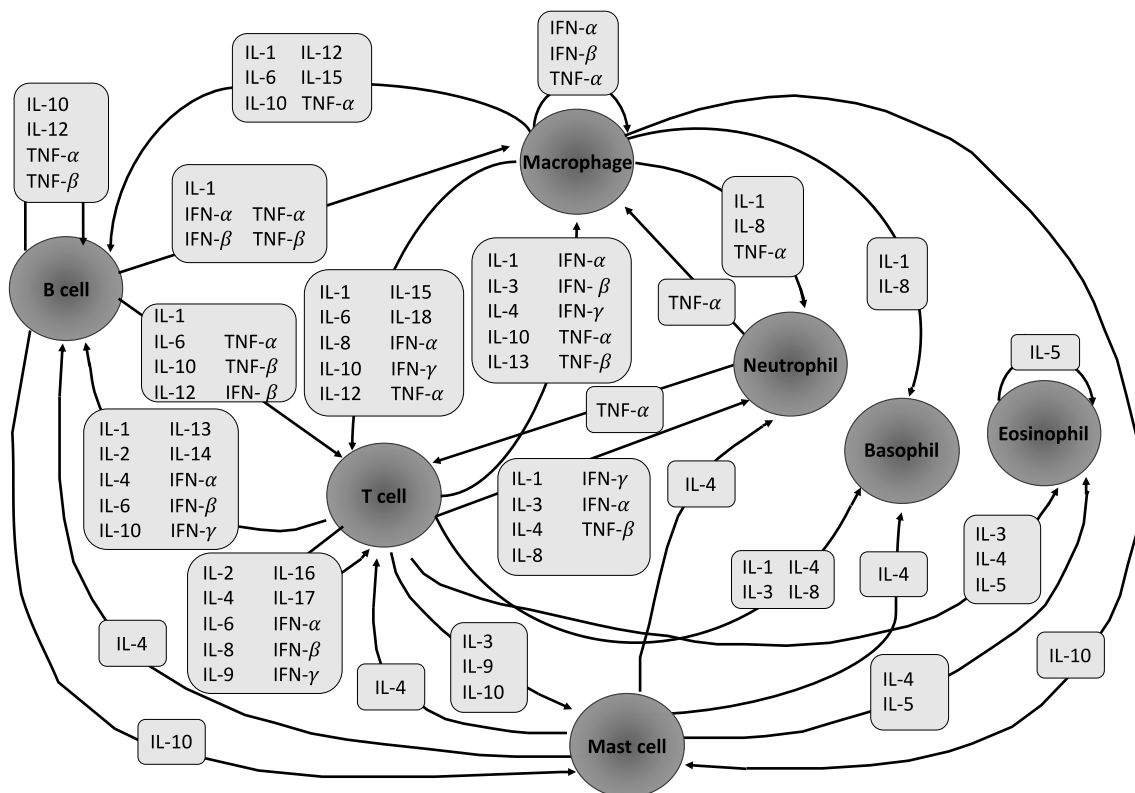


Figure 2.5 – Cytokine network. The effectors of the immune system (macrophages, neutrophils, basophils, eosinophils, mast cells, B cells and T cells) are regulated by the action of distinct cytokines and communicate with each other through these cell-signalling molecules. The proliferation and activation of immune effectors is achieved as a response to cytokine production and leads to the secretion of these proteins by other cells, in a back and forward regulation. For instance, macrophages produce cytokines to stimulate a specific antigen-dependent response by B and T cells, while it also induces non-specific responses from the other cells. In turn, T cells are able to produce a variety of cytokines that stimulate specific antigen-associated responses, such as the activation of B cells, which produce molecules to regulate the expression of macrophages. Adapted from [178]. IL: interleukin; TNF: tumour necrosis factor; IFN: interferon.

Although the response of the immune system to *S. epidermidis* infections has not been extensively studied, it was already established that invasions by this opportunistic pathogen cause an increase in the production of some cytokines. For instance, the incubation of monocyte-derived macrophages with *S. epidermidis* biofilm cells resulted in the stimulation of the production of IL-1 β , IL-13, IFN- γ and TNF- α , with overexpression of IL-13 compared to planktonic counterparts [181]. Another study revealed that the anti-inflammatory cytokine IL-10 was produced by monocyte-derived dendritic cells incubated with this bacterium [182], while other studies reported the secretion of pro-inflammatory cytokines such as IL-6, TNF- α and IFN- γ upon infection with *S. epidermidis* [183].

Overall, there are several reports of the activation of the host immune system in response to opportunistic infections caused by *S. epidermidis* bacteria. Nonetheless, the levels of cytokine production are often considered low comparing, for instance, with *S. aureus*, which showed considerably higher levels of cytokine production and activation of immune cells [182, 184].

2.3.3. Activation of the complement system

As mentioned before, the complement system is an important part of the innate immune system. This system is composed of opsonizing proteins that act as pro-inflammatory chemoattractant molecules in the recruitment of phagocytic cells to the site of infection [185], since the main purpose of the complement is to enhance phagocytosis with the opsonization of cells.

The complement system can be activated by bacterial PAMPs, leading to the sequential production and activation of protein fragments that will, for instance, stimulate immune cells to produce cytokines and modulate the activation of a proteolytic cascade, in which the cleavage of some proteins will lead to the activation and cleavage of the next protein [185, 186]. This activation can be done by three distinct pathways: (i) classical, (ii) lectin, and (iii) alternative. While the first (classical) depends on antibodies bound to bacterial surface molecules, the second (lectin) requires the binding between mannose-binding lectin or other ficolins to carbohydrate residues present in the surface of the pathogen cells [185, 187]. On the other hand, the alternative pathway can be distinguished from the previous ones by being continuously and spontaneously activated with the hydrolysis of the thioester bond in the C3 molecule [188]. Upon the activation of the complement system and, subsequent production of chemoattractant molecules, such as C3a or C5a, the phagocytic cells will move to the site of the infection and phagocytose opsonized cells [189].

Although the activation of the complement system by *S. epidermidis* invasions is not completely understood, previous studies have reported that the classical and lectin pathways seem to be the way of triggering the complement system by this bacterium [175]. Importantly, *S. epidermidis* has some particularities that allow it to avoid recognition and activation of the complement system, as will be further explored.

2.3.4. Evasion from the immune system

Several studies argue that *S. epidermidis* has the capacity to escape immune defences, mainly due to the misrecognition as a commensal species rather than a pathogenic invader. Moreover, the fact that *S. epidermidis* is frequently found living as biofilms also contributes to the decrease of the efficacy of the immune system in eradicating *S. epidermidis*, since biofilms are known to employ particular mechanisms to evade the host response. For instance, a previous study showed that the biofilm confers protection of *S. epidermidis* against opsonization by complement proteins and against neutrophil-mediated killing [190]. On the other hand, the biofilm structure *per se* can help in the evasion by hindering the diffusion of antibodies, opsonophagocytic molecules, phagocytic cells and ROS through the matrix [191–193], interfering with the process of opsonization and, more importantly, with the success of phagocytosis [4, 194, 195], as demonstrated for several species, including *S. aureus* [191, 196] and *S. epidermidis* [4, 190, 194, 195].

S. epidermidis has been shown to produce molecules that confer tolerance to AMPs, such as the SepA protease [197], and it was previously reported that this species' biofilms were able to inactivate AMPs and also the proteins of the complement system, avoiding the death of the constituting cells [190]. Moreover, *S. epidermidis* is also recognized by its ability to produce phenol-soluble modulins (PSMs), which were suggested to present cytolytic activity against human neutrophils [198, 199] and by the production of PNAG [4], important for biofilm formation, and formation of a polysaccharide capsule [200, 201] that prevents bacteria from opsonization and protects them from phagocytosis by neutrophils and macrophages [190, 202–205].

Finally, a recent review focused on how *Staphylococci* evade the host innate response emphasized that biofilm formation is the most common strategy used by *S. epidermidis* and *S. aureus* to avoid opsonization and further phagocytosis through the production of proteins that target opsonization by blocking chemoattractant molecules [206].

Taken together, it is evident that *S. epidermidis* bacteria are often able to evade or delay the action of the host immune system, contributing to the spreading of the infection, especially biofilm-associated infections.

2.4. Toxin-antitoxin systems

Among the genes differentially expressed in VBNC induced conditions, *mazE*, a gene that belongs to the *mazEF* operon, has triggered special interest since it was only detected in biofilms with a higher proportion of dormant cells [10]. Homologues of *mazEF* operons have been previously characterized as toxin-antitoxin (TA) systems in several species, such as *S. aureus* where it was found to be involved both in bacterial stress response [11] and diminished susceptibility to antibiotics [12]. Moreover, the involvement of TA complexes in biofilm dormancy and persistence have previously been reported in other species, where, for instance, it was found that *mazEF* increased the number of persister cells in *Streptococcus mutans* [207] and that the overexpression of the toxin *mazF* was related to the formation of persister cells in *E. coli* [208].

Several factors are known to facilitate the persistence of nosocomial infections, such as the formation of persister cells [209], decreased susceptibility to antimicrobials [210], inducement of a dormant state [210] and increased biofilm formation [209], mechanisms in which TA systems were suggested to play a role [211].

TA systems are genetic modules that consist, in general, of a set of two closely related genes organized in an operon, encoding a stable toxin and a labile antitoxin [212]. TA systems are located on plasmids and chromosomes of many bacteria and work as a “poison and antidote” system, since the two modules present antagonistic roles [11, 213]. Usually, the toxin targets an essential cell process and the antitoxin is capable of neutralizing or suppressing the effect of the toxin [212, 214].

Most studies addressing TA systems, including the *mazEF* system, have been performed with *E. coli* [215–217]. In this species, TA systems contribute to the bacterial epigenetic machinery that regulates and control bacterial survival, meaning that the overexpression of these genes can regulate the inhibition of cell growth and, in some cases, trigger cell death [210, 218]. An interesting feature of these systems is that, when they are contained in plasmids, only the daughter cells containing the plasmid and, consequently, the TA system, survive after cell division, a process known as post-segregational killing [219]. In the case of plasmid-encoded TA modules, when a daughter cell is replicated with no plasmid inside, the labile antitoxin, which is already present

inside the cell, will be readily degraded due to its instability upon TA activation, resulting in the free action of the stable toxin, which was also inherited during cell division, inducing cell death, what explains why cells only survive in the presence of the replicating plasmid [219, 220]. It has also been found that the inducement of low levels of toxin expression in *E. coli* systems may result in the growth arrest of a subpopulation of cells, or, when inducing the toxin at high levels, to a state of induced dormancy, which supports the involvement of TA systems in biofilms dormancy [221–224]. This presents a challenge to antimicrobial therapies and compromises the efficiency of antibiotics and, consequently, has been pointed out as one of the main causes of persistent infections [223].

Several studies, conducted especially with *E. coli*, allowed to understand that the homeostasis of bacteria can be disrupted by a series of events, as exposure to antibiotics [209, 210], phage infection [211, 225], and other stressful conditions, for instance nutrient starvation and temperature and pH alterations) [207, 210, 226]. These factors were shown to interfere with several cellular processes in which TA systems are proposed to have a role [227, 228], as represented in **Figure 2.6**. As a result, TA systems were shown to be involved in biofilm formation [209], cellular stasis [211], antibiotic-mediated programmed cell death [210], formation of persister cells [227] and antiphage defence [229]. Bacterial toxins of TA systems can be released into the host cells, threatening the host immune response by inducing death of the host innate immunity cells, increasing bacterial virulence, as discovered with the toxins ChpK and MazF in *Leptospira interrogans* [230].

Currently, there are 6 categories of TA systems, according to their nature and mode of action [231]. In types I and III, the antitoxins are RNA molecules that regulate the activity of the corresponding toxin by inhibiting the translation of the toxin mRNA, as antisense RNA (Type I) or by directly inhibiting the action of the toxin protein (Type III) [212, 231]. TA systems type II and IV are characterized by the fact that both toxin and antitoxin are proteins [209, 231]. While in type II systems the antitoxin binds directly to the toxin to inhibit its effect and toxicity [212, 232], in type IV systems, the proteins counteract without direct interaction, with the antitoxin reversing the effect of the toxin by acting on its target [220, 233]. Interestingly, the latest TA system types to be discovered present different regulatory principles compared to types I to IV [220, 231]. Type V are characterized by a protein antitoxin that has a sequence-specific activity of endoribonuclease that acts on the cleavage of the toxin mRNA, preventing its translation [234]. On the other hand, type

VI are known to have the toxin degraded by cleavage through a specific protease in a complex together with the antitoxin, rather than a direct actuation of the antitoxin itself [235].

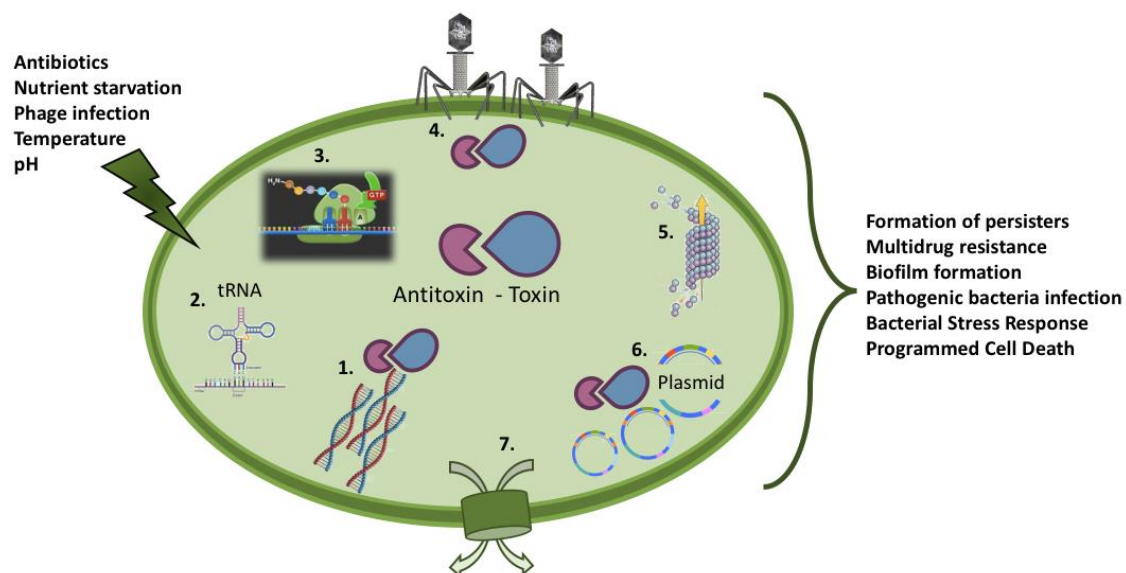


Figure 2.6 – Hypothetical involvement of TA systems in cellular processes and main functions performed by these complexes in several bacterial species, most already confirmed for *E. coli*. Stressful conditions can act as TA inducers, such as antibiotic stress, nutrient starvation, phage infection and environmental alterations, as changes in temperature and pH. TA systems can perform a diversity of functions, as regulation of: 1. DNA replication; 2. tRNA synthesis; 3. macromolecular synthesis; 4. phage infection; 5. cytoskeletal polymerization; 6. plasmid maintenance; and 7. cell wall disruption. Adapted from [227].

2.4.1. Characterization of the *mazEF* module in *E. coli*

The *mazEF* system was first described in *E. coli* as a stress-induced TA located on the chromosome, in which *mazF* codes for the stable toxin MazF and *mazE* for the labile antitoxin MazE [217]. *mazEF* is negatively autoregulated by the combined action of the toxin (MazF) and antitoxin (MazE) proteins, which are co-expressed and interact directly at the transcriptional level on the *mazEF* promoter P_2 , being characterized as a type II system [212, 228].

Stressful conditions can affect the continuous expression of *mazE* by preventing its transcription and/or translation, thereby, *mazEF* is defined as a stress-induced suicide module [236]. On the other hand, the toxin MazF is a sequence-specific mRNA endoribonuclease that can initiate the

programmed cell death (PCD) pathway in response to various stresses [210, 212, 228], as described in **Figure 2.7**.

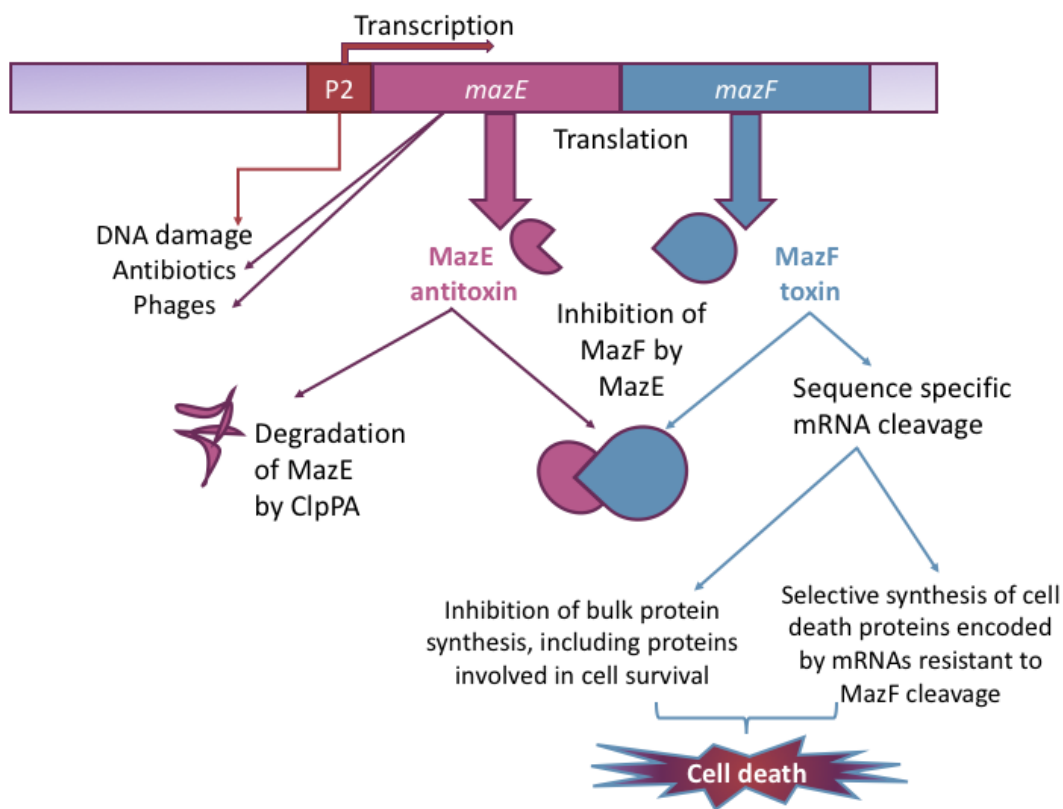


Figure 2.7 – Schematic representation of the *E. coli* *mazEF*-mediated cell death pathway. The combined action of MazE and MazF proteins on the *mazEF* promoter *P₂* leads to the negative autoregulation of *mazEF* at the transcription level. MazE antitoxin and MazF toxin act in a coordinate matter, with the MazE antitoxin inhibiting the action of the toxin MazF. Besides being labile, MazE protein can also be degraded by ClpPA, leaving the toxin to act freely and induce apoptosis. MazF toxin can inhibit protein synthesis through cleavage of mRNAs and act on the synthesis of cell death proteins that induce programmed cell death. Adapted from [210].

Although MazF is a stable long-lived toxin, the labile protein antitoxin, MazE, can be degraded by ClpPA serine protease [228]. Stressful conditions, such as antibiotics targeting the inhibition of translation and/or transcription or antibiotics able to cause DNA damage, can prevent the expression of the *mazEF* module and, consequently, cause a reduction in the concentration of MazE, allowing MazF toxin to act uncontrollably [218]. Such stress conditions, known to cause bacterial cell death, have been found to act through the *mazEF* module [236–238]. Recent studies have shown that MazE could “resuscitate” *E. coli* cells within six hours of MazF overproduction,

however, exceeded that time threshold, MazE was unable to reverse the bactericidal effect of the overexpressed MazF [218, 226].

Proteins from the TA complexes are known to lead to cell growth inhibition, which, in turn, may interfere with DNA replication and translation, cell division and ATP synthesis [227]. As so, by inhibiting important cellular processes, TA modules may decrease *E. coli* biofilm formation and hinder its maintenance [220, 227]. Previous studies have suggested that some TA modules may play an important role in infections, especially during host colonization, implying that TA systems may be considered new virulence factors [224]. Moreover, a differential effect of TA systems on cell death and biofilm formation was previously shown with *E. coli*, where a significant defect on biofilm formation was caused by the deletion of the toxin *mazF*, emphasizing the idea that biofilm formation may, indeed, be affected by *mazEF* TA mechanisms [239].

As previously explained, a continuous expression of MazE is required to prevent cell death, and this served as a basis for the development of a model to study *mazEF* as an inducer of programmed cell death in response to severe nutrient starvation, such as a specific lack of amino acids, heat shock or exposure to antibiotics [217, 230, 240]. Furthermore, *mazEF* acts as a regulator by inhibiting translation through cleavage of mRNAs at specific sites, recognizing specific amino acid sequences to induce a reversible state of bacteriostasis [228]. Nonetheless, as a result of the involvement of TA systems in PCD, it is suggested that mutations on the *mazEF* complex may result in a prominent decrease of biofilm formation, as previously reported in several studies [214, 239, 241].

2.4.1.1. Programmed cell death in *E. coli*

PCD is an active process that leads to cell death. This mechanism is essential to several microorganisms, since it is required for the elimination of harmful and/or damaged cells and regulation of the cellular population [242, 243]. The most well-known PCD processes in bacteria are related to TA systems [228]. The relation between TA and PCD has already been proven, for instance, with the activation of *mazEF*-mediated PCD as a response to DNA repair systems failure to detect damaged chromosomes, causing cells carrying genomic mutations or defects to enter the PCD pathway, thereby contributing to genomic stability within populations [210].

Although the involvement of *mazEF* in PCD may be controversial in other species [244], with some authors reporting that MazF induces a reversible stasis phenomenon instead of leading to cell

death [245, 246], *mazEF* was first described as a TA system linked to PCD in *E. coli*, being responsible for post-segregational killing [217, 228]. The expression of *mazEF* has been associated with a state of bacteriostasis in response to cellular damage [247], however, when reaching a certain threshold, for instance due to exhaustive DNA damage, the TA system may guide cells to enter the PCD pathway instead [248]. This mechanism is also associated with severe nutritional stress, during which the death of part of the population provides nutrients to the remaining surviving cells [217]. It has been shown that *mazEF*-mediated death can be triggered by thymine starvation [236, 249], mitomycin C [237], nalidixic acid [237], UV irradiation [237], antibiotics [210, 238], oxidative (H_2O_2) stresses [237, 250] and high temperatures (50 °C) [251, 252].

2.4.2. *mazEF* and persister cells

Despite some debate regarding the involvement of TA systems in the control of biofilm formation, as previously mentioned, recent studies reported that the autoregulation of these systems interferes with the formation of persister cells in *E. coli* [129, 253], either by a spontaneous [254] or stochastic [221, 255] process. Moreover, further studies explained how the redundancy of type II TA systems in bacteria is associated with an increased frequency in the formation of persister cells [256].

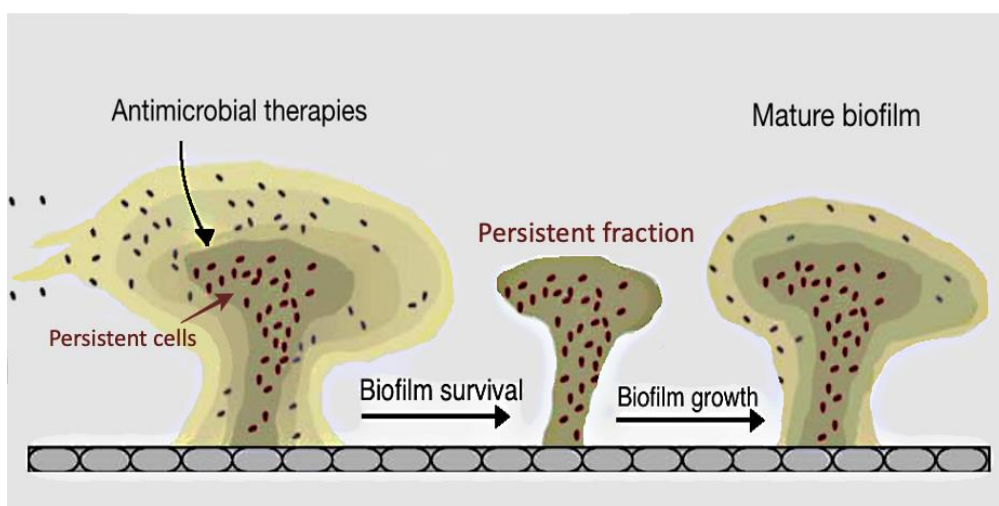


Figure 2.8 – Schematization of the persistence mechanism due to persister cells. Although antimicrobial therapies can eradicate part of the biofilm cells, some persistent variants are not affected by the antimicrobial drugs and can persist, ensuring biofilm survival. After antimicrobial therapy discontinuation, the cells within the persistent fraction are able to continue to grow and lead to a mature biofilm again. Adapted from [257].

Although a small number of cells enter the persistent state (Figure 2.8), this phenomenon is remarkably influenced by the decrease in the synthesis of some proteins, as a result of environmental and stress stimuli, such as nutritional depletion, antibiotic stress and host immune system pressure [209], known to influence the regulation of the expression of TA modules [106].

2.4.3. The role of *mazEF* system in different species

It is known that TA systems may present different sequences, mechanisms and physiological functions in gram-positive and gram-negative bacteria [258, 259]. Thereby, not all the findings reported in *E. coli* can be extrapolated for other species, especially gram-positive. Although the TA function of *mazEF* was experimentally confirmed in distinct species, such as *S. aureus* [260], *S. mutans* [261] and *Staphylococcus equorum* [262], this operon has been described as a TA system in other species based on protein homology, as, for instance, in *S. epidermidis* [263], *P. aeruginosa* [264] and *Mycobacterium tuberculosis* [265]. In fact, a recent study showed that the *S. aureus mazEF* sequence has a homologous sequence in all the 36 species of the *Staphylococcus* genus that were assessed [266]. Interestingly, it was previously found that MazE-like proteins in gram-positive species form a cluster and are more closely related than the MazE-like proteins in gram-negative bacteria [11]. Accordingly, also MazF proteins in gram-positive strains presented a significant similarity and identity between them [262].

Notwithstanding, some similarities were found between *mazEF* functions in several species, regardless of their gram staining. Earlier studies reported that TA systems did not play a role in biofilm formation in either *S. mutans* [267] or *E. coli* [268]. However, recent studies showed the involvement of TA in biofilm formation [209, 269], with diverse scientific publications linking *mazEF* regulation and biofilm formation on distinct pathogenic species, such as *E. coli* [214, 241], *P. aeruginosa* [264, 270], and *S. aureus* [270, 271]. Interestingly, it was previously reported that *mazEF* systems may be involved in the persistence of *S. mutans* bacteria [207], where overexpression of genes associated with the toxin and antitoxin modules was found in persister cells, similarly to what was described in *E. coli* [208, 253]. More similarities with *E. coli* have been described in *M. tuberculosis*, a bacteria that can be either gram-positive or -negative, as it was found that *mazEF* play a role in antimicrobial susceptibility and in stress response in this species [265, 272].

Similarly to what happens in *E. coli* (gram-negative) with the specific mRNA sequence ACA [273], in *Bacillus subtilis* (gram-positive) the mRNA interferase MazF is specific for the UACAU sequence. Moreover, in *S. aureus* (gram-positive) it was also found that *mazF* is a sequence-specific mRNA interferase that cleaves mRNA UACAU sequences [260].

Regarding *mazEF* TA complex in *Staphylococcus spp.*, previous studies with *S. aureus* [274] reported an increased transcription of *mazEF* upon exposure to antibiotics, such as tetracycline and erythromycin [275, 276], which corroborates the hypothesis that TA systems may affect antimicrobial susceptibility. It was also found that *S. aureus mazF* is able to induce bacteriostasis by targeting selective mRNAs for cleavage, which may be related to the dormant physiological state of bacteria [277, 278], and that the interaction with antibiotics triggers an increase in the expression of other TA systems contributing to its virulence [275, 276]. A recent study targeting novel TA systems in *S. aureus* confirmed that the *mazF* endogenous sequence may induce stasis rather than cell death and that the deletion of the *mazEF* operon resulted in increased sensitivity to β -lactam antibiotics [279]. Moreover, previous experiments conducted with *S. equorum* led to the suggestion that *mazEF* is involved in processes beyond pathogenicity in staphylococci [262]. However, to date, the function of *mazEF* on *S. epidermidis* is yet to be determined. Notwithstanding, it is already known that *S. epidermidis mazEF* sequence is homologous to the sequence in other species, previously described as a TA system [263, 266, 280], as outlined above. Moreover, recent studies have observed an increase in the expression of *mazF* upon biofilm formation in *S. epidermidis*, suggesting that the *mazEF* operon may be involved in the regulation of the initial phase of the biofilm lifecycle in this species [281], as also reported for other species mentioned above.

As discussed earlier, in *S. epidermidis*, *mazE* was only expressed in biofilms with induced dormancy [10], suggesting that *mazEF* may have a role in the occurrence and persistence of biofilm-associated infections.

Hence, a better study of the molecular mechanisms underlying biofilm dormancy, focusing on the role of *mazEF* as a hypothetical TA system in *S. epidermidis*, as well as in other important and concerning species, would help overcome the great number of nosocomial infections registered each year in hospitals around the world.

2.4.4. Targeting TA systems to fight infections

As discussed above, TA can regulate some virulence factors, such as the control of biofilm infections and antimicrobial susceptibility, as well as the emergence of persisters and dormant cells. This triggered interest in using them as targets for antimicrobial therapies [282]. For instance, Williams *et al.* suggested that the use of TA systems to fight bacterial infections should focus on the artificial activation of the toxin module [282].

Since TA genes have no homologues in the human genome and are ubiquitously present in several important bacterial species, the interest in exploiting these systems as potential antimicrobial targets has significantly increased over the last decade [282].

TA toxins generally interfere with biological processes that are the target of antimicrobial agents, which led to the suggestion of a new strategy, based on TA systems, for treating infections and overcoming reduced antimicrobial susceptibility. More specifically, the activation of latent toxin proteins of TA systems was proposed as an alternative or adjuvant strategy to antimicrobial therapy [270, 282–284]. For instance, the interference with the equilibrium between the toxin and antitoxin may trigger the mechanism of PCD and, consequently, TA systems may be used to control infections by inducing the death of the pathogens [285].

Although the antitoxin module of type II TA systems normally acts as a transcriptional repressor of its own operon, it can occasionally act as a repressor on the expression of other genes involved in general stress response, being suggested that the artificial induction of the antitoxin can be used to control the expression of some virulence factors and metabolic pathways involved in the infection process [209, 286, 287].

Regarding *mazEF*, it is suggested that drugs could be used to directly target the *mazEF* complex and induce PCD, resorting to the activation of the toxin either by allosteric activation of MazF or by restricting the function of the antitoxin, MazE [240, 288]. On the other hand, proteolytic degradation of the antitoxin module is another suggestion to release the toxin and, consequently, induce cell death [282, 289].

Moreover, it is possible to inhibit the transcription of the TA locus using a molecule that binds to the promoter, resulting in the obstruction of the production of both the toxin and antitoxin. Since the antitoxin has a shorter cellular half-life, it would ultimately enable the toxin to act freely inducing toxin-mediated killing of bacteria [290, 291]. A former study with *S. aureus* showed that the ectopic

expression of *mazF* decreased cell viability by more than 90%, thereby suggesting that the activation and/or induction of the MazF toxin could be used as an antibacterial strategy [292].

However, so far, no studies were found regarding the use of TA systems as specific targets for antimicrobials drugs and in the effects of the activation and/or inhibition of the toxin protein in *in vivo* situations.

Hence, more in-depth studies should be conducted to better understand the mechanisms of action of TA systems and, consequently, understand the processes by which they can modulate microbiological processes, as biofilm formation, virulence and decreased susceptibility to antibiotics, and, ultimately, their use as targets in fighting infections. Such important information would, undoubtedly, open a way to the establishment of a specific target on TA complexes aiming to control the occurrence and persistence of nosocomial infections.

2.5. Bibliography

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CHAPTER 3

Assessment of the VBNC state induction model in *S. epidermidis*

SUMMARY

A VBNC induction model has been proposed for *S. epidermidis* biofilms, however, strain-to-strain variability has been observed. With the future goal of constructing a mutant strain to test the role of the *mazEF* operon in the induction of the VBNC state, the previously developed VBNC induction model was tested in strain 1457, which is a strain known to be amenable to genetic manipulation. Surprisingly, we found that the growth media brand influenced the ability of cells to enter into the VBNC state: while strain 1457 could enter a VBNC state when grown in TSB from Merck, the same was not true when using another brand. A similar effect was confirmed with other strains.

Moreover, we aimed to explore if the same VBNC modulation model could be used in planktonic cells. To do so, two different growing approaches were used. While growing planktonic populations under the same conditions used for biofilms (24 hours of growth with 0.4% glucose, followed by 24 hours of growth with 1% glucose) resulted in similar levels of VBNC cells compared to the biofilm cultures, the growth of the planktonic cultures directly with 1% glucose for 24 hours yielded lower amounts of VBNC cells.

3.1. Brief introduction

It was previously found that biofilms are formed by bacteria in different metabolic states and phenotypes, including dormant cells [1]. While there is some controversy regarding the definition of the dormant state, in this study the term viable but non-culturable cells (VBNC) is used and defined as live cells with an intact membrane, active transcription and respiration, but with a compromised culturability on standard growth media [2]. It was previously described that *S. epidermidis* VBNC cells can be induced by growing cells in the presence of excess concentration of glucose (1%) and prevented by adding 20 mM of magnesium chloride (MgCl₂) [1]. However, it was later shown that the applicability of this model is strain-dependent [3] and no information existed regarding the ability to modulate VBNC cells in *S. epidermidis* 1457, the strain known as being the most amenable to genetic manipulation [4, 5], which would be needed for further studies in the scope of this thesis. As such, in this chapter, the ability to use the previously described model was explored, using strain 1457 as the main background. Moreover, we decided to further explore this model aiming to understand if the VBNC induction phenomenon could be replicated in planktonic cells. Thus, a variation of the VBNC induction model was applied to planktonic populations grown for up to 48 hours.

3.2. Materials and methods

3.2.1. Strains and growth conditions

Several *S. epidermidis* strains were used in the evaluation of the applicability of the VBNC state induction model. Strain 9142 [6] (isolated from a blood culture), commensal COM040A [3] (skin sample from a healthy volunteer) and clinical isolates PT11004 (isolated from bloodstream infection) and PT13003 (isolated from haemodialysis catheter) [3] were used as control strains, as it was previously confirmed that these strains were able to form high proportions of VBNC cells when grown under VBNC induced conditions [3]. Moreover, strains IE186 and 1457, isolated from an infective endocarditis [7] and from a venous catheter infection [6] were used as targets for the evaluation of the efficacy of the VBNC model.

The experiments were started by growing *S. epidermidis* strains from the 30% glycerol stocks in TSB (Liofilchem, Teramo, Italy or Merck, Darmstadt, Germany) at 37 °C with agitation at 120 rpm (orbital shaker with 10 mm orbit). Then, the overnight suspensions were diluted to an optical density, at 640 nm, (OD_{640nm}) of 0.25 ± 0.05 , which was previously found to correspond to

approximately 2×10^8 colony-forming units/mL (CFU/mL) [8]. The adjusted suspensions were used as inoculum for further experiments.

3.2.2. Biofilm formation

Biofilms were formed according to the model previously developed to modulate the induction and prevention of the VBNC state in *S. epidermidis* [1], with some modifications [9]. Briefly, 10 μ L of the previously adjusted overnight suspensions were inoculated into 1 mL of TSB supplemented either with 0.4% glucose or with 0.4% glucose and 20 mM $MgCl_2$, to further induce or prevent the VBNC state, respectively, in 24-well plates (Orange Scientific, Braine-l'Alleud, Belgium) and incubated for 24 hours at 37 °C and shaking at 120 rpm. After 24 hours, the supernatant was removed and fresh TSB supplemented with 1% glucose (induction of VBNC cells) or 1% glucose plus 20 mM $MgCl_2$ (prevention of VBNC cells) was added to the biofilms, followed by incubation under the same temperature and agitation conditions for further 24 hours. Then, the spent media was discarded, and the 48-hours biofilms were washed twice with 500 μ L of 0.9% NaCl to remove unattached cells. Finally, the biofilms were scraped from the plate surface and resuspended in 1 mL of 0.9% NaCl or 1 mL of phosphate-buffered saline 1 $\times$ (PBS) supplemented with 0.2% Tween 20, when used for flow cytometry assays.

3.2.3. Planktonic growth

Following the first experiments with biofilms, it was decided to test the VBNC model using planktonic populations. To do so, the overnight culture was adjusted to a final density of 0.25 ± 0.05 (OD_{640 nm}) and, then, two different growth approaches were tested, namely: 1) planktonic cells were grown directly in 1% glucose supplemented media for 24 hours or 2) planktonic cells were pre-grown for 24 hours in media supplemented with 0.4% glucose, followed by additional 24 hours of growth in fresh media with 1% glucose. Briefly, for the first variation of the model, 100 μ L of the previously adjusted suspension were inoculated into 10 mL of TSB (Merck) supplemented with 1% glucose or TSB + 1% glucose + 20 mM $MgCl_2$ for the prevented condition and grown for 24 hours at 37 °C and shaking at 120 rpm, to assess direct induction of a lower concentration of cells. On the other hand, to replicate the model used for biofilms, 100 μ L of the adjusted planktonic suspension were inoculated into 10 mL of TSB (Merck) supplemented with 0.4% glucose or TSB with 0.4% glucose plus 20 mM $MgCl_2$ to further induce or prevent the VBNC state and incubated at 37 °C with agitation at 120 rpm. After 24 hours, the spent media was removed by centrifugation

of the culture (10 minutes, 16000 *g*, 4 °C) and 10 mL of fresh TSB supplemented with 1% glucose or 1% glucose and 20 mM MgCl₂ were added to respectively induce and prevent the entrance into the VBNC state. Finally, the cultures were incubated under the same temperature and agitation conditions for additional 24 hours.

3.2.4. Quantification of cells

Both the 48 hours biofilm suspensions and the planktonic populations were submitted to a sonication pulse of 10 seconds at 33% amplitude (Ultrasonic Processor Model CP750, Cole-Parmer, IL, USA) to help disaggregate cells clusters prior to the analysis by flow cytometry and CFU plating. Importantly, the sonication procedure was previously optimized for *S. epidermidis* cells and it was shown that the cycle selected did not significantly affect cells viability [8].

After being homogenised, both planktonic and biofilm cells were diluted and first quantified in terms of total amount of cells by measuring the OD_{640nm}. Then, the quantification of both culturable and live cells was performed. To quantify the number of culturable cells of the biofilm populations, the suspensions were serially diluted at a 10-fold ratio and plated on tryptic soy agar (TSA) plates, which were further incubated at 37 °C overnight, until bacterial colonies reached a countable size. Knowing that the VBNC state induction model prevents part of the live cells from growing on standard culturing methods, the quantification of live cells was important to determine the ratios of VBNC cells. Thus, live cells were quantified by flow cytometry using SYBR green and propidium iodide (PI) staining, as previously optimized [10]. Briefly, a mix of PBS 1× supplemented with 0.2% Tween 20 was prepared by adding 20 µg/mL of PI (Sigma-Aldrich, St. Louis, MO, USA) and 1:80000 SYBR Green (Invitrogen, CA, USA). Then, 20 µL of bacterial suspension were diluted with 180 µL of the PBS mix and the number of cells was analysed using a flow cytometer (EC800™, Sony Biotech, CA, USA). A flow rate of 10 µL/minute was used and a total of 100000 events was acquired for each sample. FCS Express 6 was the software used to analyse data obtained and to calculate the number of live cells (positive in SYBR and both positive or negative in PI staining), according to the gating strategy defined in **Figure 3.1**. At least two technical replicates were used for each condition and three independent assays were performed.

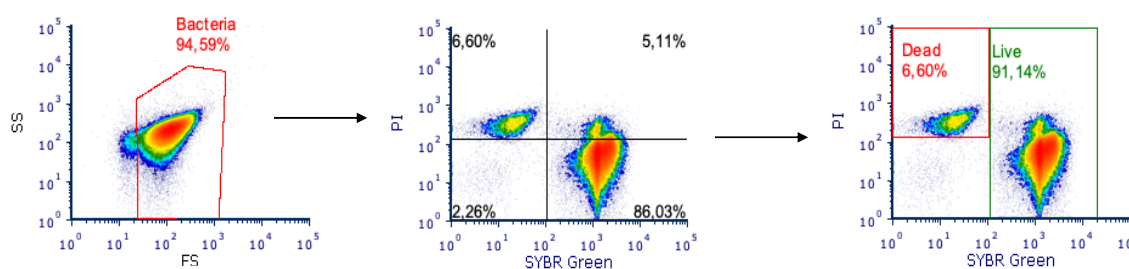


Figure 3.1 – Representative gating used for the analysis of live cells within *S. epidermidis* populations grown under VBNC modulating conditions. Forward (FS) and side (SS) scatter signals were initially used to discriminate *S. epidermidis* cells (Bacteria gate) from background and debris. Then, the bacteria population was represented in a dot plot of SYBR green vs PI fluorescence and the number of live cells determined using both SYBR+/PI- and SYBR+/PI+ populations, corresponding to live and potentially live cells, respectively. Dead cells (SYBR-/PI+) and negligible debris (not stained, SYBR-/PI-) were discarded.

3.2.5. Gene expression analysis

Biofilms and planktonic cells were grown under VBNC modulating conditions, as described in **Sections 3.2.2** and **3.2.3**. Then, 48-hours biofilms were recovered from the plates and resuspended in 1 mL of 0.9% NaCl. To extract RNA from biofilm cells, a pool of at least three independent biofilms was used to reduce biological variability [11] and 1 mL of that mix was centrifuged (10 minutes, 16000 *g*, 4 °C) and used as template. For planktonic populations, 1 mL of the 48-hours cultures was centrifuged (10 minutes, 16000 *g*, 4 °C) and further used as template. The RNA extraction protocol was performed using the kit ExtractMe RNA Bacteria & Yeast (Blirt S.A., Poland), following the manufacturer's instructions with a few optimizations, as previously described [12]. Then, RNA samples were treated with DNase I enzyme (Thermo Fisher Scientific Inc, Massachusetts, USA) and NanoDrop One (Thermo Fisher Scientific Inc) was used to quantify the concentration and assess the purity of RNA samples. Next, RNA was reverse transcribed into complementary DNA (cDNA) using 250 ng of RNA in a 10 μ L reaction volume (RevertAid H minus M-Mulv RT, Thermo Fisher Scientific, Inc.) and using random primers (Bioron, Römerberg, Germany). Finally, gene expression was analysed by qPCR using Xpert Fast SYBR Mastermix (GRiSP, Lda., Porto, Portugal), following the manufacturer's instructions and using a CFX96 (BioRad) thermal cycler with a previously optimized protocol (10 μ L reaction volume, run at 95 °C for 2 minutes, and 40 cycles of 95 °C for 5 seconds, 60 °C for 30 seconds). The primers used

for the quantification of gene expression were designed with Primer3 software [13] using *S. epidermidis* 1457 genome (Accession number CP020463.1) as a template and are described in Table 3.1.

Table 3.1 – List of primers used for qPCR analysis.

Target gene	Forward sequence (5'-3')	Reverse sequence (3'-5')	Amplicon size (bp)	Efficiency (%)
<i>16S rRNA</i>	GGGCTACACACGTGCTACAA	GTACAAGACCCGGGAACGTA	176	100.0
<i>gyrB</i>	GCATTTGGTACGGGTATTGG	CATCAACATCGGCATCAGTC	88	93.0
<i>mazE</i>	CAAAATAGAAACCACAGTCTT GAAC	AGATATTAAATGTGATTCATT GCAATC	138	91.8
<i>mazF</i>	GAAGAGGAGATGTTTATTTAG CGG	CCCAAATAATATCTAAGGCA TTATC	325	95.5

3.2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7 (Trial version, CA, USA) through all the chapters of this thesis. One-way ANOVA with Tukey's multiple comparisons test was used for the analysis of most data, except for gene expression analysis in which the unpaired T-test was used. A *p*-value below 0.05 was considered significant.

3.3. Results and discussion

3.3.1. Induction of VBNC cells in biofilms of *S. epidermidis* is dependent of variations of the growth media

The VBNC model should be able to decrease the culturability of a population, by preventing cells from growing on regular media, without impacting their viability [1]. Thereby the assessment of culturable cells (CFU), viable cells (flow cytometry) or the total amount of cells (OD_{640nm} quantification) allows the estimation of the proportion of VBNC cells. This model was first demonstrated using biofilms formed by strain 9142 and later confirmed in several strains, although not all strains were able to form VBNC cells under this experimental model [3]. Herein, in our first attempts to assess VBNC induction in *S. epidermidis* 1457, we were not able to replicate the established model, which pointed out the fact that the induction of the VBNC state in strain 1457 would not be mediated by glucose/magnesium chloride. However, when we decided to include the

original strain 9142, we observed that VBNC induction was also not occurring (**Figure 3.2**), as evaluated by the ratios between the number of culturable and live cells, assessed by CFU counting and flow cytometry, respectively.

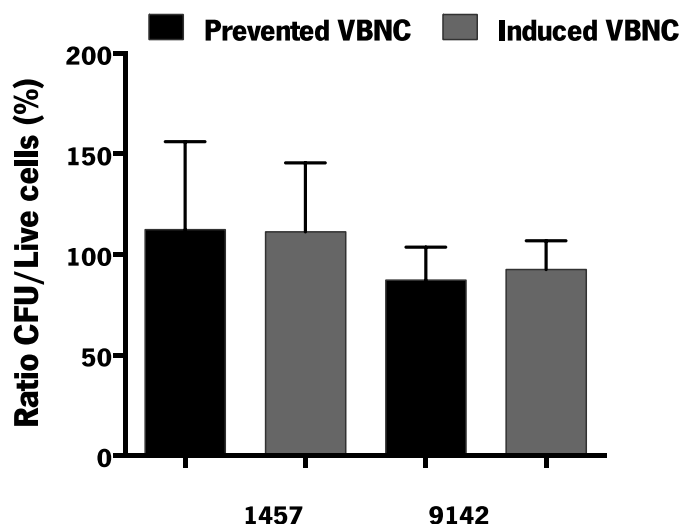


Figure 3.2 – Ratio between the number of culturable (CFU) and live biofilm cells in prevented and induced VBNC conditions, with TSB from Liofilchem. Results are displayed as the mean + standard deviation of four independent experiments. Statistical analysis was performed between the prevented and induced VBNC conditions for each strain.

Based on the previously available literature [3, 9], strain 9142 was expected to have approximately 1 log culturability reduction in induced VBNC conditions. A careful review of the published papers revealed that the growth media used (TSB) was from the brand Merck, while the experiments represented in **Figure 3.2** were performed in TSB from a different brand (Liofilchem). Since TSB is a complex medium, the exact composition between brands varies, and this can impact functional microbiology results [14]. As such, we repeated the same experiment using TSB from Merck, and we decided to increase the number of strains tested, to assess the validity of the model. For this initial screening, the total amount of cells from the biofilm was assessed by OD_{640nm} quantification and the culturable cells were assessed by CFU quantification. The ratio between the amount of cells in the induced and prevented VBNC conditions (Ratio IND/PRE) was calculated for both culturable (CFU) and total cells (OD_{640nm}).

Contrary to what was observed in **Figure 3.2**, when using TSB from Merck we were able to confirm that this experimental model was indeed able to induce the VBNC state in strain 1457 (**Figure 3.3**). As described before [3], some variability was observed, but in 5 out of the 6 strains assessed, the

reduction of culturability was statistically significant, with only *S. epidermidis* PT13003 showing a smaller decrease. Moreover, the OD_{640nm} ratios were close to 100% for all *S. epidermidis* isolates, meaning that the total amount of cells in biofilms under VBNC state induced conditions was not affected, suggesting a similar proportion of cells of biofilms in the prevented condition. Interestingly, strain 1457 showed an intermediate response to the VBNC induction model with an IND/PRE CFU ratio of circa 40%, meaning that the culturability suffered a decrease of about 60%, while strains COM040A and PT11004, previously studied by Carvalhais *et al.* [3], were the ones where the model was more effective (around 85-90% culturability decrease). Despite their higher susceptibility to VBNC induction, the fact that these strains have not been widely characterized yet, turns them into unlikely candidates for genetic manipulation.

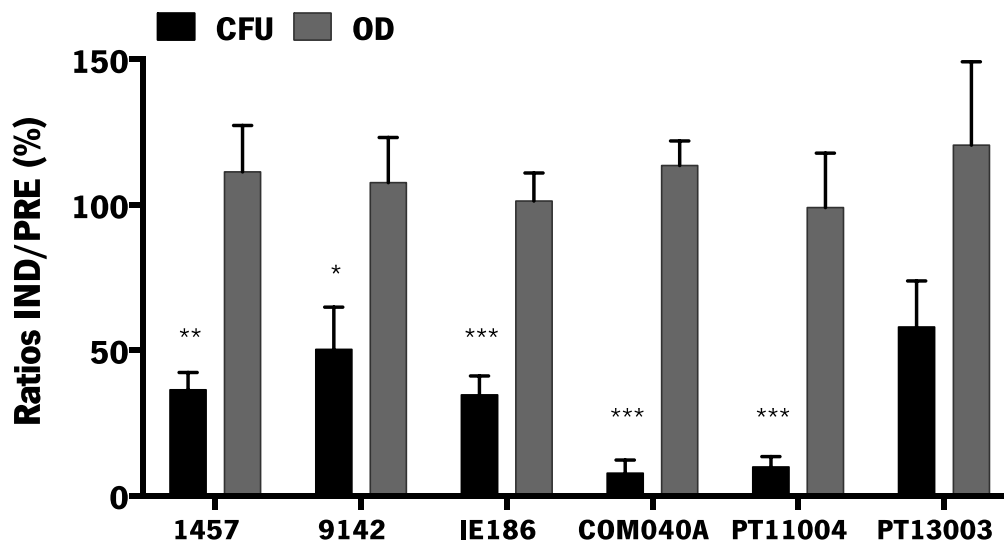


Figure 3.3 – Ratio between the number of culturable (CFU) or the total amount of cells (OD_{640nm}) found in biofilms formed by different *S. epidermidis* strains in TSB from Merck. Biofilms were grown under induced (IND) or prevented (PRE) VBNC conditions. The results are represented as the mean + standard deviation of at least three independent experiments. Statistical analysis was performed between the number of cells (CFU or total cells) on the prevented and induced VBNC state for each strain. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Despite TSB not being a chemically defined medium and its exact composition cannot be assured in distinct brands or lots, both brands (Liofilchem and Merck) have the same composition in terms of glucose (2.5 mg/L) and were supplemented with the same additional concentration of glucose (1%). However, there was an evident difference in the proportion of culturable cells in the induced VBNC state when growing biofilms of strains 1457 and 9142 with TSB from Liofilchem (**Figure**

3.2), or with TSB from Merck brand (**Figure 3.3**), suggesting the presence of a yet unidentified component that is fundamental for VBNC induction besides glucose.

Altogether, we were able to successfully implement the VBNC induction model using TSB from Merck in strain 1457, regardless of the VBNC model being slightly less effective than in some of the other strains assessed. However, *S. epidermidis* 1457 is a well-characterized strain, which genome has already been sequenced, and is more amenable to genetic manipulations, therefore, further studies were mainly conducted with this strain, using specifically TSB from Merck.

3.3.2. VBNC induction can be modulated in planktonic cells using a pre-established model for biofilms

After establishing that the previously developed model was able to induce the VBNC state in *S. epidermidis* 1457 biofilms cells, we became interested in assessing if this model could also be applied to induce VBNC cells in planktonic cultures.

We first assessed the model applicability using planktonic populations grown in similar conditions as the biofilms, wherein cells were adjusted to a concentration of around 2×10^6 CFU/mL and grown for 24 hours with 0.4% glucose or 0.4% glucose + 20 mM $MgCl_2$ and, after medium replacement, grown for additional 24 hours with 1% glucose or 1% glucose + 20 mM $MgCl_2$. In these experimental conditions, over 75% decrease in the culturability of cells was observed in the induced VBNC state (**Figure 3.4**). This finding led to the question if this model could be applied, with some alterations, to planktonic populations grown for 24 hours, which are more frequently used in scientific experiments. Thus, a second experimental setup was established, and planktonic populations were adjusted to a concentration of circa 2×10^7 CFU/mL and directly grown in TSB supplemented with 1% glucose (IND) or 1% glucose + 20 mM $MgCl_2$ (PRE) and induced for up to 24 hours.

Interestingly, the percentage of VBNC cells formed in both planktonic experimental designs was significantly different, as shown in **Figure 3.4**, wherein VBNC cells proportions was more pronounced on the 48-hours culture.

A hypothesis for the lower induction of VBNC cells on 24-hour cultures is related to the lower cellular density, since the model was applied to a cellular concentration of about 10^7 CFU/mL, while in 48-hour assays the induction started in cultures with approximately 100 times more cells (10^9 CFU/mL).

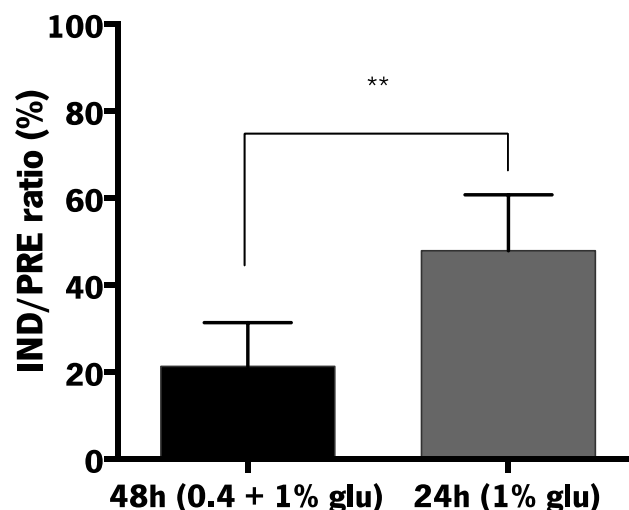


Figure 3.4 – Culturability (CFU) of *S. epidermidis* 1457 planktonic cells grown under induced (IND) or prevented (PRE) VBNC conditions using two different strategies: (i) planktonic cells were grown for 24 hours in 0.4% glucose + 24 hours in 1% glucose and (ii) planktonic cells were grown for 24 hours in 1% glucose. Results are represented as the mean + standard deviation of five independent assays. Statistical analysis was performed between the two modes of growth. ** $p < 0.01$. Glu, glucose.

It was previously shown, in *E. coli*, that RelE-induced dormancy was dependent on cellular concentration, with greater proportions of dormant cells being reached when starting the experiments with a higher concentration of cells [15]. This led to the hypothesis that nutrient consumption and further starvation is faster with higher densities of cells, which stimulates the entrance of cells into the VBNC state [15]. Another study, conducted with *P. aeruginosa*, showed that persister cells emerged in stationary planktonic cultures, but not in exponential cultures, where cell density is lower [16]. This was further confirmed in the same species in which persister cells increased in response to quorum-sensing signalling molecules, which are dependent on cell density [17]. We suggest that the same might be happening with glucose-mediated dormancy and believe a further assessment of the influence of nutrient starvation and of the release of quorum-sensing signalling molecules in the emergence of the VBNC state in *S. epidermidis* could help figure out the mechanisms behind this phenomenon.

Overall, although both models tested in planktonic populations were able to generate VBNC cells, the 48-hours model was more effective and reached similar levels of VBNC induction as in biofilm cells, suggesting that induction of the VBNC state is not dependent on the cells' phenotype.

3.3.3. *mazEF* expression is upregulated in VBNC induced condition in planktonic populations but not in biofilms of *S. epidermidis* 1457

Previous studies on the transcriptome of *S. epidermidis* 9142 biofilms grown under VBNC modulating conditions suggested a possible involvement of the *mazEF* genes in the VBNC emergence process, since it did not detect *mazE* expression in cells where the VBNC state was prevented [9]. However, on that study, RNA-Seq data was validated with a set of randomly selected genes that did not include *mazEF*. Herein, the expression of the *mazE* and *mazF* genes was determined with a different approach, analysing the genetic expression by quantitative PCR (qPCR) with the same strain of the RNA-Seq study (strain 9142). Moreover, gene expression of *S. epidermidis* 1457 was also assessed, both in biofilm and planktonic cultures.

Table 3.2 – Analysis of *mazE* and *mazF* expression on *S. epidermidis* 48 hours-old biofilms and planktonic cells of strains 9142 and 1457 grown under induced or prevented VBNC conditions. Previously obtained RNA-Seq data of strain 9142 are also presented. Results are represented as the mean $\pm$ standard deviation of at least three independent assays.

			<i>mazE</i>	<i>mazF</i>
RNA-Seq ¹	9142 biofilms	Induced	34.60 $\pm$ 10.91	122.87 $\pm$ 77.90
		Prevented	ND	115.13 $\pm$ 16.28
		Fold-change ³	N/A	1.05 $\pm$ 0.58
		Induced	8.43E-03 $\pm$ 7.77E-03	9.50E-03 $\pm$ 8.93E-03
		Prevented	2.91E-03 $\pm$ 7.48E-04	3.74E-03 $\pm$ 1.74E-04
		Fold-change ³	2.79 $\pm$ 2.59	3.19 $\pm$ 2.90
qPCR ²	1457 biofilms	Induced	4.35E-03 $\pm$ 2.89E-03	3.80E-03 $\pm$ 2.73E-03
		Prevented	3.79E-03 $\pm$ 1.82E-03	3.64E-03 $\pm$ 1.98E-03
		Fold-change ³	1.15 $\pm$ 0.60	1.05 $\pm$ 0.54
		Induced	4.44E-03 $\pm$ 2.53E-03	7.89E-03 $\pm$ 9.96E-04
	1457 planktonic	Prevented	1.17E-03 $\pm$ 6.45E-04	2.57E-03 $\pm$ 1.40E-03
		Fold-change ³	4.05 $\pm$ 2.64	2.58 $\pm$ 1.79

¹RPKM (Reads Per Kilobase of transcript per Million reads mapped) values obtained by RNA-Seq

²Normalized gene expression by qPCR (normalization to the geometric mean of *16S rRNA* and *gyrB*)

³Induced/prevented genetic expression fold-change

As shown in **Table 3.2**, we observed that RNA-Seq results were not corroborated by qPCR, wherein *mazE* was indeed expressed in VBNC prevented conditions, with an overall 3-fold upregulation in the induced condition. Because several steps are required to construct RNA-Seq libraries and the limit of detection depends on several technical aspects, some bias can occur, and, for that reason, either performing multiple RNA-Seq runs or validating the expression of target genes by qPCR are often recommended [18, 19]

An important issue when considering genetic expression is strain-to-strain variability [20] and, looking at the previous results, it is possible to observe that the expression of both *mazE* and *mazF* genes was, indeed, different between biofilms from strains 9142 and 1457. While the results obtained for the control strain (9142) suggest that the *mazEF* is affected by the modulation of VBNC cells, this was not true for strain 1457. However, when analysing the expression of *mazEF* in planktonic cultures of strain 1457, both genes were significantly upregulated in the induced condition.

3.4. Conclusions

In this study, we uncovered an unexpected phenomenon related to the previously developed VBNC induction model, wherein VBNC cells were only formed when a particular brand of TSB was used (in this case, Merck). Although we did not explore the implications of this discovery and did not assess the extension of the medium specificity (by testing a wider range of growth media), it was evident that VBNC induction is strongly affected by environmental conditions, as described in other species [21–23]. Moreover, when we attempted to validate the RNA-Seq data that suggested *mazEF* played a key role in VBNC induction, we observed that qPCR data was not in accordance with the previous data. Nevertheless, qPCR data did point out some influence of the *mazEF* operon in the modulation of the VBNC state in either strain 9142 biofilms or strain 1457 planktonic cultures.

Taken together, we achieved the aim of characterizing the VBNC induction model in several *S. epidermidis* strains, focusing particularly on *S. epidermidis* 1457. Nonetheless, the unexpected differences in the expression of the *mazEF* operon in VBNC modulation found between biofilms of strains 1457 and 9142 and between the planktonic and biofilm cultures of strain 1457, warrant further exploring of the role of this operon, as will be described in **Chapter 4**.

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CHAPTER 4

Construction of *S. epidermidis mazEF* mutants and characterization

SUMMARY

A former study focused on the emergence of the VBNC state in *S. epidermidis* biofilms found that *mazE*, a gene encoding a protein of the *mazEF* complex, was only expressed in situations where the VBNC state was induced. Based on the previous transcriptomic analysis, it was suggested that *mazEF*, a thought-to-be TA system could be important in the modulation of the VBNC state, raising interest in the study of the role of this gene in *S. epidermidis*. Thus, the initial aim of this chapter was to create an *S. epidermidis mazEF* mutant strain that allowed to study, in more detail, the function of these genes. Herein, we constructed two distinct *S. epidermidis mazEF* mutant strains, using 1457 and 9142 backgrounds, and did an initial characterization of these strains, focusing on main virulence aspects of *S. epidermidis*, such as biofilm formation, antimicrobial susceptibility and survival in human blood. Moreover, aiming to respond to a lack of information regarding the function of *mazEF* as a putative TA system, which, so far, was uniquely based on protein homology with other species, such as *S. aureus*, we complemented the *mazEF* mutant strains with *mazE*, *mazF* and *mazEF* genes and assessed the role of *mazE/mazF* as potential antitoxin and toxin counterparts, respectively. Surprisingly, *mazEF* seems not to act as a TA system in *S. epidermidis*. Moreover, the characterization results suggest that *mazEF* does not have a role in *S. epidermidis* main virulence aspects, since the mutant strains presented similar patterns of biofilm formation, antimicrobial susceptibility and survival in human blood and plasma, as the wild type strain.

4.1. Brief introduction

Due to the importance of *S. epidermidis* biofilm-associated infections and their decreased susceptibility to antimicrobial treatments and immune defences, several studies have focused on biofilm formation capacity of this opportunistic pathogen. As referred before, a former study regarding VBNC cells formation discovered a possible involvement of a *mazEF* homologue operon (*SERP1682/1681*) [1] in the modulation of these cells in *S. epidermidis*. This fact raised interest because *mazEF* is recognized as a TA system in several well-known and pathogenic species, such as *E. coli* [2], *P. aeruginosa* [3], *S. mutans* [4] and *S. aureus* [5].

Importantly, the role of *mazEF* in *S. epidermidis* has not been determined yet, although some studies reported, based on protein homology between *Staphylococci* and other strains, that it could act as a TA system where the antitoxin MazE should bind to the toxin MazF to neutralize the ribonuclease activity, as found for *S. aureus* [6] and *S. equorum* [7].

The construction of mutants in *S. epidermidis* is a time-consuming process due to the low efficiency of the protocols that are currently available, mainly related to the difficulty in overcoming the restriction system of this species. As such, herein, we faced the challenges of constructing *mazEF* mutants, following the existing standard protocols for *S. epidermidis* genetic manipulation [8], to determine the role of *mazEF* in *S. epidermidis* virulence. In addition, we aimed to explore the potential activity of *mazEF* as a TA system.

Importantly, most experiments reported herein were performed with *S. epidermidis* 1457, however, we confirmed some of the findings obtained with strain 1457 using the strain 9142 [9], where *mazE* was first identified as potentially involved in the VBNC state [1].

4.2. Materials and methods

4.2.1. Strains and growth conditions

The gene deletion protocol was started with *S. epidermidis* 1457, that was previously reported as amenable to genetic manipulation [10, 11]. This strain was used in all experiments described in this chapter. Importantly, in the course of the study, some questions regarding the reproducibility of the results among distinct *S. epidermidis* strains led to the inclusion of strain 9142 [12] to perform some confirmatory assays.

The experiments were started with a pre-inoculum of the selected *S. epidermidis* strains, which were posteriorly adjusted to a concentration of about 2×10^8 CFU/mL, as previously detailed in

Chapter 3. Of note, TSB supplemented with 10 µg/mL chloramphenicol (TSB_{CM10}) was used to grow strains harbouring the plasmids pRB473 or pRMC2.

4.2.2. Mutants construction and complementation

Genetic manipulations were based on previous protocols [8, 13], with some adaptations. Briefly, the flanking regions of the *S. epidermidis* homologue *mazEF* operon were amplified using Phusion High-Fidelity DNA Polymerase (Thermo Scientific Inc.), a proof-reading enzyme, and then linked together by an overlap PCR. The resulting fragment was cloned into the multiple cloning site (MCS) of the temperature-sensitive shuttle vector pIMAY and the result of the ligation of the insert with the plasmid was further transformed into *E. coli* DC10B, where the correct sequence and orientation of the flanking regions were confirmed by sequencing.

The knockout plasmid was then transformed into *S. epidermidis* strains by electroporation [8, 14], using 2 mm gap cuvettes (Thermo Fisher Scientific Inc., Waltham, MA, USA) and applying a pulse with the previously optimized conditions (2100 V, 25 µF and 100 Ω). Then, the clones were positively selected in plates with 10 µg/mL chloramphenicol (TSA_{CM10}) and the allelic replacement protocol was performed as previously described by Monk *et al.* [8], with some alterations. Briefly, the plasmid integration protocol was started by growing, overnight, a single colony of the previously selected clones in TSB_{CM10} at 28 °C and shaking at 250 rpm (incubator with 10 mm orbit) and the next day the overnight cultures were diluted 1:1000 in 3 mL of pre-warmed (37 °C) TSB_{CM10} and incubated overnight at 37 °C with shaking (250 rpm). On the third day, 10 µL of culture were spread on pre-warmed (37 °C) TSA_{CM10} plates and incubated overnight. The next day, the overnight single colonies were spread in fresh TSA_{CM10} plates and incubated in the same conditions. Then, the plasmid excision and eviction protocol was started by inoculating 3 mL of TSB with a single colony and incubating overnight at 28 °C with agitation at 250 rpm. At the same time, a fragment of the same colony was used to perform a colony-PCR with the plasmid screening primers, to confirm plasmid integration. Following, serial dilutions of the overnight culture were performed, reaching the dilutions of 10⁻⁵, 10⁻⁶ and 10⁻⁷, which were plated in TSA plates with 1 µg/mL anhydrotetracycline hydrochloride (TSA_{ATC}), and grown for 3 days at 28 °C. Then, the large colonies that grew were passed to both TSA_{ATC} and TSA_{CM10}. Colonies that only grew on plates with anhydrotetracycline hydrochloride, but not in plates with chloramphenicol, were grown overnight in TSB to make stocks of the clones. Finally, the deletion of the *mazEF* homologous operon in both strains was confirmed by PCR and qPCR.

Then, *S. epidermidis* 1457 $\Delta mazEF$ was transformed with two distinct plasmids: the pRB473 [15] and the pRMC2 plasmids [16], while strain *S. epidermidis* 9142 $\Delta mazEF$ was only transformed with the inducible plasmid pRMC2 for confirmatory purposes. Briefly, the homologous sequences of the *mazE* and *mazF* genes and *mazEF* operon were amplified by PCR using the genomic DNA (gDNA) of each strain and cloned into pRB473 (sequences cloned included the target genes and their natural promoter region) and/or pRMC2 (sequences cloned included a strong ribosomal binding site (RBS, or Shine-Dalgarno Sequence) and start and stop codons). While the plasmid pRB473 was first transformed into *E. coli* DC10B, the inducible plasmid pRMC2 was transformed into *S. aureus* RN4220, an important step to increase the success of transformation, since DNA methylated by strain RN4220 allows foreign DNA to be passaged, properly modified, and subsequently used for transformation of *S. epidermidis*, which is more resistant to genetic manipulations.

Finally, pRB473 and pRMC2 plasmids harbouring the respective sequences were inserted into the two *S. epidermidis* mutant strains by electroporation. Clones containing the plasmids were positively selected in TSA_{CM10} plates. The confirmation of the successful restoration of *mazE/mazF/mazEF* expression was performed by qPCR, as described before [17]. Importantly, since pRMC2 is an inducible plasmid, the confirmation of gene expression was done using an optimized concentration of anhydrotetracycline (see **section 4.2.5**). All the primers used for the genetic manipulations and PCR/qPCR confirmations are listed in **Table 4.1**.

4.2.3. Planktonic growth and biofilm formation

Both populations were initiated from overnight inocula grown in TSB, or TSB_{CM10} for strains harbouring the plasmids pRB473 and pRMC2. Planktonic cultures were started by inoculating 10 μ L of previously adjusted overnight suspensions into 10 mL of TSB/TSB_{CM10} in 25 mL Erlenmeyer flasks and incubated at 37 °C and shaking at 120 rpm for up to 24 hours. For biofilm formation, a standard and previously optimized method to grow *S. epidermidis* biofilms was used [18]. Briefly, 10 μ L of the adjusted suspensions were inoculated into 1 mL of TSB/TSB_{CM10} supplemented with 0.4% glucose in 24-well plates (Orange Scientific, Braine-l'Alleud, Belgium) and incubated for 24 hours at 37 °C and shaking at 120 rpm. After 24 hours, the supernatant was removed from all wells and 1 mL of fresh TSB supplemented with 0.4% glucose was added to the biofilms, followed by further 24 hours of incubation under the same temperature and agitation conditions. Following,

the spent media was discarded, and the biofilms were washed twice with 500 μ L of 0.9% NaCl to remove unattached cells. Finally, the biofilms were resuspended in 1 mL of the same saline solution by scraping cells from the bottom of the plate.

4.2.4. Quantification of cells

Prior to quantification, both populations of cells were submitted to a sonication process to destroy possible clusters and homogenize the suspensions, as previously described in **Chapter 3**. Similarly to what was previously described, planktonic and biofilm cells were quantified in terms of total (OD_{640nm}) and culturable (CFU) cells, at the selected time points (see **Chapter 3**).

4.2.5. Induction of *mazEF* expression

Although the complemented strain containing the pRB473 plasmid (*S. epidermidis* 1457 $\Delta mazEF::pRB473+mazEF$) is naturally expressing the *mazEF* insert, the genes cloned into the pRMC2 plasmid (*S. epidermidis* 1457 $\Delta mazEF::pRMC2+mazE/mazF/mazEF$ and *S. epidermidis* 9142 $\Delta mazEF::pRMC2+mazE/mazF/mazEF$) are only expressed upon induction of the plasmid. Therefore, the optimal conditions to induce the pRMC2 plasmid were first assessed by evaluating the effect of different concentrations of anhydrotetracycline (ATC, 0.64 and 1.28 μ g/mL) or tetracycline (TET, 0.08 and 0.16 μ g/mL), both purchased from Sigma-Aldrich (St. Louis, MO, USA), in the culturability of *S. epidermidis* 1457 $\Delta mazEF$ cells. Then, the expression of the genes cloned into the inducible plasmid (*mazE*, *mazF* or *mazEF* homologues) was started by adjusting the overnight cultures of each construct to an $OD_{640nm} = 0.5$, followed by a 1:10 dilution in 10 mL of TSB_{CM10} and incubation for 3 hours at 37 °C and 120 rpm. After the initial incubation, 0.64 μ g/mL of ATC were added to one of the tubes of each construct and incubated under the same temperature and agitation conditions for up to 24 hours. One tube without ATC was used as a control. A sample was taken from each tube at the beginning of the induction (T=0 hours), and aliquots were collected after 30, 60, 90, 120, 180 and 240 minutes and at the end of the experiment (T=24 hours) to measure the OD_{640nm} and further quantify the number of culturable cells.

Table 4.1 – List of primers used for the deletion and complementation processes. bp, base pairs; MCS, multiple cloning site.

Designation	Forward sequence (5'-3') *	Product size (bp)	Purpose
mazEF_P1	GAATGCATGGAGTTGATGCTAA	965	Amplification of flanking regions (upstream) for <i>mazEF</i> homologue knockout construction
mazEF_P2	GTTTATAATAACAAATTCCTAATCAGTG		
mazEF_P3	ttattataaaacACACTTATATAGTTGTCGATATGTCT	1023	Amplification of flanking regions (downstream) for <i>mazEF</i> homologue knockout construction
mazEF_P4	TAACGTGTTTTGGGACTCACAC		
mazEF_P1_outside	TAGCACAACATTTAATGCGTCA	WT: 2719 KO: 2100 In combination with primer mazEF_P4	<i>mazEF</i> homologue knockout confirmation
CPmazEF_F	GCTACCCGGGATGAAGAGGTTACTAACGAATTGTT	869	Amplification of <i>mazEF</i> sequence for complementation with pRB473
CPmazEF_R	GCTACCCGGGAAGCCTTAAAACTCAAGACATCC		
pIMAY_IM151	TACATGTCAAGAATAAACTGCCAAAGC	283	Primers for screening of plasmid pIMAY MCS [8]
pIMAY_IM152	AATACCTGTGACGGAAGATCACTTCG		
pRB473_Hind	CCAGTAATGACCTCAGAACTCC	212	Primers for screening of plasmid pRB473 MCS [19]
pRB473_Eco	CCCCAGGCGTTTAAGGGC		

Table 4.1 – Continued

Designation	Forward sequence (5'-3') *	Product size (bp)	Purpose
pRMC2_F	ATTCAGGCTGCGCAAC	437	Primers for screening of plasmid pRMC2 MCS [16]
pRMC2_R	TTGTTGACATTATATCATTG		
CPmazEF_F_KpnI ¥	GCTAGGTACCAAGGAGTGAAGTTATGGAGGTTCTTTTCATGTT	<i>mazE</i> : 223 <i>mazF</i> : 423 <i>mazEF</i> : 600	Amplification of <i>mazEF</i> homologue genes for complementation with pRMC2
CPmazE-R_BglII	GCTAAGATCTTAGATCATTCACTTCTTTGAATTAGATATTAAAT		
CPmazF_F_Kpn ¥	GCTAGGTACCAAGGAGTGAAGTTATGGAATGATTAGAAGAGGA		
CPmazEF_R_BglII	GCTAAGATCTTAGGTTTACTGCATATAATTATTTAAGATTT		

* Restriction enzymes recognition site (SmaI, KpnI and BglII) are in bold

¥ Shine-Dalgarno sequence was added to these primers (underlined oligonucleotides)

4.2.6. Gene expression analysis

To confirm the expression or absence of expression of the *mazEF* genes, a qPCR was performed. The expression of *mazE*, *mazF* and *mazEF* was assessed in planktonic suspensions constructs to validate the mutation and complementation procedures. Importantly, to quantify the expression of genes cloned into the inducible plasmid pRMC2, planktonic cultures were induced with 0.64 µg/mL ATC for 4 hours. Moreover, the expression of the *rsbU* gene was also assessed, since previous reports stated that *mazEF* deletions could interfere with the expression of this neighbour gene [5, 20]. Briefly, 1 mL of planktonic suspensions was centrifuged (10 minutes, 16000 *g*, 4 °C) and the following RNA extraction, cDNA synthesis and qPCR run were performed as previously detailed in **Chapter 3**.

The primers used for the quantification of gene expression are listed in **Table 4.2** and were designed with Primer3 software [21], using *S. epidermidis* 1457 genome (Accession number CP020463.1) as a template.

Table 4.2 – List of primers used for qPCR analysis of gene expression.

Target gene	Forward sequence (5'-3')	Reverse sequence (3'-5')	Amplicon size (bp)	Efficiency (%)
<i>16S rRNA</i>	GGGCTACACACGTGCTA CAA	GTACAAGACCCGGGAACGTA	176	100.0
<i>mazE</i>	CAAAATAGAAACCACAGT CTTGAAC	AGATATTAAATGTGATTCATTG CAATC	138	91.8
<i>mazEF</i>	GCAACAGAAGCTTTCCC GAT	CCCCTTGTTGACACCCTTGA	141	102.0
<i>mazF</i>	GAAGAGGAGATGTTTATT TAGCGG	CCCAAATAATATCTAAGGCA TTATC	325	95.5
<i>rsbU</i>	CTAAAGCTGCTGGAATAC CT	ATAAGCTTCAAGTCCACGAT	222	109.2

4.2.7. Antimicrobial susceptibility

A first experiment regarding antimicrobial susceptibility of *S. epidermidis* strains to rifampicin, tetracycline and vancomycin was performed to determine their minimum inhibitory concentration (MIC). In brief, overnight inocula of the strains were adjusted to a concentration of about 1×10^8 CFU/mL, followed by dilution of 2 µL of each suspension in 200 µL of TSB containing the antibiotics, in duplicates. Importantly, 2-fold dilutions of the antibiotics were performed, and the

bacterial populations were incubated in a gradient of concentrations defined for each specific antibiotic. Simultaneously, a positive control of growth was performed by adding the bacterial populations to TSB without antibiotics. The MIC was determined as the lowest concentration of antibiotic that inhibited the visual growth of the bacterial populations after 24 hours.

The antimicrobial susceptibility of planktonic cultures was evaluated upon 6 and 24 hours of incubation with antibiotics, through CFU counting and by the reduction of tetrazolium salt XTT, which estimates cells metabolic activity, as described before [18]. Briefly, 2 mL of TSB supplemented with the peak serum concentrations (PSC) of rifampicin (10 µg/mL), tetracycline (16 µg/mL) and vancomycin (40 µg/mL) (VWR) (all purchased from Sigma-Aldrich) were inoculated with 1×10^7 CFU/mL of bacteria in the stationary phase (24 hours growth) and incubated at 37 °C and 120 rpm for up to 24 hours. After 6 and 24 hours, a sample was taken to count CFUs, while at the same time points 200 µL of the cultures were collected, centrifuged (5 minutes, 16000 *g*, 4 °C) and resuspended with 200 µL of a XTT [250 mg/L] + PMS [25 mg/L] solution and incubated at 37 °C for 3 hours in the dark. After the incubation with the XTT+PMS solution, the cultures were centrifuged (5 minutes, 10000 *g*, 4 °C) and 100 µL of the supernatant were used to read the OD at 490 nm (OD_{490nm}).

4.2.8. Survival in human blood and plasma

A human *ex vivo* model [22–25], well established in our research group [26, 27], was used for the survival assays in human blood and plasma. Briefly, human blood was drawn in lithium heparin spray-coated tubes (Vacuette, Greiner Bio-one, Kremsmunter, Austria) for each experiment and pooled together in a single tube.

Plasma was separated by centrifugation of a portion of the whole blood for 20 minutes at 1440 *g* and 4 °C. The co-incubation of bacteria with either whole blood, plasma or TSB supplemented with heparin (TSB+heparin, control) was performed as described before [28, 29]. Briefly, 50 µL of bacterial suspensions with 2×10^5 CFU/mL were mixed with 450 µL of whole blood, plasma or TSB+heparin and incubated at 37 °C and 80 rpm for up to 4 hours. Cells culturability was assessed at the beginning of the assay (T=0 hours) and 1, 2 and 4 hours after incubation. Due to donor-to-donor variability, the blood of at least 3 male and 3 female donors was used.

4.2.8.1. Ethics statement

Human blood was collected from adult healthy volunteers under an active protocol approved by the Institutional Review Board of the University of Minho (SECVS 002/2014 (ADENDA)), which is in strict accordance with the Declaration of Helsinki and Oviedo Convention. All donors gave written informed consent to have blood taken.

4.2.9. Statistical analysis

Statistical analysis was performed with one-way ANOVA with Tukey's multiple comparisons test for all experiments. *p*-values below 0.05 were considered significant. At least three independent experiments were performed for each assay presented.

4.3. Results and discussion

4.3.1. Construction of *S. epidermidis* 1457 mutant and complemented strains

Herein, we first constructed an *S. epidermidis* 1457 *mazEF* mutant and its respective complemented strain, by transforming them with engineered plasmids.

Following the deletion of the *mazEF* operon, that was confirmed by qPCR, the next step was to perform the complementation of *S. epidermidis* 1457 $\Delta mazEF$. Herein, the plasmid pRB473 was used and genetically manipulated to incorporate the *mazEF* sequence including the natural promoter region of the operon. The plasmid backbone (control) and the plasmid with the *mazEF* sequence were successfully transformed into the mutant strain by electroporation. A positive selection of clones was made in TSA_{CM10} plates and plasmids were isolated from both *S. epidermidis* 1457 $\Delta mazEF::pRB473$ and *S. epidermidis* 1457 $\Delta mazEF::pRB473+mazEF$ strains. Then, a confirmatory PCR (**Figure 4.1A**) was performed, confirming the correct size of the empty and *mazEF*-ligated pRB473 plasmids. Finally, a qPCR run was performed to analyse the expression of *mazEF* and *rsbU* genes in the WT, $\Delta mazEF$ and $\Delta mazEF::pRB473+mazEF$ strains.

As expected, data from **Figure 4.1B** showed that no expression of the *mazEF* operon was found in the mutant strain, confirming the successful knock-out of the gene, while the complementation of the *S. epidermidis* *mazEF* mutant with the plasmid pRB473+*mazEF* restored the expression of the operon. The expression of *mazEF* was significantly higher in the complemented strain, which can be explained by multiple copies of the plasmid inside the bacteria, leading to the expression of multiples copies of the *mazEF* genes simultaneously. Moreover, the analysis of *rsbU* expression

confirmed that the deletion of *mazEF* did not cause important alterations in this contiguous gene [5, 20].

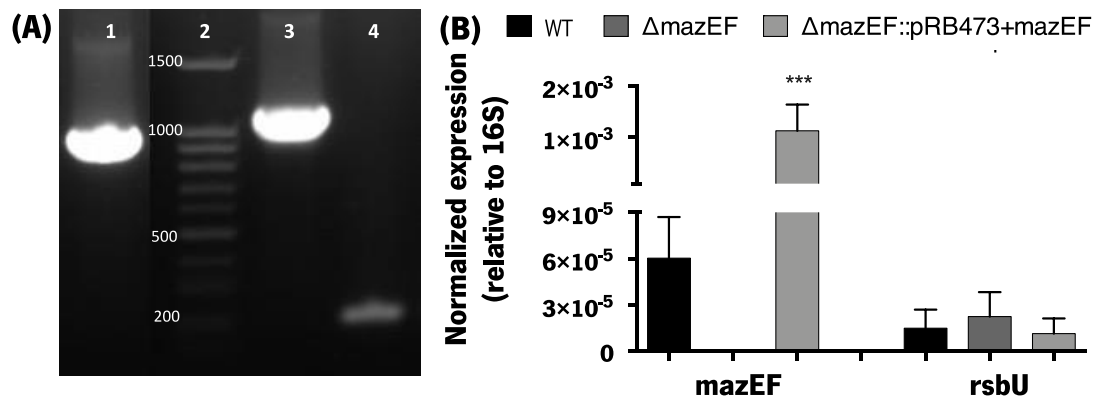


Figure 4.1 – (A) Result of electrophoresis run upon PCR with plasmids cloned into *S. epidermidis* 1457 $\Delta mazEF$. 1. pRB473+*mazEF* (screening with primers CPmazEF_F and CpmazEF_R, expected size: 869 bp); 2. 100 bp ladder; 3. pRB473+*mazEF* (amplified with pRB473_Hind and pRB473_Eco screening primers, expected size: 1067 bp); 4. Empty pRB473 plasmid (amplified with pRB473_Hind and pRB473_Eco screening primers, expected size: 212 bp); **(B)** Normalized expression (to *16S rRNA*) of *mazEF* homologue and *rsbU* genes of *S. epidermidis* 1457 WT and constructs. The results **(B)** are displayed as the mean + standard deviation of three independent experiments. Statistical analysis was performed between the WT and other strains for each gene. *** $p < 0.001$.

4.3.1.1. Complementation with plasmid pRMC2

To understand the role of each gene separately and further explore the putative role of the *mazEF* operon as a TA system, the genes were individually cloned into the inducible plasmid pRMC2 and transformed into the mutant strain. The successful transformation of the plasmids was confirmed by PCR with pRMC2 screening primers.

After the confirmation that the correct plasmids and inserts were transformed into the mutant strain (**Figure 4.2**), we needed to define the optimal concentration of antibiotic necessary to induce the pRMC2 plasmids without interfering with bacterial viability, since the use of this plasmid was only described in *S. aureus* [16]. For that reason, a pilot assay was performed with *S. epidermidis* 1457 $\Delta mazEF$ with different concentrations of tetracycline (TET) and anhydrotetracycline (ATC) and the viability (measured by CFU) and growth (quantified by OD_{640nm}) of the strains induced was compared with the control (CT, not induced).

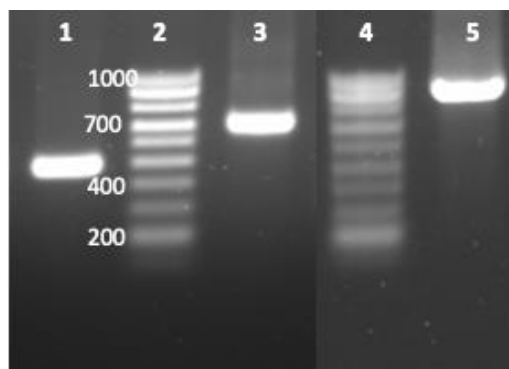


Figure 4.2 – Result of electrophoresis run upon PCR with plasmids cloned into *S. epidermidis* 1457 $\Delta mazEF$. 1. pRMC2+*mazE* insert (expected size: 491 bp); 2. 100bp ladder; 3. pRMC2+*mazF* insert (expected size: 688 bp); 4. 100bp ladder; 5. pRMC2+*mazEF* insert (expected size: 868 bp).

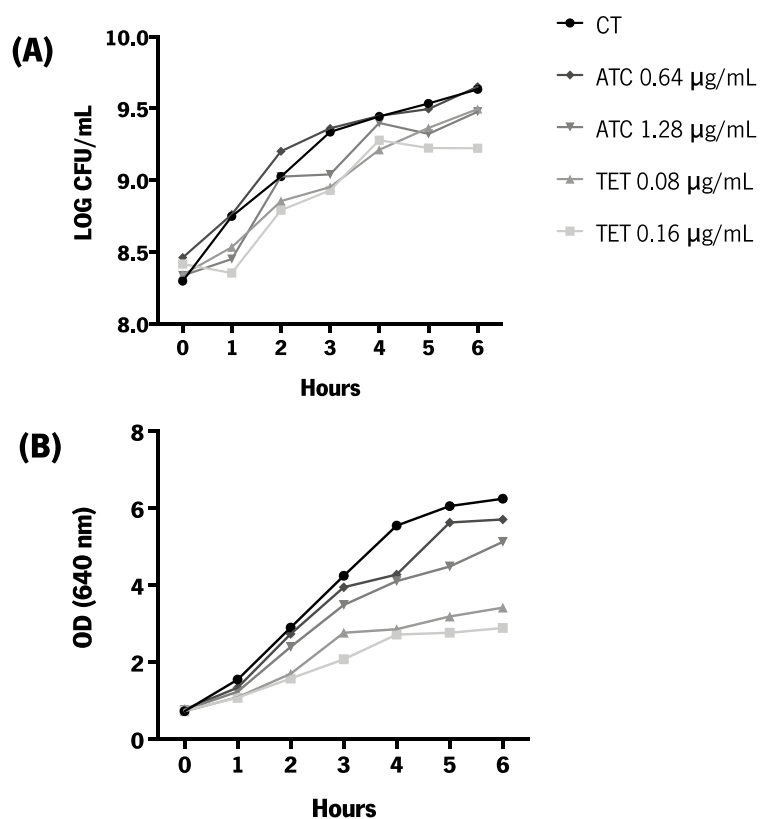


Figure 4.3 – Growth curve of *S. epidermidis* 1457 $\Delta mazEF$ planktonic cells upon incubation with different concentrations of anhydrotetracycline (ATC) or tetracycline (TET), assessed in terms of **(A)** culturability (CFU/mL) and **(B)** total amount of cells (OD_{640nm}). The control (CT) data refers to the population without any induction; Results are represented as a single value for each time point (pilot assay).

As can be seen in **Figure 4.3**, while tetracycline caused a slight decrease in cells growth and culturability, 0.64 $\mu\text{g/mL}$ of anhydrotetracycline had a minor impact on the growth of *S. epidermidis* 1457 $\Delta mazEF$. Therefore, we selected 0.64 $\mu\text{g/mL}$ of ATC to induce the expression of the inserts cloned into pRMC2 in further assays.

4.3.2. Role of *mazEF* as a putative TA system in *S. epidermidis* 1457

Since *mazEF* operon has been described as a TA system in some staphylococcal species [7, 30], we aimed to experimentally determine if it has the same function in *S. epidermidis*. To ensure that *mazE/F/EF* genes were being expressed under the selected inducing conditions (0.64 $\mu\text{g/mL}$ of ATC), a qPCR analysis was performed.

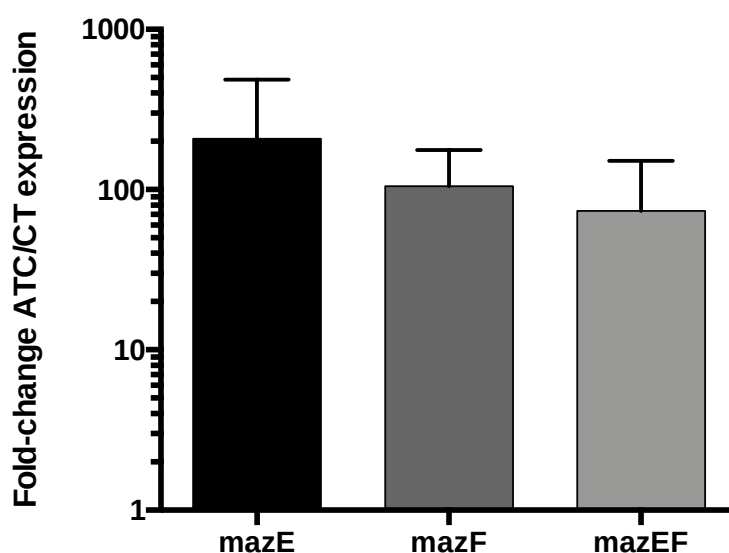


Figure 4.4 – Fold-change expression of the *mazEF* homologue genes in the pRMC2 inducible plasmid cloned into *S. epidermidis* 1457 $\Delta mazEF$, after 4 hours of induction with ATC 0.64 $\mu\text{g/mL}$, compared with the uninduced cultures grown for the same period. Results are presented as the mean + standard deviation of at least three independent assays.

As shown in **Figure 4.4**, our data allowed us to confirm that the *mazEF* homologous genes were being significantly expressed in *S. epidermidis* 1457 constructs upon induction of the plasmid. Although at different levels, all constructs were successfully expressed with at least 10-fold increased expression when compared with non-induced cells.

To analyse the putative role of *mazEF* as a TA system in *S. epidermidis*, the total amount of cells, determined by $\text{OD}_{640\text{nm}}$, and cell culturability, determined by CFU counting, were assessed in the

mutant and complemented strains upon induction of *mazE/mazF/mazEF* expression. Curiously, no significant differences of growth or culturability ($p < 0.05$) were found among *S. epidermidis* 1457 constructs, neither between the strains nor when comparing the induced strains with their respective controls (Figure 4.5).

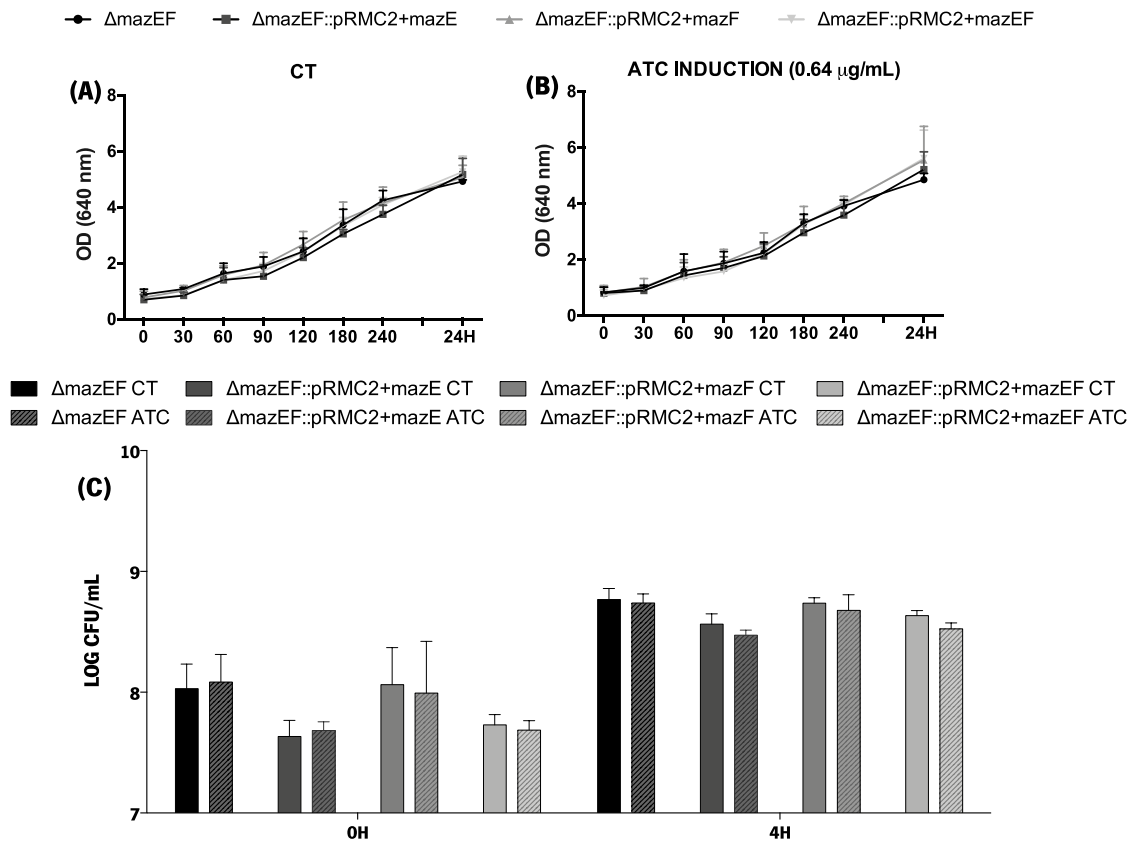


Figure 4.5 – Growth curve of *S. epidermidis* 1457 $\Delta mazEF$ constructs upon induction of *mazE/mazF/mazEF* genes, assessed by OD_{640nm} of the (A) control condition (no ATC) and (B) strains induced with 0.64 $\mu g/mL$ of ATC for up to 24 hours (24H); (C) culturability of the strains under control or inducing conditions, for up to 4 hours of growth (4H). Results are represented as the mean + standard deviation of at least three independent assays. Statistical analysis was performed between the control and induced condition for each strain and time point.

Having into consideration that the production of the toxin, without the antitoxin to counteract its toxic effect, should lead to cells death, it would be expected that the strain containing the putative toxin (*mazF* homologue) would lose culturability over time, while the mutant strains complemented both with the putative antitoxin (*mazE* homologue) or the full operon (*mazEF* homologue) would maintain its culturability. Altogether, these results suggest that the *mazEF* homologue operon is

not acting as a TA system in *S. epidermidis* strain 1457, as initially hypothesized. To exclude the possibility of this finding being a strain-dependent observation, we confirmed these results with strain 9142.

4.3.3. Construction of *S. epidermidis* 9142 mutant and complemented strains

Herein we were able to construct a mutant strain in the *S. epidermidis* 9142 background and the deletion of the *mazEF* operon was confirmed by qPCR, as represented in **Figure 4.6**.

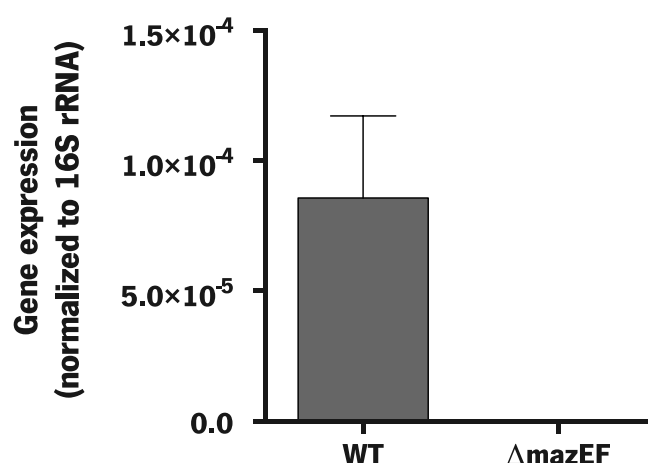


Figure 4.6 – Gene expression of *mazEF*, normalized to the expression of *16S rRNA* in *S. epidermidis* 9142 WT and mutant ($\Delta mazEF$) strains. The results are displayed as the mean + standard deviation of three independent experiments.

4.3.4. Role of *mazEF* as a putative TA system in *S. epidermidis* 9142

S. epidermidis 9142 *mazEF* mutant strain was complemented with the inducible plasmid (pRMC2) cloned with the *mazE*, *mazF* and *mazEF* genes as performed in strain 1457. Herein, the expression of *mazE/F/EF* genes under the selected inducing conditions (0.64 $\mu\text{g/mL}$ of ATC) was confirmed by qPCR.

Data represented in **Figure 4.7** confirmed that the *mazEF* homologous genes were being significantly expressed upon induction with ATC when compared to the uninduced condition, although at lower induction levels than compared with strain 1457.

To assess the role of *mazEF* as a putative TA system in strain 9142, cells culturability was analysed upon induction of the expression of the *mazE/mazF/mazEF* genes.

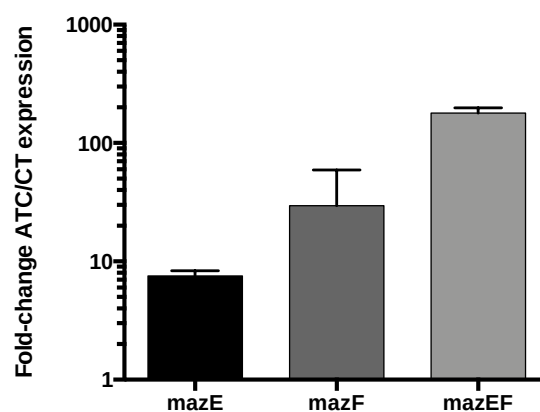


Figure 4.7 – Fold-change expression of the *mazEF* homologue genes in the pRMC2 inducible plasmid cloned into *S. epidermidis* 9142 $\Delta mazEF$, after 4 hours of induction with ATC 0.64 $\mu\text{g/mL}$, compared with the uninduced cultures grown for the same period. Results are presented as the mean + standard deviation of at least three independent assays.

As can be seen in **Figure 4.8**, very similar results were obtained among the different constructs, with the induction of the inserts resulting in similar culturability rates to the ones obtained in the uninduced condition, even when the putative *mazF* toxin was induced. These results imply that *mazF* and *mazE* are not acting as a toxin and antitoxin in *S. epidermidis*, respectively, as was previously found for strain 1457, suggesting that this finding is strain-independent.

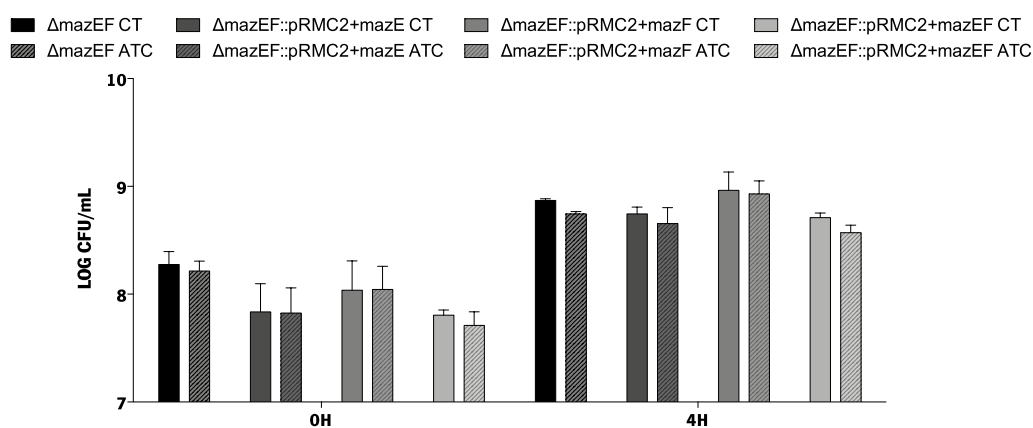


Figure 4.8 – Culturability of *S. epidermidis* 9142 $\Delta mazEF$ constructs in the control and induced (0.64 $\mu\text{g/mL}$ ATC) conditions for up to 4 hours of growth (4H). Results are represented as the mean + standard deviation of at least three independent assays. Statistical analysis was performed between the control and induced condition for each strain and time point.

4.3.5. *mazEF* is described as a putative TA system in other species

Since the previous results suggest that *mazEF* role is not related to TA functions in *S. epidermidis* strains 1457 and 9142, we aimed to assess how different the sequence of this operon is compared to other bacteria, especially Staphylococcal species (Table 4.3), but also in other species in which *mazEF* was already described as a TA system (Table 4.4). Overall, the *S. epidermidis* 1457 *mazEF* operon was found to be highly conserved within the *Staphylococcus* genus, with the *mazE* component presenting circa 70 to 90% nucleotide and amino acid identity between the distinct species, while *mazF* nucleotide sequence presents a similarity of approximately 70 to 90% nucleotide identity and over 80% amino acid identity with the remaining strains of the genus (excluding other *S. epidermidis* strains). Concretely, among the *S. epidermidis* strains, the *mazE* and *mazF* genes showed a homology of about 87 to 100% and 82 to 100%, respectively, which translates into more than 90% of protein homology.

Conversely, the similarity with non-staphylococcal species is substantially lower, with less than 53% and 45% identity for the *mazE* nucleotide and amino acid sequences, respectively. In the case of the *mazF* sequence, a maximum identity of 68% and 72% was found for the nucleotide and amino acid sequences, respectively. Importantly, the similarity with *E. coli*, the species where the *mazEF* TA is better studied, is considerably low, with circa 30 to 47% of nucleotide identity and only 11 to 36% amino acid correspondence with the *mazEF* operon.

Table 4.3 – Blast results for strains with high homology to *S. epidermidis* 1457 *mazEF* sequence (80% homology threshold to *mazE* or *mazF* sequences). All gene descriptions were derived by automated computational analysis using gene prediction method: Protein Homology.

Genus/Gram	Species (Alphabetic order) Described as TA system?	Blast with <i>S. epidermidis</i> 1457 <i>mazE</i> sequence			Blast with <i>S. epidermidis</i> 1457 <i>mazF</i> sequence		
		Gene description on GenBank - PUBMED	Nucleotide homology (%)	Amino acid homology (%)	Gene description on GenBank - PUBMED	Nucleotide homology (%)	Amino acid homology (%)
<i>Staphylococcus</i> , Gram-positive	<i>S. agnetis</i> No	Antitoxin MazE	83.19	73.21	PemK family transcriptional regulator	81.23	87.07
	<i>S. argenteus</i> No	Type II toxin-antitoxin system antitoxin MazE	85.38 – 84.80	83.93 - 85.71	Type II toxin-antitoxin system PemK/MazF family toxin	83.19	91.67
	<i>S. arlettae</i> No	Antitoxin MazE	83.09	73.21	Endoribonuclease MazF	83.43	91.53
	<i>S. aureus</i> Yes [6, 31]	Antitoxin MazE; Programmed cell death YdcD	85.38 – 86.67	85.71 – 88.24	Toxin MazF; Programmed cell death toxin YdcE; Endoribonuclease MazF; PemK family transcriptional regulator	79.89 – 85.24	90 – 95.58

Table 4.3 – Continued

<i>Staphylococcus</i> , Gram-positive	<i>S. auricularis</i> No	Programmed cell death toxin YdcD	82.50	75	Programmed cell death toxin YdcE	84.55	88.33
	<i>S. capitis</i> No	Type II toxin-antitoxin system antitoxin MazE; Antitoxin MazE.	87.13	91.07	Type II toxin-antitoxin system PemK/MazF family toxin; Toxin MazF; mRNA interferase MazF	87.54	96.61
	<i>S. caprae</i> No	Putative antitoxin MazE; Type II toxin-antitoxin system antitoxin MazE; Antitoxin MazE	90.12	92.86	Type II toxin-antitoxin system PemK/MazF family toxin; Toxin MazF	89.26	96.67
	<i>S. carnosus</i> No	Antitoxin MazE; Conserved hypothetical protein	80.41 – 81.08	85.71	PemK family transcriptional regulator; Hypothetical protein	82.17	89.38
	<i>S. chromogenes</i> No	Antitoxin MazE;	77.42	76.79	PemK family transcriptional regulator; Type II toxin-antitoxin system PemK/MazF family toxin	81.79 – 82.10	87.93
	<i>S. cohnii</i> No	Antitoxin MazE	78.79 – 80.00	78.57 – 76.79	Type II toxin-antitoxin system PemK/MazF family toxin	80.35 – 81.23	86.67 – 87.50

Table 4.3 – Continued

<i>Staphylococcus</i> , Gram-positive	<i>S. condiment</i> No	Antitoxin MazE; Programmed cell death antitoxin YdcD	80.41	85.71	PemK family transcriptional regulator; Programmed cell death toxin YdcE	82.17 – 82. 48	88.50 – 89.47
	<i>S. delphini</i> No	Antitoxin MazE	76.43	73.21	Toxin MazF	80.35	88.98
	<i>S. epidermidis</i> (other strains) No	Type II toxin-antitoxin system antitoxin MazE; Antitoxin MazE; Programmed cell death antitoxin YdcD	87.13 - 100	89.29 - 100	Type II toxin-antitoxin system PemK/MazF family toxin; mRNA interferase MazF; PemK family DNA-binding protein	82.34 - 100	91.67 - 100
	<i>S. equorum</i> Yes [7]	Type II toxin-antitoxin system antitoxin MazE; Antitoxin MazE	85.38 – 86.15	76.79	Type II toxin-antitoxin system PemK/MazF family toxin. PemK family transcriptional regulator;	83.87 – 84.16	88.33
	<i>S. felis</i> No	Antitoxin MazE	78.15	73.68	Type II toxin-antitoxin system PemK/MazF family toxin;	81.90	90.18
	<i>S. fleurettii</i> No	Antitoxin MazE	76.65	73.21	PemK family transcriptional regulator.	80.75	89.83

Table 4.3 – Continued

<i>Staphylococcus</i> , Gram-positive	<i>S. haemolyticus</i> No	Type II toxin-antitoxin system antitoxin MazE; Antitoxin MazE	84.76 – 85.37	83.93 – 85.71	PemK family transcriptional regulator; Type II toxin-antitoxin system PemK/MazF family toxin; mRNA interferase MazF; PemK family of DNA-binding protein	84.23	92.98
	<i>S. hominis</i> No	Antitoxin MazE	84.80 – 85.96	85.71	PemK family transcriptional regulator; Type II toxin-antitoxin system PemK/MazF family toxin	86.90 – 87.80	90
	<i>S. hyicus</i> No	Antitoxin MazE	84.03	73.21	Toxin MazF; mRNA interferase mazF	81.29	87.07
	<i>S. kloosii</i> No	Antitoxin MazE	85.50	76.79	Type II toxin-antitoxin system PemK/MazF family toxin	87.65	92.44
	<i>S. lugdunensis</i> No	Antitoxin MazE; Programmed cell death antitoxin YdcD	79.53 – 80.12	85.71	Type II toxin-antitoxin system PemK/MazF family toxin; PemK family DNA-binding protein; PemK family transcriptional regulator; Programmed cell death toxin YdcE	82.55 – 83.97	93.04 – 93.91

Table 4.3 – Continued

<i>Staphylococcus</i> , Gram-positive	<i>S. lutrae</i> No	Antitoxin MazE;	78.43	73.21	PemK family transcriptional regulator;	80.64	88.14
	<i>S. muscae</i> No	Antitoxin MazE;	81.51	71.43	PemK family transcriptional regulator; Toxin MazF	79.94	91.89
	<i>S. nepalensis</i> No	Antitoxin MazE;	79.39	76.79	PemK family transcriptional regulator;	82.99	86.67
	<i>S. pasteurii</i> No	Antitoxin MazE	87.13 - 87.72	89.29	Type II toxin-antitoxin system PemK/MazF family toxin. PemK family transcriptional regulator;	82.62 – 83.47	91.67
	<i>S. pettenkoferi</i> No	Antitoxin MazE	77.91	78.57	Type II toxin-antitoxin system PemK/MazF family toxin;	84.94	91.96
	<i>S. piscifermentans</i> No	Programmed cell death antitoxin YdcD	78.72	85.71	Programmed cell death toxin YdcE	82.48	89.38
	<i>S. pseudintermedius</i> No	Antitoxin MazE; Programmed cell death antitoxin YdcD	76.36	73.21	PemK family transcriptional regulator; Type II toxin-antitoxin system PemK/MazF family toxin; Programmed cell death toxin YdcE	80.06 - 80.64	88.98

Table 4.3 – Continued

<i>Staphylococcus</i> , Gram-positive	<i>S. saprophyticus</i> No	Antitoxin MazE; Type II toxin-antitoxin system MazE; Programmed cell death antitoxin YdcD	77.30 - 77.91	71.43 – 73.21	Type II toxin-antitoxin system PemK/MazF family toxin; Toxin MazF	82.94 – 83.24	86.67 – 88.33
	<i>S. schleiferi</i> No	Antitoxin MazE; Hypothetical protein;	76.65 – 77.25	73.21	PemK family transcriptional regulator; PemK family protein;	80.40 - 80.75	89.83
	<i>S. schweitzeri</i> No	Antitoxin MazE	85.38	85.71	mRNA interferase MazF	83.19	90.83
	<i>S. sciuri</i> No	Antitoxin MazE; Programmed cell death antitoxin YdcD	73.24 – 74.65	70.59 – 72.55	Type II toxin-antitoxin system PemK/MazF family toxin; Programmed cell death toxin YdcE; PemK family transcriptional regulator;	80.13 – 81.49	84.68
	<i>S. simiae</i> No	Programmed cell death antitoxin YdcD	84.21	87.50	Programmed cell death toxin YdcE	86.03	92.44
	<i>S. simulans</i> No	Antitoxin MazE	78.95	82.00	Toxin MazF; PemK family transcriptional regulator. Type II toxin-antitoxin system PemK/MazF family toxin;	82.14 – 82.44	89.38 - 89.47
	<i>S. stepanovicii</i> No	Antitoxin MazE	70.59	70.59	Toxin MazF	82.41	80.53

Table 4.3 – Continued

<i>Staphylococcus</i> , Gram-positive	<i>S. succinus</i>	Nd	82.01	82.61	Nd	81.76	90.27
	No						
	<i>S. warner</i>	Antitoxin MazE; Hypothetical protein	86.55 – 87.13	89.29	Type II toxin-antitoxin system PemK/MazF family toxin. PemK family transcriptional regulator;	82.34 – 82.6	91.67
	<i>S. xylosus</i>	Antitoxin MazE	80.61	75	Type II toxin-antitoxin system PemK/MazF family toxin; mRNA interferase MazF; PemK transcriptional regulator	81.77 – 82.04	88.33
<i>Sulfitobacter</i> , Gram- negative	<i>S. donghicola</i>	Antitoxin MazE	87.72	89.29	Type II toxin-antitoxin system PemK/MazF family toxin	82.62	91.67
	No						

Table 4.4 – Blast with *S. epidermidis* 1457 *mazE* or *mazF* sequence homologous with strains where sequences were described as part of TA systems

Gram staining	Species (Alphabetic order) + Reference description as TA system	Blast with <i>S. epidermidis</i> 1457 <i>mazE</i> sequence			Blast with <i>S. epidermidis</i> 1457 <i>mazF</i> sequence		
		Gene description on GenBank -PUBMED	Nucleotide homology (%)	Amino acid homology (%)	Gene description on GenBank -PUBMED	Nucleotide homology (%)	Amino acid homology (%)
Gram-negative	<i>Escherichia coli</i> [32]	MazF-MazE toxin- antitoxin system antitoxin MazE; AbrB/MazE/SpoVT family DNA-binding; Programmed cell death antitoxin MazE.	39.7 - 45.8	11.2 – 22.3	Endoribonuclease MazF; PemK-like cell growth regulatory protein ChpA; MazF family transcriptional regulator; Programmed cell death toxin MazF;	30.4 – 46.9	17.3 – 35.9
	<i>Leptospira interrogans</i> [33]	Transcriptional regulator/antitoxin, MazE; Addiction module antidote protein; AbrB/MazE/SpoVT family DNA-binding domain-containing protein	43.3 – 48.0	11.1 – 23.7	ppGpp-regulated growth inhibitor ChpA/MazF; endoribonuclease MazF; Type II toxin- antitoxin system PemK/MazF family toxin	49.0 – 51.8	25.8 – 32.7

Table 4.4 – Continued

Gram-variable	<i>Mycobacterium tuberculosis</i> [34]	Type II toxin-antitoxin system antitoxin MazE; Antitoxin MazE; AbrB/MazE/SpoVT family DNA-binding domain-containing protein;	31.8 – 44.0	36.9 – 44.4	Type II toxin-antitoxin system PemK/MazF family toxin; mRNA interferase MazF.	9.1 – 22.1	16.9 – 37.7
Gram-positive	<i>Bacillus anthracis</i> [35]	AbrB/MazE/SpoVT family DNA-binding domain-containing protein; Hypothetical protein;	28.7 – 42.5	14.8 – 25.5	PemK family transcriptional regulator;	45.0 – 67.5	68.6 – 71.6
	<i>Bacillus subtilis</i> [36]	Type II toxin-antitoxin system antitoxin EndoA1; Antitoxin EndoA1; CopG family ribbon-helix-helix protein; AbrB/MazE/SpoVT family DNA-binding domain-containing protein;	36.7 – 43.4	10.6 – 21.3	Type II toxin-antitoxin system endoribonuclease NdoA; mRNA interferase EndoA; PemK family transcriptional regulator; Toxin mazF;	63.6 – 66.2	67.2

Table 4.4 – Continued

Gram-positive	<i>Listeria monocytogenes</i> [37]	AbrB/MazE/SpoVT family DNA-binding domain-containing protein; Transition state regulator Abh; AbrB family transcriptional regulator; Transition state regulatory protein.	34.6 – 46.5	12.8 – 19.6	PemK family transcriptional regulator; Type II toxin-antitoxin system PemK/MazF family toxin.	58.8 – 65.1	56.5 – 65.2
	<i>Streptococcus mutans</i> [38]	MazE; Cell growth regulatory protein; AbrB/MazE/SpoVT family DNA-binding domain-containing protein; Antidote-toxin recognition MazE family protein; Cell division protein FtsW	43.2 – 52.4	11.0 – 23.0	PemK; ppGpp-regulated growth inhibitor; Type II toxin-antitoxin system PemK/MazF family toxin	39.7 – 43.2	18.1 – 26.6
Not applicable (NA)	Human Genome NA	NA	Not detected	Not detected	NA	Not detected	Not detected

Curiously, the similarity with *S. aureus*, where *mazEF* was also experimentally determined as a TA system, was significantly higher, with more than 85% homology of the *mazE* sequence (both nucleotide and amino acids) and from circa 80 to more than 90% of nucleotide and amino acids correspondence of the *mazF* sequence.

Nonetheless, the homology between the *S. epidermidis* and *S. aureus* sequences triggered the interest in exploring the main similarities and differences and further exploring the impact in the translation into proteins. As such, an alignment of the *S. epidermidis* 1457 and *S. aureus* Newman sequences was performed.

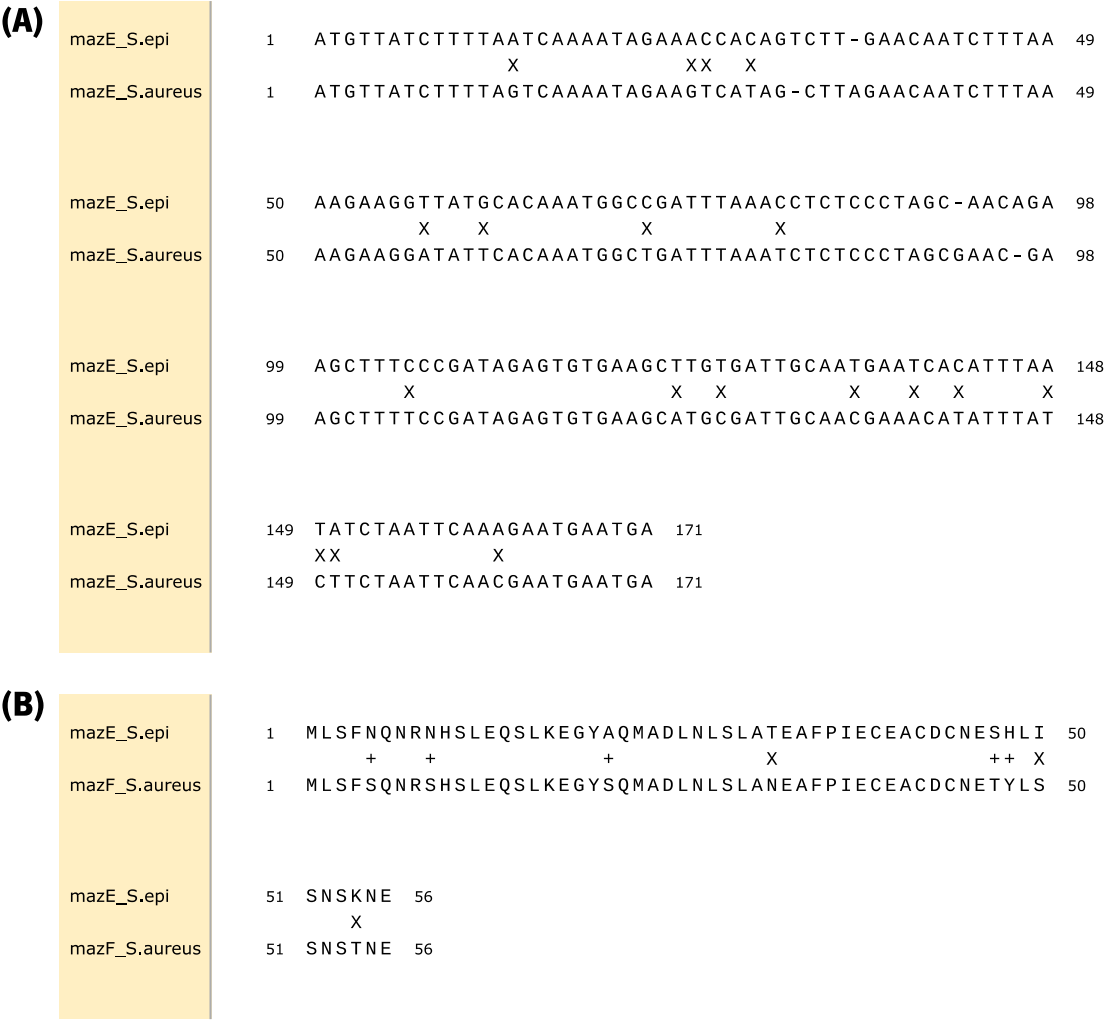


Figure 4.9 – Alignment of *mazE* sequences of *S. epidermidis* 1457 (upper row) and *S. aureus* Newman (lower row). **(A)** Alignment of *mazE* DNA sequences; **(B)** Alignment of *mazE* amino acid sequences. “-” represents a gap between the sequences, “X” represents different nucleotides/amino acids and “+” represents an alteration of amino acid by a similar one.

(A)	mazF_S.epi	1	ATGATTAGAAGAGGAGATGTTTATTTAGCGGATTTATCACCAGTTCAAGG	50
	mazF_S.aureus	1	ATGATTAGACGAGGAGATGTTTATTTAGCAGATTTATCACCAGTACAGGG	50
			X X X X X X	
	mazF_S.epi	51	GTCGAAACAAGGGGGAGTAAGACCTGTAGTTATCATTCAAAATGATACTG	100
	mazF_S.aureus	51	ATCTGAACAAGGGGGAGTCAGACCTGTAGTCATAATTCAAAATGATACTG	100
			X X X X X X	
	mazF_S.epi	101	GTAATAAATATAGTCCAACCTGTAATTGTAGCTGCGATTACTGGTAGGATT	150
	mazF_S.aureus	101	GTAATAAATATAGTCCTACAGTTATTGTTGCGGCAATAACTGGTAGGATT	150
			X X X X X X X X	
	mazF_S.epi	151	AATAAAGCGAAAAATACCAACCCACGTAGAAATTGAAAAGAAAAAGTATAA	200
	mazF_S.aureus	151	AATAAAGCGAAAAATACCGACACATGTAGAGATTGAAAAGAAAAAGTATAA	200
			X X X X	
	mazF_S.epi	201	ATTAGACAAAGATTCAAGTTATTCTTCTT-GAACAAATTAGAACACTAGAT	249
	mazF_S.aureus	201	GTTGGATAAAGACTCAGTTA-TATTATTAGAACAAATTCGTACACTTGAT	249
			X X X X X X X X X X	
	mazF_S.epi	250	AAAAAGCGTTTAAAAAGAAAAATTAACATTTTTATCAGA-GAGTAAATGA	298
	mazF_S.aureus	250	AAAAAACGATTGAAAGAAAACTGACGTACTTATCCGATGA-TAAATGA	298
			X X X X X X X X X	
	mazF_S.epi	299	TAGAGGTTGATAATGCCTTAGAT-ATTAGTTTGGGATTAAATAACTTTGA	347
	mazF_S.aureus	299	AAGAAGTAGATAATGCACTA-ATGATTAGTTTAGGGCTGAATGCAGTAGC	347
			X X X X X X X X X X	
	mazF_S.epi	348	TCATCATAAATCTTA	362
	mazF_S.aureus	348	TCACCAGAAAAATTA	362
			X X XX	
(B)	mazF_S.epi	1	MIRRGDVYLADLSPVQGSEQGGVRPVVIIQNDTGNKYSPTVIVAAITGRI	50
	mazF_S.aureus	1	MIRRGDVYLADLSPVQGSEQGGVRPVVIIQNDTGNKYSPTVIVAAITGRI	50
	mazF_S.epi	51	NKAKIPTHVEIEKKKYKLDKDSVILLEQIRTLDDKRLKEKLTLFLESKMI	100
	mazF_S.aureus	51	NKAKIPTHVEIEKKKYKLDKDSVILLEQIRTLDDKRLKEKLTYLSDDKMK	100
			+ +X X	
	mazF_S.epi	101	EVDNALDISLGLNFDHHS	120
	mazF_S.aureus	101	EVDNALMISLGLNAVAHQKN	120
			X XXX X +	

Figure 4.10 – Alignment of *mazF* sequences of *S. epidermidis* 1457 (upper row) and *S. aureus* Newman (lower row). **(A)** Alignment of *mazF* DNA sequences; **(B)** Alignment of *mazE* amino acid sequences. “–” represents a gap between the sequences, “X” represents different nucleotides/amino acids and “+” represents an alteration of amino acid by a similar one.

The analysis of **Figure 4.9** confirmed the high homology between the *mazE* sequences of both strains assessed, with few differences being found: DNA sequences only showed 18 different nucleotides and 4 gaps, which resulted in an amino acid sequence with an identity of 48 out of 56 amino acids, but a similarity of 53/56 amino acids. On the other hand, data from **Figure 4.10** showed that the *mazF* DNA sequences presented 56 distinct nucleotides and 6 gaps, resulting in an amino acid sequence with an identity of 110/120, in which a total of 112 amino acids were considered similar.

Moreover, to explore the effect of the amino acid differences in the translation into a protein, a prediction of the protein structure of MazE and MazF was performed and represented in **Figure 4.11**. Although the main structure of the proteins in the *S. epidermidis* and *S. aureus* strains is similar, there are some “low” and “very low” confidence levels for some areas of the protein structure prediction, which may indicate structural differences that could affect the activity of these proteins, which should be further experimentally determined.

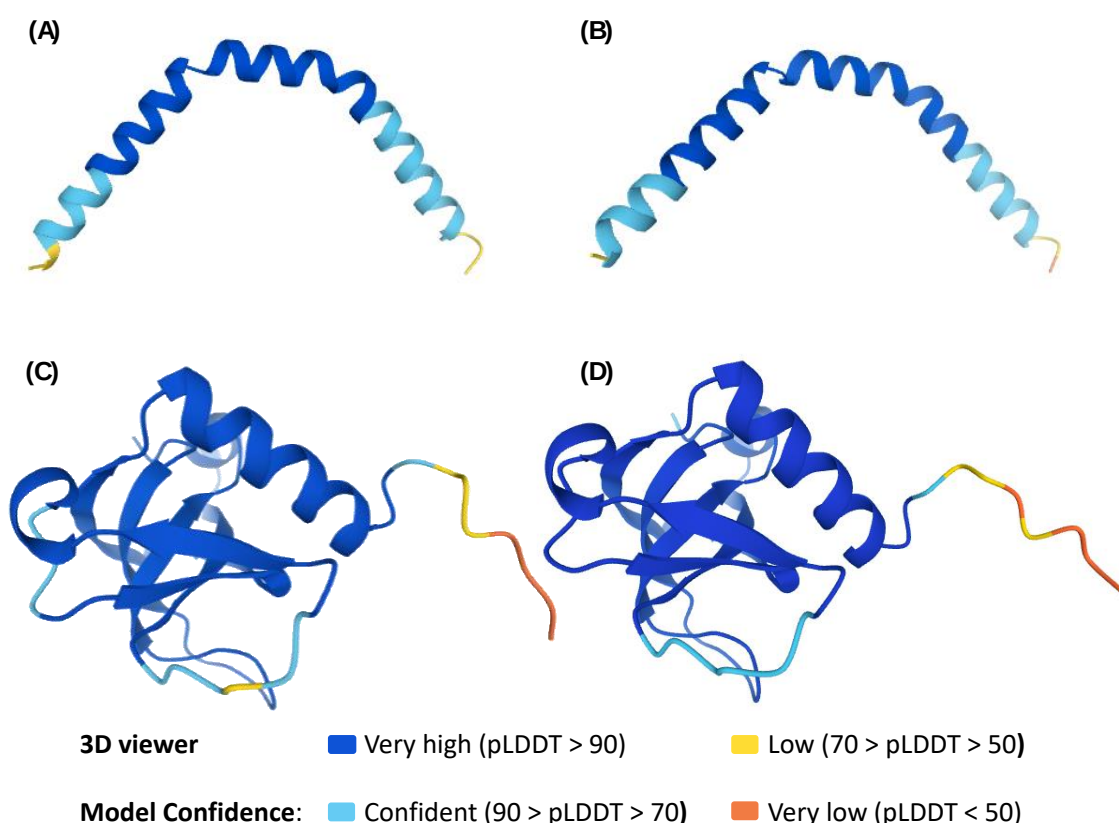


Figure 4.11 – Prediction of MazEF protein structure of *S. epidermidis* and *S. aureus* strains. (A) *S. epidermidis* 1457 MazE structure prediction; (B) *S. aureus* Newman MazE structure prediction; (C) *S. epidermidis* 1457 MazF structure prediction and (D) *S. aureus* Newman MazF structure prediction. Structure prediction was obtained with “AlphaFold Protein Structure Database”[39, 40].

Taken together, since no toxin and antitoxin outcome of the *S. epidermidis* *mazF* and *mazE* genes was found in strains 9142 or 1457, we suggest that the *mazEF* operon in the *S. epidermidis* species has a different role, despite the high homology found with *S. aureus*.

4.3.6. Effect of *mazEF* in bacterial growth and biofilm formation

We further characterized the *mazEF* mutant in the physiology and virulence potential of *S. epidermidis*. Therefore, to understand if the *mazEF* mutation affected cell growth, planktonic populations growth kinetics of all *S. epidermidis* 1457 strains (WT, $\Delta mazEF$ and $\Delta mazEF::pRB473+mazEF$) was assessed for up to 24 hours.

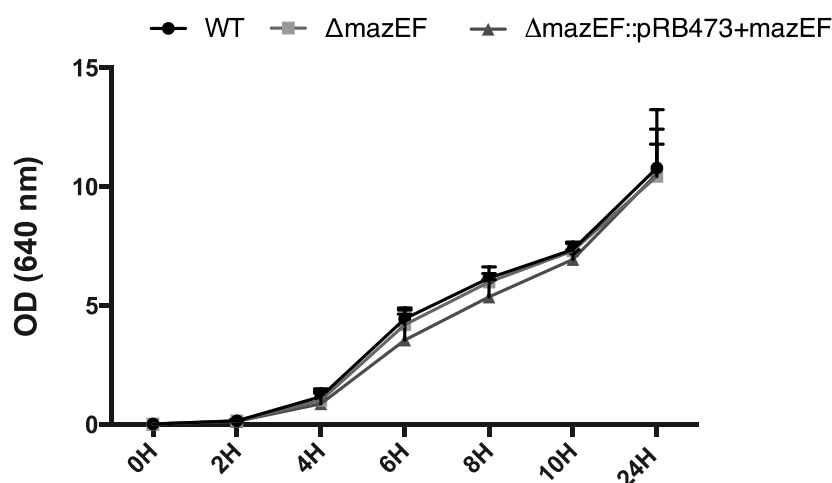


Figure 4.12 – Growth curve of planktonic populations of *S. epidermidis* 1457 WT, $\Delta mazEF$ and $\Delta mazEF::pRB473+mazEF$ strains, determined by OD_{640nm}; Results are presented as the mean + standard deviation of three independent assays. Statistical analysis was performed between the WT and the other strains for each time point.

As assessed by the quantification of the total amount of cells represented in **Figure 4.12**, all strains presented a similar growth profile in the planktonic mode, either during the exponential or stationary phases, despite their different genetic makeover.

Moreover, we aimed to understand if the *mazEF* deletion could affect biofilm formation. To do so, we grew 48-hours biofilms of both *S. epidermidis* 1457 and *S. epidermidis* 9142 and their respective constructs and evaluated the number of culturable biofilm cells.

Overall, the results from **Figure 4.13** show that similar biofilms, in terms of bacterial culturability, were produced by the distinct WT and constructs of each strain.

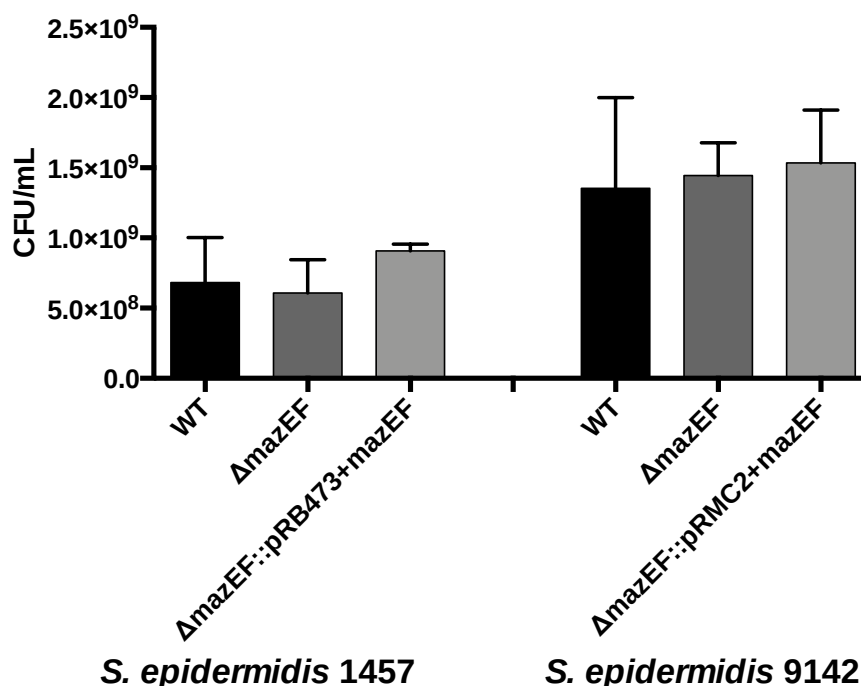


Figure 4.13 – Quantification of 48-hours biofilms formed by *S. epidermidis* 1457 WT, $\Delta mazEF$ and $\Delta mazEF::pRB473+mazEF$ and *S. epidermidis* 9142 WT, $\Delta mazEF$ and $\Delta mazEF::pRMC2+mazEF$, assessed by the number of culturable cells (CFU/mL). Results are presented as the mean + standard deviation of three independent assays. Statistical analysis was performed between the WT and constructs for each strain.

Altogether, these results suggest that *mazEF* does not present an important function in general planktonic growth or biofilm formation in *S. epidermidis*, at least under our experimental conditions. Nonetheless, the growth of planktonic and biofilm populations was also assessed in conditions where the VBNC state was either induced or prevented, since previous data suggested the involvement of *mazEF* in the modulation of this state and will be presented in **Chapter 6**.

4.3.7. Effect of *mazEF* in antimicrobial susceptibility

We further assessed the role of *mazEF* in *S. epidermidis* virulence by performing an antimicrobial susceptibility study, using three antibiotics with distinct mechanisms of action, namely, rifampicin (RNA synthesis inhibitor), tetracycline (protein synthesis inhibitor) and vancomycin (cell wall synthesis inhibitor). First, the minimal inhibition concentration (MIC) of vancomycin, rifampicin and

tetracycline was determined, and the susceptibility of the strains was classified according to EUCAST standards.

Table 4.5 – Minimum inhibitory concentration (mg/L) of rifampicin, tetracycline and vancomycin against *S. epidermidis* 1457 WT and constructs.

Strain:	Rifampicin $S \leq 0.06$; $R > 0.5$ mg/L	Tetracycline $S \leq 1$; $R > 2$ mg/L	Vancomycin $S \leq 4$; $R > 4$ mg/L
WT	0.008 – 0.016 Susceptible	0.5 – 1 Susceptible	4 – 8 Intermediate
$\Delta mazEF$	0.008 – 0.016 Susceptible	0.5 – 1 Susceptible	4 – 8 Intermediate
$\Delta mazEF::pRB473+mazEF$	0.008 – 0.016 Susceptible	0.5 – 1 Susceptible	4 – 8 Intermediate

As can be seen in **Table 4.5**, no differences were found in the MIC of any antibiotic determined for the distinct strains. Importantly, the MIC range found for vancomycin indicated an intermediate susceptibility, as the lower value of the range obtained coincides precisely with the breaking point, while all isolates were considered susceptible to rifampicin and tetracycline.

We then evaluated the susceptibility of planktonic cultures to the PSC of the antibiotics with different cells targets. The culturability, assessed by CFU quantification, and metabolic activity, assessed by the reduction of tetrazolium salt (XTT) were measured after 6 and 24 hours of incubation with antibiotics.

As depicted in **Figure 4.14**, it is evident that all antibiotics induced an important reduction of culturability in *S. epidermidis* but that *mazEF* constructs presented a similar behaviour compared to the WT strain, with no statistically differences in neither the CFU nor the XTT experiments, independently of the antibiotics or time of incubation.

Previous studies have shown that *mazEF* could be involved in the alteration of susceptibility to antimicrobial substances. For instance, a study focused on the role of *mazF* in *S. aureus* revealed that *mazEF*TA system is involved, among others, in the susceptibility to antibiotics [41]. Moreover, the influence of *mazEF* expression in the antimicrobial susceptibility of *P. aeruginosa* and some staphylococcal species was also reported, leading to decreased susceptibility to gentamicin,

ciprofloxacin, and clindamycin [42]. Nonetheless, herein, no differences were found when the *mazEF* operon was deleted, suggesting a distinct role of this operon in *S. epidermidis*.

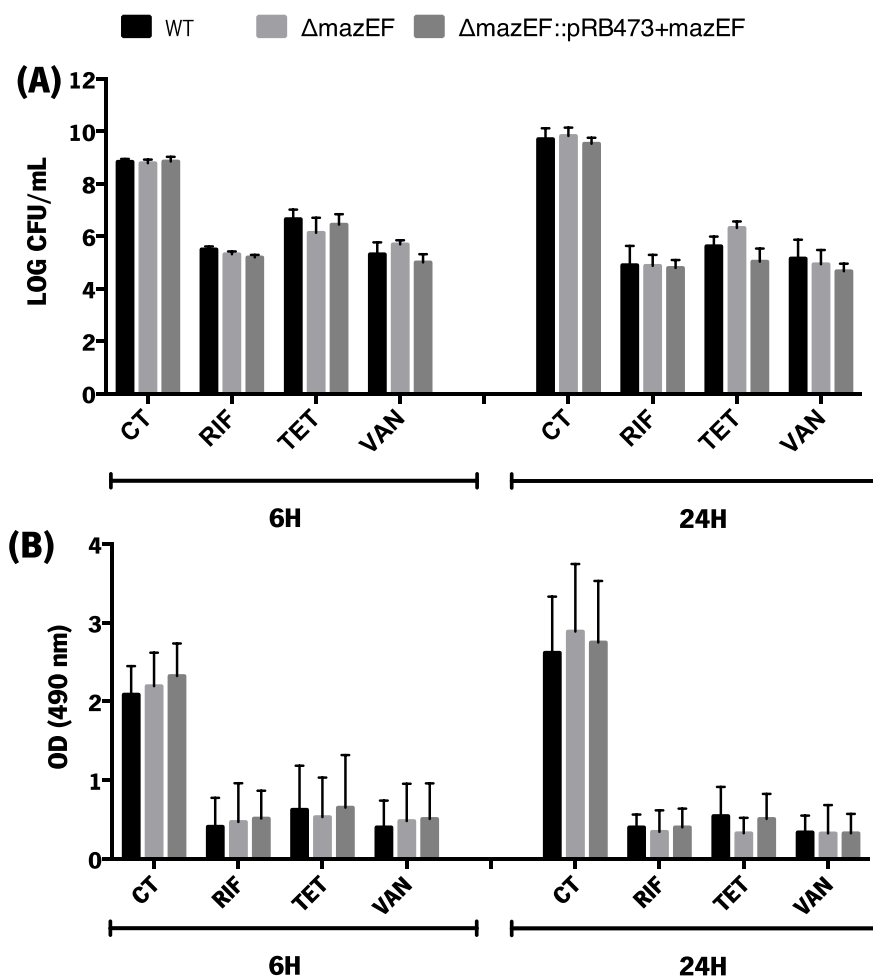


Figure 4.14 – Antimicrobial effect of rifampicin (RIF), tetracycline (TET) and vancomycin (VAN) on 24 hours-old planktonic cultures of *S. epidermidis* strains after 6 and 24 hours of incubation as determined by **(A)** culturability (LOG CFU/mL) and **(B)** metabolic activity (XTT, OD_{490nm}). Results are presented as the mean + standard deviation of at least three independent experiments. Statistical analysis was performed between the WT and constructs for each time point and antibiotic.

4.3.8. Role of *mazEF* in the survival in human blood and plasma

In order to enhance our understanding on how *mazEF* impacts virulence, it is fundamental to assess if the deletion of the *mazEF* operon could affect *S. epidermidis* ability to survive in the host. Therefore, we aimed to understand if there were differences in the survival of the *S. epidermidis* 1457 WT, $\Delta mazEF$ and $\Delta mazEF::pRB473+mazEF$ strains when exposed to human blood and plasma, obtained from healthy donors, for up to 4 hours.

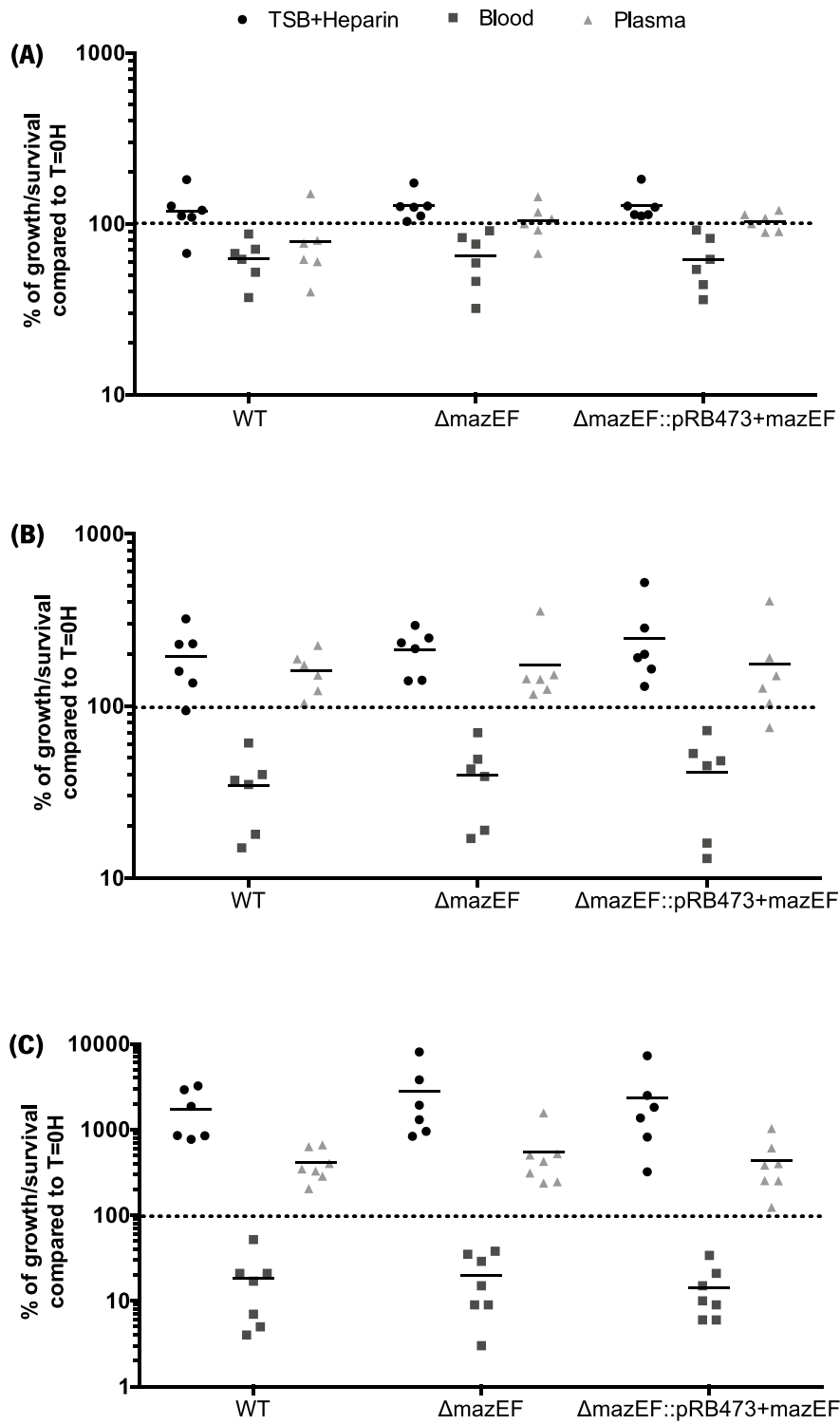


Figure 4.15 – Survival of *S. epidermidis* strains in human blood and plasma. TSB+Heparin was used to evaluate the heparin effect on bacterial growth. Results are represented as the percentage of growth/survival (%) compared to T=0H for each donor upon incubation with TSB+Heparin, blood and plasma for **(A)** 1 hour, **(B)** 2 hours and **(C)** 4 hours. The horizontal bars represent the mean of 6 independent experiments performed with blood collected from different donors (3 female and 3 male).

Importantly, since it was previously shown that human plasma alters gene expression in *S. epidermidis* [29] and to understand if the antimicrobial effect of blood was indeed due to phagocytic processes and not caused by soluble factors present in plasma, we separated human plasma from the whole blood and studied the effect of incubation with this fluid under the same conditions.

Moreover, to ensure the potential antimicrobial effect of human fluids and discard the effect of the anticoagulant used during blood collection, a control was made with growth media containing heparin at the same concentration (TSB+Heparin). These assays were started with a concentration of cells of about 1×10^5 CFU/mL (T=0H), that was previously optimized [28], and the results of culturable cells quantification are presented as the % of survival or growth compared to the initial bacterial load (T=0H).

From the analysis of **Figure 4.15** it is noticeable that there are minor differences between the strains when exposed to human blood, plasma, or TSB+Heparin, in all the time points assessed (1, 2 and 4 hours of incubation). As expected, incubation with human blood led to a significant decrease of bacterial culturability and the antimicrobial effect was more pronounced in the longer incubation periods, with a reduction of the initial bacterial concentration of up to 90%. Moreover, taking into account that incubation with plasma resulted in a partial bacteriostatic effect compared to growth in TSB+Heparin and that the incubation with blood led to a significant decrease of the culturability of bacteria, it is suggested that the antimicrobial effect detected upon incubation with human blood was caused by phagocytic processes performed by polymorphonuclear cells and monocytes that circulate in human blood [43, 44], but are not present in the human plasma.

4.4. Conclusions

The *mazEF* operon was first identified as a TA system in *E. coli*, and further confirmed as a TA complex in other gram-negative and gram-positive bacteria, with homology studies suggesting that this operon could have a TA function also in *S. epidermidis* [45, 46].

Curiously, despite the homology between *mazEF* sequences in *S. epidermidis* 1457 and *E. coli* not being very high, a significant homology was found with the *mazEF* sequences of *S. aureus*, which is a closely-related species where this operon was recently confirmed to act as a TA system [41]. For this reason, it was unexpected to find that the *S. epidermidis mazEF* operon does not seem to function as a TA system, a discovery that seems not to be strain-dependent, as an additional strain (*S. epidermidis* 9142) was used to confirm these findings. Nonetheless, it was previously shown

that the regulation of some important genes is different in *S. epidermidis* and *S. aureus* strains, despite their significant homology [47], and our data suggest that the same happens with the *mazEF* operon. Moreover, since up to 10% of differences in the amino acid sequence of the distinct *S. epidermidis* strains was found, we could not exclude intra-strain structural differences of the MazE and MazF proteins and, consequently, a different role of the *mazEF* operon among other strains.

We further explored if the physiological changes induced by the deletion of the *mazEF* in *S. epidermidis* 1457 and 9142 could affect the growth of the constructs, either as planktonic or biofilm populations. Of note, no significant differences were found between the WT and constructs of each strain in any of the experiments performed, which, altogether, suggests that the *mazEF* operon is not fundamental in the growth and biofilm formation processes of *S. epidermidis* 9142.

Interestingly, other biological roles have been linked with *mazEF*, whereby we focused on the study of the *mazEF* influence in the main virulence aspects of *S. epidermidis*, such as antimicrobial response and survival in human blood and plasma. Curiously, the same was not found for strain 1457, since no differences in the antimicrobial susceptibility or survival in human blood and plasma were found between the WT strain and the constructs.

Altogether, the first characterization of the *mazEF* mutant and complemented strains demonstrated that this operon does not seem to have a significant impact on the general virulence factors of this bacteria, at least under the tested conditions and in the two strains tested.

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CHAPTER 5

Importance of *mazEF* in the immune response

SUMMARY

The activation of the innate immune response upon pathogenic invasions has previously been described for a wide variety of species, including the commensal bacteria *S. epidermidis*, and extensively reported in the literature. *mazEF* homologue operons have formerly been shown to influence the host immune response to bacterial infections, raising the interest in the study of the impact of *mazEF* deletion on the activation of the host immune system. Herein we aimed to understand if the expression of the *mazEF* operon could modulate the response of the host by analysing the production of pro- and anti-inflammatory cytokines by human monocyte-derived macrophages (MDMs) and dendritic cells (MDDCs).

The activation of MDDCs upon interaction with the distinct *S. epidermidis* strains was also evaluated. The study of the interaction of *S. epidermidis* with host immune cells was completed with the study of the production of reactive oxygen species (ROS) by polymorphonuclear leukocytes (PMNs). Overall, the analysis of the results obtained for the individual strains revealed a very similar response by the immune cells in all the parameters assessed, suggesting that the deletion of the *mazEF* operon from *S. epidermidis* 1457 genome did not have a significant impact on the activation of the innate immune response. Notwithstanding, our results confirm that the *S. epidermidis* 1457 strain induces the activation of the assessed immune cells and production of cytokines by human-derived M1- and M2-type MDMs and MDDCs, both as the production of ROS by human-isolated neutrophils.

5.1. Brief introduction

S. epidermidis cells have the ability to evade the host immune response, resorting to some immune evasion molecules [1, 2], hindering its elimination by the host. Importantly, this ability has been previously shown to be more evident in biofilms, where *in vitro* studies have reported enhanced tolerance of *S. epidermidis* biofilms to the host immune response [3, 4]. Being a commensal and opportunistic pathogen, usually accounting for increased morbidity rates rather than mortality [5, 6], may be one of the reasons why there has not been an extensive focus on the study of the immune response to this species. Thereby, the literature on the activation of the host innate immune system by *S. epidermidis* is rather scarce.

Curiously, the *mazEF* operon has been associated with alterations in the response of the host to infections caused by *Mycobacterium tuberculosis* [7] and the modification of immune cells with the *mazF* endoribonuclease from *E. coli* has been shown to interfere with their immunogenicity [8]. Hence, we could not discard a possible modulatory role of *mazEF* in the host immune response induced by *S. epidermidis* bacteria and aimed to understand the effect of this operon on the bacterial interaction with innate immune cells.

Herein, human monocyte-derived macrophages (MDMs) and dendritic cells (MDDCs) were differentiated from peripheral blood mononuclear cells (PBMCs), since these are professional phagocytic cells that have a fundamental role in the innate immune response [9]. Moreover, neutrophils were isolated from blood samples for the assessment of the production of reactive oxygen species (ROS), as this cell population is also a major player in the innate immune response, able to phagocytose and kill extracellular bacteria through the production of ROS and antimicrobial peptides (AMPs) [10].

5.2. Materials and methods

5.2.1. Strains and growth conditions

To understand the role of the homologous *mazEF* operon in the response of the immune system, *S. epidermidis* 1457 WT, $\Delta mazEF$ and $\Delta mazEF::pRB473$ were grown overnight in TSB at 37 °C with 120 rpm agitation in an orbital shaker (10 mm orbit). The concentration of the overnight suspensions was then adjusted to approximately 2×10^8 CFU/mL by adjusting the OD_{640nm} of the suspensions to 0.25 ± 0.05 , for further incubation with immune cells.

5.2.2. Isolation of polymorphonuclear leukocytes (PMNs) and differentiation of MDMs and MDDCs

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats through density gradient centrifugation using the SepMate™ PBMC isolation tubes (Stemcell Technologies) and following the manufacturer's protocol. To obtain monocyte-derived cells, Histopaque-1077 (Sigma-Aldrich) was used as the density gradient medium and the SepMate™ tube was centrifuged at 1200 *g* for 15 minutes. The cells obtained were magnetically labelled and sorted to be able to selectively collect monocytes (CD14+ cells), using anti-human CD14 MicroBeads (Miltenyi Biotec), according to the manufacturer's instructions. The monocytes selected were resuspended in RPMI (RPMI 1640, Sigma-Aldrich) supplemented with 10% FBS (Biowest), 4 mM L-glutamine (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), 50 μ M β -mercaptoethanol and 1% penicillin-streptomycin, from now on designated as complete RPMI (cRPMI). Then, cells were counted with trypan blue and seeded in 6-well culture plates at a final concentration of 1×10^6 cells/mL. Specific cytokines were added to the culture to promote the differentiation of the monocytes into MDMs and MDDCs. More specifically, GM-CSF (50 ng/mL) or M-CSF (50 ng/mL) were added to originate M1- or M2-type MDMs, respectively, while GM-CSF (50 ng/mL) and IL-4 (50 ng/mL) were added as a mean to differentiate cells into MDDCs. Plates with the cell cultures were incubated at 37°C with 5% CO₂ for up to 7 days. Every 3 days, half of the cell culture medium was removed and replaced by fresh medium. The isolation of PMNs from buffy coats was completed by a double gradient technique using solutions with distinct densities, namely Histopaque-1077 (Sigma-Aldrich) and Histopaque-1119 (Sigma-Aldrich), according to the SepMate™ protocol. The cells obtained upon this procedure were resuspended in cRPMI for further use.

5.2.2.1 Ethics statement

Buffy coats (fraction of anticoagulated blood sample containing most the white blood cells and platelets) from healthy blood donors used for M1/M2 MDMs and MDDCs differentiation were acquired at the Immunohemotherapy Department of Centro Hospitalar São João from Porto, Portugal. All donors gave have given informed written consent for part of their blood samples to be used for scientific purposes, according to Protocol reference 260/11, under ethical approval of the service. The principles of the Declaration of Helsinki and Oviedo Convention were respected during

the procedures used to obtain peripheral blood mononuclear cells (PBMCs) from the buffy coats donated.

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

On day 7, the non-adherent and loosely adherent MDDCs were harvested from bottom the of the plate through gentle up and down pipetting movements, while both M1- and M2-type MDMs were detached from the plate with 5 mM EDTA. Cells were then recovered from the 6-well plates and resuspended in supplemented RPMI (sRPMI, media supplemented with 10% FBS, 2% L-glutamine, 1% HEPES and 50 μ M β - mercaptoethanol), followed by counting with trypan blue to estimate the concentration of cells. Next, MDDCs were seeded in 96-well plates with a round bottom, while MDMs were seeded in flat-bottom plates. Both types of monocyte-derived cells were infected with the strains (WT, $\Delta mazEF$ and $\Delta mazEF::pRB473+mazEF$) at a multiplicity of infection (MOI) of 1MDDC/MDM:5Bacteria or 1MDDC/MDM:10B. Controls were added to a better estimation of the level of cytokine production, namely a negative control where cells were not stimulated and a positive control with stimulation of cells with 2 μ g/mL lipopolysaccharide (LPS) from *E. coli* (Sigma-Aldrich). Then, the plates were incubated at 37°C and 5% CO₂. After 2 hours, 50 μ g/mL gentamicin were added to each well to stop bacterial growth, and the plates were incubated back in the same conditions (37°C and 5% CO₂) for up to 24 hours. After the incubation period, the plates were centrifuged at 300 *g* for 10 minutes and the supernatants were collected and stored at -80°C for further measurement of cytokine secretion by ELISA. Finally, cells were prepared and stained for flow cytometry detection and analysis of cell surface activation.

5.2.4. Cytokine release evaluation by sandwich ELISA

The secretion of specific pro-inflammatory (TNF- α , IL-6, IL-8 and IL-12p70) and anti-inflammatory (IL-10) cytokines by MDMs and MDDCs upon infection with *S. epidermidis* strains was quantified by sandwich ELISA. A commercial kit, specific for each cytokine, was used to analyse the cytokine level in cell culture supernatants (Human TNF- α DuoSet® ELISA Development System, Human IL-6 DuoSet® ELISA Development System, Human IL-8 DuoSet® ELISA Development System, Human IL-1 β DuoSet® ELISA Development System, Human IL-10 DuoSet® ELISA Development System and Human IL- 12p70 DuoSet® ELISA Development System from R&D systems) and the manufacturer's protocol was followed.

5.2.5. Evaluation of MDDCs activation by fluorescence-activated cell sorting (FACS)

The detection of cell surface activation markers from MDDCs relied on flow cytometry analysis. Briefly, the cells infected with the *S. epidermidis* strains were collected after 24 hours of incubation, washed, and stained with a fixable viability dye (eFluor 780) and with cell surface antibodies against the activation markers: anti-human CD11c APC-conjugated (clone BU15), anti-human HLA-DR 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), all from eBiosciences. Then, cells were fixed with paraformaldehyde and resuspended in FACS buffer (PBS 1× supplemented with 1% bovine serum albumin and 0.05% sodium azide). Finally, samples were analysed by flow cytometry, using a BD FACSCanto™ II and data was analysed with FlowJo™ 10.7.1 Software (BD Life Sciences), according to the gating strategy defined in **Figure 5.1**.

5.2.6. Evaluation of reactive oxygen species (ROS) production

After adjusting the concentration of freshly isolated PMNs to 1×10^6 cells/mL, the human-derived cells were incubated with *S. epidermidis* with 2 distinct MOI, namely 1PMN:5B or 1PMN:10B in 96-well plates. To analyse the production of ROS, the samples were stained with 2',7' – dichlorofluorescein diacetate (DCFDA/H2DCFDA- Cellular ROS Assay Kit, Sigma-Aldrich) according to the manufacturer's instructions. A positive control of the reaction was done by incubating PMNs with 50 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich).

5.2.7. Statistical analysis

Data obtained from the results of this chapter were analysed with one-way ANOVA with Tukey's multiple comparisons test. Differences between the WT and the other strains were regarded as statistically significant when a *p*-value lower than 0.05 was found.

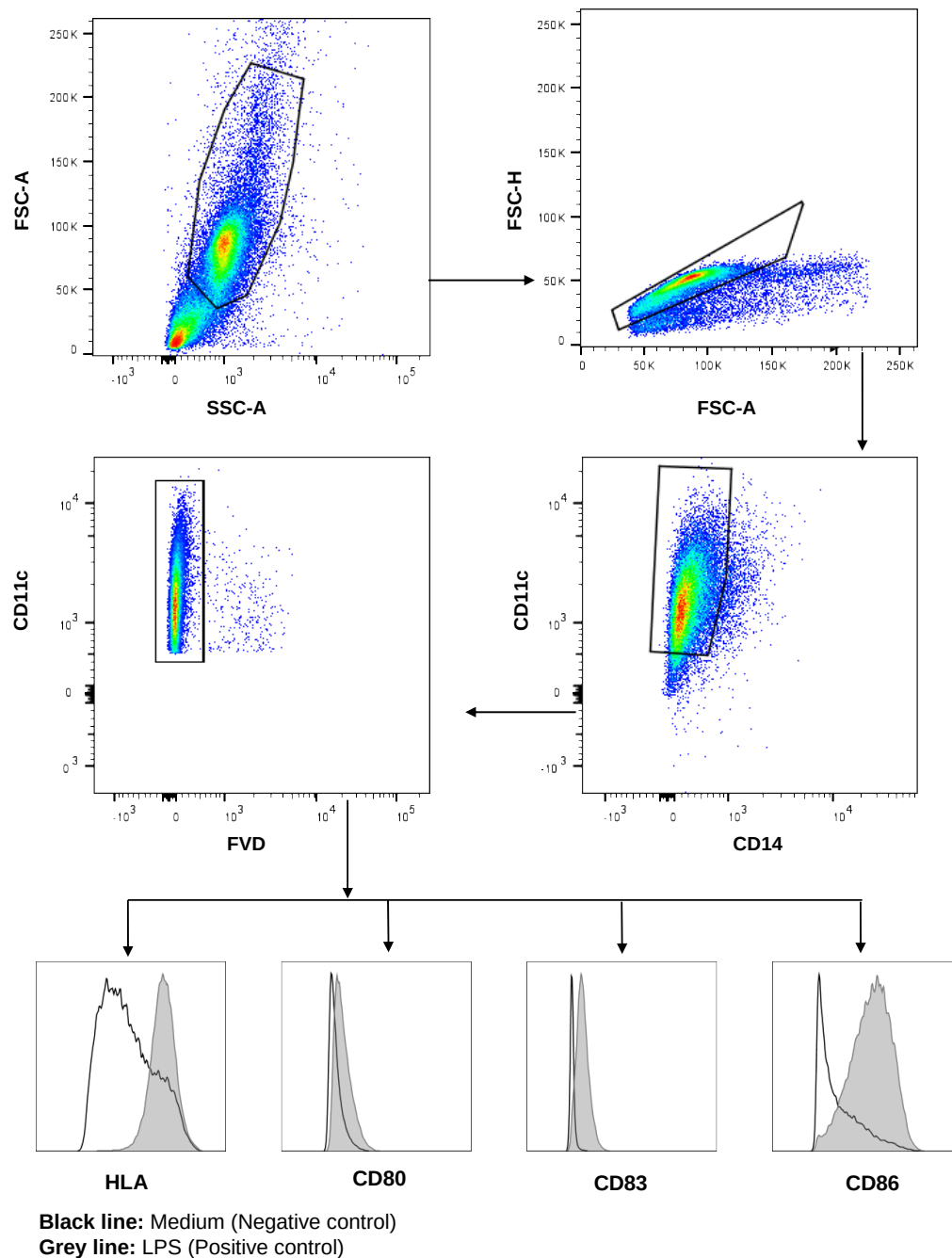


Figure 5.1 – Representative gating used for the analysis of activation makers expression upon incubation of MDDCs with *S. epidermidis* strains. The samples were first filtered by size and complexity by representing them in a dot plot with forward (FSC-A) vs side (SSC-A) scatter. Then, using FSC-H (height) vs FSC-A (area) representation it was possible to select single cells and eliminate duplets for further analysis. Subsequently, the CD11⁺ population was selected at the same time that CD14⁺ cells (MDDC that did not differentiate) were excluded. Finally, dead cells that incorporated fixable viability dye (FVD) were also excluded from the analysis and the remaining CD11⁺ cells were used to measure the mean of intensity fluorescence (MIF) of the distinct surface markers.

5.3. Results and discussion

5.3.1. Quantification of secreted cytokines by human MDMs and MDDCs

Neutrophils, macrophages and dendritic cells are professional phagocytic cells. When the host senses a pathogenic invasion, it reacts via the innate immune response with the production of antimicrobial substances, activation of the complement system and production of other pro-inflammatory mediators, such as cytokines [9]. Both M1- and M2-type MDMs were used in this study, since the “classically activated” macrophages (M1) are generally involved in proinflammatory and phagocytic processes, while the “alternatively activated” ones (M2) generally present an anti-inflammatory profile [11].

Herein, we assessed the production of cytokines by M1- and M2-type MDMs and MDDCs, namely pro-inflammatory interleukins IL-12p70, IL-6, IL-8 and tumour necrosis factor (TNF- α) and anti-inflammatory IL-10.

As shown in **Figure 5.2**, no differences were found in the production of any of the assessed cytokines induced by the distinct strains, suggesting that the action of *mazEF* operon on the stimulation of M1-type MDMs is not relevant. M1-type MDMs are generally considered pro-inflammatory. Therefore, they are expected to produce more pro-inflammatory cytokines upon contact with pathogens. Herein, we can see that the anti-inflammatory IL-10 cytokine (**Figure 5.2 E**) was slightly less produced than all the pro-inflammatory cytokines (**Figure 5.2 A-D**), especially with the lower multiplicity of infection (MOI 1:5), as compared with the control (LPS-induced cytokines).

Overall, by analysing the levels of cytokines in the cell culture supernatants normalized to the positive control (LPS), it is evident that *S. epidermidis* 1457 strains induced the production of cytokines by the M1-type MDMs at a similar rate to the positive control (LPS), with the exception of IL-12, where the expression of cytokine in response to incubation with the strains was around 2-fold higher than in the LPS control. Importantly, the higher multiplicity of infection (MOI 1:10) did not significantly impact the production of cytokines, with similar or slightly higher levels of production. On the other hand, M2-type MDMs are frequently viewed as anti-inflammatory and are expected to be more involved in the production of anti-inflammatory cytokines, such as IL-10.

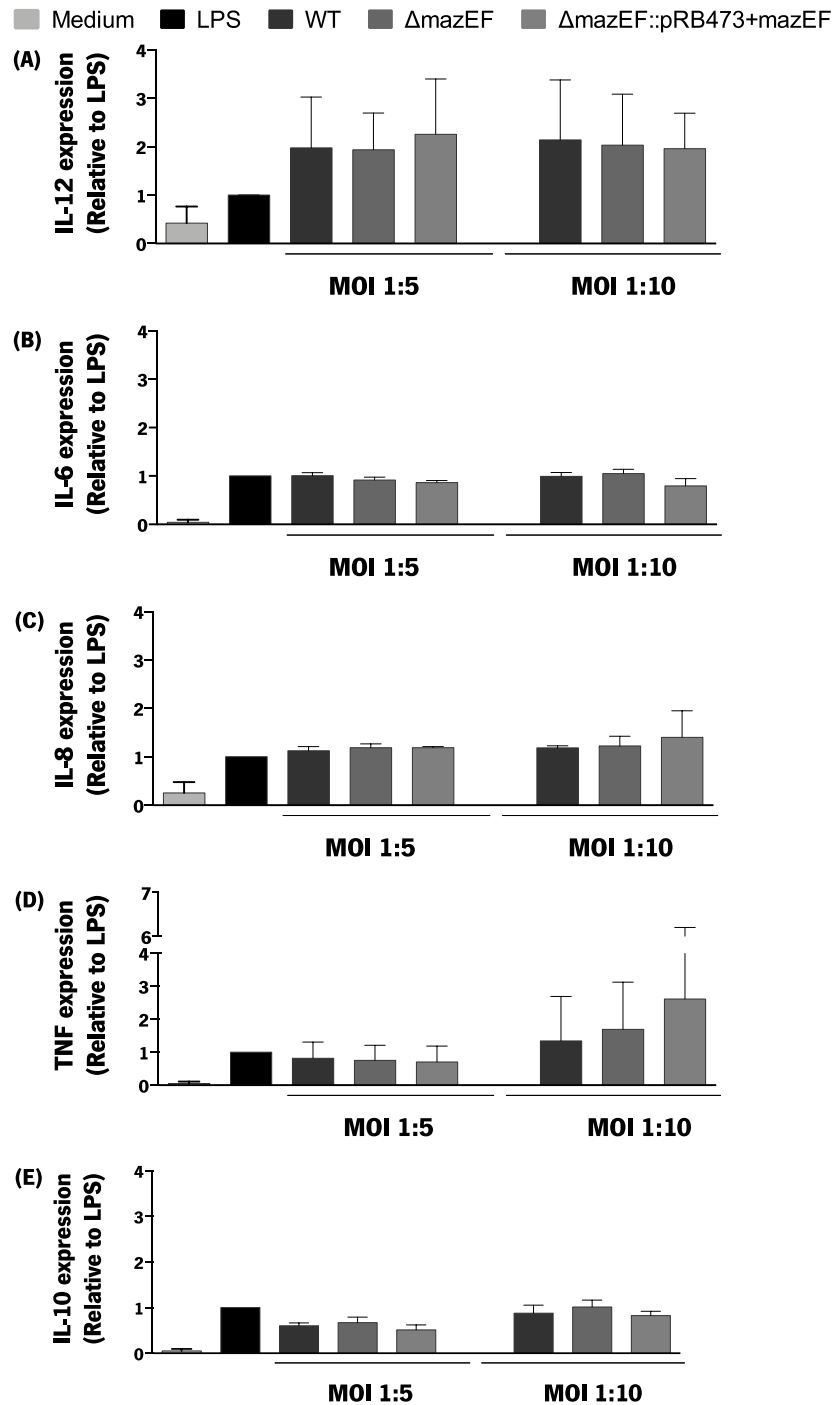


Figure 5.2 – Effect of *mazEF* deletion on the response of M1-type MDMs, assessed by the quantification of cytokines secreted upon 24 hours of infection with *S. epidermidis* 1457 strains at a MOI of 1MDM:5B and 1MDM:10B. The levels of (A) IL-12p70, (B) IL-6, (C) IL-8, (D) TNF- α and (E) IL-10 released into the supernatants were measured by ELISA and the results are presented as the mean plus standard deviation of the data normalized to the positive control samples (LPS). Three independent experiments were performed, in which each condition was performed in duplicate. Statistical analysis was performed between the WT and constructs for each condition.

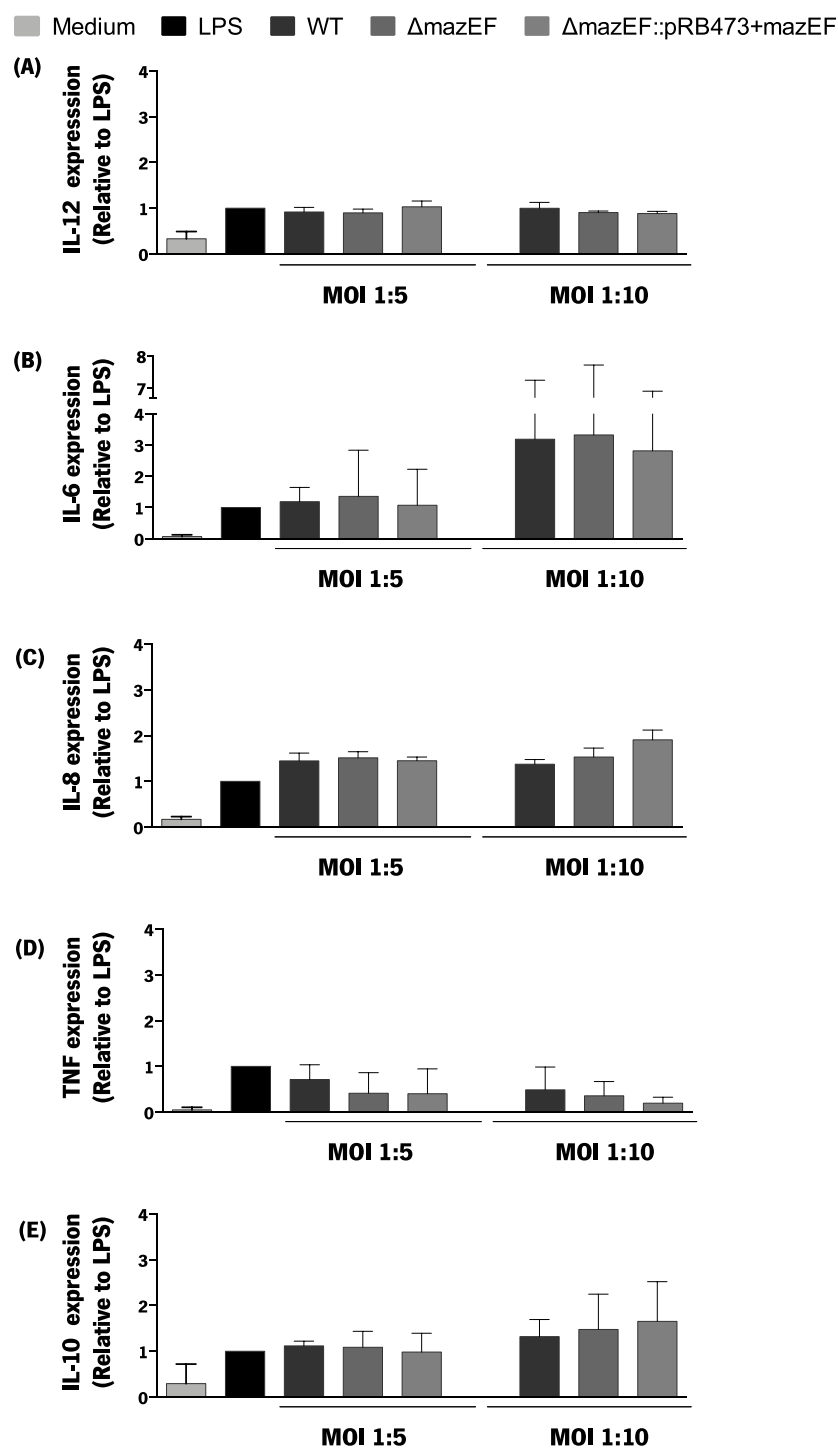


Figure 5.3 – Effect of *mazEF* deletion on the response of M2-type MDMs, assessed by the quantification of cytokines secreted upon 24 hours of infection with *S. epidermidis* 1457 strains at a MOI of 1MDM:5B and 1MDM:10B. The levels of (A) IL-12p70, (B) IL-6, (C) IL-8, (D) TNF-α and (E) IL-10 released into the supernatants were measured by ELISA and the results are presented as the mean plus standard deviation of the data normalized to the positive control samples (LPS). Three independent experiments were performed, in which each condition was performed in duplicate. Statistical analysis was performed between the WT and constructs for each condition.

As depicted in **Figure 5.3**, no differences were found for the response of the M2-type MDMs to the distinct *S. epidermidis* strains, suggesting that *mazEF* genes did not cause significant differences in the expression of cytokines by these cells. Moreover, *S. epidermidis* strains led to a production of cytokines that was similar or marginally higher than when the MDMs were incubated with LPS, except for TNF- α where the cytokine levels were found to be below the positive control, although none of the differences were statistically significant.

Importantly, combining the analysis of **Figure 5.2** and **Figure 5.3**, it is suggested that the polarization of the MDMs (M1- and M2-type) did not interfere with the response to infection with this particular *S. epidermidis* strain.

Finally, the production of cytokines by MDCCs was also assessed and the levels of cytokines released to the supernatants are represented in **Figure 5.4**. Overall, the production of cytokines by MDCCs incubated with *S. epidermidis* strains reached lower levels compared to MDCCs stimulated with LPS. Curiously, a lower expression of TNF- α in the WT compared to mutant strain was found with both multiplicities of infection, nonetheless, it was not considered statistically significant. On all the other cytokines assessed, the WT, mutant ($\Delta mazEF$) and complemented ($\Delta mazEF::pRB473+mazEF$) strains led to similar levels of expression upon 24 hours of infection.

5.3.2. Activation/maturation of human MDCCs

Upon activation, phagocytic cells express specific activation markers, including antigen-presenting and costimulatory molecules, such as HLA and CD80, CD83 and CD86, respectively [12]. Moreover, the CD83 marker can also be seen as an identity marker, as it gives information on the maturation of MDCCs [12, 13]. Herein, we aimed to understand if the different strains and/or distinct multiplicities of infection could impact the activation of human MDCCs, by assessing the upregulation of surface markers, such as CD80, CD83, CD86 and HLA-DR, by flow cytometry.

While some variability was found in the expression of these activation markers upon stimulation with all the strains tested, the results were very similar among strains, regardless of the multiplicities of infection, and no significant differences were found between the strains harbouring or not the *mazEF* operon. Looking at the expression levels with the negative control (medium alone), we can see that there is a basal expression of all markers analysed, as expected [14, 15]. Moreover, data shows that all strains led to an intermediate level of expression, ranging between the positive (LPS) and the negative controls.

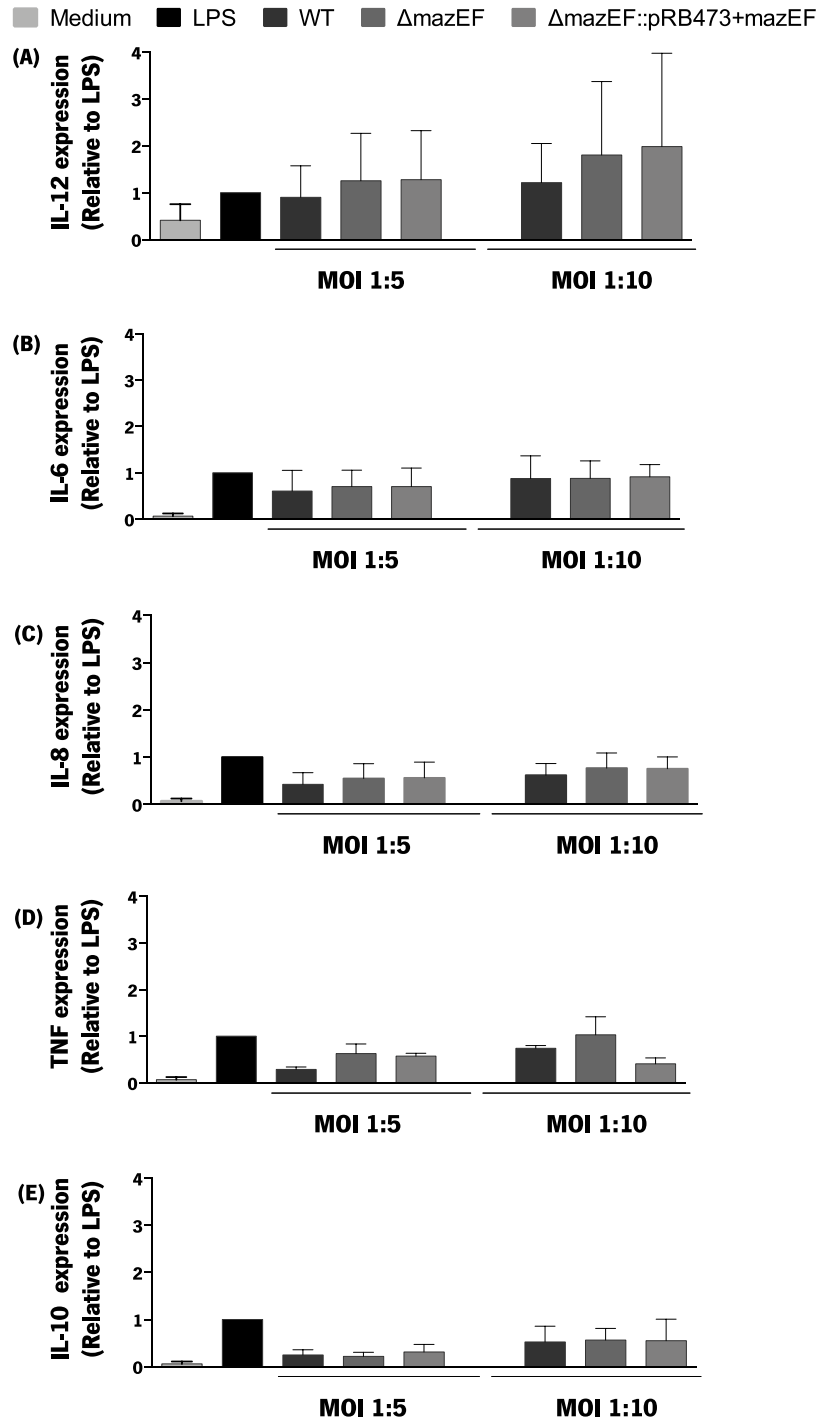


Figure 5.4 – Effect of *mazEF* deletion on the response of MDDCs, assessed by the quantification of secreted cytokines upon 24 hours of infection with *S. epidermidis* 1457 strains at a MOI of 1MDDC:5B and 1MDDC:10B. The levels of (A) IL-12p70, (B) IL-6, (C) IL-8, (D) TNF- α and (E) IL-10 released in the supernatants were measured by ELISA and the results are presented as the mean plus standard deviation of the data normalized to the positive control samples (LPS). Three independent experiments were performed, in which each condition was performed in duplicate. Statistical analysis was performed between the WT and constructs for each condition.

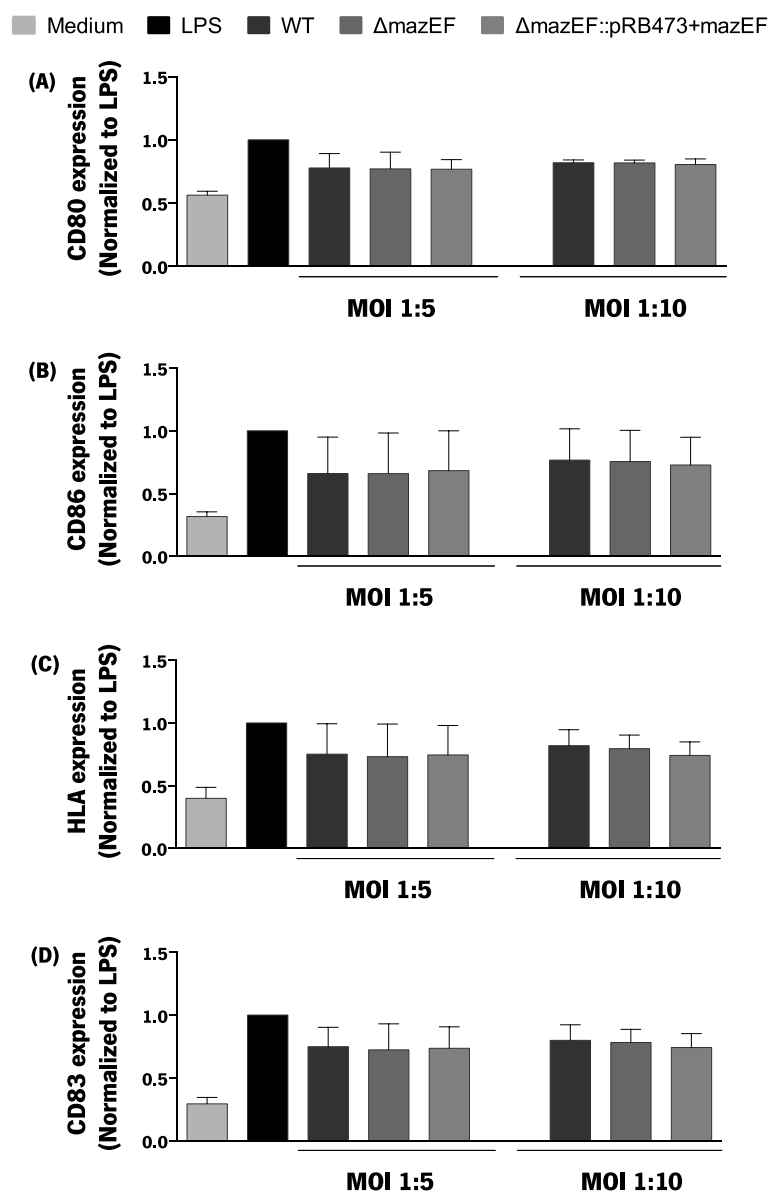


Figure 5.5 – Effect of *mazEF* deletion on the activation and maturation of MDDCs, assessed by the quantification of the expression of activation markers upon 24 hours of infection with *S. epidermidis* 1457 strains at a MOI of 1MDDC:5B and 1MDDC:10B. The expression levels of **(A)** CD80, **(B)** CD86, **(C)** HLA-DR and **(D)** CD83 were measured by flow cytometry. Results are presented as the mean plus standard deviation of the data normalized to the positive control samples (LPS). Three independent experiments were performed, in which each condition was performed in duplicate. Statistical analysis was performed between the WT and constructs for each marker and MOI.

5.3.3. Production of ROS by PMNs

Besides macrophages and dendritic cells, the professional phagocytes group also includes neutrophils. These polymorphonuclear cells present the particularity of producing specific molecules upon pathogen phagocytosis to intracellularly kill them. Despite no differences were

found between the wild-type and mutant strains in the tested interactions with MDMs and MDDCs, we decided to perform an additional experiment, since neutrophils are the cells with the higher capacity to produce reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), which are capable of killing invading pathogens. Herein we aimed to explore the effect of *mazEF* deletion on the production of ROS by human neutrophils over time.

Again, no significant differences were found among the strains. Nevertheless, as represented in **Figure 5.6**, it is possible to see that as little as 15 minutes were enough for neutrophils to start producing ROS upon *S. epidermidis* 1457 challenge, and that the production of ROS increased at a continuous rate over time until the assay was terminated (105 minutes). These results suggest that *mazEF* genes do not play an important role in activating ROS production in neutrophils.

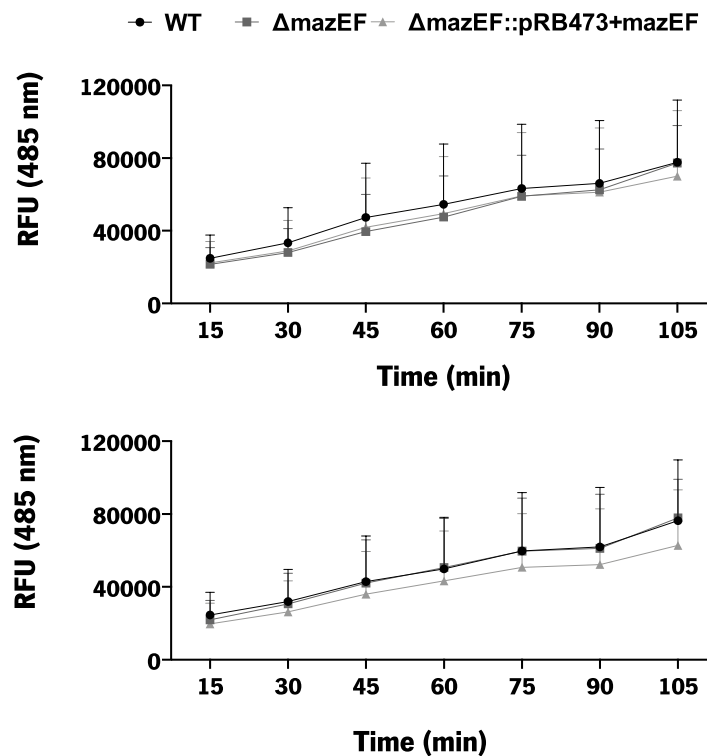


Figure 5.6 – Effect of *mazEF* deletion on the production of ROS by PMNs upon infection with *S. epidermidis* 1457 strains at a MOI of **(A)** 1PMN:5B and **(B)** 1PMN:10B. The relative fluorescence units (RFU) of ROS from 15 to 105 minutes (with 15 minutes intervals) were measured by ELISA and the results are presented as the mean plus standard deviation of three independent experiments. Statistical analysis was performed between the WT and constructs for each time point.

5.4. Conclusions

Herein, we aimed to understand whether *mazEF* could play a role in modulating the innate immune response to *S. epidermidis*, resorting to a physiologically relevant model using primary human macrophages, dendritic cells and neutrophils derived from human peripheral blood cells.

Firstly, regarding the activation of the innate immune response, it was found that the deletion of the *mazEF* homologue did not significantly impact the production of pro-inflammatory (IL-12, IL-6, IL-8 and TNF- α) and anti-inflammatory (IL-10) cytokines by M1- or M2-type MDMs and MDDCs upon incubation with *S. epidermidis* strains, with either multiplicities of infection (5 or 10 bacteria to each M1/M2/MDDC).

Given the results obtained for the quantification of cytokines produced by MDDCs, we expected beforehand that the *mazEF* deletion would not significantly impact the activation of MDDCs, which was further confirmed with the quantification of activation and maturation markers. Herein, we showed that all *S. epidermidis* strains were able to activate MDDCs and non-representative differences were found among the strains.

Finally, the analysis of the production of ROS by neutrophils revealed that a similar effect was induced by the distinct strains.

Altogether, these results strongly suggest that the ability of *S. epidermidis* 1457 to withstand the host immune response, specifically concerning the activation of MDDCs, release of some important cytokines by MDMs and MDDCs and production of ROS by neutrophils, does not rely on *mazEF* homologous genes.

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CHAPTER 6

The role of *mazEF* in modulating VBNC cells

SUMMARY

Herein, we studied the role of *mazEF* in the induction/prevention of VBNC cells in biofilms and planktonic populations, using wild-type (WT) *S. epidermidis* strain 1457, its mutant ($\Delta mazEF$) and complemented ($\Delta mazEF::pRB473+mazEF$) strains. Importantly, contrarily to what was previously proposed, no significant differences were found in the ability of the WT, mutant or complemented strains to form biofilms or to induce the VBNC state.

Nonetheless, we could not discard a putative role of *mazEF* in the virulence potential of *S. epidermidis*. Therefore, we aimed to understand the impact of the *mazEF* deletion in the antimicrobial susceptibility and in the expression of some important genes associated with the virulence of *S. epidermidis* 1457, both when the VBNC state was induced or prevented.

Altogether, it seems that *mazEF* may not be essential in the modulation of the VBNC condition in *S. epidermidis* 1457.

6.1. Brief introduction

A previous RNA-Seq study suggested that *mazEF* operon could have been involved in VBNC modulation [1], which raised interest in the study of this operon in *S. epidermidis*. Despite the high homology of the *S. epidermidis* 1457 *mazEF* sequence with other *mazEF* homologues described as TA systems in *S. aureus*, we have shown in **Chapter 4** that *mazF* and *mazE* expression are not involved in toxin and antitoxin functions in this strain.

Nonetheless, we could not discard its potential role in the modulation of VBNC cells formation and, to test this hypothesis, the VBNC induction model previously validated for strain 1457 (**Chapter 3**) was used herein with the *mazEF* mutant and complemented strains.

6.2. Materials and methods

6.2.1. Strains and growth conditions

Most experiments of this study were conducted with the wild-type strain 1457 (WT), the genetically manipulated strain where the *mazEF* operon was deleted ($\Delta mazEF$) and the strain complemented with a plasmid (pRB473) cloned with the *mazEF* sequence ($\Delta mazEF::pRB473+mazEF$) and including the gene natural promoter, as described in **Chapter 4**. *S. epidermidis* 9142 wild-type (9142 WT), mutant (9142 $\Delta mazEF$) and complemented (9142 $\Delta mazEF::pRMC2+mazEF$) strains were also used for the validation of the results regarding the role of *mazEF* in the modulation of VBNC cells, since this was the strain previously used in the studies that identified a potential involvement of the *mazEF* operon in the emergence of the VBNC state [1].

Similarly to the previous chapters, the experiments were started with a pre-inoculum of *S. epidermidis* strains grown from the 30% glycerol stocks in TSB (Merck, Darmstadt, Germany) and incubated overnight at 37 °C with agitation at 120 rpm (orbital shaker with 10 mm orbit). Then, the concentration of the overnight suspensions was adjusted to approximately 2×10^8 CFU/mL by diluting the suspensions until an OD_{640nm} of 0.25 ± 0.05 was reached, as previously optimized for *S. epidermidis* [2], to be used for further experiments.

6.2.2. Biofilm formation and planktonic growth

Biofilms and planktonic cultures were prepared as described in **Chapter 3**, sections 3.2.2 and 3.2.3, respectively. Importantly, for the planktonic growth, the 48-hours model was followed to replicate the protocol used for biofilm growth. Of note, for confocal analysis (CLSM), biofilms were

formed on Nunc™thermanox™ coverslips (Thermo Fisher Scientific) that were placed inside 24-well plates (Orange Scientific, Braine-l'Alleud, Belgium). Then, the spent media was discarded, and biofilms were washed twice with 500 µL of 0.9% NaCl to remove unattached cells and further staining for confocal analysis.

Regarding antimicrobial assays, biofilms were formed in 96-well plates (Orange Scientific, Braine-l'Alleud, Belgium), as previously optimized [3], by incubating 2 µL of overnight suspensions into 200 µL of TSB/ TSB_{CM10} supplemented with 0.4% glucose or 0.4% glucose + 20 mM MgCl₂ and incubated at 37 °C and 120 rpm. After 24 hours, the supernatant was removed from all wells and 200 µL of fresh TSB_{CM10} supplemented with 0.4% glucose or 0.4% glucose + 20 mM MgCl₂ were added to the biofilms, to induce or prevent the VBNC state, respectively. After incubating the plate for 24 hours under the same conditions of temperature and agitation, spent media was removed, and biofilms were washed twice with 100 µL of NaCl 0.9% for further incubation with antibiotics.

6.2.3. Quantification of cells

The number of total cells in planktonic and biofilm cultures was quantified by OD_{640nm}, live cells by flow cytometry and culturable cells by assessing CFU, as previously described in **Chapter 3**.

6.2.4. Confocal laser scanning microscopy (CLSM) analysis

CLSM analysis used 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI), which is a stain that binds to nucleic acids allowing the visualization of individual cells, and wheat germ agglutinin (WGA) staining that allows the detection of the biofilm matrix by binding to PNAG, as previously optimized [4]. Briefly, after forming biofilms in coverslips, as detailed in **section 6.2.2.**, biofilms were stained using 10 µg/mL WGA (Thermo Fisher Scientific) and 100 µg/mL of DAPI (Sigma-Aldrich), according to the manufacturer's instructions. Then, stained biofilms were observed with Olympus FluoView FV1000 (Olympus, Lisboa, Portugal), using a water-immersion objective (40×/1.15 W) and different regions of the biofilms were analysed. Representative images of two independent experiments were obtained for further analysis with FluoView FV1000 Software (version 4.2).

6.2.5. Antimicrobial assays

The antimicrobial susceptibility assays of biofilms were performed as described for planktonic cells (**Chapter 4, section 4.2.7**), with some modifications. Briefly, 200 μ L of TSB supplemented with the PSC of rifampicin (10 μ g/mL) and tetracycline (16 μ g/mL) were added to the 48-hours biofilms, in triplicates for both CFU and XTT analysis, and incubated at 37 °C and 120 rpm, for up to 24 hours. After 6 and 24 hours, the media was removed from the wells and biofilms washed once with 100 μ L of NaCl. Half of the biofilms were resuspended in 200 μ L NaCl for further CFU quantification, while 200 μ L of the XTT [250 mg/L] + PMS [25 mg/L] solution were added to the remaining biofilms and the plate was incubated at 37 °C for 3 hours in the dark. After the incubation with the XTT+PMS solution, the supernatants were recovered, centrifuged (5 minutes, 10000 *g*, 4 °C) and 100 μ L of the supernatant were used to read the OD_{490nm}.

6.2.6. Gene expression analysis

To understand the transcriptomic alterations of cells grown under VBNC inducing conditions, RNA was isolated from biofilm and from planktonic cells where the VBNC state was either induced or prevented, allowing the analysis of gene expression in these particular conditions. To do so, 1 mL of a pool of at least three independent resuspended-biofilm cells or 1 mL of planktonic suspensions were used to extract RNA, as detailed in **Chapter 4 (Section 4.2.6)**.

Importantly, further analysis with a wider range of genes was performed with biofilms formed by the WT, $\Delta mazEF$ and $\Delta mazEF::pRB473+mazEF$ strains. Primer3 software [6] was used to design the primers for quantification of gene expression described in **Table 6.1**, using *S. epidermidis* 1457 genome as the template (Accession number CP020463.1).

6.2.7. Statistical analysis

Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test for most the assays, except for the comparison between the WT and mutant strains regarding gene expression results, in which statistical differences were analysed using unpaired T-test. At least three independent assays were performed for each experiment and differences between analysed conditions were considered significant when a *p*-value lower than 0.05 was obtained.

Table 6.1 – List of primers used for qPCR analysis.

Target gene	Forward sequence (5'-3')	Reverse sequence (3'-5')	Amplicon size (bp)	Efficiency (%)
<i>16S rRNA</i>	GGGCTACACACGTGCTACA A	GTACAAGACCCGGGAACGT A	176	100.0
<i>clpP</i>	GTTGCTTGTCCTTGTGCACC	CTGTATTGGTATGGCAGCGT C	129	105.7
<i>codY</i>	TTCGCCACCACCTAAAATTG G	AGAAGATCGACATATACCGC GTGA	166	98.3
<i>gyrB</i>	GCATTTGGTACGGGTATTGG	CATCAACATCGGCATCAGTC	88	93.0
<i>icaA</i>	TGCACTCAATGAGGGAATCA	TAAGTGCCTAATTTTGGAT T	134	93.1
<i>mazE</i>	CAAAATAGAAACCACAGTCT TGAAC	AGATATTAAATGTGATTCATT GCAATC	138	91.8
<i>mazEF</i>	GCAACAGAAGCTTTCCCGAT	CCCCTTGTTTCAGACCCTTGA	141	102.0
<i>mazF</i>	GAAGAGGAGATGTTTATTTA GCGG	CCCAAATAATATCTAAGGC ATTATC	325	95.5
<i>pdhA</i>	GCTTCTTGTCCTGCTGTTGG TG	TTGGATGAAGACGGAAATGT CG	173	106.5
<i>psmβ</i>	TTGCAAATACAGTAAAAGCA GCA	TCCGAAGATTTTACCTAATA CGC	107	94.7
<i>psmγ</i>	ATGGCAGCAGATATCATTTT TAC	AACAAAGTTACAAAAGTTAC AATAGACTC	116	95.4

6.3. Results and discussion

6.3.1. *mazEF* does not alter biofilm formation and VBNC cells induction rates in *S. epidermidis* strains

To evaluate *mazEF* potential role in VBNC state modulation in *S. epidermidis* biofilms grown under VBNC prevented and induced conditions [7], total, live and culturable cells of the biofilms were quantified by OD_{640nm} measurement, flow cytometry and CFU counting, respectively.

From the analysis of **Figure 6.1** it is noticeable that no statistical differences ($p > 0.05$) were observed among the strains, as assessed by the quantification of total and live cells, either in the prevented or induced VBNC state. Nevertheless, the mutant strain exhibited a slightly lower concentration of live cells in both VBNC modulating conditions compared to the WT strain.

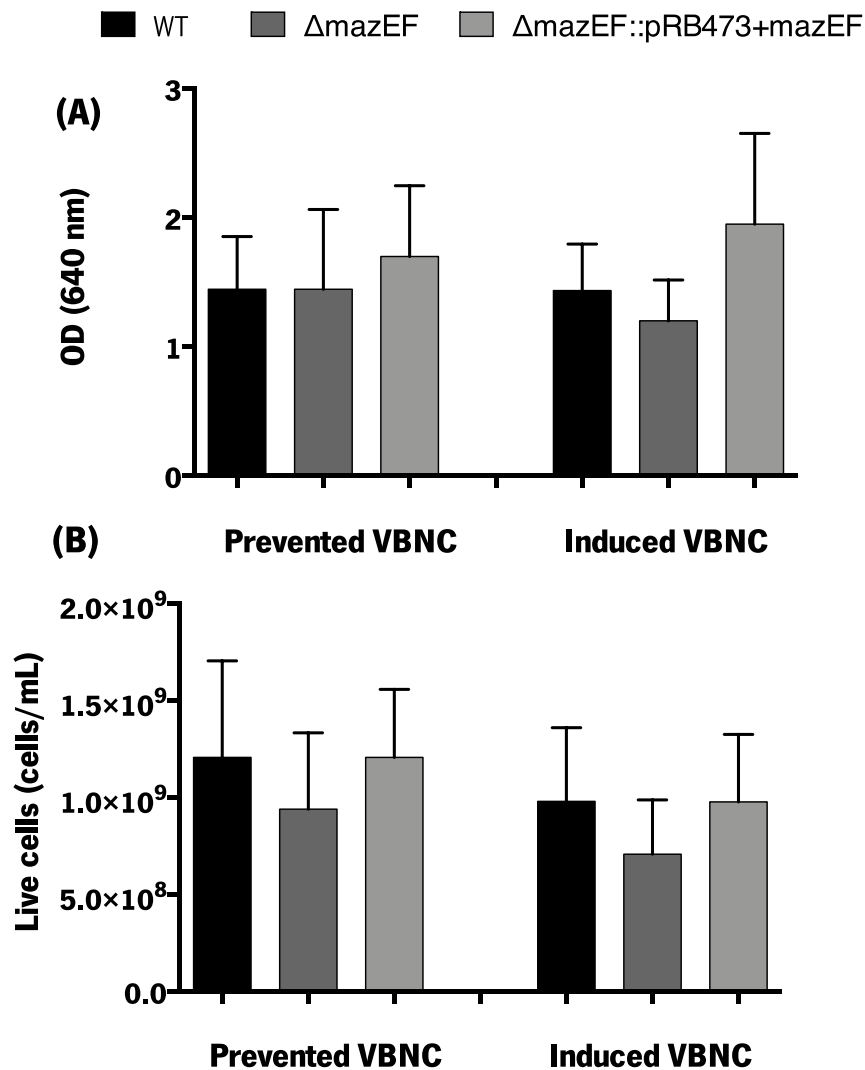


Figure 6.1 – Comparison of *S. epidermidis* 1457 biofilms grown under prevented or induced VBNC conditions. **(A)** OD_{640nm} and **(B)** concentration of live cells (cells/mL). Results are represented as the mean + standard deviation of at least four independent experiments. Statistical analysis was performed between the WT and constructs for each condition.

Although some variability was found for the parameters assessed regarding the quantification of biofilms for all strains tested, this was not surprising since biofilm formation is known to be greatly variable and dependent on the exact conditions of growth, where a small difference in the temperature or aeration of the biofilms can deeply impact their structure [8].

Additionally, we aimed to study the effect of *mazEF* in the emergence of VBNC cells. Thus, the number of culturable cells was also assessed.

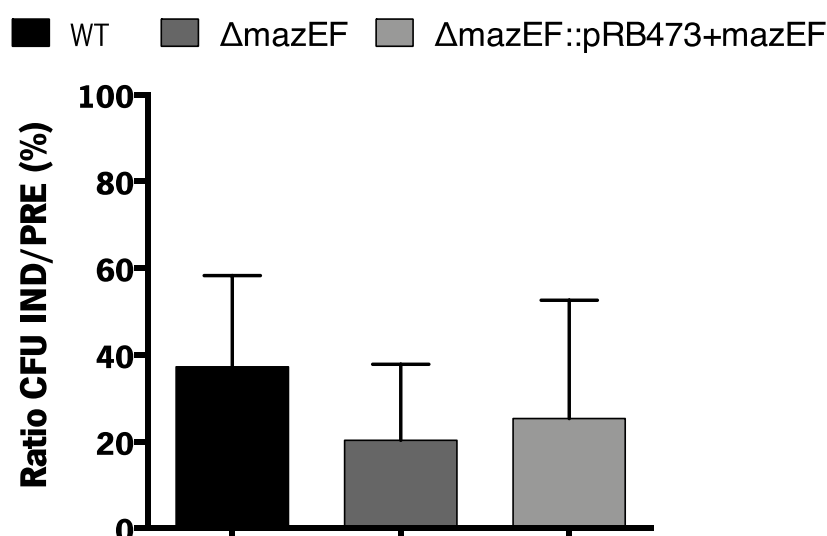


Figure 6.2 – Quantification of biofilms of *S. epidermidis* 1457 strains under VBNC modulating conditions, represented as the ratio (%) between the number of culturable cells in the induced (IND) and prevented (PRE) VBNC state; Results are represented as the mean + standard deviation of at least four independent experiments. Statistical analysis was performed between the WT and constructs.

Not surprisingly, the analysis of data from **Figure 6.2** revealed that the culturability of cells significantly decreased when the VBNC state was induced, as assessed by the CFU IND/PRE ratios being lower than 40%, finding that was consistent for all the strains. However, no significant differences were found between the mutant ($\Delta mazEF$) or complemented ($\Delta mazEF::pRB473+mazEF$) strains when compared to the WT in any of the parameters assessed, strongly suggesting that *mazEF* does not have a pivotal role in the modulation of the VBNC state.

Even though no significant differences were previously detected in *mazEF* role between strains 1457 and 9142, herein we included both strains to characterize under VBNC modulating conditions, since it was previously shown that VBNC induction is strain-dependent [9]. We conducted a brief characterization of strain 9142 and its respective constructs, using biofilms grown under VBNC-modulated conditions and assessing the extent of biofilm formation in terms of culturable cells (CFU).

Data presented in **Figure 6.3** shows that all strains had a similar decrease in the number of culturable cells in the VBNC induced condition, confirming the previous results found for *S. epidermidis* 1457 strains and, thus, suggesting that the model used herein to induce the VBNC

state in biofilms is, after all, independent on the *mazEF* operon, contrary to what was previously hypothesized.

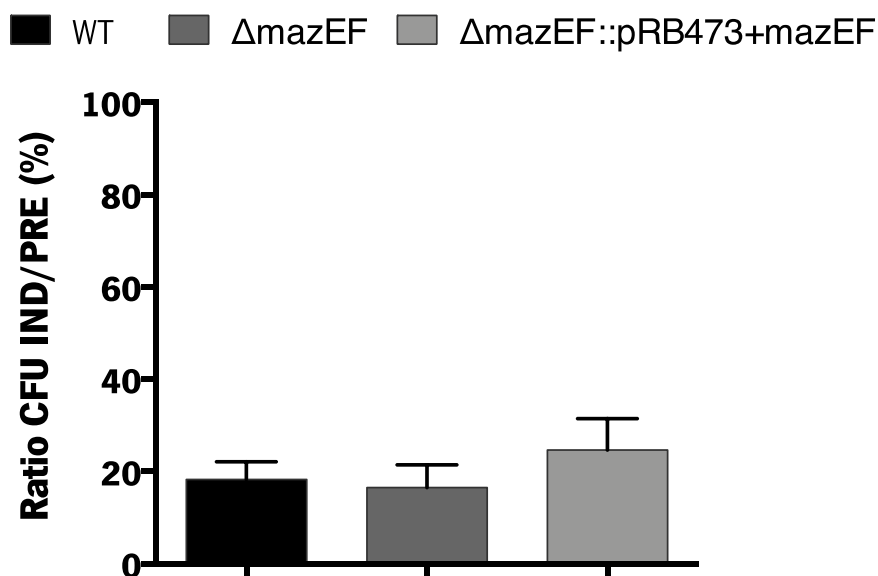


Figure 6.3 – Quantification of biofilms of *S. epidermidis* 9142 strains, assessed as the ratio (%) of culturable (CFU) cells in the induced (IND) and prevented (PRE) VBNC state. Results are represented as the mean + standard deviation of at least three independent experiments. Statistical analysis was performed between the WT and constructs.

6.3.2. *mazEF* does not alter planktonic growth and VBNC cells induction in *S. epidermidis* 1457 strains

Despite no significant differences were found in the induction of the VBNC state in biofilms of *S. epidermidis* 1457 strains, we also decided to characterize the role of *mazEF* operon in VBNC state induction in planktonic cultures. To accomplish this goal, we quantified the amount of total (OD_{640nm}), live (flow cytometry) and culturable cells (CFU) of cultures grown under the induced and prevented VBNC conditions.

Our results show that planktonic cultures reached an equivalent proportion of cells after 24 hours of growth (**Figure 6.4**), with all strains showing similar levels of both total and live cells within the two conditions assessed, with no statistically significant differences observed ($p > 0.05$).

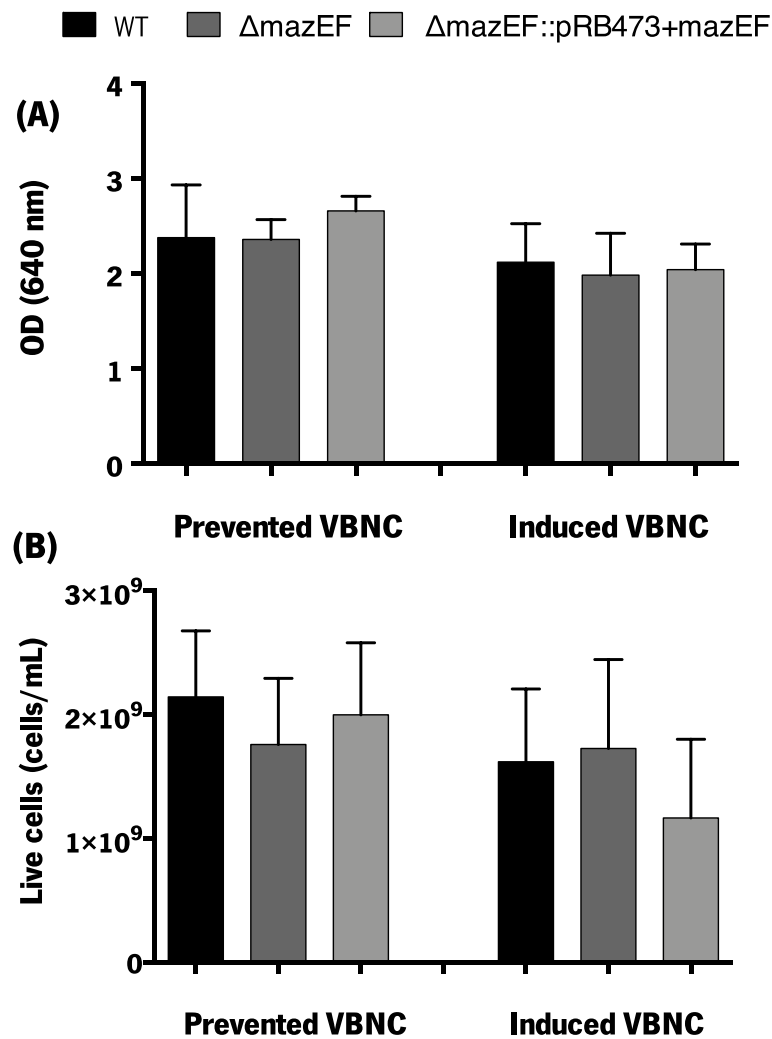


Figure 6.4 – Comparison of planktonic cultures of *S. epidermidis* 1457 WT, mutant ($\Delta mazEF$) and complemented ($\Delta mazEF::pRB473+mazEF$) strains grown for 24 hours under prevented or induced VBNC conditions. **(A)** OD_{640nm} and **(B)** concentration of live cells (cells/mL). Results are represented as the mean + standard deviation of at least three independent experiments. Statistical analysis was performed between the WT and constructs for each condition.

While it seems evident that the deletion or further complementation of the *mazEF* operon in *S. epidermidis* did not affect the extent of planktonic growth under VBNC modulating conditions, we also assessed the ratio of culturable cells between the two conditions.

Not surprisingly, the ratios of culturable cells between the induced and prevented VBNC state populations were similar to all tested strains (**Figure 6.5**), further ruling out the hypothesis that *mazEF* is the main regulator of VBNC state modulation in *S. epidermidis*.

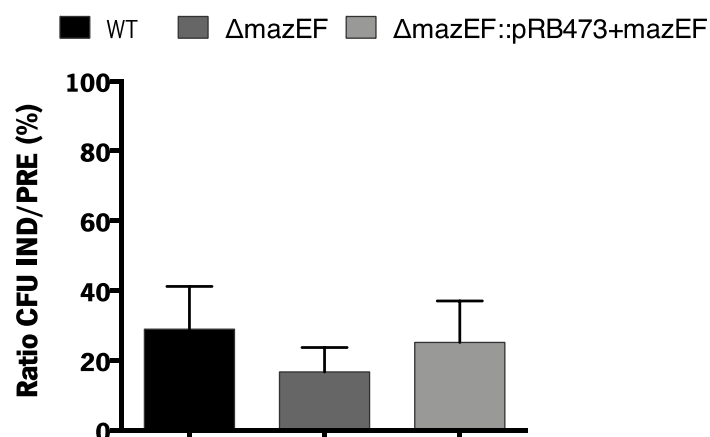


Figure 6.5 – Quantification of planktonic cultures of *S. epidermidis* 1457 strains grown under VBNC modulating conditions, represented as the ratio (%) between the number of culturable cells in the induced (IND) and prevented (PRE) VBNC state. Results are represented as the mean + standard deviation of at least three independent experiments. Statistical analysis was performed between the WT and constructs.

6.3.3. Biofilm structure of *S. epidermidis* 1457 strains in induced or prevented VBNC conditions seems not to be affected by *mazEF* deletion

Although our data pointed to a negligible role of *mazEF* in *S. epidermidis* 1457 VBNC state induction, we decided to study if the deletion of this operon presented an impact on biofilm structure. To determine this, confocal scanning laser microscopy (CLSM) was used to perform a qualitative analysis of the biofilms formed by the WT, mutant and complemented strains, grown under VBNC modulating conditions.

By analysing the images obtained by CLSM, no noticeable differences were found among the microcolonies of biofilms formed by the WT, mutant and complemented strains (as exemplified in **Figure 6.6**). Moreover, when comparing prevented or induced VBNC states, similar biofilm structures were observed for each isolate. Although the results suggest some differences in the thickness of the biofilms formed, we speculate that the variations observed are due to the widely variable topography of these structures. However, to be able to perform a robust evaluation of the thickness of the biofilms formed by each strain, the analysis of a large volume of images, covering a significant surface of the biofilm, would be necessary. Taken together, the qualitative analysis of biofilms structure suggests not only that *mazEF* does not have a central role in biofilm architecture, but also that the entrance of biofilms into the VBNC induced state does not substantially alter their structure, as found for the different strains assessed.

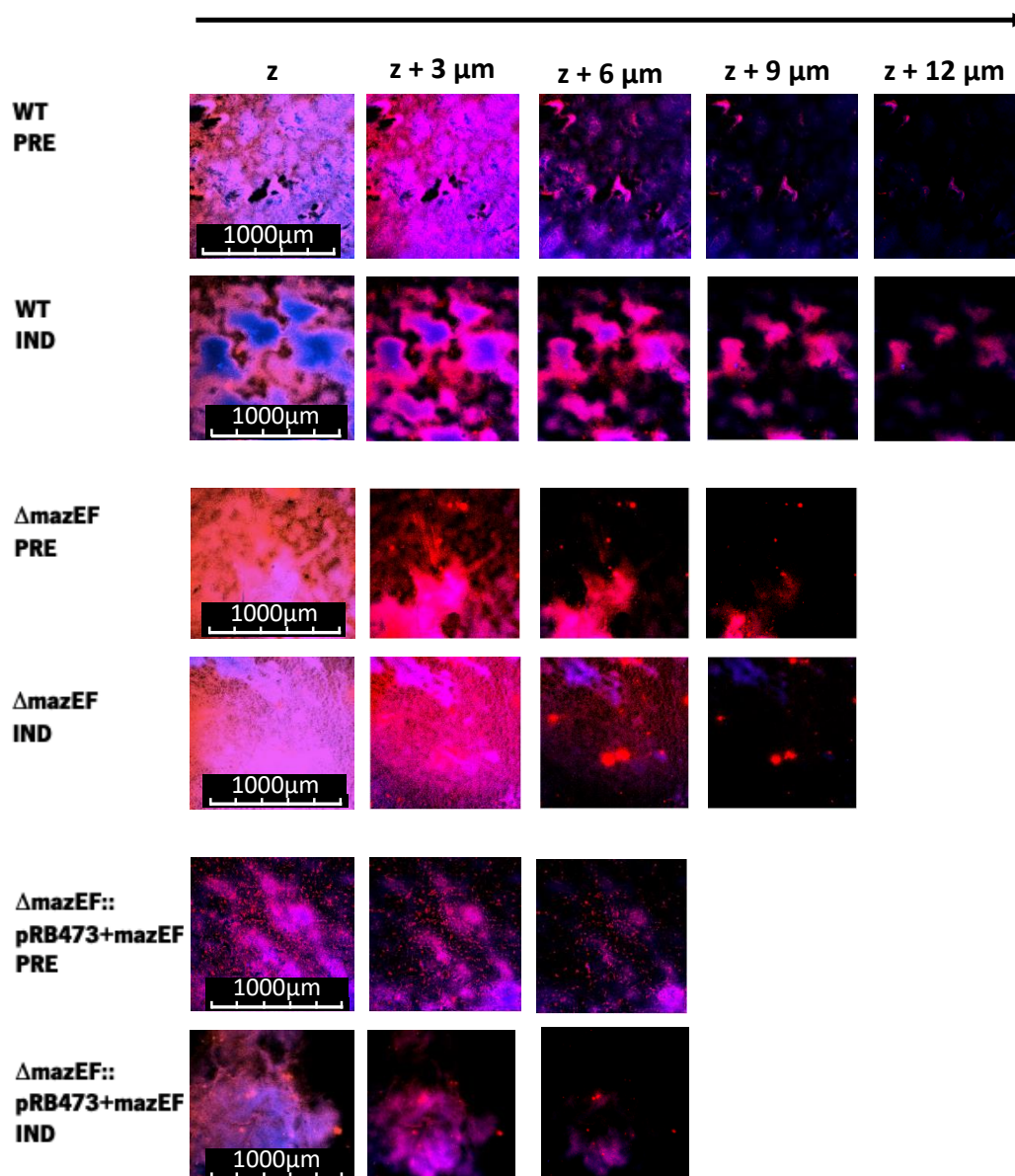


Figure 6.6 – *S. epidermidis* 1457 biofilm structure analysis by CLSM. Example of biofilms grown under VBNC modulating conditions; PRE: Prevented VBNC state; IND: Induced VBNC state. The images shown are representative of two independent experiments.

6.3.4. *mazEF* deletion has a minor effect on antimicrobial susceptibility of biofilms in induced and prevented VBNC conditions to rifampicin and tetracycline

After having determined that *mazEF* deletion does not affect the MIC and susceptibility of planktonic cultures to some antibiotics (**Chapter 4**), herein we aimed to understand how the biofilm phenotype could influence the susceptibility to antibiotics of the wild-type and mutant strains. Thus, the antimicrobial effect of rifampicin and tetracycline on biofilms with VBNC modulated conditions was assessed. Importantly, it was previously demonstrated that *S. epidermidis* 1457 biofilms were

susceptible to treatment with rifampicin and tetracycline, but not with vancomycin [9], therefore we herein addressed the susceptibility of biofilms to the first antibiotics, while vancomycin was excluded from these experiments.

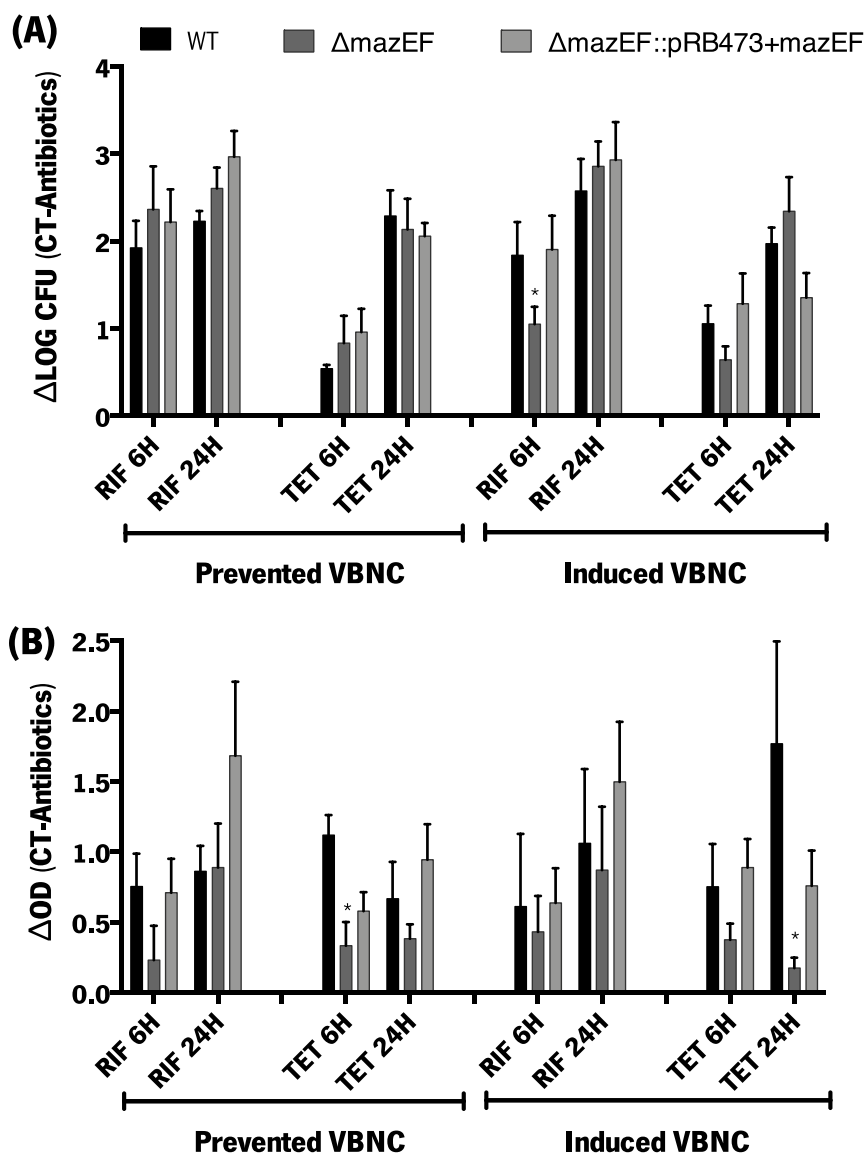


Figure 6.7 – Antimicrobial effect of rifampicin (RIF) and tetracycline (TET) on 48 hours-old biofilms of *S. epidermidis* strains grown under VBNC modulating conditions, after 6 and 24 hours of incubation as determined by **(A)** differences in culturability (LOG CFU/mL) or **(B)** metabolic activity (XTT, OD_{490nm}) between controls and biofilms challenged with antibiotics. Results are presented as the mean + standard deviation of at least three independent experiments and expressed as the difference to the CT (no antibiotic). Statistical analysis was performed between the mutant and complemented strain compared to the WT strain for each antibiotic and time point. * $p < 0.05$.

As shown in **Figure 6.7A**, we observed a significant decrease on the susceptibility to rifampicin, in biofilms formed by the mutant strain under VBNC inducing conditions, after 6 hours of incubation. However, this difference was no longer observed at 24h of incubation. Curiously, when analysing the antimicrobial effect on cell metabolism, as assessed by XTT colorimetry quantification (**Figure 6.7B**), incubation with tetracycline led to significant differences between the WT and mutant strains in biofilms grown under prevented VBNC conditions (after 6 hours of incubation), and in biofilms where the VBNC state was induced (after 24 hours of incubation).

It has been previously shown that tetracycline can induce the VBNC state in *S. epidermidis* biofilms [9]. As such, these results point to some indirect relation between *mazEF* deletion and metabolic activity in the presence of tetracycline. However, the extent of this association, both as the molecular mechanisms involved, were not explored in this work and should be addressed in the future.

6.3.5. *mazEF* deletion does not impact the expression of selected virulence genes in *S. epidermidis* 1457 biofilms grown under VBNC modulated conditions

We have previously shown that *mazEF* expression in biofilms of the WT strain was not affected by the VBNC modulation conditions (**Chapter 3**). Herein, we aimed to understand if the deletion of the *mazEF* operon from *S. epidermidis* 1457 could affect the expression of other relevant virulence genes, when grown under VBNC modulating conditions.

From the analysis of **Figure 6.8**, we can see that the mutant strain presented a similar expression of all genes when compared to the WT, both under the prevented and induced VBNC conditions, suggesting that the deletion of the *mazEF* operon did not significantly interfere with the expression of the virulence genes assessed. Curiously, when testing the expression of gene *psm γ* , the complementary strain presented a significantly higher expression than the WT.

Moreover, the entrance into the dormant state did not significantly affect the expression of the selected genes in most of the tested conditions, with the exception of genes *pdhA*, *psm β* , and *psm γ* for the WT strain and *pdhA* and *psm β* genes for the complemented strain.

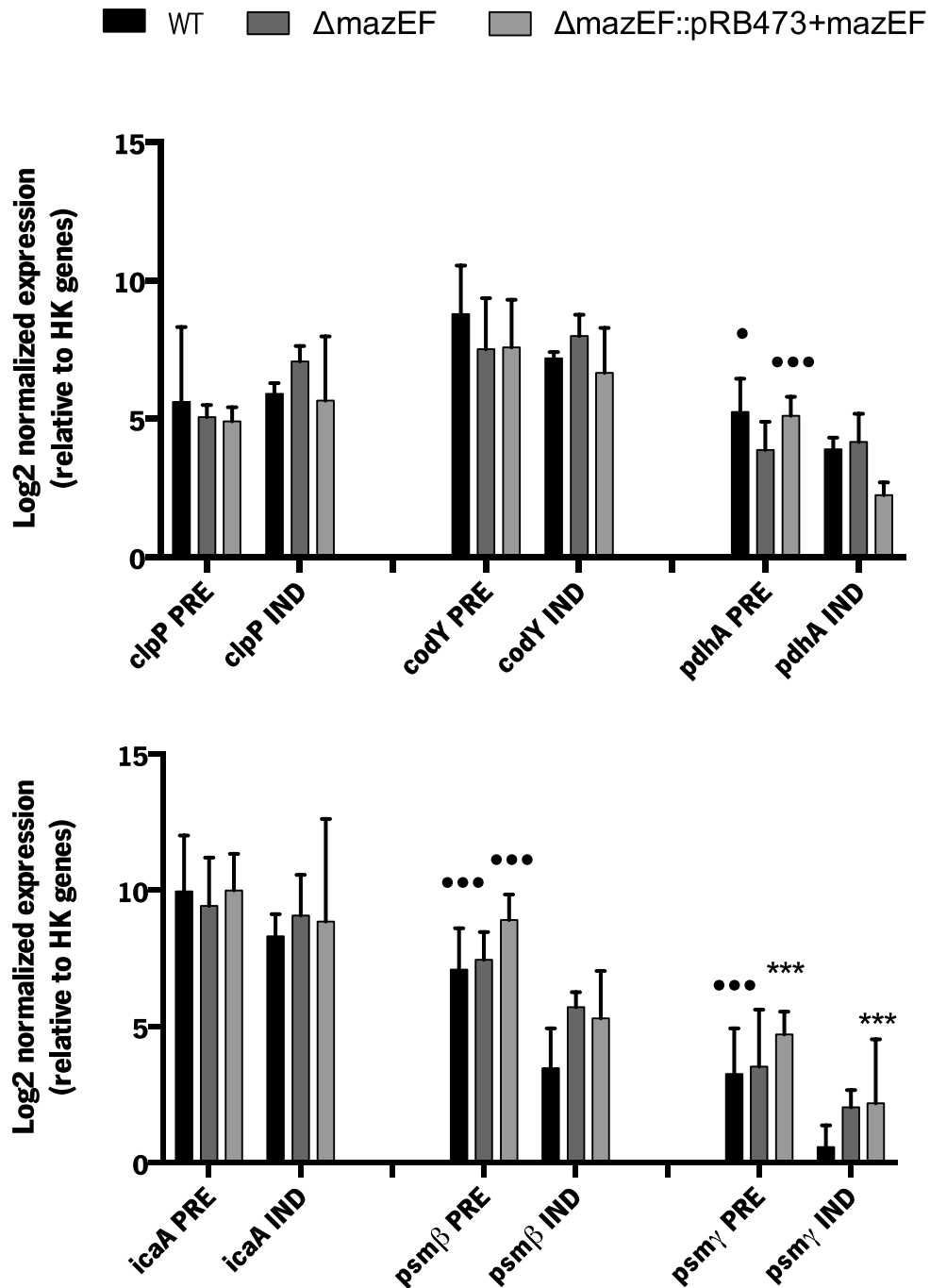


Figure 6.8 – Log2 fold expression of *clpP*, *codY*, *pdhA*, *icaA*, *psmβ* and *psmγ* in *S. epidermidis* 1457 WT, $\Delta mazEF$ and $\Delta mazEF::pRB473+mazEF$ biofilms. Results are presented as the Log2 normalized expression relative to the reference genes (geometric mean of *16S rRNA* and *gyrB*) and the mean + standard deviation of at least four independent experiments was used. Statistical differences between the mutant or the complemented strains compared to the WT were identified with *, while statistical differences between the induced and prevented VBNC state for each strain and gene were identified with •. *** $p < 0.001$; • $p < 0.05$; *** $p < 0.001$. PRE: prevented VBNC state; IND: induced VBNC state.

6.4. Conclusions

Previous studies on the ability of distinct *S. epidermidis* strains to produce VBNC cells within biofilms have already shown that the VBNC induction model used herein is strain-dependent [9]. Nonetheless, strain 1457 was confirmed to be susceptible to VBNC induction (**Chapter 3**), despite having a lower VBNC emergence rate than other strains.

Herein, we aimed to understand the role of *mazEF* in the growth of biofilms and planktonic cells under modulating VBNC conditions and, more importantly, understand the potential involvement of the *mazEF* operon in preventing the emergence of VBNC cells in those populations.

Although some minor differences were found in the concentration of total and live cells of the WT and mutant strains, especially when the VBNC state was prevented, these differences were not considered significant, which led us to conclude that *mazEF* may not be essential to the modulation of the VBNC state in neither biofilm nor planktonic populations, at least on the strains tested herein.

Moreover, the impact of the *mazEF* deletion or further complementation on the structure of biofilms, antimicrobial susceptibility to rifampicin and tetracycline and in the expression profile of some virulence genes was also assessed.

Interestingly, the mutant strain showed a diminished susceptibility to rifampicin, as assessed by the culturability upon 6 hours of incubation, that was not found with the higher incubation period. Moreover, tetracycline significantly decreased the metabolic activity of the mutant strain in two distinct cases, while not significantly affecting the reduction of culturability, which is an interesting finding that should further be explored.

Regarding genetic expression analysis, our results showed a similar expression of all genes assessed in the WT, mutant and complemented strains, with the exception of *psmγ* gene, which was significantly upregulated in the complemented strain. Moreover, the mutant strain was the only where the expression of *pdhA* and *psmβ* was similar in biofilms where the VBNC state was induced or prevented, suggesting a possible relation between the *mazEF* and *pdhA* and *psmβ* genes.

Overall, the *mazEF* operon seems to have a minor role in the modulation of the VBNC state of both biofilm and planktonic populations, while influencing the antimicrobial profile of *S. epidermidis* 1457 to some degree and affecting the expression of some virulence genes.

6.5. Bibliography

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CHAPTER 7

Main conclusions and future perspectives

SUMMARY

This chapter presents the main conclusions of this thesis, describing the major findings and limitations found throughout this study. Moreover, future perspectives are proposed, aiming to answer new research questions that emerged during the development of this thesis.

7.1. Main conclusions

This thesis comprehends the first study of the role of the putative toxin and antitoxin *mazEF* homologous genes in *S. epidermidis* virulence, with a special focus on its influence on the modulation of the VBNC state. Herein, we used a previously described VBNC biofilm induction model and were able to demonstrate, for the first time, that it can also induce VBNC cells in planktonic cultures, being this somewhat related to cellular densities. Importantly, this thesis started with the hypothesis that *mazEF* could act as a TA system in *S. epidermidis*, based on homology with other bacterial species. To experimentally determine this hypothesis, *mazEF* mutants were constructed and complemented with genes: *mazE*, the putative antitoxin, and *mazF*, the putative toxin. Additionally, the effect of *mazEF* deletion on the antimicrobial susceptibility profile of *S. epidermidis* strains was evaluated, as well as its ability to survive in human blood. Moreover, the importance of *mazEF* in the immune response was assessed by the infection of human-derived macrophages, dendritic cells and neutrophils with the distinct *S. epidermidis* strains. Altogether, the questions established at the beginning of this study were all addressed.

1. Does *mazEF* act as a toxin-antitoxin system in *S. epidermidis*?

Collectively, the results obtained focusing on the putative toxin and antitoxin role of the *mazEF* genes system did not support the predicted TA function of this operon, at least not on the 2 strains tested (1457 and 9142). Importantly, when comparing the sequences of the *mazE* and *mazF* genes of *S. epidermidis* 1457 with *S. aureus* Newman, where the *mazEF* homologue was experimentally determined as a TA system, high homology was found. Nonetheless, and despite the genomic redundancy, the nucleotide differences lead to a distinct amino acid chain sequence, resulting in a predicted different protein, which may possibly justify why an operon with more than 80% of nucleotide homology may feature a distinct role in those species.

2. Does *mazEF* have an influence on VBNC modulation on *S. epidermidis* biofilms?

Taking into consideration the previous hypothesis that *mazEF* could be involved in VBNC state modulation in biofilms of *S. epidermidis*, it was evaluated whether the *mazEF* mutants would present a distinct VBNC phenotype, in comparison with the WT strains. However, when we first attempted to validate the expression of *mazEF* under induced or prevented VBNC conditions by

qPCR analysis, we observed minimal and somewhat variable differential expression, not supporting a key involvement of *mazEF* in VBNC induction.

Although being evident that the VBNC induction is strongly dependent on environmental conditions and of the strains used, the same was not true regarding the presence or absence of the *mazEF* operon. Such conclusion arose from the observation that under our experimental conditions, the *mazEF* mutant strains (1457 $\Delta mazEF$ and 9142 $\Delta mazEF$) presented a similar ability to generate VBNC cells within the biofilms compared to the wild-type strains. Moreover, although the qPCR analysis suggested a possible involvement of *mazEF* in the modulation of the VBNC state of planktonic populations in strain 1457, the same was not verified experimentally, where all the constructs showed similar VBNC proportions compared to the WT strain.

3. Is the susceptibility to antimicrobials affected by the deletion of the *mazEF* complex?

An important issue related to biofilm-associated infections is their great ability to survive antimicrobial treatment, especially when higher proportions of VBNC cells are present. Moreover, previous studies have associated *mazEF* with a decreased susceptibility to some antibiotics in other species. For that reason, we aimed to understand if the deletion of the *mazEF* operon from the *S. epidermidis* 1457 genome could impact the susceptibility to antibiotics of both planktonic and biofilm cells. The analysis of the antimicrobial response of planktonic populations to rifampicin, tetracycline and vancomycin revealed no differences between the WT strain and the constructs assessed, either in the culturability or metabolic activity of cells.

On the other hand, when studying antimicrobial susceptibility of biofilms, we did find differences among strains, with the mutant strain showing a significant decrease of the metabolic activity upon treatment with tetracycline, while not affecting the culturability of cells, when compared to the WT strain. Moreover, the mutant strain was also found to be significantly less susceptible to rifampicin after 6 hours of treatment, a difference that did not perpetuate to the 24 hours incubation time point. Overall, these results suggest a minor implication of *mazEF* deletion on antimicrobial susceptibility.

4. Can innate cell immunity be more effective against *mazEF* mutants than to the wild-type strain?

Aiming to assess the impact of *mazEF* deletion on the innate immune response, the activation of macrophages, dendritic cells and neutrophils derived from human peripheral blood cells was evaluated. M1- and M2-type macrophages and dendritic cells were infected with the bacteria and the elicited production of pro- and anti-inflammatory cytokines was evaluated. No evident differences between the WT and the constructs were found, which suggests that *mazEF* does not affect the activation of the innate immune response to *S. epidermidis*. Moreover, the production of ROS by neutrophils infected with the distinct *S. epidermidis* strains revealed similar results, collectively supporting the hypothesis that the immune response to WT and *mazEF* mutant strains is equally effective.

Overall, it was expected that answering those questions would contribute to the understanding of the role of *mazEF* in *S. epidermidis* infections. However, so far, we could only determine minor roles of the *mazEF* operon on the virulence of this species.

7.2. Major limitations and future work

Despite having successfully answered the initially proposed questions, some limitations were found in the course of the studies conducted herein.

The first limitation was found in an attempt to induce a VBNC state in *S. epidermidis* biofilms with TSB from Liofilchem, a brand different from the one used to develop the VBNC modulation model. More specifically, VBNC state induction was only achieved with TSB from Merck, suggesting that the composition of the media strongly affects the emergence of VBNC cells. For that reason, we believe that a further investigation of the chemical composition of this complex media could help to elucidate the molecular mechanisms behind the process of VBNC state modulation.

During the study of the putative TA function of the *mazEF* module some adjustments and optimizations had to be performed to be able to express, separately, the *mazE* and *mazF* genes in the mutant strain, using an inducible plasmid (pRMC2). Herein, an initial limitation was found since the natural ribosomal binding sequence (RBS) of the *mazEF* operon could not be included, as the *mazE* and *mazF* counterparts are partially overlapped in the *S. epidermidis* 1457 genome and, for that reason, an “artificial” RBS had to be chosen and added during the cloning process, to ensure

the translation of the mRNA molecules. Although we were able to induce the expression of *mazE* and *mazF* upon induction with anhydrotetracycline, the activity of the MazE and MazF proteins was not experimentally determined, therefore, we were not able to determine if the expressed proteins had activity. This is relevant since some predicted structural differences between *S. epidermidis* 1457 MazE and MazF and *S. aureus* Newman were found. Future efforts should be placed into the characterization of these proteins of *S. epidermidis* to determine their role in this species and in the confirmation of whether these proteins can act as antitoxin and toxin, respectively.

Regarding the role of *mazEF* in antimicrobial susceptibility of *S. epidermidis* strains, a deeper study on the role of this operon in cells' culturability and metabolism should be further addressed, by assessing the mechanisms involved behind this association.

Finally, another limitation of this study was the use of a reduced number of *S. epidermidis* strains. Despite having found that distinct strains present more than 80% and 90% of homology, respectively, in the nucleotide and amino acid sequences of the *mazEF* operon, the use of a higher number of strains could contribute to a better knowledge on the role of the *mazEF* operon.

Altogether, we suggest that future work on this matter should focus on:

1. Study of the MazE and MazF proteins activity and conformation;
2. Assessment of the influence of *mazEF* deletion with a wider range of antibiotics and study of the mechanisms behind the association of *mazEF* and antimicrobial susceptibility;
3. Selection of a more representative number of *S. epidermidis* isolates to address *mazEF* role both as a putative TA system and influence in other virulence factors of this species.