VIRGINIA MARIA DINIS CARVALHAIS

EFFECT OF MODULATING *S. EPIDERMIDIS* BIOFILMS DORMANCY IN THE BIOFILM PHYSIOLOGY AND HOST IMMUNE RESPONSE

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Nuno Cerca
Categoria – Investigador Principal
Afiliação – Centro de Engenharia Biológica, LIBRO – Laboratório de Investigação em Biofilmes Rosário Oliveira, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal.

Co-orientador – Rui Vitorino
Categoria – Professor Auxiliar Convidado
Afiliação – Seção Autónoma de Ciências da Saúde, Universidade de Aveiro, 3810-193 Aveiro

Co-orientador – Manuel Vilanova
Categoria – Professor Associado
Afiliação – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal.

O trabalho desenvolvido nesta tese foi realizado no Laboratório de Investigação em Biofilmes Rosário Oliveira do Centro de Engenharia Biológica da Universidade do Minho, no Channing Labs do Brigham and Women's Hospital (Boston), no grupo de Espetrometria de Massa da Unidade de Investigação de Química Orgânica, Produtos Naturais e Agroalimentares da Universidade de Aveiro, e no Laboratório de Imunologia Mário Arala Chaves do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Este trabalho foi financiado pela FEDER, sob os projetos FCOMP-01-0124-FEDER-014309, NORTE-07-0124-FEDER-000027 e FCOMP-01-0124-FEDER-027462 e, pela FCT, pelos projetos PTDC/BIA-MIC/113450/2009 e PEst-OE/EQB/LA0023/2013 e, pela bolsa de doutoramento individual SFRH/BD/78235/2011.

PRECEITOS LEGAIS

De acordo com o disposto no n.º 1 do artigo 34.º do Decreto-Lei n.º 74/2006, publicado em Diário da República, 1.ª série, n.º 60 de 24 de Março de 2006, e republicado pelo Decreto-Lei n.º 115/2013, publicado em Diário da República, 1.ª série, n.º 151 de 7 de Agosto de 2013, que procede à terceira alteração ao Decreto-Lei n.º 74/2006, de 24 de março de 2006, constam nesta tese os artigos já publicados, que a seguir se discriminam:

Chapter 2:

Carvalhais V, França A, Cerca F, Vitorino R, Pier GB, Vilanova M, Cerca N. (2014) Dormancy within *Staphylococcus epidermidis* biofilms: a transcriptomic analysis by RNA-seq. *Applied Microbiology and Biotechnology*; 98(6): 2585-96

Chapter 3:

Carvalhais V, França A, Pier GB, Vilanova M, Cerca N, Vitorino R (2015) Comparative proteomic and transcriptomic profile of *Staphylococcus epidermidis* biofilms grown in glucose-enriched medium. *Talanta*; 132: 705-712

Chapter 4:

Carvalhais V, Cerca N, Vilanova M, Vitorino R (2015) Proteomic profile of dormancy within *Staphylococcus epidermidis* biofilms using iTRAQ and label-free strategies. *Applied Microbiology and Biotechnology*, 99(6):2751-2762

Chapter 5:

Carvalhais V, Cerveira F, Vilanova M, Cerca N, Vitorino R (2015) An immunoproteomic approach for characterization of dormancy within *Staphylococcus epidermidis* biofilms. *Molecular immunology*, 65(2):429-435

AGRADECIMENTOS

Aos meus orientadores Nuno Cerca, Rui Vitorino e Manuel Vilanova pela orientação, partilha de conhecimentos e oportunidade de integrar as suas equipas.

Ao Gerald Pier pela disponibilidade para me aceitar no seu laboratório de investigação.

A todos os membros do “grupo Cerca” por me terem acolhido e integrado no seu grupo.

A todos os que em todos os laboratórios que frequentei, partilharam os seus conhecimentos e tempo e, que contribuíram para a minha aprendizagem e desenvolvimento.

Às minhas antigas orientadoras de Mestrado pelo seu apoio constante.

À Fundação para a Ciência e Tecnologia pelo financiamento.

À minha família, por tudo.

MUITO OBRIGADO!

RESUMO

Apesar de ser um colonizador da pele e mucosas humanas, a bactéria *Staphylococcus epidermidis* tem a capacidade de aderir à superfície de biomateriais, sendo um dos principais agentes responsáveis pelas infeções associadas a biofilmes. Bactérias em estado de dormência podem ser encontradas entre a população metabolicamente heterogénea de um biofilme. Estas células apresentam baixa atividade metabólica, que tem sido associada à sobrevivência das bactérias no hospedeiro por longos períodos de tempo, ao aumento da tolerância aos antibióticos e à evasão ao sistema imune do hospedeiro. Para estudar a dormência em biofilmes de *S. epidermidis*, usou-se um modelo *in vitro* desenvolvido previamente para variar a proporção de bactérias dormentes no biofilme. De acordo com este modelo, a acidificação do meio de cultura causada pelo metabolismo da glucose, é o responsável por induzir a dormência nas bactérias. No entanto, este efeito pode ser prevenido pela adição de magnésio ao meio de cultura, durante a formação do biofilme.

Neste trabalho, para caracterizar o estado de dormência em biofilmes de *S. epidermidis* foi usada uma análise que integrou múltiplas abordagens. Primeiro, foi realizada uma análise do transcriptoma total de biofilmes com e sem dormência. Os dados mostraram que em biofilmes com dormência induzida, a tradução do mRNA está diminuída, enquanto que houve um aumento da expressão dos transcriptos envolvidos nos processos de oxidação-redução e no metabolismo do piruvato. Para determinar a relação entre os transcriptos e as proteínas de biofilmes dormentes de *S. epidermidis*, testaram-se diferentes métodos de extração e correlacionaram-se os dados obtidos. A melhor correlação (r = 0.37) foi obtida pela combinação da lise mecânica com *FastPrep* e a solução de extracção contendo *CHAPS*. Por fim, foi usada uma metodologia quantitativa para determinar o proteoma de biofilmes de *S. epidermidis* com e sem dormência induzida. De forma similar aos resultados obtidos com a transcriptómica, as proteínas com atividade catalítica e com atividade GTPase foram encontradas com expressão aumentada quando a dormência foi induzida, enquanto que proteínas envolvidas no processo de tradução encontram-se menos expressas.

Numa tentativa de avaliar a relação entre a dormência no biofilme e o hospedeiro, determinámos o impacto da dormência em biofilmes de *S. epidermidis*, analisando o padrão de reatividade ao soro humano. Curiosamente, a proteína CodY foi apenas reativa ao soro em biofilmes dormentes, enquanto que a proteína ClpP foi apenas reativa quando a dormência foi evitada. A expressão da proteína CodY está aumentada no caso de limitação nutricional uma vez que é sensível à disponibilidade nutricional. A ausência de ClpP foi previamente associada com a redução da capacidade para formar biofilmes em *S. epidermidis* e com virulência diminuída num modelo animal com infeção por biofilmes desta bactéria. Estes resultados sugerem que o magnésio poderá ser um fator nutricional importante nos biofilmes de *S. epidermidis*.

Por último, foi analisada a relevância da dormência em isolados comensais e clínicos de *S. epidermidis*. Vários isolados foram capazes de entrar em dormência. Contudo, a indução de dormência não pareceu ter afetado de forma diferente a sobrevivência as células dos biofilmes quando expostas a sangue total humano. Adicionalmente, foi avaliado o efeito de três antibióticos diferentes contra biofilmes de *S. epidermidis* com e sem dormência. A vancomicina não causou nenhum efeito nos biofilmes formados. Curiosamente, a tetraciclina levou a uma redução da culturabilidade da bactéria no entanto, não se verificou redução da viabilidade celular, sendo que teve um maior efeito sobre os biofilmes com dormência induzida. A rifampicina foi capaz de reduzir a culturabilidade e viabilidade bacteriana, mas em diferentes escalas. Apesar de a rifampicina causar morte das bactérias, o desenvolvimento de um estado de viabilidade mas não cultivável nos biofilmes foi induzido pela tetraciclina e rifampicina.

De forma geral, demonstrou-se que este modelo de dormência caracteriza-se por um perfil proteómico e transcriptómico próprio, uma interação com o hospedeiro distinta e com um impacto clínico relevante. No entanto, a caracterização global da dormência aqui realizada, fornece informação quanto à totalidade do biofilme e não apenas à subpopulação de bactérias dormentes. Muito provavelmente, as diferenças entre bactérias cultiváveis e dormentes são maiores que as aqui referidas.

ABSTRACT

Despite being a common colonizer of human skin and mucosae, *Staphylococcus epidermidis* has a strong ability to adhere to biomaterial surfaces. Therefore, *S. epidermidis* is among the most common causative agents of biofilm-associated infections. Dormant bacteria may be found among the metabolically heterogeneous cells within biofilms. These cells present a low metabolic activity which has been associated with long-term bacterial survival, increased cellular tolerance to antibiotics and evasion of the host immune system. To study dormancy within *S. epidermidis* biofilms, we used an *in vitro* model to modulate the proportions of dormant bacteria within a biofilm. According to this model, the culture medium acidification, due to glucose metabolism, was responsible for inducing bacteria into a dormant state. In contrast, this effect was found to be prevented by the addition of magnesium to the culture medium.

In the present work, an integrative' *omics* analysis of dormancy within *S. epidermidis* biofilms was used to characterize dormancy state. First, a whole-transcriptome analysis of biofilms with prevented and induced dormancy was conducted. The results highlighted that in dormancy, mRNA translation was decreased, while transcripts involved in oxidation-reduction processes and in the pyruvate metabolism were upregulated. To determine the relationship between transcripts and proteins of *S. epidermidis* biofilms with induced dormancy, different protein extraction methods were tested and the obtained data was correlated with the transcriptomic profile. The best correlation was obtained by a combination of FastPrep mechanical lysis and detergent extraction buffer containing CHAPS. In this case, the correlation between *S. epidermidis* biofilms with induced dormancy mRNA transcripts and protein abundance was 0.37. Then, a quantitative proteomics approach was used to characterize the whole proteome profile of *S. epidermidis* biofilms with prevented and induced dormancy. Similarly to transcriptome analysis, it was found that expression of proteins involved in catalytic activity and GTPase activity was overexpressed in induced dormancy. Additionally, proteins involved in translation processes were found less expressed in dormancy.

In an attempt to evaluate the interplay between dormant biofilms and the host, it was assessed whether dormancy could have an impact on the host immune reactivity to *S. epidermidis* biofilms, by analyzing their immunoreactivity pattern to human sera. Interestingly, CodY protein was only reactive to sera in dormant biofilms and ClpP protein was only reactive when dormancy was prevented. The expression of CodY is increased when cells experience nutrient deprivation since it senses nutrient availability. The ClpP deletion was previously associated with reduced ability to form *S. epidermidis* biofilms and with reduced virulence in a rat model of biofilm-associated infection. These results suggest that magnesium seems to be an important nutrient in *in vitro S. epidermidis* biofilms.

Lastly, the clinical impact of dormancy among *S. epidermidis* isolates was studied. Curiously, several clinical and commensal isolates were able to trigger dormancy. However, induced dormancy did not seem to affect differently the survival of biofilms when exposed to whole human blood. Then, the effect of three different antibiotics against *S. epidermidis* biofilms with induced and prevented dormancy was assessed. Vancomycin did not show any effect on mature biofilms. Tetracycline led to a reduction of bacteria culturability but not to a reduction of bacteria viability. Rifampicin was able to decrease bacteria culturability and viability, but in a different scale. Interestingly, despite rifampicin was also causing bacteria death, these results point out to the development of a viable but-non culturable state within biofilms induced by tetracycline and rifampicin.

Overall, using a multiple combined strategy, it was demonstrated that this dormancy model has a particular transcriptomic and proteomic profile, a distinct interplay with host and a relevant clinical impact. Importantly, the global characterization of dormant biofilms here performed, provides information regarding the whole biofilm and not only about the dormant bacterial cells biofilm subpopulation. Most probably, differences among culturable and dormant bacteria are greater than those referenced here.

TABLE OF CONTENTS

α-CHCA	α-cyano-4-hydroxycinnamic acid
2-DE	two-dimensional electrophoresis
ACN	acetonitrile
AMPs	antimicrobial peptides
CDC	Center for Disease Control and Prevention
cDNA	complementary DNA
CFUs	colony forming units
CHAPS	3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate
CLSM	confocal laser scanning microscopy
eDNA	extracellular DNA
emPAI	exponentially modified protein abundance index
FDR	false discovery rate
GO	gene ontology
GRAVY	grand average of hydropathy
IEF	isoelectric focusing
IPG	immobilized pH gradient
iTRAQ	isobaric tags for relative and absolute quantitation
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	liquid chromatography
MALDI-TOF	matrix assisted laser desorption/ionization–time of flight
MS	mass spectrometry
MSCRAMMs	microbial surface components recognizing adhesive matrix molecules
NIH	U.S. National Institute of Health
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PGA	poly-γ-glutamic acid
p*I*	isoelectric point
PI	propidium iodide
PIA	polysaccharide intercellular adhesin
PNAG	poly-N-acetylglucosamine
ppGpp	alarmone guanosine 5'-diphosphate-3'-diphosphate
PRRs	pattern recognition receptors
PSM	phenol-soluble modulins
PTMs	post-translational modifications
qPCR	quantitative polymerase chain reaction
RNA-seq	RNA-sequencing
RPKM	Reads per Kilobase per Million Mapped Reads

SCV	small-colony variant
SDS	sodium dodecyl sulfate
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TA	Toxin-Antitoxin
TBS-T	Tris-Buffered Saline-Tween 20
TCA	tricarboxylic acid cycle
TFA	trifluoroacetic acid
TLR	toll-like receptor
TSA	trypticase soy agar
TSB	tryptic soy broth
VBNC	viable but non-culturable

LIST OF TABLES

CHAPTER 1.

GENERAL INTRODUCTION

1.1. *Staphylococcus epidermidis*

Staphylococcus epidermidis is a coagulase-negative Gram-positive bacterium, which colonizes skin and mucosa as a commensal microbe [1]. However, *S. epidermidis* may turn into an opportunistic bacterium, being one of the most frequent causes of nosocomial infections [2-5]. Infections caused by *S. epidermidis* are widely associated with a medical device implantation, such as intravenous catheters [6-9], prosthetic vascular valves [10-14], joint prostheses [15-17], peritoneal dialysis catheters [18], cardiac pacemakers [19,20], cerebrospinal fluid shunts [21,22], endotracheal tubes [23], genitourinary tract catheters [24], intraocular lenses [25,26], breast implants [27-29], and other polymer and metal implants [30].

The Center for Disease Control and Prevention (CDC) estimates that approximately half of hospital-acquired infections, in USA, are attributed to indwelling medical devices [31]. Among them, coagulase-negative Staphylococci are the leading cause of biomaterials-related infections, with nearly 80% of cases attributed to *S. epidermidis* [32]. *S. epidermidis* is responsible for approximately 30% to 40% of nosocomial bloodstream infections, associated with intravascular catheter or other prosthetic medical device [30,33]. World Health Organization official reports are lacking. Nevertheless, it is believed that, in Europe, it is not a different reality, compared to the USA. For example, in Germany, where more than 2.5 million of medical devices are used annually, it was estimated that up to 100000 patients suffer major complications from their implantation [34]. In countries such as the UK, France, Italy and Germany, it was estimated that catheter-related bloodstream infections resulted in 8400 to 14400 episodes of catheter-related bloodstream infections per year, with 1000 to 1584 deaths in the same period and an annual burden of 35.9 to 163.9 million euros [35]. In Portugal, hospital-acquired infections had a 10.6% incidence, in 2012 [36]. These infections were mainly localized in respiratory airways (29.3%), urinary tract (21.1%), surgery tissue (18%), and bloodstream (8.1%). Gram-positive bacteria were responsible for about half of the bloodstream infections (48.4%). Additionally, statistically significant differences were found when an indwelling medical device was used. More precisely, the presence of a vascular central catheter increased the number

of infections to 31.3% (9.7% in absence of catheter vascular central), and urinary tract device increased the development of an infection to 32.9% (9.7% in absence of urinary tract device).

Due to the difficulties in obtaining a successful treatment of biomaterials-related infections caused by *S. epidermidis*, these infections are associated with a significant morbidity and expensive costs [37]. Often, the removal of the infected device is required, affecting patients' life quality [38]. Additionally, the most affected patients by *S. epidermidis* infections are those immunocompromised, long-term hospitalized and critically ill [2].

1.2. *Staphylococcus epidermidis* biofilms

Biofilms are defined as "a structured community of cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface" [39]. They can be formed in abiotic and biotic surfaces [40]. *S. epidermidis* has a strong ability to adhere to surfaces and to form biofilms, which represents the main *S. epidermidis* virulence factor [41].

Consequently, according to the U.S. National Institute of Health (NIH), 80% of the chronic bacterial infections diagnosed are biofilm-associated (or around 65% according to the CDC) [42], such as dental caries, periodontitis, cystic fibrosis lung infection, chronic otitis media, chronic rhinosinusitis, chronic tonsillitis, chronic wounds, musculoskeletal infections/osteomyelitis, endocarditis, urinary tract infections and infectious kidney stones/biliary tract infections [43]. The most prevalent *S. epidermidis* biofilm-associated infections are bacteremia, endocarditis, orthopedic prosthetic devices infections, dialysis-catheter associated infections and genitourinary infections [30].

The extracellular matrix, where biofilm cells are embedded, contributes for a protected mode of growth that allows survival in a hostile environment [44]. The *S. epidermidis* extracellular matrix contains several components which are absolutely essential for biofilm structure [45,46], such as cell surface and secreted proteins [47,48], polysaccharides such as poly-N-acetylglucosamine (PNAG, a product of *icaADBC* operon [49]) [50], teichoic acids [51], poly-γ-glutamic acid (PGA, a

product of *capBCAD* locus) [52] and extracellular DNA (eDNA) [53]. It was shown that PGA is involved in the protection to antimicrobial peptides (AMPs) and, in the neutrophil killing and virulence in a mouse model of device-related infection [52]. However, the major exopolysaccharide found in *S. epidermidis* biofilm matrix is polysaccharide intercellular adhesin (PIA) or PNAG, responsible for mediating intercellular adhesion in *S. epidermidis* biofilm formation and the most important virulence factor of *S. epidermidis* biofilms [49,54]. However, even *ica* negative strains are able to establish biofilms [55-57] and induce a biofilm infection [57,58]. Therefore, biofilm formation may be mediated by other surface proteins, such as Bhp [59] and Aap [60]. Also, PNAG has the ability to reduce the activity of AMPs and the killing by human neutrophils, since phagocytosis and killing by human polymorphonuclear leucocytes was significantly increased in a mutant strain lacking PIA production in comparison with a wild-type strain [44,61].

Biofilm formation consists in a coordinated sequence of events (Figure 1.1), that consists on: (a) primary attachment and adhesion of bacteria to a surface; (b) intercellular aggregation and accumulation of bacteria leading to maturation of the biofilm; (c) dispersion of single cells or clusters of cells, which may start the whole process of biofilm formation elsewhere [39,40,62-66].

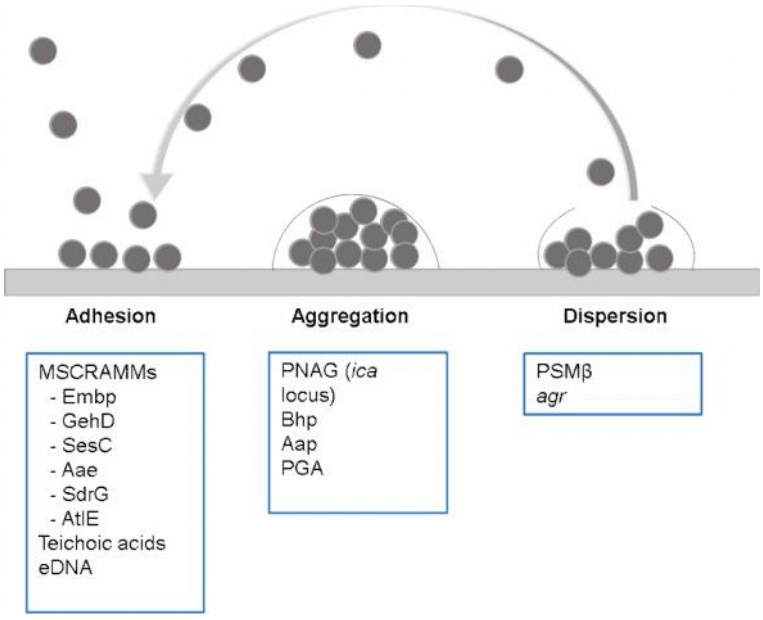

Figure 1.1.: Biofilm formation stages and the most important components involved in each step.

After the insertion of an implant, the external surface is rapidly coated with host proteins, such as fibronectin, fibrinogen, vitronectin, collagen, albumin and immunoglobulins [67-70]. During the initial attachment/adhesion of bacteria to the indwelling medical device, several *S. epidermidis* proteins, known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [71], interact with the coated biomaterial [72,73]. MSCRAMMs comprise bacteria surface adhesion proteins that mediate the attachment to the referred host proteins anchored onto the biomaterial surface [74]. Consequently, host proteins coating the biomaterial have been demonstrated to enhance bacterial colonization [75]. Among the most studied MSCRAMMs are SdrG/Fbe (affinity to fibrinogen) [76,77], Aae (affinity to vitronectin) [78], AtlE (affinity to vitronectin) [79], Embp (affinity to fibronectin) [80,81], and GehD (affinity to collagen) [82]. Other components were shown to be involved also in the initial steps of biofilm formation, such as teichoic acids [83] and eDNA [53,84].

The bacterial accumulation/aggregation step is mainly mediated by exopolysaccharides and proteins which participate in intercellular aggregation of bacteria, such as PNAG [85], Aap [60], Bhp [59], and also PGA [86]. Finally, the dispersion of cell clusters from a biofilm contribute to dissemination of the microorganism extending a biofilm infection and represent an important source of *S. epidermidis* infection [87]. The disruption of cell-to-cell interactions within the biofilm may be due to the phenol-soluble modulins β (PSMβ) [88,89]. PSM are regulated by *agr* quorum-sensing system [90,91]. The *agr* quorum-sensing system has been implicated in the control of biofilm thickness and biofilm structuring [91], and it is known to coordinate the communication between bacteria within a biofilm [92]. In *Staphylococcus aureus*, the PSM pathogenic activity has been further studied, with implication in cytolysis, immunomodulation and biofilm development [93].

Biofilm bacteria are distinct from their free-floating counterparts. More precisely, biofilm differs from planktonic cells in metabolic activity [94], induced host immune response [95], transcriptomic expression profile [94,96], protein expression profile [97], substrate uptake [98], and tolerance to antibiotics [99]. A hallmark of bacterial biofilms is their higher capacity to tolerate antimicrobial agents in comparison with planktonic cells [100], revealing the huge clinical impact of this finding. Several

mechanisms have been enumerated as responsible for tolerance to therapeutics in infections caused by biofilms, most recently reviewed by Olsen [101]. The biofilm matrix may limit the penetration and diffusion of some antibiotics [102,103], despite diffusion through matrix is no longer believed to be a significant factor [104-106]. Also, antibiotic action may be influenced by different metabolic physiological states of cells within a biofilm such as in persister cells, other subpopulations and by slow growth rate [100,102,107-116,116-126]. Additionally, environmental factors may contribute to antibiotic efficacy, such as oxygen gradient [127,128], stress response to nutrients limitation, excess of waste products, low oxygen levels and antimicrobials [123,129-132].

Despite the limitations in using antibiotics against established biofilms, they are still the main approach to treat biofilm-infections before more drastic procedures, such as removal of an implanted device. New treatment proposals are being introduced and tested [133]. However, it is important to develop a strategy that takes into account the pathogenesis of biomaterial-associated infections, which depends on the interaction of the bacteria, the device and the host [134].

1.3. The interaction of biofilms with the host immune system

The immune response to *S. epidermidis* biofilms has been assessed using *ex vivo*, *in vivo* and *in vitro* models. Cytokine production and expression of cell activation markers [135-139], phagocytic activity [95,140,141], immunoreactivity [142] and interaction with whole blood [143,144] were already evaluated in the context of *S. epidermidis* infections. However, the knowledge on how the host immune system interacts and how may be potentiated to eliminate *S. epidermidis* biofilm infections is still insufficient.

Biofilm formation can be facilitated by the host inflammatory response since host inflammatory molecules may facilitate adhesion to the surface of medical devices [41]. Cardille *et al.*, described that human plasma enhanced *S. aureus* biofilm formation, *in vitro*, leading to an increase of MSCRAMMs expression [145]. Cells of the human innate immune system also recognize pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs),

important for the initial bacteria-host contact. Several PAMPs of *S. aureus* were already described, such as lipoproteins, peptidoglycan fragments, formylated peptides and PSM peptides [146]. Interestingly, additionally to PSM role in *S. epidermidis* biofilm detachment [89], PSM represent an important immunomodulator and virulence factor [147]. However, the function of secreted PSM over immune evasion is not yet known [148].

In *S. epidermidis*, toll-like receptor 2 (TLR2) and TLR9 seem to be the most important innate immune recognition receptors of staphylococcal species biofilms, which recognize lipoteichoic acid [149] and peptidoglycan from staphylococci cell wall [150]. More specifically, PNAG was reported to stimulate TLR2 [151] and this was important in clearance of *S. epidermidis* bacteremia [136]. The potential of *S. epidermidis* in activating TLR2 seems to be so strong, that this interaction was further used to induce expression of antimicrobial peptides in order to enhance a response to other pathogens [152].

The multilayered and high density structure of biofilms also provides protection to bacteria against host-defense mechanisms, such as phagocytosis [39,153,154]. Foster [155] and more recently Hanke and Kielian [156] reviewed the host immune system evasion mechanisms of staphylococci. Despite the lack of information in how the host immune system interacts and responds to *S. epidermidis* biofilms, production of PNAG [95] and formation of a polysaccharide capsule [52] to hide the bacteria from opsonins and phagocytic immune cells, such as neutrophils and macrophages [44,140,141,157-162] were enumerated as the main evasion mechanisms, that result in tolerance to the innate immune response [95,96,154].

Regarding complement system activity against *S. epidermidis* biofilms, serum C5 factor levels in patients exposed to biofilm forming isolates were higher as compared to those of patients exposed to non-biofilm forming isolates [144]. Moreover, it was demonstrated that *S. epidermidis* biofilms activated more complement factors than planktonic cells, despite C3b and IgG deposition was lower on bacteria from biofilms, which may contribute to evasion of polymorphonuclear neutrophils killing [141]. However, opsonization of *S. epidermidis* biofilms with IgG and C3b was not required for polymorphonuclear neutrophils activation [163]. This suggests the existence of other molecules that can lead to the *in vitro* activation of polymorphonuclear neutrophils, such as

proteins [163] and polysaccharides [164] from the *S. epidermidis* biofilm matrix. Analysis of the bacteria secreted factors and surface-exposed proteins contribute to understand the host-pathogen interaction. With that intention, the comparison of virulent and commensal *S. epidermidis* strains exoproteome provided evidences suggesting that more virulent factors such as hemolysin, antigens and urease, may contribute to virulence and adaptation of *S. epidermidis* strains [165].

In addition to phagocytosis, the production of peptides with antimicrobial activity is an important host innate mechanism to avoid microbial evasion. AMPs are mainly found in saliva, skin, blood cells, among others. The mode of action of AMPs is disrupting membrane integrity (via interaction with negatively charged cell membrane) [166], by inhibiting proteins, DNA and RNA synthesis, or by interacting with certain intracellular targets, repressing processes such as protein folding, cell wall synthesis and metabolic turnover [167,168]. However, staphylococci developed strategies to evade antimicrobial peptides such as surface charge alterations, extracellular proteases, synthesis of exopolymers and efflux pumps proteins [169]. Despite the announced disadvantages of using AMPs as treatment [170], a few were tested against *S. epidermidis* biofilms, such as human cathelicidin peptide LL37 which was reported as a potent inhibitor of *in vitro* biofilm formation [171], chimeric peptide composed of a dermaseptin derivative, which prevented *in vivo* staphylococcal biofilm infections [172], and Ltx peptides that eliminated metabolic activity in *in vitro* biofilms [173].

In an attempt to identify potential markers of infection and define possible therapeutic targets in *S. epidermidis* biofilm infections, proteomics has been used to identify immunogens. Therefore, immunoproteomics combines proteomics and detection of immunoreactive antigens using a biologic fluid [174]. Sellman *et al*. identified 29 immunoreactive or serum binding proteins of *S. epidermidis* using serum from rabbits previously immunized by intraperitoneal injection [142]. In the referred work, the authors identified five vaccine candidates, namely acetyl-CoA C-acetyltransferase, Na+/H+ antiporter, alanine dehydrogenase, lipoate ligase and cysteine synthase [142]. Later, Pourmand *et al*. identified autolysin AtlE, lipase GehD and surface protein ScaB as candidate antigens for prophylaxis or immunotherapy to control *S. epidermidis* infection, since they were opsonic *in vitro* [175]. However, a similar work using *S. epidermidis* biofilms was lacking.

Additionally, differences between *in vivo* and *in vitro* biofilms have been enumerated [176-178]. Despite all of this knowledge, *in vivo* immune response against *S. epidermidis* biofilms may vary according to localization of biofilm infection, how the foreign device is inoculated and the amount of inoculum [156].

1.4. Heterogeneity of cells within a biofilm

Biofilms are a structurally complex and dynamic system (Figure 1.2).

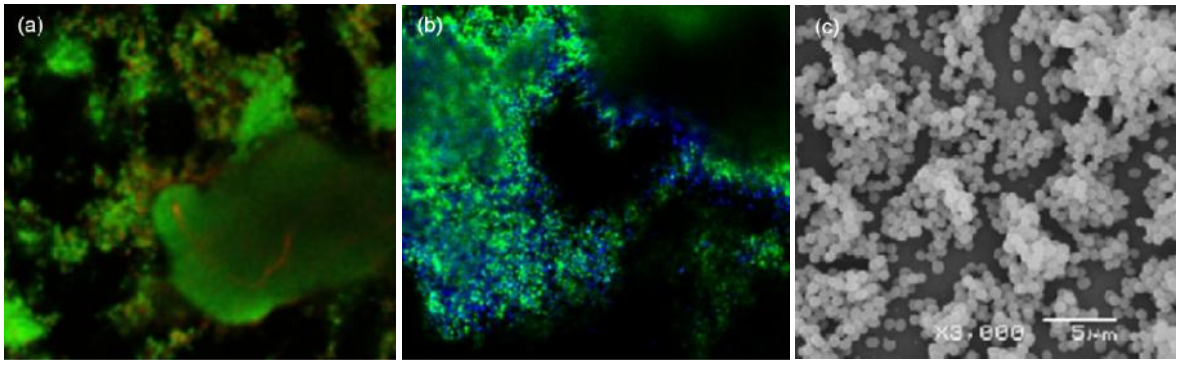

Figure 1.2.: Staphylococci biofilms. (a) Confocal laser scanning microscopy of *S. epidermidis* biofilm (Z = 30µm) stained with LIVE/DEAD [179]; (b) Confocal laser scanning microscopy of *S. epidermidis* RP62A biofilms (Z = 24µm) stained with 4',6-diamidino-2-phenylindole (DAPI) and wheat germ agglutinin (WGA) [180]; (c) Scanning electron micrograph of *S. epidermidis* biofilms [181].

Bacteria physiological state within a biofilm is heterogeneous [182,183] and this may affect sensitivity to antibiotics and host immune response [184]. Different sub-populations have been described, such as, growing aerobically, growing fermentatively, dead and dormant [182] (Figure 1.3).

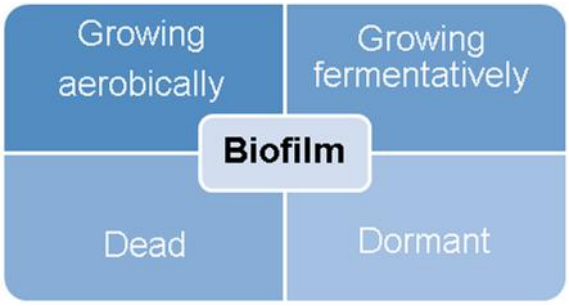

Figure 1.3.: Physiological heterogeneity within a biofilm.

Environmental conditions are the main perturbation that can lead to heterogeneity. Factors such as, concentrations of nutrients (carbon [185,186], nitrogen [187], phosphate [188], iron-sulfur [189,190], ion sources [191,192]) and the growth conditions (pH [193,194], temperature [195-200], membrane-acting agents [110,201], oxygen availability [131,182,202-204], oxidative stress [131,205-208], osmotic stress [209-211], and chemical stress [212,213]) are sensed by microorganisms that adapt to local environment conditions.

Likely, stochastic expression of genes and genetic variations (mutations, genetic recombination) also generate phenotypic and genetic diversity within biofilms [214,215]. Consequently, all these factors may influence transcriptionally, physiologically and morphologically the bacterial cells, in order to adapt and survive [216].

The structural, chemical and biological heterogeneity within a biofilm seems to depend on the spatial location of the cells inside the community [215]. Structurally, cells at the top of *Pseudomonas aeruginosa* biofilms have increased expression of genes involved in general metabolic functions, while these genes have decreased expression in cells at the bottom of the biofilms, suggesting that bacteria in the upper layer of a biofilm are more metabolically active than those in the lower layers [114,131]. Dormant sub-population is in a critical physiological state since most of antimicrobials used in therapeutic require metabolically active cells to be effective [1,217-221]. In consequence, the presence of dormant bacteria within a biofilm has been associated with recurrent infections [222,223].

Nowadays, the concepts of cells in viable but non-culturable (VBNC), dormant (also referred as latent, in *Mycobacterium* spp. [224,225]), small-colony variant (SCV) and persister state are very often used to describe a sub-population that presents tolerance to antimicrobial treatment. However, there is some controversy in the definition of these terms and they may not have been used properly in all situations (Figure 1.4).

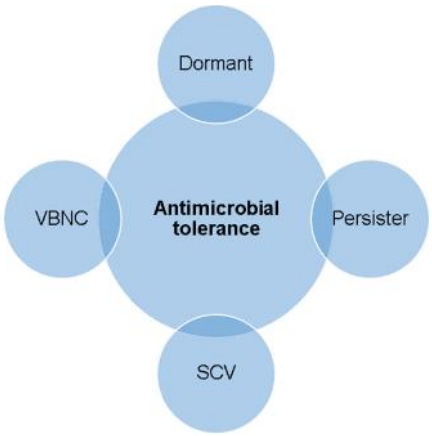

Figure 1.4.: Terms used to define bacteria that present tolerance to antimicrobial treatment.

In 1944, Joseph Bigger described a small population of *Staphylococcus* spp. that persisted to penicillin action [226]. Latter, the concept of persister cells was improved by Kim Lewis, describing these cells as “dormant variants of regular cells that form stochastically in microbial populations and are highly tolerant to antibiotics” that consisted in a small fraction of a bacterial population [221]. More recently, the definition of persister cells was adjusted by Orman and Brynildsen. These authors showed that not every persisters are dormant and finished suggesting that “persistence is far more complex than dormancy” [227], similarly to what Wakamato *et al.* described for mycobacteria persisters [228]. These cells are frequently referred as non-growing dormant cells. In general, persisters are characterized by: low levels of translation [229,230], lack of protein synthesis to become tolerant to antibiotics [231] and dowregulation of genes involved in metabolic and biosynthetic pathways, and energy production [229,232,233]. Importantly, the number of persister bacteria represent about 1% of the cells in the stationary phase and in biofilm cultures of *Escherichia coli* [229], but represent a lower number in an exponential state (about 0.001%) [234,235]. However, the expression profile of persisters was different from the expression profile of non-growing stationary phase cells which may suggest a distinct physiological state [229]. Interestingly, it was observed that inhibition of respiration during stationary phase reduced persister levels [236]. Curiously, the rate of persister cells formation was shown to be dependent on the inoculum age [237]. The protocol that has been widely used to isolate persister cells [235] consists in collecting bacteria (persisters) by centrifugation, after incubation with high doses of

antibiotics. Another protocol was developed using a green fluorescent protein reporter gene to sort out cells based on the lack of expression of degradable green fluorescence protein [229]. Interestingly, the degree of persistence was shown to vary according to the used antibiotic [237,238]. The existence of persister cells after *S. epidermidis* biofilms treatment with levofloxacin and vancomycin was also demonstrated by Shapiro *et al.*, in a higher proportion than in exponential growth phase [239]. Due to the most recent information on persisters behavior, more and more researchers defend a new characterization of these cells [227,228,240].

A very close relation between the concept of persister and dormant cells was announced. It was hypothesized that all persisters are dormant, but not all dormant cells are persisters [241]. Persisters are referred as the bacteria that grow after exposure and treatment with high doses of antibiotics, without undergoing genetic changes [214,235]. However, viability and culturability are different terms [242]. Dormant bacteria are viable and present low metabolic activity. Consequently, laboratorial detection of dormant cells may induce misleading interpretations about the infection status due to absence of growth [243]. In order to overcome this problem, it is important to assess the number of viable and culturable cells [138]. The number of culturable cells is easily available by routine methods, such as counting of colony forming units (CFUs). But CFUs may not correspond to the real number of viable cells. Thus, fluorescence-based approaches based on nucleic acids binding and/or exclusion should be employed, using techniques such as flow cytometry, fluorescence microscopy, and even real time-PCR experiments targeting 16S rDNA [201,244-246]. As referred above, dormancy brings implications in therapeutic efficacy and recurrence of biofilm infections.

Despite the latest improvements, distinction between persisters and VBNC cells is not yet clear [247-250]. Recent experiments show that VBNC, unlike persisters, do not form colonies when incubated in enriched medium, but both do not grow under antibiotic stress [248,251]. Additionally, Orman and Brynildsen assumed that the main difference between persister and VBNC cells is the ability of persisters to restart normal growth after antibiotic treatment [251]. Curiously, it was also shown that VBNCs are more abundant than persisters in untreated antibiotic samples [249,251]. In a deeper study, it was verified that VBNC and persister cells coexist

and are induced by the same conditions [249]. VBNC was a concept introduced by JD Oliver, after Xu *et al.* publication [252]. VBNC bacteria was defined as “bacteria metabolically active, while being incapable of undergoing the sustained cellular division required for growth in or on a medium normally supporting growth of that cell” [253]. In general, VBNC has been defined as a state where cell is metabolically active despite their incapacity in forming a colony on a plate [253]. This condition emerged after the underestimation of viable populations of microorganisms in aquatic environment by culture methods, but detected by immunofluorescent microscopy, acridine orange direct counting and direct viable counting [252]. Since then, environmental and food microbiological analysis became more complex than microorganism culture monitoring, due to the underestimation of viable cells by CFU counting. This represents a risk to public health [247] and constitute an important reservoir of pathogens in the environment [220].

Nevertheless, the VBNC state of bacteria was previously referred as dormancy. Distinct opinions have been published regarding differences between VBNC and dormant bacteria. Some authors defend that in VBNC cells, the metabolic activity is measurable, contrary to dormant cells that have low metabolic activity [254-257]. Others argue that dormant and VBNC are the same cells, since VBNC do not show metabolic activity [258,259]. Also, Oliver *et al*. defend that VBNC and dormant physiological states are the same, presenting low metabolic activity [249,260]. However, it remains unclear whether VBNCs exhibited significant metabolic activity and if VBNC and dormant are synonymous. Additionally, it is defended that VBNC cells may regain culturability, in opposite to Orman *et al.* data [251], and are still virulent, capable of causing infection [261-264]. The presence of VBNC was already observed in *S. aureus* biofilms subjected to antibiotic pressure [201]. More precisely, antibiotics and nutrient depletion were used to induce VBNC, that reduced the culturability of the cultures despite cells were still viable.

Lastly, SCV are defined as slow-growing cells that form smaller colonies on agar plates (about 10 times smaller than those of normal phenotype) [265], due to deficiencies in electron transport and in thymidine biosynthesis [266,267]. The reduced metabolism of SCV [268] and the ability to survive intracellularly [269,270], also explain the decreased susceptibility to antibiotics. Additionally,

SCV present a decreased expression of toxins, increased expression of adhesins and intracellular growth [271]. It was suggested that the SCV phenotype may be a defense mechanism against environmental stresses [272] and associated with persistence in the host environment [273]. The existence of SCV in *S. epidermidis* biofilms was already demonstrated [274-279].

Despite difficulties to establish correct definitions, the most important consequence of the existence of VBNC or dormant or persister or SCV cells, is their tolerance to stress factors and increased tolerance to antibiotics. Consequently, this leads to bacteria long-term survival in host. In the present thesis, “dormant” and “dormancy” were the chosen terms to describe the sub-population of cells that are viable but do not grow in an appropriate solid medium.

1.5. *Staphylococcus epidermidis* biofilm dormancy model

Previously, Cerca *et al*. described an *in vitro* model to induce dormancy in *S. epidermidis* biofilms [138] (Figure 1.5).

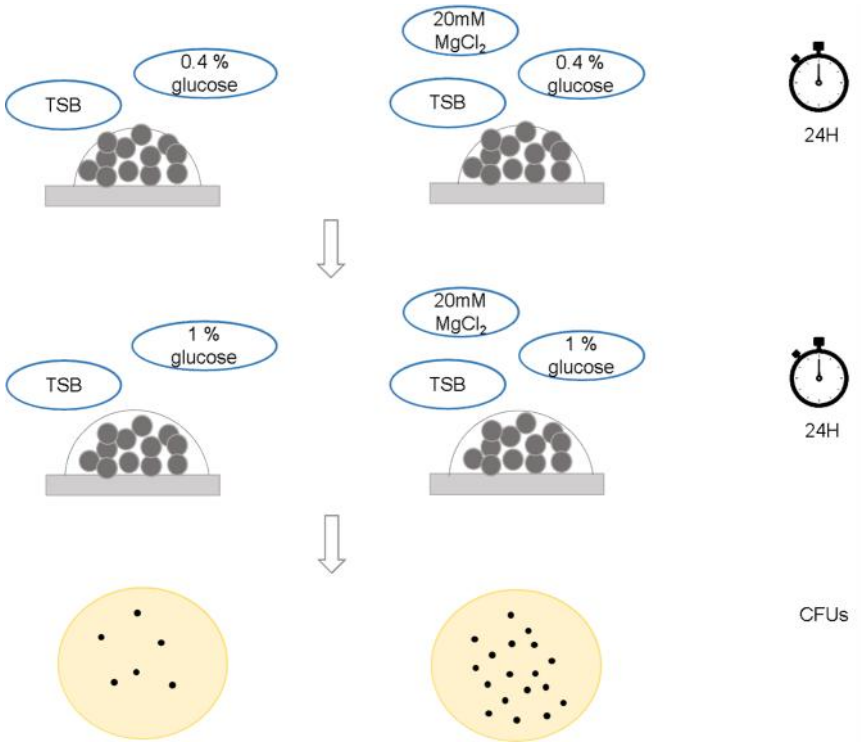

Figure 1.5.: Model to induce and counteract dormancy in *S. epidermidis* biofilms.

In the referred model, modulation of dormant bacteria within *S. epidermidis* biofilms was performed by adding divalent ions, such as Calcium (Ca^{2+}) and Magnesium (Mg^{2+}), to biofilm culture growth in a glucose-enriched medium. The accumulation of acidic compounds, produced by glucose metabolism, induced cells to enter in a dormant state, which was counteracted by high extracellular levels of divalent ions. Acidic pH conditions have the power to inhibit bacterial growth [280], and Dunne and Burd showed the importance of pH in primary adhesion of *S. epidermidis* to surfaces [281]. However, the *S. epidermidis* ATP activity at acidic pH showed that this species has the ability to succeed under acidic pH [282]. Cerca *et al.* showed that buffering the accumulation of acidic compounds with hydrogen phosphate to maintain pH conditions prevented the glucose-dependent accumulation of dormant bacteria [138]. Additionally, a higher amount of PNAG was found in maintained pH conditions [283]. Those results proved that the decreased culture pH affected the *S. epidermidis* biofilms physiology. The physiological state of *S. epidermidis* biofilm bacteria was assessed by staining cells with SYBR Green I and propidium iodide (PI) viability dyes, using flow cytometry [284], similarly to other works that intended to determine the number of viable and culturable cells [285-288]. Both PI (red fluorochrome) and SYBR Green fluorochromes have affinity to DNA, however, PI only penetrates compromised membranes and displaces SYBR Green due to a higher affinity [289].

The use of divalent cations to counterbalance the pH effect did not lead to a decrease of culturable cells, as in *S. epidermidis* biofilms only grown in glucose excess [138]. Divalent cations, such as Mg^{2+} and Ca^{2+}, can bind to teichoic acid from cell wall [290,291], contributing to maintain the integrity and stability of bacteria cell wall [292]. However, despite no consensus [293], differences in the binding affinity of divalent cations to cell wall components of *S. epidermidis* were reported [294,295]. Interestingly, Cerca *et al.*, demonstrated that Mg^{2+} alone (without Ca^{2+}) could account for preventing the glucose-induced cell dormancy [296]. Both divalent cations can influence biofilm formation [297] and already in the 1950's, it was demonstrated that magnesium was needed for bacterial growth [298,299]. In *S. epidermidis* biofilms, Mg^{2+} was shown to increase adhesion and slime production in biofilm formation [281,300,301]. Also, in *Pseudomonas*

fluorescens biofilms, Mg^{2+} increased initial attachment and altered subsequent biofilm formation and structure [302]. Later, Mulcahy and Lewenza showed that magnesium limitation promotes the transition from planktonic to biofilm formation in *P. aeruginosa* [303].

Metal ions play an important role in bacteria homeostasis and viability, acting as co-factors for enzymes, facilitating protein-protein and protein-nucleic acid interactions and, are involved in membrane stability, stabilizing tertiary structures of proteins as well as nucleic acids, by regulation of gene transcription, DNA replication, and protein synthesis [304,305]. The host environment leads to metal ion fluctuations which contribute for killing of invading pathogens [191]. The mean serum concentration of Mg^{2+} in human body is approximately 0.76 to 1.15 mM [306-308] and is required by DNA and RNA polymerases, as well as many ATPases and kinases [309]. Thus, the availability of free Mg^{2+} in the host is thought to be an important signal for pathogenic bacteria to select in which compartment they reside [310]. For example, Mg^{2+} limitation was recognized as an important environmental trigger to develop *P. aeruginosa* biofilm [303]. Interestingly, *S. epidermidis* biofilms that grew without magnesium induced a lower response of murine immune cells, such as macrophages [138] and dendritic cells [296], indicating that *S. epidermidis* with induced dormancy were less inflammatory than biofilms grown with Mg^{2+}, contributing to evasion of the immune system. It was shown that production of TNF-α, IL-1 and IL-6 in murine bone-marrow-derived macrophage cell cultures, as well as that resulting from *in vivo* macrophage activation were found decreased when *S. epidermidis* biofilms grown in the absence of Mg^{2+} were used as stimulus [138]. Moreover, although bone marrow-derived dendritic cells did not significantly alter their surface expression of co-stimulatory and antigen-presenting molecules upon contact with bacteria from biofilms with induced dormancy, these induced a lower production of TNF-α, IL-12 and IL-6 upon contact with dendritic cells [296].

Up to now, only MgtE (Mg^{2+} transporter) and CorA protein (Mg^{2+}channel) were identified in *S. epidermidis* as Mg^{2+} transport proteins. Despite no association to *S. epidermidis* virulence, the CorA channel in *Salmonella* spp. was associated with virulence [311-313]. A RNA-based metal sensor in bacteria was suggested due to the regulation of MgtE in response to Mg^{2+} via M-box riboswitch [314].

Therefore, differences in growth under different conditions may lead to physiology changes that may be reflected in a distinct phenotypic profiles.

1.6. Proteomic and transcriptomic crosstalk to disclose molecular pathways in bacterial dormancy

Despite environmental conditions [207,315], a stochastic basis was proposed as a mechanism of bacterial persisters formation, independent of drug factors (gene expression affected by cellular, physical and biochemical processes) [124,230,235,316-318]. Consequently, several genes have been associated to persistence and dormancy, in an attempt to unravel their molecular genomic relevant pathways. The analysis of altered expression of genes and proteins can pinpoint specific pathways in bacterial persistence within biofilms. Different "*omics*", such as genomics, transcriptomics and proteomics, have been employed in an attempt to characterize dormancy. Together, these approaches give a distinct coverage of the condition, essential for the elucidation of the molecular pathways.

Table 1.1 is a compilation of genes, proteins and biological pathways associated with persistence, dormancy and VBNC. An exhaustive overview of genes, proteins and biological pathways linked to persisters formation was already performed by several authors, for different organisms, such as *Pseudomonas* spp., *E. coli* and *Mycobacterium* spp. [222,319,320]. The most widely studied genes associated with persisters are the Toxin-Antitoxin (TA) loci, since it was demonstrated that persister cells contained high levels of TA mRNAs [321] and were involved in pathogenicity of *E. coli* and *Mycobacterium tuberculosis* bacteria [322].

Table 1.1.: Compilation of genes, proteins and biological pathways associated with persistence, VBNC, dormancy and resuscitation from dormancy.

Gene/Protein or Pathway	Specie	Observation	Reference
TCA cycle	*E. coli*	Deletion of TCA cycle enzymes reduce persister formation in stationary phase metabolism	[236]
ClpP protease	*S. aureus*	Mutation of *clpP* leads to increased susceptibility to antibiotic killing	[115]
dinJ yoeB/yefM	*E. coli*	Genes likely to contribute to dormancy. However, a knockout of *yoeB* had no effect on persister formation	[229]
dnaK	*S. aureus*	Deletion of *dnaK* produced decreased number of persisters	[323]
oxyR	*E. coli*	Prominent gene involved in persistence to multiple antibiotics	[324]
DosP	*E. coli*	Phosphodiesterase DosP increases persistence	[325]
GhoT toxin	*E. coli*	GhoT increases persistence	[326]
glpD	*E. coli*	GlpD is a G3P dehydrogenase involved in the utilization of glucose as a carbon source. Its deletion drastically decreased persister development	[327]
HipA toxin	*E. coli*	*hipA7* mutants have increased rates of persister formation.	[328,329]
katG clpC2 rsbW sigF pip	*M. tuberculosis*	Genes upregulated in persisters	[233]
Lon protease	*E. coli*	Necessary for persister cell formation	[330]
MazEF RelBE	*Streptococcus mutans*	Type II toxin-antitoxin systems caused an increase in the number of persisters	[331]
MazF	*E. coli*	Overexpression of MazF toxin, leads to the formation of persisters	[332]
MqsR/MqsA	*E. coli*	Overproduction of MqsR toxin is increased in persistence	[333]
NadE	*M. tuberculosis*	NadE was required for persistence during starvation and under hypoxic conditions	[334]
Obg	*E. coli* *P. aeruginosa*	Obg levels control persistence, mediated by (p)ppGpp	[335]
phoU	*E. coli*	Deletion of *phoU* decreases the frequency of persisters	[336]
ppGpp (encoded by *relA* and *spoT*)	*E. coli*	Persisters have high levels of ppGpp	[238,318]
relA	*E. coli*	Deletion of *relA* decreases the frequency of persisters	[337]
relE toxin	*E. coli*	Overexpression is increased in persisters	[338]

relE homologs	*M. tuberculosis*	Ectopic expression of three RelE homologs also produces drug-tolerant cells	[339]
rpoS	*E. coli*	Involved in the persistence of *E. coli* in the VBNC state	[340]
rpoS *gadB* and *gadX* *mdtF* *osmY*	*E. coli*	Deletion of these genes increased persistence	[341]
TA modules (*relBE*, *mazEF*, *dinJ/yafQ*, *yefM/yoeB*)	*E. coli*	Overexpressed genes in persisters	[232]
TisB toxin	*E. coli*	Overexpression led to persisters formation	[110]
yafQ toxin	*E. coli*	May play a role in the formation of biofilm persister cells	[342]
fba *tsf* *tuf*	*E. coli*	Genes up-regulated in VBNC	[343]
katG	*Vibrio vulnificus*	Gene down-regulated in VBNC under *in vitro* conditions	[344]
tuf *rpoS* *relA*	*Vibrio cholerae*	Genes down-regulated in VBNC	[345]
RMF (Ribosome Modulation Factor)	*E. coli*	RMF inactivates *E. coli* ribosomes, therefore is associated with a dormant phenotype	[346]
rmf	*P. aeruginosa*	May be required for maintenance of cell integrity in older dormant cells, localized at the bottom of the biofilm	[131]
Rpf (resuscitation-promoting factor)	*Micrococcus luteus* *Mycobacterium smegmatis*	Rpf was found to stimulate the growth of dormant cells when added extracellularly	[259,347]

The first gene to be linked to increased tolerance to antibiotics was *hipA* gene in *E. coli* [328,329] that encodes the toxin part of *hipBA* TA system. The *hipA7* mutant, decreases the affinity of the toxin to the antitoxin [348], leading to a 1000-fold increased persister level, as compared to that of the wild type strain, with increased tolerance toward beta-lactams, fluoroquinolones and aminoglycosides [232,329]. Later, a greater interest on TA modules/systems occurred due to their possible link to persisters formation. TA systems consist of a stable toxin, which inhibits an essential cellular function, and a labile antitoxin, which neutralizes the activity of its associated toxin [338,349,350]. Under normal conditions, the

antitoxin establishes a complex with the toxin, inactivating it. However, in stress conditions, the antitoxin is degraded and the stable toxin is no longer neutralized [321]. Disregulation of TA systems under stress conditions occurs mainly by degradation or depletion of the antitoxin by RNAses or Lon protease (ATP-dependent protease), that may affect DNA replication, translation, peptidoglycan synthesis, cell division and ATP production, according to the affected TA system [351]. In case of persistence, studies were mainly developed in *E. coli* and *M. tuberculosis*, showing as main consequence multidrug tolerance [352]. Interestingly, it was shown that in *E. coli*, the bacteria become dormant if the toxin level was higher than a threshold, and that the amount by which the threshold was exceeded determined the duration of dormancy [348]. Additionally, Lon protease is also required for persistence [330]. When Lon protease was overproduced in *E. coli*, persisters levels increased [330], possibly due to the degradation of antitoxin, the Lon protease target [321].

Until now, five TA systems classes were described, varying in the mode of action of the antitoxin, involving a broad range of cellular processes [351,353]. Particularly in staphylococcal species, TA modules were already described [354-358]. SprA1 toxic peptide (PepA1) and the SprA1 RNA antitoxin (type I TA system that consists on a toxic peptide and an antisense RNA that inhibits toxin translation [359]) are within a pathogenicity island in *S. aureus* and induce lytic cell death [354]. The MazEF TA module (a type II TA system) was described in *Staphylococcus equorum* and experiments suggested that in staphylococci, TA system may also control processes beyond pathogenicity [357]. Also in *S. aureus*, MazF toxin was implicated in regulation of staphylococcal virulence factors [356] and a *mazEF* orthologue termed *pemIK* was found in *S. aureus* plasmid [358]. Lastely, two YoeB homologues of *E. coli yefM-yoeB* TA system, were found in *S. aureus*, with translation initiation inhibitors function [355].

Using Rapid Automated Scan for Toxins and Antitoxins in Bacteria (RASTA-Bacteria) tool [360], we can predict TA systems in *S. epidermidis*. Sorted by a confidence score above 70%, we identified the following genes in the *S. epidermidis* genome (strain RP62A or ATCC 35984 (biofilm-forming) and ATCC 12228 (non-biofilm forming)) (Table 1.2).

Table 1.2.: Predicted TA systems in *S. epidermidis* ATCC 35984 and ATCC 12228 by using the RASTA-Bacteria tool.

Gene	Score (%)	Possible partners	Protein product
ATCC 35984			
SERP1550	90.15	*SERP1549* and *SERP1551*	hypothetical protein
SERP1549	89.57	*SERP1550*	death-on-curing family protein
SERP1681	76.34	*SERP1682*	endoribonuclease MazF
SERP0849	75.34	*pgsA*	cro/ci family transcriptional regulator
SERP0029	72.59	*Rorf_692*	cro/ci family transcriptional regulator
ATCC 12228			
SE1672	76.34	*SE1673*	endoribonuclease MazF
SE0959	75.34	*SE0960*	hypothetical protein
SE2386	72.59	*Rorf_68138*	hypothetical protein

Additionally to TA systems, a stringent response may be activated when bacteria respond to stress, potentiating the transition to a dormant state. In a stringent response, the alarmone guanosine 5'-diphosphate-3'-diphosphate (ppGpp) and guanosine 5'-triphosphate-3'-diphosphate (pppGpp), usually referred as (p)ppGpp, are produced via *relA* and *spoT* (which can also degrade ppGpp) during a carbon and aminoacid limitation [361]. The synthesis of ppGpp reprograms and adjusts the gene expression of the cells to survive to nutritional limitations [321], having implications in the DNA replication and proteins synthesis, leading to a decrease of these processes [361]. Therefore, (p)ppGpp has been implicated in a number of non-stringent processes, including virulence [361,362], persister cell formation [238,241] and biofilm formation [363]. (p)ppGpp has been described as an important persistence mediator and required for persistence [318,321], both in stochastically and environment-induced persistence [364], since Korch *et al.* reported that *E. coli* K-12 cells deficient in (p)ppGpp synthesis display a reduced level of persisters [337]. Additionally, it has been suggested that Lon protease is required for the ppGpp-dependent persister formation [318]. During starvation, the high levels of (p)ppGpp destabilize the antitoxin that leads to increased synthesis rates of both toxin and antitoxin [364].

When staphylococci undergo a nutrient limitation environment, the activity of tricarboxylic acid cycle (TCA) increases and the bacteria catabolize non-preferred carbon sources such as acetate [365]. Under aerobic conditions, *S. aureus* utilizes glycolysis, pentose phosphate pathway, and the TCA to catabolize glucose [366]. Under glucose excess there is a high expression of glycolytic enzymes, whereas expression of TCA enzymes are repressed [367-369]. The repression of TCA cycle genes is a common response to high concentrations of glucose in culture media, a response mediated by CcpA in Gram-positive bacteria [65]. Also, several enzymes involved in TCA cycle require iron, which means that under low iron availability, TCA cycle activity is reduced [370,371]. Particularly in *S. epidermidis*, TCA cycle inactivation leads to an alteration of the metabolic status, resulting in derepression of PNAG biosynthetic genes [372] .

Clearly, formation of persisters and dormant cells must be linked to a set of different factors [373]. This phenomenon is not dependent on one or two specific “persister genes”. Instead, the process is very complex and under multigenic control. Variation in the expression of a large number of other genes can also affect persistence or the ability of bacteria to grow in the presence of an antimicrobial drug. These genes may include global regulators, genes involved in metabolism, and stress-response components [240].

1.7. Goals

Due to the impact of dormant bacteria in chronic and relapsing infections, it is of major importance to better understand this physiological state.

Therefore, the main goals of this thesis were to provide a characterization of *S. epidermidis* biofilms with induced dormancy by using complementary approaches, such as:

I. Describing transcriptomic and proteomic profiles of biofilm dormancy;

II. Assessing the correlation of trancriptome and proteome profiles of *S. epidermidis* biofilms;

III. Determining the immunoreactivity profile of biofilm dormancy to human sera;

IV. Evaluating the clinical relevance of dormancy.

CHAPTER 2.

DORMANCY WITHIN *S. EPIDERMIDIS* BIOFILMS: A TRANSCRIPTOMIC ANALYSIS BY RNA-SEQ

DORMANCY WITHIN *S. EPIDERMIDIS* BIOFILMS: A TRANSCRIPTOMIC ANALYSIS BY RNA-SEQ

Summary

In an attempt to identify the major transcriptomic differences between *S. epidermidis* biofilms with prevented and induced dormancy, biological triplicates from *S. epidermidis* biofilms were assessed by RNA-sequencing. Significant differences were found in the expression of 147 genes. A detailed analysis of the results was performed based on direct and functional gene interactions. Biological processes among the differentially expressed genes were mainly related to oxidation-reduction processes and acetyl-CoA metabolic processes. Gene-set enrichment revealed that the translation process is dependent upon the presence of dormant bacteria. Transcription of mRNAs involved in oxidation-reduction processes was associated with induced dormancy within *S. epidermidis* biofilms. These results suggest that dormancy within *S. epidermidis* biofilms lead to a distinct transcriptomic profile.

2.1. Introduction

The purpose of this study was to describe the major transcriptomic features of *S. epidermidis* biofilms with induced or prevented dormancy, using high-throughput RNA-sequencing (RNA-seq) technique. RNA-seq has been used for transcriptome analysis as an alternative to other transcriptomic technologies such as microarrays, due to advantages such as the large dynamic range, high technical reproducibility and a reference transcriptome is not a requirement [374-376]. RNA-seq has been applied to prokaryotic transcriptome studies [377,378] and some efforts have been made to use next-generation sequencing in clinical microbiology to test the properties of growing bacterial pathogens [379]. RNA-seq studies in *E. coli* and *M. tuberculosis* dormant persister sub-population have been previously carried out without biological replicates, being reported that linked to the dormant state were changes in transcription of genes involved in metabolism, biosynthetic pathways or oxidative stress [229,233].

To achieve our goal, we performed RNA-seq of three biological triplicates from *S. epidermidis* biofilms with prevented and induced dormancy and we used a data analysis approach based on direct and functional gene interactions analysis, namely, gene set-enrichment and cluster analysis.

2.2. Materials and Methods

2.2.1. Biofilm growth conditions

S. epidermidis strain 9142 (isolated from blood culture by [380]) was used to establish biofilms with prevented and induced dormancy, as previously described [138]. One colony of strain *S. epidermidis* 9142 was inoculated in Tryptic Soy Broth (TSB) (Becton, Dickinson and Company) and incubated at 37 ºC in a shaker at 120 rpm for 18 h. The overnight culture was adjusted to an optical density at 640 nm of 0.250 (±0.05) with TSB and 10 µL of the suspension was transferred into a 24-well plate (COSTAR, Corning Incorporated) containing 1 mL of TSB supplemented with 0.4% glucose (v/v) (TSB 0.4%G) (American Bioanalytical) or TSB 0.4%G enriched with 20 mM magnesium chloride ($MgCl_2$) (American

Bioanalytical). The culture plates were then incubated at 37 ºC in a shaker at 120rpm for 24 h. After this period, the culture medium covering the biofilm was removed and replaced by fresh TSB supplemented with 1% glucose (v/v) (1%G) or TSB 1%G containing 20 mM $MgCl_2$ (1%G + Mg^{2+}). Biofilms were then allowed to grow in these same conditions for 24 additional hours. Next, biofilm culture medium was removed and biofilms were washed twice with 1× phosphate buffered saline (PBS) (Boston BioProducts). Then, bacteria within the biofilms were recovered in 1 mL of PBS. All tubes were placed on ice to inhibit cell growth. Prior to preparation of the RNA-seq library, biofilm dormancy was assessed as previously described [138]. Briefly, biofilms from both conditions were grown with the same initial amount of cells. Number of CFU/mL in each condition was determined using the spread plate method in Trypticase™ Soy Agar with 5% Sheep Blood (TSA II™) (Becton, Dickinson and Company).

2.2.2. RNA extraction

Total RNA was extracted using the RNeasy Mini kit (Qiagen). To remove genomic DNA, Ambion® TURBO DNA-free™ kit (Invitrogen) was used followed by acid-phenol:chloroform precipitation (Ambion), both following the manufacturer's instructions. RNA integrity was determined using the Bioanalyzer 2100 (Agilent Technologies), and samples with RNA Integrity Number (RIN) above 8 were selected for complementary DNA (cDNA) library preparation. Three independent biological experiments, each one from a pool of four biofilms, were performed.

2.2.3. cDNA library preparation and Sequencing

Before library preparation, ribosomal RNA (rRNA) was depleted from total RNA samples using the Ribo-Zero™ Magnetic kit for Gram-Positive bacteria (Epicentre), following the manufacturer's specifications. An Illumina® TruSeq™ RNA Sample Preparation kit (Illumina) was used with the already purified mRNA, following the manufacturer's protocol. The construction of the libraries was rigorously validated by quantitative PCR and Hi-Sensitivity D1K TapeStation (Agilent 2200 TapeStation). Sequencing data were generated from paired-end

reads (2 × 150 bp) on a MiSeq® system (Illumina) using a RNA-seq library of 10 nM.

2.2.4. RNA-sequencing data analysis

The removal of sequence adapters, mapping to reference genome and normalization of gene expression was performed using CLC Genomics Workbench software (V.5.5.1, CLC Bio.). RNA-seq reads were aligned to the reference genome of *S. epidermidis* strain RP62A (Genebank accession number NC_002976). Gene expression was normalized by calculating Reads per Kilobase per Million Mapped Reads (RPKM) using the methods described by Mortazavi and colleagues [381] in which normalization is adjusted by the counts of reads per kilobase per million mapped and gene length. Since consistency between experiments is a natural concern in RNA-seq, Pearson correlation was used to compare RPKM values between triplicates. To test for significant gene expression changes, Baggerley's test (beta-binominal) [382] with false discovery rate (FDR) correction was applied [383]. A p-value ≤ 0.05 was considered statistically significant. Venn diagram was generated using the Venny tool from BioinfoGP [384] to identify the transcripts uniquely expressed in each condition.

2.2.5. Biological interactions

Gene Ontology (GO) [385] and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways [386] analysis were performed to determine the function of differentially expressed genes, using STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins) (version 9.05) [387]. Classes with a p-value ≤ 0.05 were considered statistically significant. Further analysis was carried out using Cytoscape software (version 2.8.3) [388], namely a gene-set enrichment analysis for over- and down-representation in the set of genes with a 1.50 fold-expression change and their neighbors [389] and a cluster analysis with MCODE plugin [390]. Biological interpretation was carried out using STRING bioinformatic tool.

2.2.6. Quantitative PCR

In order to validate some differentially expressed genes, quantitative PCR (qPCR) was performed to quantify the mRNA transcripts of *pgi*, *gapA-2*, *acnA*, *ureA*, *ctsR* and *rplE.* The primers were designed with Primer3 and are shown in Table 2.1.

Table 2.1.: Primers used in qPCR amplification.

Target gene	Primer sequence (5' to 3')	Melting temperature (ºC)	Amplicon size (bp)
acnA	FW catattggcctaccggagaa RV tcacgagaagatcccattcc	60	118
pgi	FW tactacgacagaaccagcag RV catcaggtacaacaaacgtc	59	170
gapA-2	FW agcacctgttgacgttgga RV accagttgcaaaagtcctcaat	60	171
16S rRNA	FW gggctacacacgtgctacaa RV gtacaagacccgggaacgta	60	176
ureA	FW gttgtagctgctgaggttgc RV agctacgccatccatgacat	59	183
ctsR	FW tacaacgcgctcatattgct RV cgccaccacgtttactttct	60	117
rplE	FW acgtgacttccaaggtgtttc RV tcctcgtcagtgttagcagtt	60	162

1 µg of total RNA was reverse transcribed using RevertAid First strand cDNA synthesis (ThermoScientific) using random primers (NZYTech). The experiment was performed using iQ SYBR 2× green supermix (Bio-Rad) and CFX96™ thermocycler (Bio-Rad) with the following cycling parameters: 10 min at 95 °C followed by 40 repeats of 5 s at 95 °C, 10 s at 60 °C, and finally 20 s at 72 °C. The cycle threshold values (C_T) were determined and the relative fold differences were calculated by the $2^{(C_T \text{ reference gene} - C_T \text{ target gene})}$ method [391] using 16S rRNA as the reference gene. Three independent experiments were run in triplicate. Statistical significance was analyzed using unpaired t-test (with GraphPad Prism version 6).

2.3. Results

2.3.1. Transcriptome analysis

We used RNA-seq technology to characterize the transcriptome of *S. epidermidis* biofilms with induced and prevented dormancy. Total RNA was isolated from three biologic replicates of cells within *S. epidermidis* biofilms grown in excess glucose with (prevented dormancy) or without Mg^{2+} (induced dormancy).

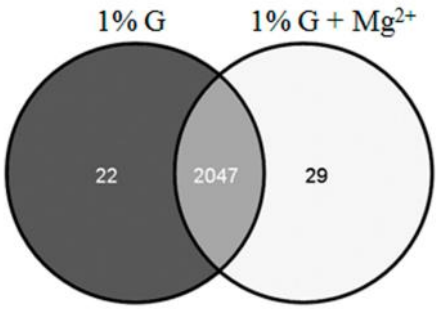

Figure 2.1.: Venn diagram summarizing the overlap between genes with RPKM value above 1.00 in *S. epidermidis* biofilms with prevented (1%G + Mg^{2+}) or induced (1%G) dormancy.

After depletion of rRNA, cDNA libraries were constructed using Illumina® TruSeq™ RNA Sample Preparation kit and MiSeq® sequencing system. An average of 3029921 (1%G) and 2650647 (1%G + Mg^{2+}) sequencing reads were obtained for each cDNA library. A global comparison of genes between biological triplicates showed a high correlation ($r > 0.98$, Pearson's Correlation Coefficient for induced dormancy and $r \geq 0.95$ for prevented dormancy). Prior to trimming the raw data, a Venn diagram was constructed (Figure 2.1) to summarize the overlap between genes expressed in each condition.

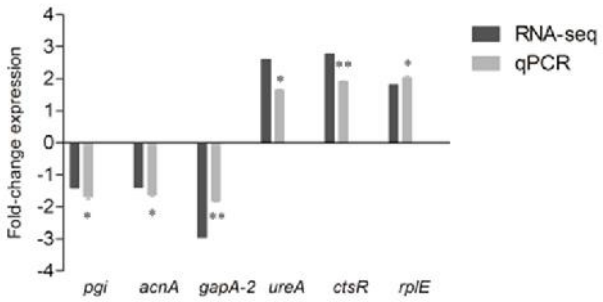

Figure 2.2.: qPCR validation of some differentially expressed genes. Fold-change expression is related to *S. epidermidis* biofilms grown in 1%G plus 20 mM of Mg^{2+}. The bars represent the mean and the error bars the standard deviation. Statistical significance was analyzed using unpaired *t*-test (GraphPad Prism v.6) $*p < 0.05$, $**p < 0.01$

The expressed genes uniquely found in each condition with a RPKM above 1.00 encoded many uncharacterized proteins with unknown functions. Nevertheless, among identified genes with higher RPKM values only found in *S. epidermidis* biofilms with induced dormancy was *mazE*, a gene which encodes an antitoxin component of a toxin-antitoxin module (*mazEF* module) and is related with the bacterial stress responses through mRNA degradation [392,393].

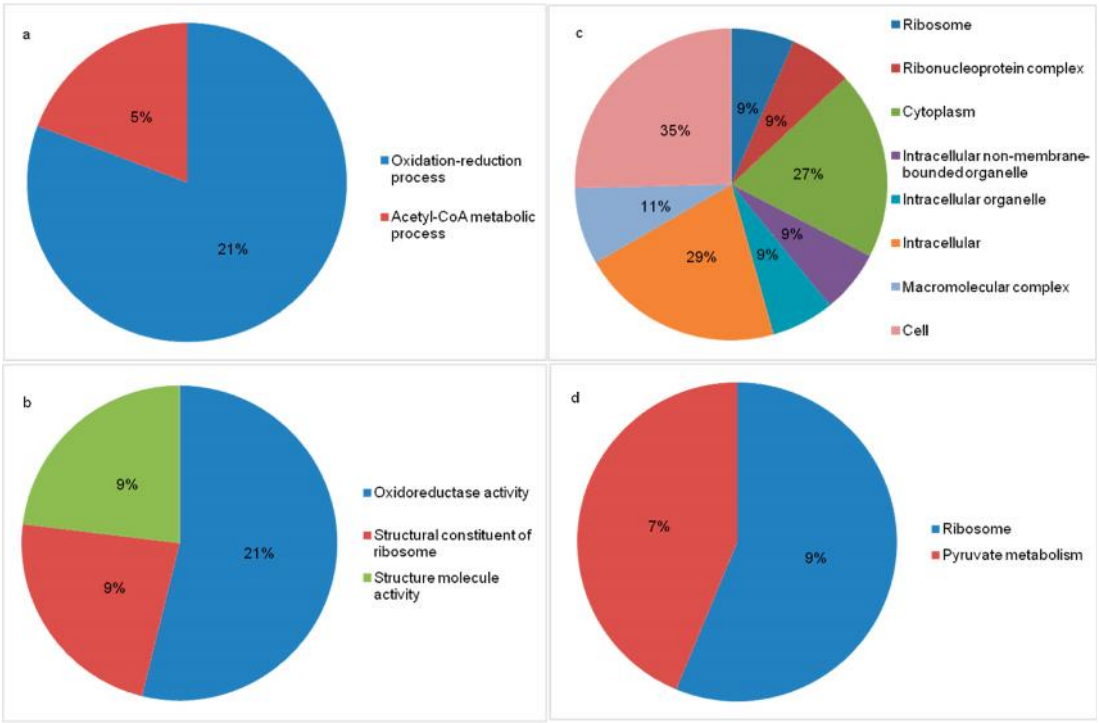

Figure 2.3.: Significant GO annotation and KEGG pathways of differentially expressed genes ($p < 0.05$, FDR corrected). Biological processes (a), molecular functions (b), cellular components (c) and KEGG pathways (d).

For downstream analysis the minimum limit for inclusion of a gene was a RPKM above 1.00 in both conditions. All genes with RPKM values under 1.00 were discarded. Thus, among the 2047 genes identified as being transcribed in both conditions, 147 genes (7%) were differentially expressed between *S. epidermidis* biofilms with prevented and induced dormancy, wherein 28 (1%) were up-regulated and 119 (6%) were down-regulated. Additionally, the distribution of the $\log_{10}$ RPKM among the differentially expressed genes of each biological triplicate also showed high agreement between the independent experiments, as

determined by a r^2 value near 1. To further validate our data, qPCR was performed using a selection of up, or down-regulated genes, as shown in Figure 2.2.

GO annotation was used to classify the functions of the 147 differentially expressed genes and a biological pathway-based analysis was performed using the KEGG pathway. All statistically significant categories are represented in Figure 2.3, including the oxidation-reduction and acetyl-CoA metabolic biological processes (Figure 2.3a), oxido-reductase functions and ribosome activity as the main molecular functions (Figure 2.3b). Cytoplasm and intracellular components were the main cellular components represented in the differently expressed genes (Figure 2.3c). The enriched KEGG pathways were for the ribosome synthesis pathway and pyruvate metabolism (Figure 2.3d).

In order to provide a more detailed analysis through pyruvate pathway, KEGG metabolic network was consulted and pyruvate cycle scheme was drawn to identify which enzymes involved in this pathway are encoded by differentially expressed genes (Figure 2.4).

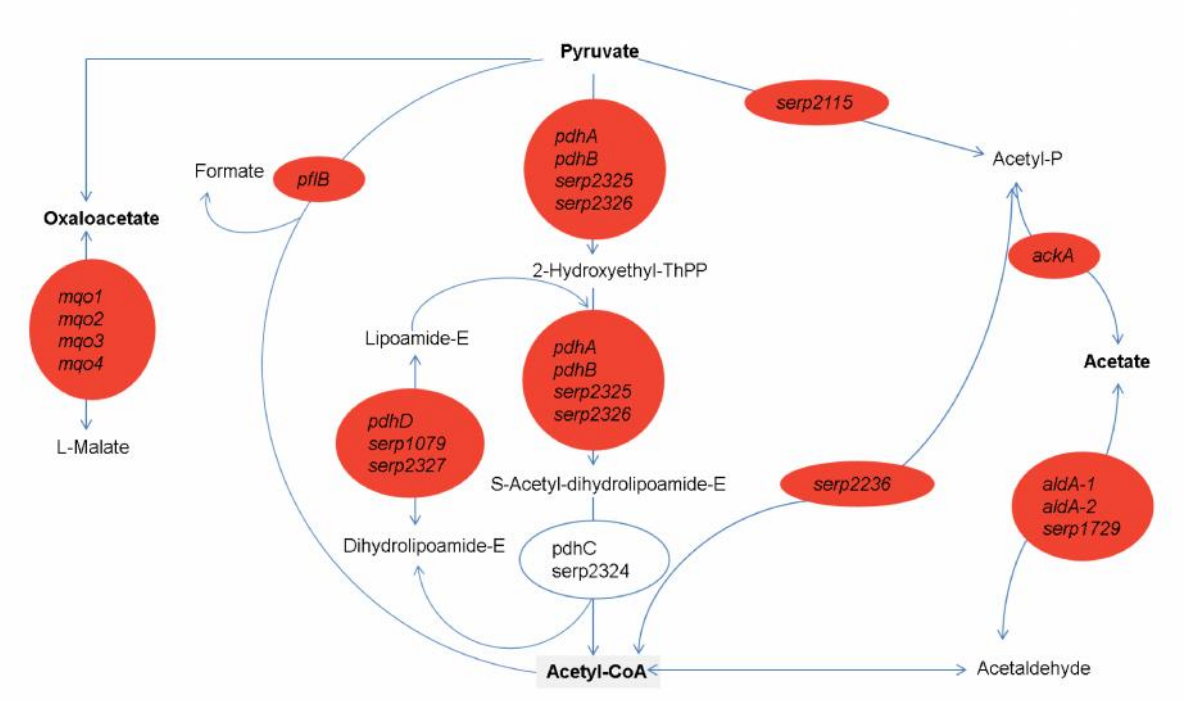

Figure 2.4.: Scheme of pyruvate metabolism and related products including genes which encode enzymes in each specific reaction. Red circles represent enzymes encoded by differentially expressed down-regulated genes in *S. epidermidis* biofilms with prevented dormancy.

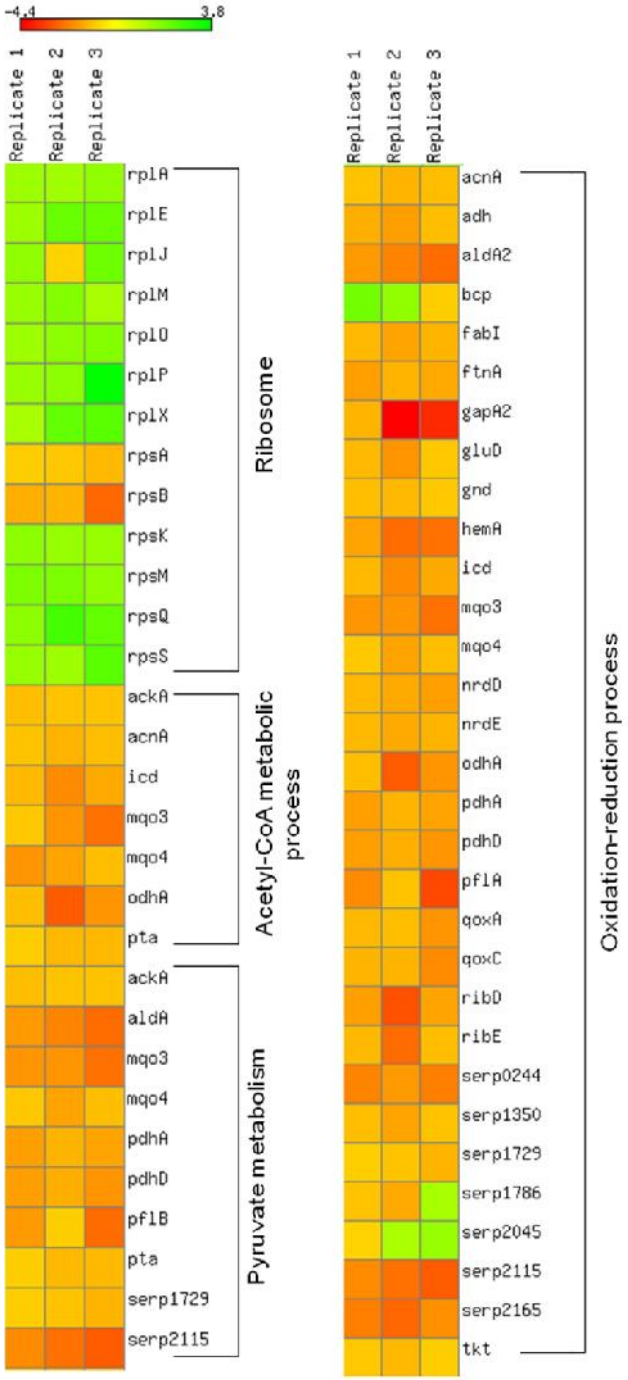

Figure 2.5.: Heat map of main biological processes and KEGG pathways statistical significant in differentially expressed genes between induced and prevented dormancy of *S. epidermidis* biofilms. Values represent fold-change expression among biological triplicates. Red and green colors correspond to decrease and increase expression, respectively. Heat map was constructed using Matrix2png.

Relevant biological processes and KEGG pathways within differentially expressed genes on replicates are shown in a heat map (Figure 2.5), constructed to depict fold-change values, by using Matrix2png [394]. Gene expression showed down-regulation of oxidation-reduction process, pyruvate metabolism and acetyl-CoA metabolic process in *S. epidermidis* biofilm with prevented dormancy compared to biofilms with induced dormancy. Conversely, pathways involved with ribosomes were up-regulated in biofilms with prevented dormancy. Twenty eight of the differentially expressed genes encoded uncharacterized proteins with unknown functions. In an attempt to find homology with known proteins, we performed a BLAST analysis, a search in the Pfam database (version 27.0) for Pfam domains [395] and PSORTb program (v. 3.0) [396] to predict their sub-cellular localization (Table 2.2). Despite BLAST analysis indicate some homology with genes encoding uncharacterized proteins from other species, most of the translated proteins would have a cytoplasm or cytoplasmic membrane localization, whereas 15 other proteins contained annotated domains in the Pfam database

indicating these were primarily domains with unknown functions. Table 2.3 lists the 10 most highly transcribed genes based on RPKM values from *S. epidermidis* biofilms with prevented dormancy.

Table 2.2.: Differentially expressed genes representing uncharacterized proteins with significant Pfam domain, including predicted localization by PSORTb.

Name	Function	Predicted localization	Protein family (Pfam) domain match
serp0087	Uncharacterized protein	Cytoplasm	Uncharacterized conserved protein
serp0121	Uncharacterized protein	Unknown	Protein of unknown function
serp0581	UPF0413 protein	Cytoplasm	Thioredoxin
serp0701	UPF0358 protein	Unknown	Protein of unknown function
serp0707	Uncharacterized protein	Cytoplasmic membrane	Protein of unknown function
serp0741	Uncharacterized N-acetyltransferase	Cytoplasm	Acetyltransferase (GNAT) domain
serp1053	Uncharacterized protein	Cytoplasmic membrane	Protein of unknown function
serp1180	Putative Holliday junction resolvase[a]	Unknown	Uncharacterized protein family
serp1210	Uncharacterized protein	Cytoplasm	Protein of unknown function
serp1402	Uncharacterized protein	Cytoplasmic membrane	Bacterial protein of unknown function
serp1720	UPF0340 protein	Cytoplasm	Protein of unknown function
serp1754	Uncharacterized protein	Cytoplasm	NADH(P)-binding
serp1771	Uncharacterized protein	Cytoplasmic membrane	Domain of unknown function: Predicted membrane-bound metal-dependent hydrolase
serp1783	Uncharacterized protein	Cytoplasmic membrane	Domain of unknown function: Small integral membrane protein
serp2066	Uncharacterized protein	Cytoplasm	Domain of unknown function: Uncharacterized protein conserved in bacteria

[a] Could be a nuclease that resolves Holliday junction intermediates in genetic recombination

Among the down-regulated and up-regulated transcripts in these *S. epidermidis* biofilms were genes mostly associated with oxidation-reduction and metabolic processes, respectively. Interestingly, most of the differentially expressed genes had a relatively small fold-change (-5.60 to 2.92), of which 13 of the up-regulated genes had a fold-change above 1.50 and 80 genes had a fold-change under -1.50.

Figure 2.6 shows RPKM values of a few *S. epidermidis* virulence genes [1] among the two distinct conditions.

Table 2.3.: List of 10 genes with higher RPKM values, among the differentially expressed genes, in *S. epidermidis* biofilms with prevented and induced dormancy. Only genes with a fold-change expression ≤ -1.50 or ≥ 1.50 were included. All genes had a FDR-corrected $*p < 0.05$

	Gene	Definition	RPKM 1%G	RPKM 1%G + Mg^{2+}	Fold-change
Rank down-regulated					
1	*serp1782*	Alkaline shock protein 23	18172.55	7034.27	-2.58
2	*serp0244*	Aldo/keto reductase	5885.19	2759.36	-2.13
3	*pdhD*	Dihydrolipoamide dehydrogenase	4352.90	2433.65	-1.79
4	*pdhA*	Pyruvate dehydrogenase complex E1 component, alpha subunit	3908.68	2361.75	-1.65
5	*recA*	Recombinase A	4788.63	1654.38	-2.89
6	*serp1784*	Uncharacterized protein	4967.70	1624.00	-3.06
7	*mqo-4*	Malate:quinone oxidoreductase	3457.27	1590.09	-2.17
8	*lexA*	LexA repressor	3307.97	1571.21	-2.11
9	*aldA-2*	Aldehyde dehydrogenase	3645.71	1569.85	-2.32
10	*ptsI*	Phosphoenolpyruvate-protein phosphotransferase	2156.90	1350.52	-1.60
Rank up-regulated					
1	*serp0419*	Ribosomal subunit interface protein	4191.31	12241.98	2.92
2	*ctsR*	CtsR family transcriptional regulator	2279.57	6328.79	2.78
3	*serp0163*	UvrB/UvrC domain-containing protein	1885.05	4870.32	2.58
4	*serp0164*	ATP:guanido phosphotransferase	1775.17	4289.27	2.42
5	*ppaC*	Manganese-dependent inorganic pyrophosphatase	443.67	861.75	1.94
6	*rpsM*	30S ribosomal protein S13	425.78	698.09	1.64
7	*serp1180*	Putative Holliday junction resolvase	368.63	583.88	1.58
8	*rplE*	50S ribosomal protein L5	279.81	505.85	1.81
9	*ureA*	Urease subunit gamma	182.26	473.11	2.60
10	*rpsQ*	30S ribosomal protein S17	225.01	456.69	2.03

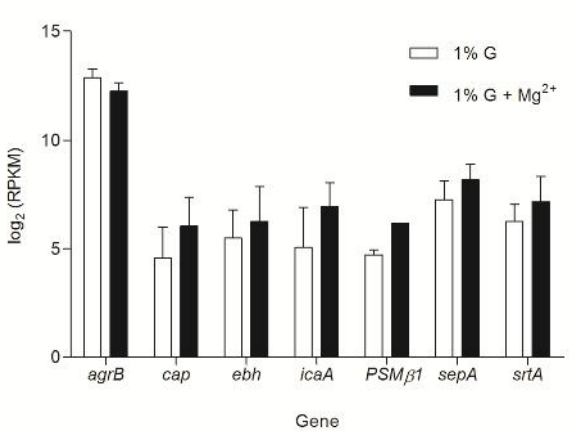

Figure 2.6.: Variation of log_2(RPKM) among some known virulence genes in *S. epidermidis* biofilms with prevented (1%G + Mg^{2+}) and induced (1%G) dormancy. The bars represent the mean and the error bars the standard error of the mean.

Although differences were not statistically significant, in general, virulence genes seemed to be slightly more transcribed in prevented dormancy.

2.3.2. Enrichment map analysis and cluster analysis

Downstream analysis of transcripts was based in direct and functional gene interactions with Cytoscape [397]. STRING was used to create a gene interaction network including all differentially expressed genes and neighbors, yielding a total of 1442 nodes and 10295 edges. The GO analysis from the gene set-enrichment is given in Table 2.4. Enrichment map analysis of the up-regulated and neighbor genes associated with *S. epidermidis* biofilms with prevented dormancy revealed distinct functions involved in translation and biosynthetic processes, such as macromolecular synthesis. Conversely, down-regulated genes encoded catabolic processes and oxidoreductase activity. Gene clusters were obtained in Cytoscape with MCODE plugin. Among the differently expressed genes we found 2 gene clusters with a score value above 2.0 and at least 4 nodes (Figure 2.7). The main biological processes of each cluster were in accordance with the results obtained in the gene set-enrichment.

Table 2.4.: Statistically significant biological processes from gene set-enrichment analysis.

Gene-set enrichment	Biological process	*p*-value *
Up-regulation		
GO:0006412	Translation	4.56E-15
GO:0044267	Cellular protein metabolic process	2.52E-10
GO:0010467	Gene expression	3.75E-09
GO:0034645	Cellular macromolecule biosynthetic process	4.68E-09
GO:0019538	Protein metabolic process	8.31E-09
GO:0009059	Macromolecule biosynthetic process	8.58E-09
GO:0044249	Cellular biosynthetic process	2.89E-05
GO:0015986	ATP synthesis coupled proton transport	7.83E-05
GO:0009058	Biosynthetic process	1.84E-04
GO:0006754	ATP biosynthetic process	5.53E-04
GO:0044260	Cellular macromolecule metabolic process	1.07E-03
GO:0043170	Macromolecule metabolic process	4.97E-03
GO:0009142	Nucleoside triphosphate biosynthetic process	1.14E-02
GO:0034220	Ion transmembrane transport	1.14E-02
GO:0015992	Proton transport	2.65E-02
Down-regulation		
GO:0008152	Metabolic process	9.76E-04
GO:0055114	Oxidation-reduction process	1.44E-03
GO:0006006	Glucose metabolic process	2.16E-02

* Bonferroni correction

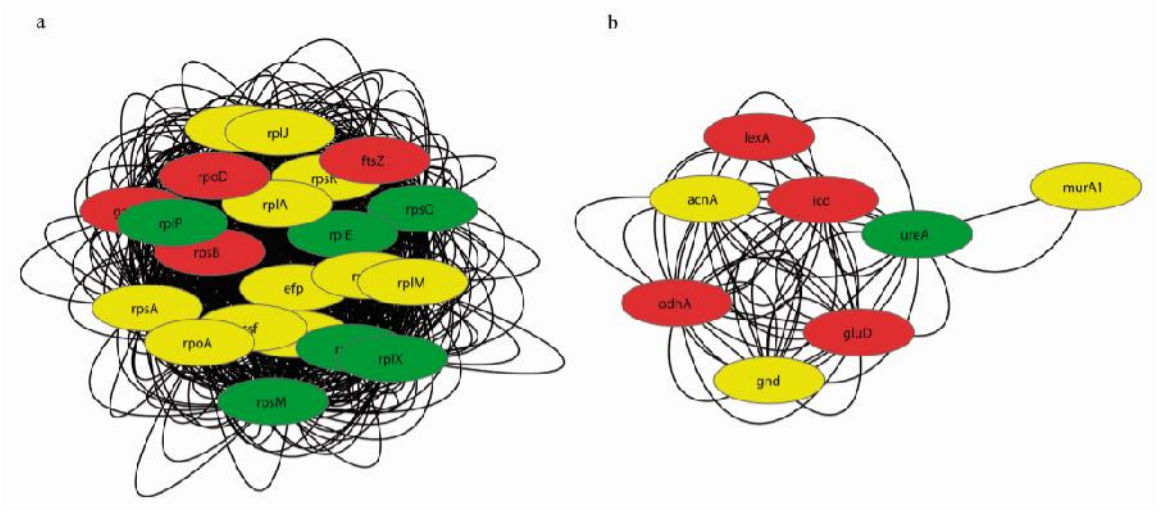

Figure 2.7.: Clusters generated by MCODE plug-in in Cytoscape, including fold-change expression. Red, green and yellow circles represent fold-change values under -1.50, above 1.50 and, between -1.50 and 1.50, respectively. Biological processes are translation and biosynthetic processes (a); and tricarboxylic acid cycle, acetyl-CoA catabolic process, coenzyme catabolic process, cofactor catabolic process, acetyl-CoA metabolic process, aerobic respiration, cellular respiration, energy derivation by oxidation of organic compounds, coenzyme metabolic processes (b)

2.4. Discussion

It has been reported that cells in the deeper layers of the biofilms are less active than the ones in the upper layers, although they are still viable but with a slow growth and a low metabolic rate [131,217]. Bacterial phenotypes can change according to environmental conditions and chemical-concentration gradients, generating distinct populations and contributing to heterogeneity within biofilms [215]. Dormancy is considered an adaptive response to adverse environmental conditions. Since the survival capacity of dormant bacteria in biofilm-related infections is increased [217], it is of utmost importance to characterize *S. epidermidis* biofilms dormant sub-population. In order to improve mRNA quantitation by RNA-seq technology, many transcriptomic studies have tried to increase the information content by depleting rRNA [398]. Previous bacterial RNA-seq studies suggest that a minimum of 2–5 million reads from an rRNA-depleted library are required for accurate coverage [398]. Moreover, paired-end sequencing is a more efficient strategy to characterize and quantify the transcriptome of bacteria without annotated genomes [399]. Besides biological variability, technical issues like coverage [378], sequence depth [400], variation from one flow cell to another [399], variation between the individual lanes within a flow cell [399] and library preparation effect [401] may also affect sequencing and cause variability within replicates. In order to overcome these limitations, we ran 3 biological replicates consisting of pools of 4 biofilms for each replicate in order to obtain more accurate results. This approach contributed to the observed consistency in our data.

GO analysis has been used in genome-wide expression studies to reduce complexity and highlight biological processes [402]. GO analysis of the 147 differentially expressed genes between the two studied conditions identified ribosome activity, oxidation-reduction and acetyl-CoA metabolic processes as the major classes with differences in mRNA transcripts comparing biofilms with induced and prevented dormancy. The main biological processes and KEGG pathways showed consistency within gene expression levels among biological triplicates and evidence that genes involved in oxidation-reduction, acetyl-CoA metabolic processes and pyruvate metabolism were less expressed in biofilms

with prevented dormancy as opposed to when their development did not include addition of Mg^{2+} to the culture medium. In the opposite, ribosome pathway was more expressed in *S. epidermidis* biofilms with prevented dormancy. Differentially expressed genes encoded transcripts mostly localized to the intracellular compartments, suggesting that the major changes related to the development of dormancy are occurring within the cell cytoplasm. These results were expected since *S. epidermidis* biofilms grown in the presence of Mg^{2+} have a lower number of dormant bacteria and consequently more metabolically active cells. In addition, half of the most down-regulated gene transcripts in prevented dormancy are involved in oxido-reductase activity, such as *serp0244*, *pdhA*, *pdhD*, *aldA* and *mqo3* (Table 2.3). Conversely, the most up-regulated genes are mainly related to metabolic processes. Previously, the impact of virulence of dormant *S. epidermidis* biofilm was demonstrated using *in vitro* and *in vivo* models [138,296]. In the present work, biofilms with prevented dormancy had an increased, but not significant, expression of some of the main virulence genes.

Glucose has a key role in biofilm physiology [96,380]. The major pathways for glucose catabolism in Staphylococci are glycolysis, the pentose-phosphate pathways and the TCA cycle [366]. Recently, it was proposed a metabolic-based strategy to target and eradicate bacterial persisters. Allison *et al.* showed that carbon source metabolites (such as glucose, mannitol and fructose) may be used to generate proton motive forces, potentiating the aminoglycosides activity against persister cells [403]. Similarly, Peng *et al.* showed the use of exogenous glucose and alanine to stimulate the central metabolic pathways and therefore increase the uptake of antibiotic through resistant bacteria [404]. Not surprisingly, glucose has influence in biofilm physiology since glucose metabolism leads to accumulation of acidic products decreasing the pH of the culture medium [405]. The model that we previously described showed that dormancy was a consequence of excess of glucose catabolism that lead to the acidification of the culture medium and consequently to the accumulation of dormant bacteria within *S. epidermidis* biofilm [138]. This phenomenon was prevented by the presence of high extracellular levels of magnesium which did not change the pH of the culture medium, indicating that the Mg^{2+} effect on preventing cell dormancy was not related to the pH of the culture, since low pH was still maintained in the presence of Mg^{2+} [406].

Low pH was previously associated with a loss of activity of glycolytic enzymes and damage to cell membrane and macromolecules such as proteins and DNA, and was related to changes in biogenesis and maintenance of cell membrane integrity [407]. Additionally, it was shown that pH-mediated potentiation of aminoglycosides is effective against both *in vitro* and *in vivo* Gram-positive of nosocomial pathogens [408].

Piddington *et al.* demonstrated that Mg^{2+} is essential to *M. tuberculosis* growth in acidic conditions and it could not be replaced by other divalent cations [409]. A possible influence of Mg^{2+} in protein structure and physical properties was also described in *M. tuberculosis* [410]. Interestingly, Mg^{2+} levels in biofilm cultures were maintained over time [406]. In addition, genes related to the Mg^{2+} transport system, such as *corA*, *mgtE* and *serp1967* [411] were not differentially expressed, suggesting that high Mg^{2+} levels culture are also not stimulating expression of magnesium transport genes.

Previous work with dormant bacterial cultures demonstrated decreased levels of macromolecule synthesis, nutrient transport and respiration rates [260]. Moreover, earlier transcriptomic studies of dormant persister cells of several microorganisms showed a down-regulation of energy and metabolism pathways [233], increased reactive oxygen species levels [412] and down-regulation of genes involved in energy production [229]. Toxin overexpression from toxin-antitoxin modules has been linked to the persister fraction in *E. coli* and *M. tuberculosis* cultures [413]. The toxin-antitoxin module *mazEF* is highly conserved in *Staphylococcus* species and was described in detail in *S. aureus*, where antitoxin MazE binds toxin MazF to neutralize ribonucleases activity [414]. Despite no alterations in *mazF* expression between S. *epidermidis* biofilms grown in the absence or presence of Mg^{2+}, the *mazE* gene had a RPKM superior to 1.00 in dormant biofilms.

Taken together, these results reveal that transcription of genes involved in oxidation-reduction processes and glucose metabolism, including the acetyl-CoA and pyruvate pathways, was more active in dormant *S. epidermidis* biofilms, despite no differences in glucose consumption, probably due to lower metabolism of dormant bacteria. On the other hand, genes related to ribosome activity were up-regulated in *S. epidermidis* biofilms when Mg^{2+} levels were higher in the growth medium. Additionally, these results provide evidence that the genes directly

involved in Mg^{2+} transport do not seem to have a direct role in the shift to dormancy. The global changes found in this work do not provide information concerning gene expression confined only to the dormant bacterial cells. Most probably, differences among culturable and dormant bacterial cells are greater than those referenced here. Single-cell analysis methods are being developed and may allow a more accurate comparison between these physiological states [415]. Yet, dormant bacterial cell isolation is still a meaningful challenge.

Overall, these results contribute to a better understanding of the mechanisms underlying the dormancy phenomena. Nevertheless, these findings also raise questions regarding the role of dormant *S. epidermidis* biofilms in virulence, since it is still debatable if there is a lower virulence potential due to lower metabolism or if expression of a specific gene or a set of genes could lead to the suppression of the host immune response.

CHAPTER 3.

COMPARATIVE PROTEOMIC AND TRANSCRIPTOMIC PROFILE OF *S. EPIDERMIDIS* BIOFILMS GROWN IN GLUCOSE-ENRICHED MEDIUM

COMPARATIVE PROTEOMIC AND TRANSCRIPTOMIC PROFILE OF *S. EPIDERMIDIS* BIOFILMS GROWN IN GLUCOSE-ENRICHED MEDIUM

Summary

Contrary to some advances made in the transcriptomic field, proteome characterization of *S. epidermidis* biofilms is less developed. To highlight the relation between transcripts and proteins of *S. epidermidis* biofilms, we analyzed the proteomic profile obtained by two mechanical lysis methods (sonication and bead beating), associated with two distinct detergent extraction buffers, namely SDS and CHAPS. Based on gel electrophoresis-LC-MS/MS, we identified a total of 453 proteins. While lysis with glass beads provided greater amounts of protein, CHAPS extraction buffer allowed identification of a higher number of proteins compared to SDS. Our data shows the impact of different protein isolation methods in the characterization of the *S. epidermidis* biofilm proteome. Furthermore, the correlation between proteomic and transcriptomic profiles was evaluated. The results confirmed that proteomic and transcriptomic data should be analyzed simultaneously in order to have a comprehensive understanding of a specific microbiological condition.

3.1. Introduction

The availability of the complete genome of *S. epidermidis* strains RP62A (ATCC 35984) [411] and ATCC 12228 [416] has led to the development of proteomic studies, since it has been suggested that the genome sequence is not sufficient to elucidate the biological functions of an organism [417]. Although there have been major advances in the molecular characterization of the pathogenic mechanisms of *S. epidermidis* biofilms [1], much less is known regarding the proteome. A few proteomic studies, however, have been performed in order to identify specific features associated with the pathogenicity and physiology [418-421]. In *S. epidermidis*, 2-Dimensional Electrophoresis (2-DE) analysis of proteomic patterns showed several differentially expressed proteins when comparing commensal and invasive strains [418]. To ensure high quality and reliable proteomic results, an appropriate sample preparation is fundamental [422,423]. Due to the complex structure of biofilms [46], it is necessary to develop an effective lysis method in order to obtain maximum coverage of the biofilm proteome and minimal protein losses, similar to the approach optimized for total RNA extraction from *S. epidermidis* biofilms [424]. Different protein extraction methods, including enzymatic, chemical, mechanical and other methods available via commercial extraction kits have been tested to obtain the highest number of proteins in *Staphylococcus* spp. [97,425]. Although the majority of these studies were performed with cell suspensions, relatively harsh techniques have been shown to be rapid and efficient to disrupt and lyse biofilms of Gram-positive bacteria, such as mechanical methods like bead beating with glass beads (FastPrep) or sonication [97,426,427]. Often, to optimize protein recovery, enzymes and detergents may be used in conjugation with mechanical lysis. Since lysostaphin efficiently cleaves staphylococcal cell wall peptidoglycan [428,429], it may be used to disrupt staphylococcal biofilms [430]. However, due to high costs of lysostaphin, detergents are frequently used to enhance protein isolation and solubilisation [431].

In an attempt to determine the relation between protein and mRNA levels, several studies have shown that often the correlation is surprisingly low, and differs widely among organisms [432]. Correlation coefficients were found to vary from 0.09 to

0.46 in multi-cellular organisms, from 0.34 to 0.87 in yeasts, whereas in bacteria the correlations ranged from 0.20 to 0.47 [433]. Up to now, no correlation analysis between the transcriptome and proteome of *S. epidermidis* biofilms has been conducted.

Hence, herein, since distinct lysis and extraction methods may yield different protein recoveries, we assessed different lysis methods to obtain proteins from *S. epidermidis* biofilms grown in glucose-enriched medium. Then, we compared the proteomic profile with the gene expression profile obtained by RNA-seq technology. We undertook a gel-based method to determine protein isolation efficiency, using total protein extracted with a detergent-based extraction (SDS (sodium dodecyl sulfate) or CHAPS ((3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate))) coupled with mechanical lysis (sonication or bead beating). A detailed analysis of proteomic data was performed in each condition. Label-free relative protein abundance, determined by exponentially modified protein abundance index (emPAI) was used for the relative quantitation of the proteome and was compared to transcriptomic profile. The overall goal was to characterize and correlate both proteomic and transcriptomic profiles of *S. epidermidis* biofilm-grown cells.

3.2. Materials and methods

3.2.1. Growth conditions

Biofilm forming *S. epidermidis* strain 9142 was used as a model [380]. One colony was inoculated into 1 mL of TSB (Liofilchem) and incubated at 37 ºC at 120 rpm for 18 h. The overnight culture was adjusted to an optical density at 640 nm of 0.250 (±0.05) with TSB and 10 µL of the suspension was transferred into a 24-well plate (Thermo Fisher Scientific) containing 1 mL of TSB supplemented with 0.4% glucose (v/v) (Thermo Fisher Scientific). The culture plate was then incubated at 37 ºC at 120 rpm for 24 h. After this period, the culture medium covering the biofilm was removed and replaced by fresh TSB supplemented with 1% glucose (v/v). Biofilms were allowed to grow in these same conditions for 24 additional

hours. Thereafter, biofilm culture medium was removed and biofilms were washed twice with PBS.

3.2.2. Protein preparation

The same number of biofilms (twelve biofilms for each condition) were directly scraped and suspended in detergent extraction buffers: 25 mM Tris-HCl (pH = 7.2) (Pharmacia Biotech), 10 mM CHAPS (Sigma-Aldrich), 0.5 M NaCl (VWR), 5% glycerol (Sigma-Aldrich) and 1 mM PMSF (Sigma-Aldrich) or 60 mM Tris-HCl (pH = 6.8), 10% glycerol, 5% SDS (USB Corporation) and 1 mM PMSF. Mechanical lyses was performed in a sonicator (Cole-Parmer® 750-Watt Ultrasonic Homogenizer, Cole Parmer) (10 min, 30 s running, 10 s pause, 40% amplitude) or by bead beating, using glass beads of 0.1 mm (Sigma-Aldrich) in a FastPrep® cell disruptor (BIO 101, ThermoElectron Corporation, Thermo Fisher Scientific) (3 cycles of 30 s and 6.5 m/s). After lyses, cell debris were removed by centrifugation (15,000 *g* for 15 min at 4 ºC) and proteins precipitated with cold acetone [434]. Then, protein quantification was performed using the RC-DC assay (Bio-Rad), following the manufacturer's instructions.

3.2.3. One-dimensional gel electrophoresis (SDS-PAGE) in gel protein digestion and protein identification

Forty µg of protein were incubated with SDS 10% (w/v), 0.5 M Tris-HCl (pH 6.8), glycerol, mercaptoethanol and bromophenol blue (w/v) for 5 min at 100 ºC. Then, samples were loaded on Novex NuPAGE® 4-12% Bis-Tris gel (Life Tecnologies) and proteins were separated at a constant voltage (200 V). The gel was stained with colloidal Coomassie G-250 and all the lanes were manually excised into 16 gel slices for in-gel digestion with trypsin TPCK (AB Sciex). Peptide extraction was made with 10% formic acid/ 50% acetonitrile. Dried peptides were dissolved in 5% acetonitrile (ACN) (VWR), 0.1% formic acid (Sigma-Aldrich) and 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich). Separation of tryptic peptides by nano-HPLC was performed on the module separation Ultimate 3000 (Dionex, Thermo Fisher Scientific) using a capillary column (Pepmap100 C18; 3 µm particle size, 0.75 µm internal diameter, 15 cm in length). A gradient of solvent A,

(water/ACN/TFA (98:2:0.05, v/v/v)) to solvent B (water/ACN/TFA (10:90:0.045, v/v/v)) was used. The separation of 2 µg/µL sample was performed using a linear gradient (5-50% B for 30 min, 50-70% B for 10 min and 70-5% A for 5 min) with a flow rate of 0.3 µL/min. The eluted peptides were mixed with a continuous flow of α-cyano-4-hydroxycinnamic acid (αCHCA) matrix solution (270 nL/min, 2 mg/mL in 70% ACN/0.1% TFA and internal standard Glu-Fib at 15 ftmol) and applied directly on a MALDI plate in 20 s fractions using an automatic fraction collector Probot (Dionex, Thermo Fisher Scientific). Mass spectra were obtained on a matrix assisted laser desorption/ionization–time of flight MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems) in the positive ion reflector mode and obtained over the mass range from 700-4500 Da with 900 laser shots. A fragmentation voltage of 2 kV was used throughout the automated runs. The spectra were processed and analyzed by the T2S (v1.0, Matrix Science Ltd.) and submitted in Mascot software (v.2.3.0.2, Matrix Science Ltd.) for protein/peptide identification based on MS/MS data using the following criteria: trypsin as enzyme; a maximum of two missed cleavages; mass tolerances of 40 ppm for peptide precursors, mass tolerance of 0.6 Da was set for fragment ions. Simultaneously, phosphorylation of Threonine (T), Serine (S) and Tyrosine (Y) were searched as variable modifications. The local FDR was calculated by searching the spectra against SwissProt (*Firmicutes*, release date 06.02.2013) decoy (random) database. Protein identification was considered reliable when the individual ion score for each peptide had a minimum individual score of 95% and a minimum sequence tag of four aminoacids. Relative quantitation was determined by emPAI. Gene ontology [385] analysis was performed with proteins identified in each extraction condition using STRING database (version 9.1) [387] (statistically significant specific GO terms, FDR-adjust $p < 0.05$). Two independent experiments were performed with pools of *S. epidermidis* biofilms.

3.2.4. RNA sequencing

RNA extraction from *S. epidermidis* biofilms, cDNA library preparation and RNA-seq, were performed as described in Chapter 2. Gene expression was normalized by calculating RPKM using the methods described by Mortazavi and colleagues [381], in which normalization is adjusted by the counts of reads per kilobase per

million mapped and gene length. All genes with RPKM value under 1.00 were not considered for analysis. Three independent experiments, each one from a pool of four biofilms, were performed.

3.3. Results and Discussion

3.3.1. Proteomic characterization

In the present study, a proteomic and a transcriptomic analysis of *S. epidermidis* biofilms grown in glucose-enriched medium was performed using gel-based proteomic separation and LC-MS/MS analysis along with RNA-seq technology, respectively. The proteomic profile obtained is highly dependent on the extraction procedure used, with quickness, handling and robustness of protein extraction methodologies being important criteria [425,435-437]. Before protein concentration, we used SDS-PAGE to compare the lysis efficiency of sonication and glass bead disruption of *S. epidermidis* biofilm cells (Figure 3.1).

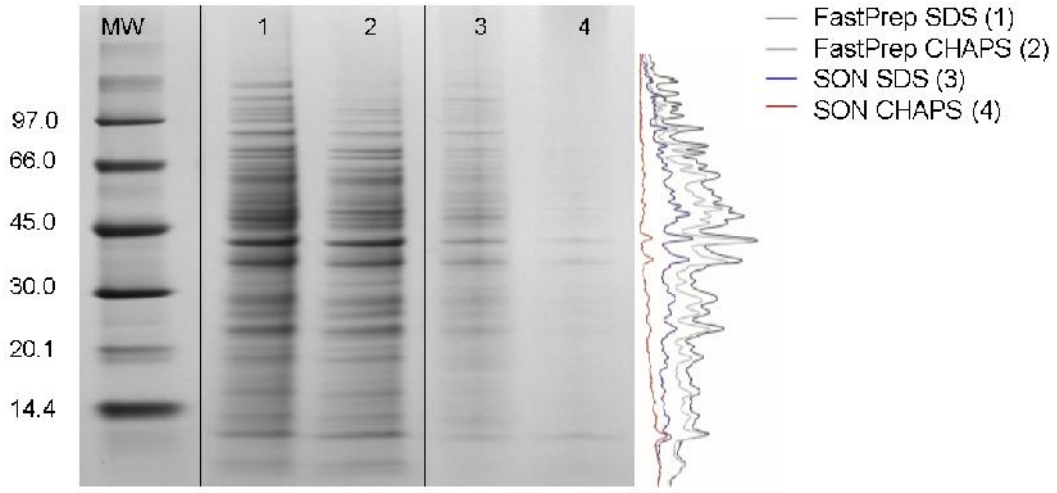

Figure 3.1.: SDS-PAGE before protein concentration and respective optical density traces. 1 - Protein profile obtained with FastPrep and SDS buffer extraction; 2 - Protein profile obtained with FastPrep and CHAPS buffer extraction; 3 - Protein profile obtained with sonication and SDS buffer extraction; 4 - Protein profile obtained with sonication and CHAPS buffer extraction. MW – molecular weight (KDa).

Cellular lysis by the glass bead disruption method produced a higher protein yield compared to the performed sonication cycle. Due to sample and protein

properties, precipitation may be required as a treatment to reduce or eliminate substances which could interfere with protein identification, as well as to concentrate proteins [438]. However, it is known that protein precipitation leads to specific changes in the protein composition of proteomic samples. These modifications depend, mainly, on the specific structure of the protein rather than the precipitating agent used [439]. Nevertheless, proteins recovered in each extraction method were precipitated, solubilised and quantified, followed by SDS-PAGE and LC-MS/MS analysis.

A total of 453 *S. epidermidis* proteins were identified, which corresponds to 17.96% of the predicted proteome (total of 2522 for *S. epidermidis* RP62A strain in UniProtKB database, at 29.11.2013) with an increased confidence (FDR was below 5.5% among replicates). Venn diagram [384] demonstrated that 158 proteins were common to all extraction conditions (Figure 3.2) .

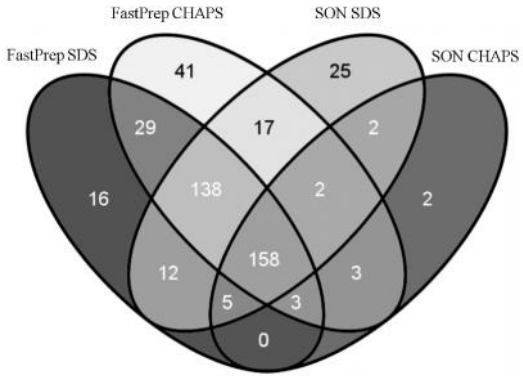

Figure 3.2.: Overlap of proteins found in each tested condition determined using Venn diagram. FastPrep – extraction with bead beating; SON – sonication.

A total of 361 proteins were identified in FastPrep lysates with SDS, 391 in FastPrep with CHAPS, 359 in sonication lyses with SDS and 175 proteins after sonication in the CHAPS extraction buffer. The FastPrep protocol seemed to be the most efficient to lyse cells within *S. epidermidis* biofilms. Additionally, the CHAPS extraction buffer resulted in the identification of a higher number of proteins, compared to the SDS extraction buffer. Interestingly, this last finding, diverges from the work described by Encheva *el al.* [440], where it was shown that SDS gave a higher protein yield as compared to CHAPS, in an analysis of the

Streptococcus pneumoniae proteome. SDS is an anionic detergent, used as a surfactant in protein denaturation, breaking lipid-lipid interactions and lipid-protein interactions rather than protein-protein interactions [441]. SDS is known to be very effective at solubilising almost all proteins [442,443]. However, it denatures them, causing loss of their native conformations and functions [444]. Alternatively, zwitterionic detergents such as CHAPS minimize protein-protein interactions [431,445]. Differences in extraction efficiency support the importance of extraction buffer to optimize protein yield or to increase yields of groups of proteins.

Next, GO annotation, incorporating molecular function, biological processes and cellular component of proteins recovered from each extraction condition was analyzed. The main differences were observed in molecular function enriched categories (Figure 3.3).

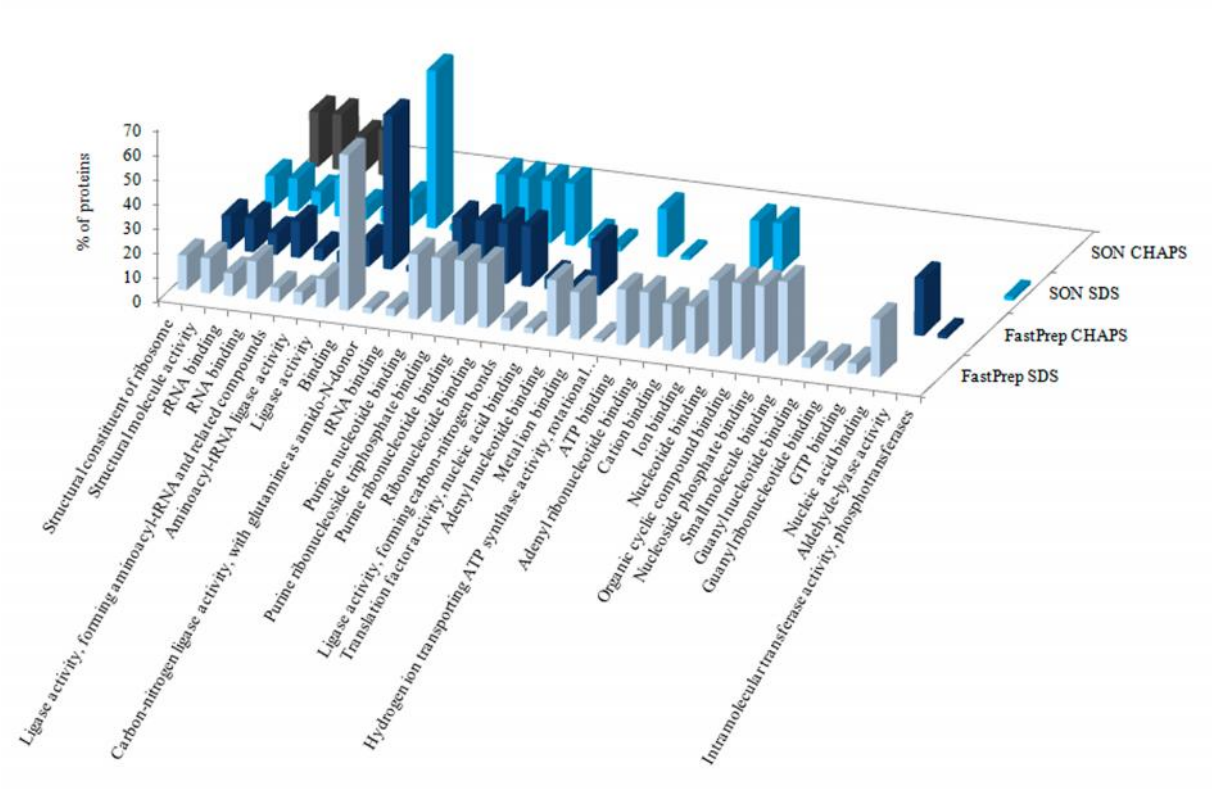

Figure 3.3.: GO classification. Molecular function specific terms with statistically significant enrichment ($p < 0.05$, FDR-corrected).

The GO terms adenyl nucleotide binding (GO:0030554) and nucleic acid binding (GO:0003676) were only found in protein extraction performed with FastPrep lysis. Similarly, the GO terms for metal ion binding (GO:0046872), cation binding

(GO:0043169) and ion binding (GO:0043167) were only found enriched in extractions performed with SDS buffer. These findings may be explained by the interaction established by SDS and cationic metal ions, due to the anionic head group of SDS [446]. While some studies showed that mechanical disruption of bacterial cells enhances the identification of cytoplasmic proteins [447,448], a number of cell membrane, secreted and cell surface proteins were also identified here. Membrane proteins are hydrophobic and they are present in low levels, since they are unstable and often aggregate, causing problems in analyzing membrane protein extractions [449]. However, within a heterogeneous protein properties set, it is difficult to find the conditions which will solubilise all proteins, including most membrane proteins, since not all are equally hydrophobic [450].

In an attempt to search for a methodological impact in the intrinsic properties of identified proteins, GRAVY score (grand average of hydropathy), molecular weight and isoelectric point (p*I*) were analyzed (Figure 3.4).

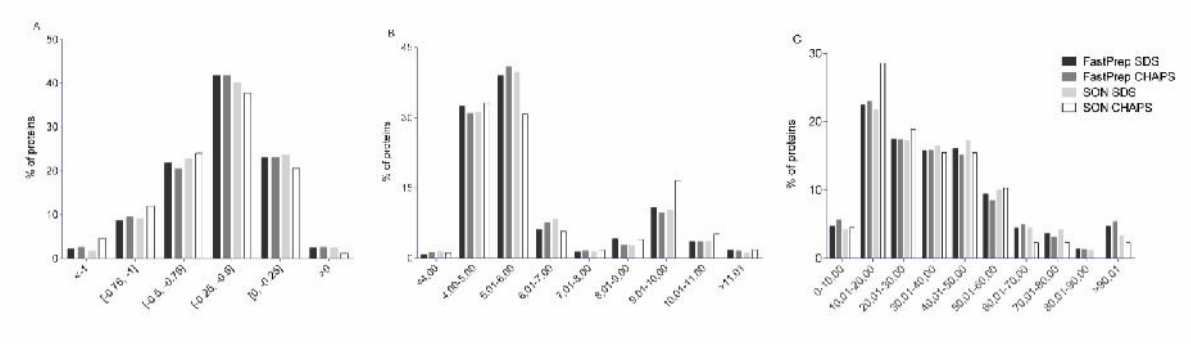

Figure 3.4.: Intrinsic properties of proteins. GRAVY (A), p*I* (B) and molecular weight (C) for each condition.

Overall, p*I*, protein molecular weight and GRAVY score presented a similar tendency, among the tested conditions, with a few exceptions. Most of the identified proteins had a GRAVY score between -0.5 and -0.25 (Figure 3.4A). CHAPS extraction buffer allowed separation of a higher number of hydrophilic proteins (GRAVY score lower than -1.00) compared to SDS extraction buffer. Although gel-based approaches, e.g. SDS-PAGE, may allow recovery of a significantly higher number of hydrophobic proteins, these extraction methods seemed to be less efficient at isolating hydrophobic proteins (positive GRAVY score). However, a few cell membrane proteins were identified, despite having

high hydrophobicity. Our approach allowed us to identify only 3.3% of hydrophobic proteins. In proteins inferred from homology or with evidence at protein level (total of 997 proteins in UniProtKB/Swiss-Prot at 29.11.2013), the number of hydrophobic proteins corresponds to 13.04%. Concerning p*I*, most of the isolated proteins showed a value between 4-6 or 9-10, similar to proteins inferred from homology or with evidence at protein level. More than 60% of proteins had a p*I* between 4 and 6 (Figure 3.4B). Gumber et *al.* [451] showed that non-ionic detergents, such as Tween20 and Triton X-100 were more efficient at extracting proteins with high p*I*. Conversely, CHAPS associated with urea was shown to extract more proteins with low p*I* [451]. Regarding molecular weight, most of the proteins presented a mass between 10 and 50 KDa (Figure 3.4C), which is also the most common range among the proteins inferred from homology or with evidence at the protein level. The FastPrep protocol allowed separation of higher numbers of proteins with more than 90 KDa compared to sonication. Moreover, the CHAPS extraction buffer combined with FastPrep seemed to be more efficient than SDS in obtaining proteins of less than 10 KDa. Independent of the methodological approach, a predominance of hydrophilic proteins, with 10-50 KDa and a p*I* between 4 and 6 was observed. Overall, major differences were not clearly found in molecular weight, GRAVY and p*I*, and only the GO analysis showed evident differences between SDS and CHAPS detergent extraction buffers.

3.3.2. Transcriptomic characterization

Whole transcriptomic profile of *S. epidermidis* biofilm cells was obtained by RNA-seq. This methodology represents a valuable method to measure mRNA expression levels, since RPKM normalization allows comparison of transcript levels among and within samples [381]. A total of 2069 genes were found with a RPKM value above 1.00, which corresponds to 77.72% of the *S. epidermidis* genome (total of 2662 for RP62A strain in GenBank). More than 50% of transcripts presented RPKM values up to 250. The most represented GO slim terms in each RPKM group (RPKM < 150, 151 > RPKM < 250 and RPKM > 250) are represented in Figure 3.5.

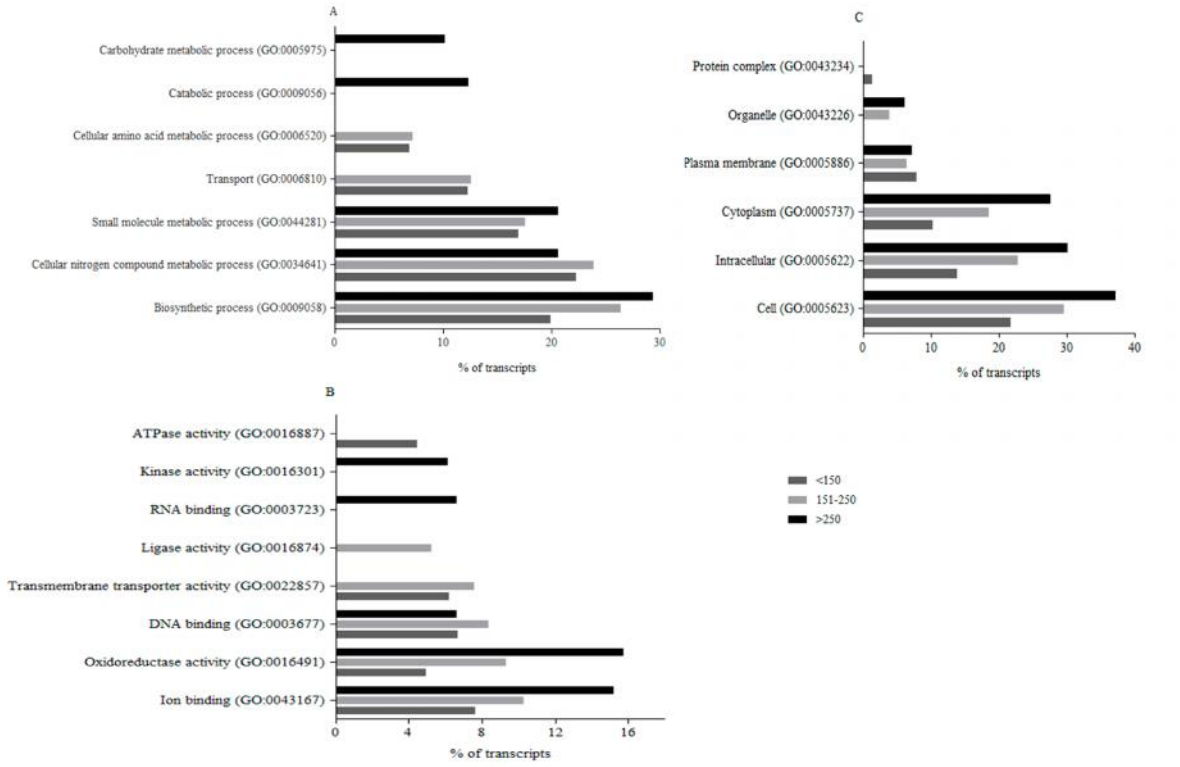

Figure 3.5.: GO classification of RNA-seq data. Five most represented GO slim terms of biological processes (A), molecular functions (B) and cellular components (C).

Additionally to common GO terms, RPKM values above 250 showed a predominance of catabolic (GO:0009056) and carbohydrate metabolic processes (GO:0005975) within the biological process GO category (Figure 3.5A). In contrast, transcripts with RPKM lower than 250 were associated with transport (GO:0006810) and cellular amino acid metabolic processes (GO:0006520), in addition to common GO terms. Molecular functions related to RNA binding (GO:0003723) and kinase activity (GO:0016301) were only found in transcripts with RPKM higher than 250 (Figure 3.5B). Regarding cellular components (Figure 3.5C), protein complex category (GO:0043234) was amongst the most represented transcripts with RPKM lower than 150. Amongst the 10 most expressed transcripts are genes involved in response to stimulus, translation, catabolic processes and non-coding RNAs (Table 3.1).

Table 3.1.: List of the 10 genes with higher RPKM values found in transcriptomic analysis and respective function.

Gene	RPKM	Gene function
SERP_SeSRP1	299510.4	Non-coding RNA involved in protein export
SERP_SetmRNA1	372738.7	Transfer-messenger RNA
SERP1273	28468.9	Universal stress protein
SERP2379	27311.7	Diacetyl reductase [(S)-acetoin forming] which catalyzes the irreversible reduction of 2,3-butanediol to (S)-acetoin in the presence of NADH
asp23	24019.2	Alkaline shock protein 23 which may play a key role in alkaline pH tolerance
arcC	11927.2	Carbamate kinase
SERP2220	13204.3	Universal stress protein
SERP_SernpB1	15829.8	Non-coding RNA
spoVG	13016.0	Septation protein
SERP2343	11865.1	Uncharacterized protein

These were expected results since during growth, bacteria need to obtain energy. Moreover, increased expression of certain classes of genes may be related to an adaptive response [110,452-455]. It is well known that glucose has a key role in biofilm physiology since is generally used to promote biofilm growth *in vitro* [96,380]. However, high concentrations of glucose affect biofilm gene expression [380].

3.3.3. Comparative proteomic and transcriptomic analysis

In order to compare proteomic and transcriptomic profiles, mass spectrometry and RNA-seq data were compared. Large scale proteomic experiments have been conducted using emPAI to determine, in a single LC-MS/MS experiment, the relative quantitation of proteins based on protein coverage by the peptide matches in a database search result [456-458]. Since the emPAI value has a linear correlation with protein concentration, it allows a more accurate estimation of protein abundance, as compared to simple peptide or spectral count [458,459]. In order to obtain an estimation of protein abundance, two independent experiments were performed. The emPAI values presented the same tendency among proteins found in each replicate, as indicated by Pearson correlation (Pearson's r correlation), which varied between 0.81 and 0.99. Based on these results, we

chose to calculate the emPAI average for further analysis. Extremely abundant proteins may affect the efficiency of protein identification because of ionization suppression and detector saturation as well as the limited loading capacity of LC columns [458]. We observed that bead beating lysis led to a lower percentage of identified proteins with low emPAI (0-0.20) compared to those identified following sonication (Figure 3.6).

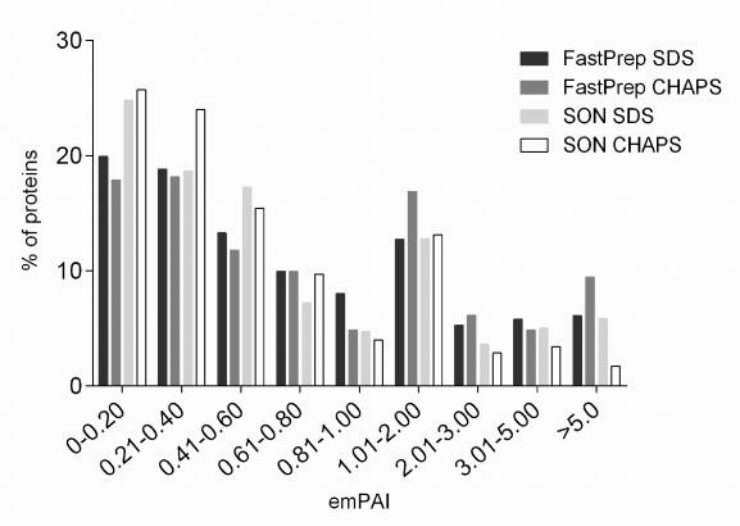

Figure 3.6.: emPAI distribution among the distinct conditions.

Moreover, a combination of bead beating and CHAPS extraction buffer showed more proteins with higher emPAI values (> 5.00). Protein separation by SDS-PAGE and *in-gel* digestion decreased sample complexity and, consequently, contributed to avoiding suppression by extremely abundant proteins. Since proteins with similar abundances may functionally be correlated, proteins common to all conditions were further analyzed using CLUSTER 3.0 [460] (Figure 3.7). We found four main clusters which resulted from the hierarchical clustering (two main biological processes are represented in Figure 3.7.). Proteins with lower emPAI tended to be involved in biosynthetic and cellular nitrogen compound metabolic processes (cluster A and D). Clusters B and C include mainly proteins with higher emPAI values. Protein cluster B is mainly related to biosynthetic processes and translation. Protein cluster C is related to biosynthetic and catabolic processes. Catabolic process was also found as one of the most prevalent GO term in transcripts with higher RPKM (Figure 3.5A).

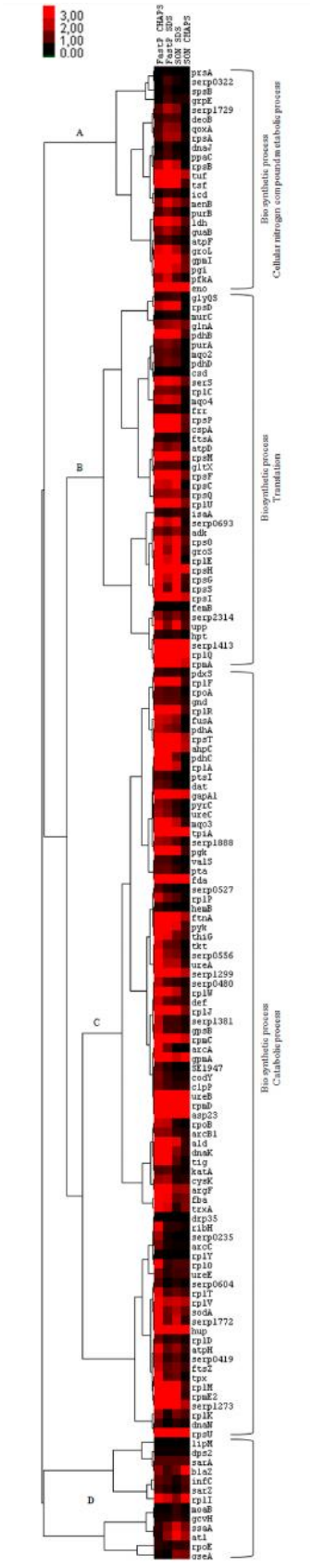

Figure 3.7.: emPAI hierarchical clustering. Clusters and two most represented biological function of the common proteins in each of the tested conditions generated in CLUSTER 3.0 and based on emPAI average from replicates (Slim terms).

Interestingly, proteins with the highest emPAI values presented translation among the main biological processes. In accordance, molecular functions, such as structural constituent of ribosome and RNA binding, were found among these proteins. However, these biological functions were not evidenced in transcripts with the highest RPKM. More precisely, instead of translation, response to stress was a biological process found significant in these transcripts, among others. In relation to molecular functions, only oxidoreductase activity was a significant class. Despite a biological condition is not characterized by the most expressed transcripts or proteins, it is of utmost importance to undertake a whole bacteriome analysis using multi-approaches. RPKM obtained by RNA-seq was next compared to emPAI values obtained from protein extraction experiments performed with FastPrep and sonication (Figure 3.8).

Correlations between mRNA transcripts and protein abundance obtained with FastPrep lyses were 0.37 and 0.36 (Pearson coefficient) in SDS and CHAPS extraction buffer, respectively. A somewhat smaller correlation coefficient was observed in sonication lyses with SDS (r = 0.30) and CHAPS extraction buffer (r = 0.22). Although this is not a strong correlation, our results are in agreement with correlation data previously described in bacteria [433]. When we zoomed Figure 3.8A and Figure 3.8B, we found that several proteins did not correlate with mRNA expression level. The main biological processes of these proteins are translation (when emPAI is higher than RPKM) and response to stress (when

emPAI is lower than RPKM). Interestingly, dormancy is associated with low metabolic activity, leading to a decrease expression of transcripts involved in translation [214]. This suggests that these biological processes, in particular, may contribute to a lower correlation between transcriptomic and proteomic data. In almost every organism, transcript abundances only partially predict protein abundances, which suggests that other modes of regulation must be involved to explain how the levels of proteins are set within cells [432]. Also, the localization of proteins may display different correlations when compared to global correlation [461].

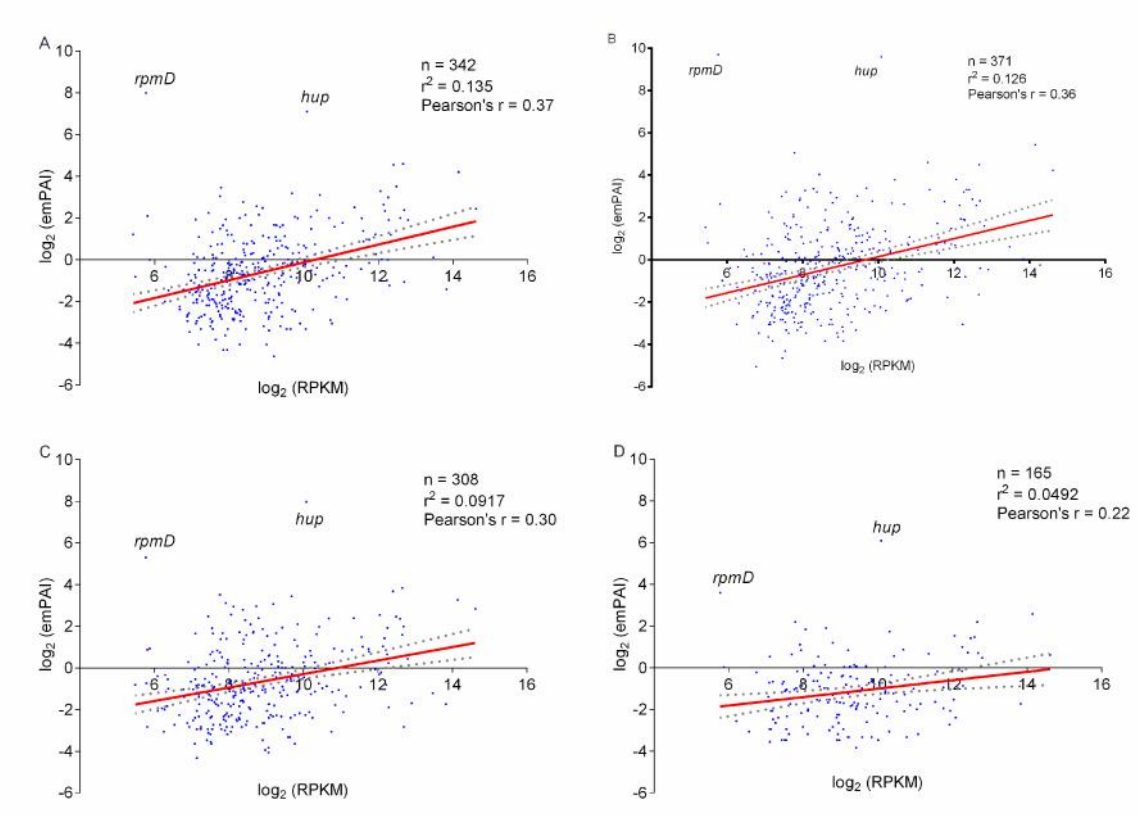

Figure 3.8: Protein and transcript correlation in *S. epidermidis* biofilms grown in glucose enriched medium. FastPrep SDS (A), FastPrep CHAPS (B), SON SDS (C), SON CHAPS (D). Linear regression (R^2), Pearson correlation (Pearson's r) and number of proteins (n) are shown. The 95% confidence interval is shown is each condition, represented in grey dashed line. All correlations were significant ($p < 0.01$). SON - sonication

Despite direct correlation between proteins and mRNA is lower than 0.50, besides noise and experimental errors [462], several levels of regulation of transcripts and proteins may cause variability, such as transcription, post-transcription and post-

translation regulation. Protein folding and post-translational modifications as phosphorylation, glycosylation, acetylation, proteolysis, methylation, lipidation, among others, are the most significant modifications [463-466]. Phosphorylation, such as found in LRTQKIVFS(Phospho)IGLCYSILMALSR peptide (Q5HLC3), corroborate that protein modifications increase phenotypic variability. These regulation mechanisms may explain why transcripts and proteins levels have a low correlation, among bacteria. Previously, it was shown that, in *S. aureus*, serine and threonine phosphorylations was associated with metabolic activity, virulence and translation [467]. This addresses the biological importance of regulation processes and modifications over proteins.

3.4. Conclusion

Similar to other studies, we demonstrated that protein extraction methods affect the proteome characterization of *S. epidermidis* biofilms. It is assumed that an efficient protein extraction method is crucial to ensure successful proteome analysis. A combination of mechanical and chemical lyses or enzymatic lysis provides a better methodology to extract proteins from *S. epidermidis* biofilms. The ideal choice of a detergent is still empirical, although, optimization of sample preparation is fundamental to correctly analyze the cell proteome. Our data suggest that a higher number of total proteins from *S. epidermidis* biofilm can be extracted and recovered by a combination of glass beads mechanical lysis with CHAPS extraction buffer. However, only mildly hydrophobic proteins can be solubilised with SDS or CHAPS. Also, the use of SDS extraction buffer led to an enhancement of metal ion binding proteins. A few lyse buffers have been used to enhance protein extraction, but one buffer is not sufficient for all purposes. This study emphasizes the need for a detailed study over extraction buffers that should be used for each experiment in order to identify the most efficient extraction methodology.

More important, we provide evidences that transcriptomic and proteomic data present a similar correlation to other bacteria, despite the detergent extraction buffer used. Interestingly, proteins involved in translation or response to stress

appear to be those which less correlate with the correspondent transcript. Several *"omics"* may provide useful information instead of individual transcriptome or proteome analysis, since it gives an integrative view of a specific physiological state.

CHAPTER 4.

PROTEOMIC PROFILE OF DORMANCY WITHIN S. *EPIDERMIDIS* BIOFILMS USING ITRAQ AND LABEL-FREE STRATEGIES

PROTEOMIC PROFILE OF DORMANCY WITHIN *S. EPIDERMIDIS* BIOFILMS USING ITRAQ AND LABEL-FREE STRATEGIES

Summary

Here, we provide a detailed characterization of the quantitative proteomic profile of *S. epidermidis* biofilms with induced and prevented dormancy. A total of 427 and 409 proteins were identified by label-free and label-based quantitative methodologies, respectively. From these, 29 proteins were found to be differentially expressed between *S. epidermidis* biofilms with prevented and induced dormancy. Proteins overexpressed in *S. epidermidis* with prevented dormancy were associated with ribosome synthesis pathway, which reflects the metabolic state of dormant bacteria. In the opposite, underexpressed proteins were related to catalytic activity and ion binding, with involvement in purine, arginine and proline metabolism. Additionally, GTPase activity seems to be enhanced in *S. epidermidis* biofilm with induced dormancy. The role of magnesium in dormancy modulation was further investigated with bioinformatics tool based in predicted interactions. The main molecular function, of proteins which strongly interact with magnesium, was nucleic acid binding. Different proteomic strategies allowed to obtain similar results and evidenced that prevented dormancy led to an expression of a markedly different repertoire of proteins in comparison to the one of dormant biofilms.

4.1. Introduction

The transcriptomic profile of *S. epidermidis* biofilms with different proportions of dormant bacteria confirmed that the translation process was related to the proportion of dormant bacteria (Chapter 2). Transcriptomic data analysis also suggested that oxidation-reduction processes were associated with dormancy. However, the expression level of a gene does not necessarily indicate the amount of protein produced, nor its biological activity [468]. Similar to other bacteria [433], *S. epidermidis* transcripts and proteins showed a modest correlation (Chapter 3), which suggests that transcript regulation and protein modifications may determine the biological importance of a gene [463,464,466].

Currently, proteomic tools allow simultaneously the identification and comparison of protein expression in different pathophysiological conditions. Quantitative proteomic analysis based on mass spectrometry may be achieved, among others, by stable isotope labeling, such as iTRAQ (isobaric tags for relative and absolute quantitation) or by label-free spectral counting, such as emPAI [469]. In one experimental setup, iTRAQ has the capability of multiplexing up to eight samples [470] where proteolytic peptides are chemically labeled with amine-specific isobaric tags. Then, upon induced fragmentation in the mass spectrometer, peptides yield different reporter ions in 113–121 *m*/*z* range (in the case of a 8-plex experiment) [470]. Alternatively, the relative protein abundance, emPAI, offers label-free relative quantitation of a protein in a complex sample, based on protein coverage by the peptide matches which is proportional to the protein abundance in a cell [458]. The ability to accurately quantify proteins by label-free spectral counting depends on the number of spectra obtained and the sample coverage [469]. Thus, apart from relative quantitation, label-free also allows a qualitative analysis based on the number of identified proteins. Conversely, iTRAQ methodology do not allow to identify unique proteins, since the protein ratio is only calculated when the protein is present in both tested conditions.

The purpose of our study was to describe quantitative profile of *S. epidermidis* biofilms with induced and prevented dormancy, using label-free and label-based proteomic approaches. To achieve this, total proteins from *S. epidermidis* biofilms

with different proportion of dormant cells were quantified by iTRAQ coupled with 2-dimensional LC-MS/MS and by SDS-PAGE-LC-MS/MS (GeLC-MS/MS).

4.2. Materials and Methods

4.2.1. Biofilm growth conditions

One colony of *S. epidermidis* 9142 (isolated from blood culture [380]) was inoculated in TSB (LiofilChem) and incubated at 37 ºC in an orbital shaker at 120rpm for 18 h. The overnight culture was adjusted to an optical density at 640 nm of 0.250 (±0.05) with TSB and 10 µL of the suspension was transferred into a 24-well plate (Orange Scientific) containing 1 mL of TSB supplemented with 0.4% glucose (v/v) (Fisher Scientific) or TSB 0.4%G enriched with 20 mM magnesium chloride ($MgCl_2$) (Merck), to form biofilms. The culture plates were then incubated at 37 ºC in an orbital shaker at 120 rpm for 24 h. After this period, the culture medium was removed and replaced by fresh TSB supplemented with 1% glucose (v/v) or TSB 1%G containing 20 mM $MgCl_2$. Biofilms were then allowed to grow in these same conditions for an additional 24 h. Next, biofilm culture medium was removed and biofilms were washed twice with PBS. Then, bacteria within the biofilms were resuspended in 1 mL of PBS. Biofilm dormancy was assessed as previously described [138]. Briefly, the number of CFU/mL in each biofilm growth condition was determined using the spread plate method in TSA (LiofilChem). A reduction of about one log difference is typically expected in similarly grown biofilms without addition of Mg^{2+}.

4.2.2. Preparation of protein extracts

For total protein extraction, biofilms were directly scrapped and resuspended with detergent extraction buffer, 25 mM Tris-HCl (pH = 7.2) (Pharmacia Biotech), 10 mM CHAPS (Sigma-Aldrich), 0.5 M NaCl (VWR), 5% glycerol (Sigma-Aldrich) and 1 mM PMSF (Sigma-Aldrich). Then, mechanical lysis was performed in a bead beating using glass beads of 0.1 mm (Sigma-Aldrich) in a FastPrep® cell disruptor (BIO 101, ThermoElectron Corporation) (3 cycles of 30 s and 6.5 m/s). After lysis, cell debris were removed by centrifugation (15,000*g* for 15 min at 4 ºC). Then,

proteins were precipitated with 20% of TCA-cold acetone and quantified using the RC-DC assay (Bio-Rad), following the manufacturer's instructions.

4.2.3. Gel electrophoresis (SDS-PAGE), in-gel protein digestion and protein identification

40 µg of protein were incubated with SDS 10% (w/v) (USB Corporation), 0.5 M Tris-HCl (pH 6.8), glycerol, mercaptoethanol (Sigma-Aldrich) and bromophenol blue (w/v) for 5 min at 100 ºC. Then, samples were loaded on a SDS-PAGE and proteins were separated at a constant voltage (150 V) for 50 min. The gel was stained with colloidal Coomassie G-250 and entire gel lanes were manually excised into 16 gel slices for in-gel digestion with trypsin TPCK treated (ABSciex). Peptides extraction was made with 10% formic acid/ACN. Peptides were dried in a SpeedVac (Thermo Savant, Thermo Scientific) and were dissolved in 5% ACN (MS grade, VWR), 0.1% formic acid (Fluka Analytical, Sigma-Aldrich) and 0.1% TFA (Sigma-Aldrich). Separation of tryptic peptides by nano-HPLC was performed on the module separation Ultimate 3000 (Dionex, Thermo Fisher Scientific) using a capillary column (Pepmap100 C18; 3 µm particle size, 0.75 µm internal diameter, 15 cm in length). A gradient of solvent A, (water/ACN/TFA (98:2:0.05, v/v/v)) to solvent B (water/ACN/TFA (10:90:0.045, v/v/v)) was used. The separation of 2 µg/µL sample was performed using a linear gradient (5-50% B for 30 minutes, 50-70% B for 10 min and 70-5% A for 5 min) with a flow rate of 0.3 µL/min. The eluted peptides were mixed with a continuous flow of α-CHCA matrix solution (270 nL/min, 2 mg/mL in 70% ACN/0.1% TFA and internal standard Glu-Fib at 15 ftmol) and were applied directly on a MALDI plate in 20 s fractions using an automatic fraction collector Probot (Dionex). Mass spectra were obtained on a matrix assisted laser desorption/ionization–time of flight MALDI TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems) in the positive ion reflector mode and obtained in the mass range from 700-4500 Da with 900 laser shots. A fragmentation voltage of 2 kV was used throughout the automated runs. The spectra were processed and analyzed by the T2S (v1.0, Matrix Science Ltd.) and submitted in Mascot software (v.2.1.0.4, Matrix Science Ltd.) for protein/peptide identification based on MS/MS data using the following criteria: trypsin as enzyme; a maximum of two missed cleavages; mass tolerances of 40

ppm for peptide precursors, mass tolerance of 0.6 Da was set for fragment ions. The local FDR was calculated by searching the spectra against SwissProt (*Firmicutes*, release date 06.02.2013) decoy database (false positive peptides/(false positive peptides + total peptides))*100). Protein identification based on a single peptide hit had a minimum individual score of 29 (95%) and a minimum sequence tag of four amino acids (five consecutive peaks in the MS/MS spectrum). Quantification was performed using emPAI. GO terms [385] and KEGG pathways [386] in each condition were determined using STRING database (version 9.1) (accessed at 10.12.2014) [387]. Independent replicates were performed with 22 pools of *S. epidermidis* biofilms, for each condition.

4.2.4. iTRAQ labeling

An in-solution digestion was performed for iTRAQ labeling, as previously described [471]. Briefly, 100 µg of protein was precipitated with 6 volumes of cold acetone at -20 ºC for 3 h. After samples centrifugation and acetone decantation, pellets were resuspended with triethylammonium bicarbonate buffer (TEAB) (0.1 M, pH 8.5) (Sigma-Aldrich) and 2% SDS to achieve a final concentration of 0.05%. Samples were then reduced with 50 mM tris(2-carboxyethyl)phosphine (TCEP) (Sigma-Aldrich) for 1 h at 37ºC with agitation. Then, samples were alkylated with 10 mM S-methyl methanethiosulfonate (MMTS) (Sigma-Aldrich) for 10 min at room temperature with agitation. Trypsin was added to each sample and the digestion was performed for 18 h at 37 ºC. Digested sample peptides were subsequently labeled with the iTRAQ® reagent - 8plex (ABSciex) following the protocol provided by the manufacturer. Briefly, labels were reconstituted in 60% isopropanol, added to each sample peptides and incubated for 2 h at room temperature with agitation. Peptides from *S. epidermidis* biofilms with induced dormancy (1%G) were labeled with 119 isobaric reagent and peptides from biofilms with prevented dormancy (1%G + Mg^{2+}) were labeled with 121 isobaric reagent. The reaction was stopped by adding water and acidification with formic acid. Labeled samples were then combined and dried in SpeedVac.

Then, labelled peptides were separated from an adapted multidimensional LC approach, as described by Vitorino *et al.*, based on high pH for first dimension

peptide chromatography with a C18 reverse phase HPLC column and acidic pH for second one [472]. Thus, sample loading was performed at 200 µL/min with buffers (A) 2% ammonium hydroxide and 0.014% formic acid in water, pH 10 and (B) 2% ammonium hydroxide and 90% ACN in water, pH 10. After 5 min of sample loading and washing, peptide fractionation was performed with linear gradient to 70% B over 85 min. Sixty fractions were collected, dried in a SpeedVac and resuspended in 5% ACN and 0.1% TFA. Collected fractions were then separated by LC. Briefly, peptides loaded onto a C18 pre-column (5 mm particle size, 5 mm; Dionex) connected to a reverse phase column PepMap100 C18 (150 mm_75 mm i.d., 3 mm particle size). The flow rate was set at 300 nl/min. The mobile phases A and B were 2% ACN and 0.05% TFA in water, and 90% ACN with 0.045% TFA in water, respectively. The gradient started at 10 min and ramped to 60% B till 50 min and 100% B at 55 min and retained at 100% B till 65 min. The separation was monitored at 214 nm using a UV detector (Dionex/LC Packings). Using the micro-collector Probot (Dionex/LC Packings) and, after a lag time of 5 min, peptides eluting from the capillary column were mixed with a continuous flow of α-CHCA matrix solution (in internal standard Glu-Fib) and were directly deposited onto the LC-MALDI plates. The spectra were generated and processed with 4800 MALDI-TOF/TOF. Protein identification based on MS/MS data were performed with ProteinPilot^{TM} software (v.4.04, AB Sciex) using Paragon search method. SwissProt from *S.epidermidis* strain RP62A (*Firmicutes*, release date 06.02.2013) was used as protein database. Default search parameters used were: trypsin as the digestion enzyme with 2 missed cleavages, 40 ppm tolerance, carbamidomethyl modification on cysteine residue, iTRAQ 8-plex modification of N-terminal and lysine peptide residues as fixed modification. Additionally, biological modifications with emphasis on methionine oxidation, deamidation and iTRAQ 8-plex modification of tyrosine residue and deamidation, were considered variable modifications. Bias correction was applied and proteins were identified with a confidence level of 95%. Proteins were found to be differentially expressed when $p < 0.05$ (FDR-corrected). Proteins were considered overexpressed in prevented dormancy when the iTRAQ ratio was above 1, and underexpressed when iTRAQ ratio was lower than 1. GO and KEGG were determined using STRING as described in *4.2.3.* section.

4.2.5. Immunodetection and identification of oxidized proteins

Carbonylated proteins were assayed as previously described [473,474]. Briefly, whole cell proteins from both conditions were separated by isoelectric focusing with immobilized pH gradient (IPG) strips non-linear pH 3–10 gradient (GE Healthcare). Then, each strip was incubated with 12% SDS for 30 min at room temperature and derivatized with 20 mM DNPH (2,4-dinitrophenylhydrazine) (Acros Organics) in 10% TFA for 30 min at room temperature, in the dark. The reaction was stopped with equilibration buffer (2% (w/v) SDS, 6 M urea, 30% glycerol, 0.05 M Tris–HCl (pH 8.8) and 20 mg/ml DTT for 30 min at room temperature. The second dimension was obtained by SDS-PAGE. Then, gels were stained with colloidal Coomassie G-250 or gels were transferred onto a nitrocellulose membrane. Immunodetection was performed using anti-2,4-dinitrophenol (DNP) antibody (Merck) and detected by enhanced chemiluminescence ECL (Amersham Pharmacia Biotech) according to the manufacturer's procedure. Images were recorded using X-ray films (GE Healthcare) and the films and the gels were scanned in Molecular Imager Gel Doc XR^+ system (Bio-Rad) and analyzed with QuantityOne software (v. 4.6.9, Bio-Rad). Protein spots reactive to anti-DNP antibody were manually excised and an *in-gel* digestion was performed as described in *4.2.3.* section. Peptides were resuspended and directly deposited onto 384-well MALDI plates (Applied Biosystems) with α-CHCA matrix solution. α-CHCA matrix solution was prepared by diluting 2.5 mg/mL of α-CHCA in ACN 70%/ TFA 0.3%. Experiments were carried in duplicate. Peptide mass spectra were obtained as referred in *4.2.3.* section. Mass spectra were processed and analyzed by the Global Protein Server (GPS) Workstation (Applied Biosystems). Searches were performed against the SwissProt protein database for *Firmicutes* (release date 06.02.2013). The database search parameters were: mass tolerance of 40 ppm for precursor ions and 0.4 Da for fragment ions; trypsin digestion with two missed cleavages. Protein identity was accepted at the 95% confidence level.

4.3. Results

4.3.1. Label-free data analysis

The work reported here represents a multi-approach to determine the protein changes in dormancy within *S. epidermidis* biofilms, using a previously described model based in Mg^{2+} [138].

After GeLC-MS/MS separation and identification of proteins from induced (1%G) and prevented (1%G + Mg^{2+}) dormancy in *S. epidermidis* biofilms, label-free qualitative and relative quantitation analysis were performed from two independent replicates. For qualitative analyses, Venn diagram was generated with identified proteins in each condition, using Venny tool from BioinfoGP [384] (Figure 4.1).

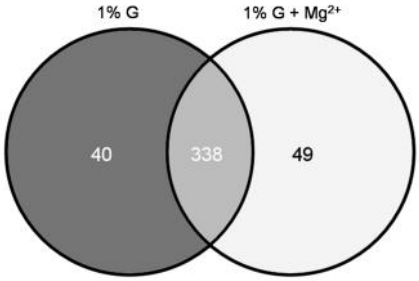

Figure 4.1.: Venn diagram summarizing the overlap between proteins identified by label-free methodology in *S. epidermidis* biofilms with prevented (1%G + Mg^{2+}) or induced (1%G) dormancy.

A total of 378 and 387 proteins (FDR < 5%) were identified in induced and prevented dormancy, respectively. Statistically significant GO terms (including biological processes and metabolic functions) for proteins found in each condition are detailed in Figure 4.2.

Despite no major differences were found, cell wall organization or biogenesis (GO:0071554) was a biological process only found statistically significant in *S. epidermidis* biofilms with induced dormancy (including the MurC, Ddl, MurG, LtaS, Atl, DltC, MurA1, FemB, FemA, DltA, MurB, FemX and GlmU proteins). GTPase activity (GO:0003924) was a molecular function only found statistically significant in *S. epidermidis* biofilms with induced dormancy. Examples of such proteins included SERP0696, InfB, PrfC, Obg, MnmE, UreG, Era, LepA, FtsZ, FusA and EF-Tu. Among them, only seven proteins were common to the proteome of *S.*

epidermidis biofilm with prevented dormancy. Interestingly, these proteins are involved in the nucleotide metabolic process and present ion binding activity.

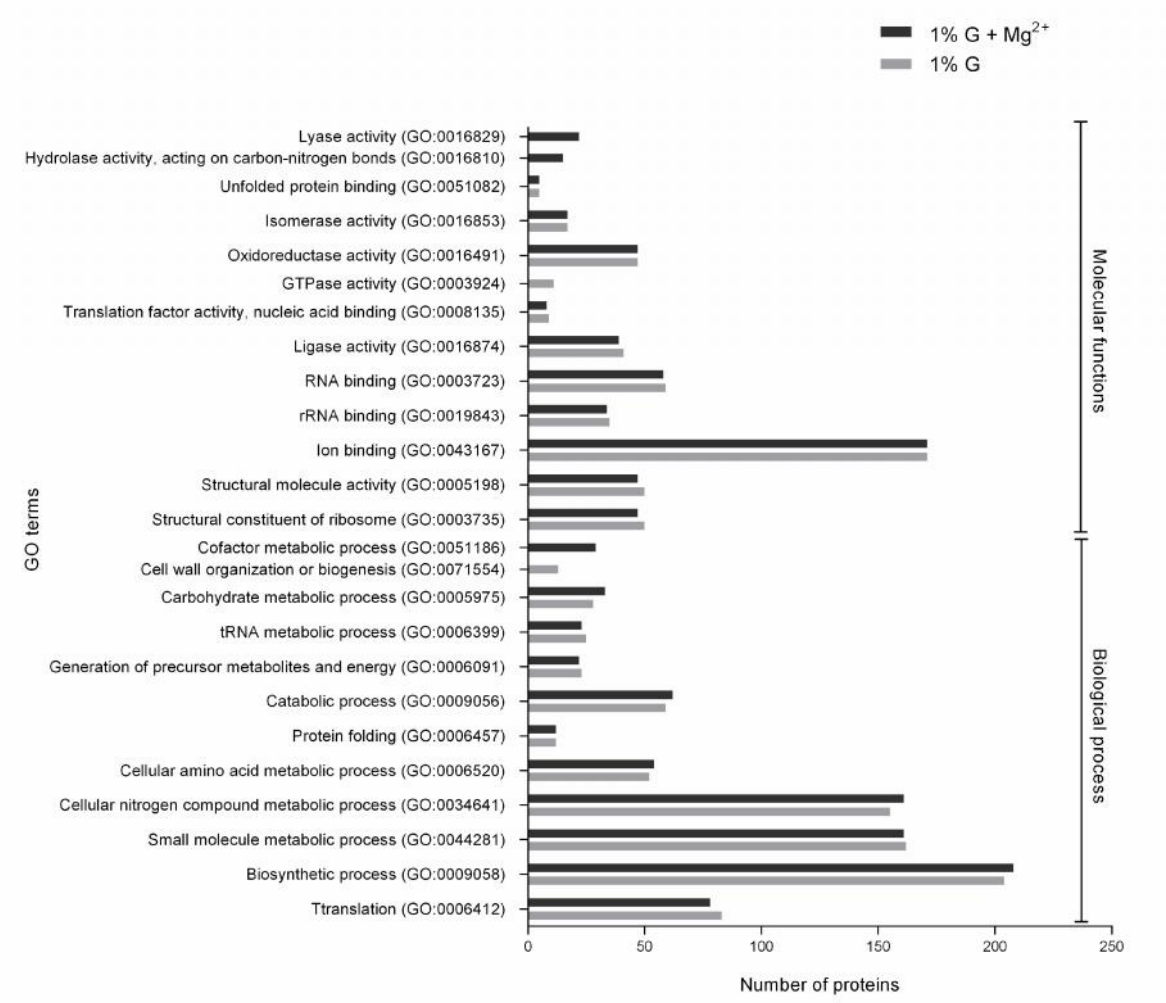

Figure 4.2.: Significant biological processes and molecular functions terms in *S. epidermidis* biofilms proteome with prevented (1%G + Mg^{2+}) and induced (1%G) dormancy ($p < 0.05$, FDR-corrected). Data obtained by label-free methodology. Classes with a *p*-value ≤ 0.05 were considered statistically significant.

Furthermore, cofactor metabolic process (GO:0051186) was a biological process only significant in prevented dormancy. Additionally, hydrolase activity, acting on carbon-nitrogen bonds (GO:0016810) and lyase activity (GO:0016829) were molecular function categories only found significant in prevented dormancy. Among all the proteins identified in both conditions, only 40 proteins were exclusively assigned to dormancy in *S. epidermidis* biofilms (Figure 4.1). Conversely, only 49 proteins were exclusively assigned to *S. epidermidis* biofilms with prevented dormancy (Figure 4.1). Thus, Figure 4.3 shows the three most

represented biological processes and molecular functions in unique proteins found in each condition.

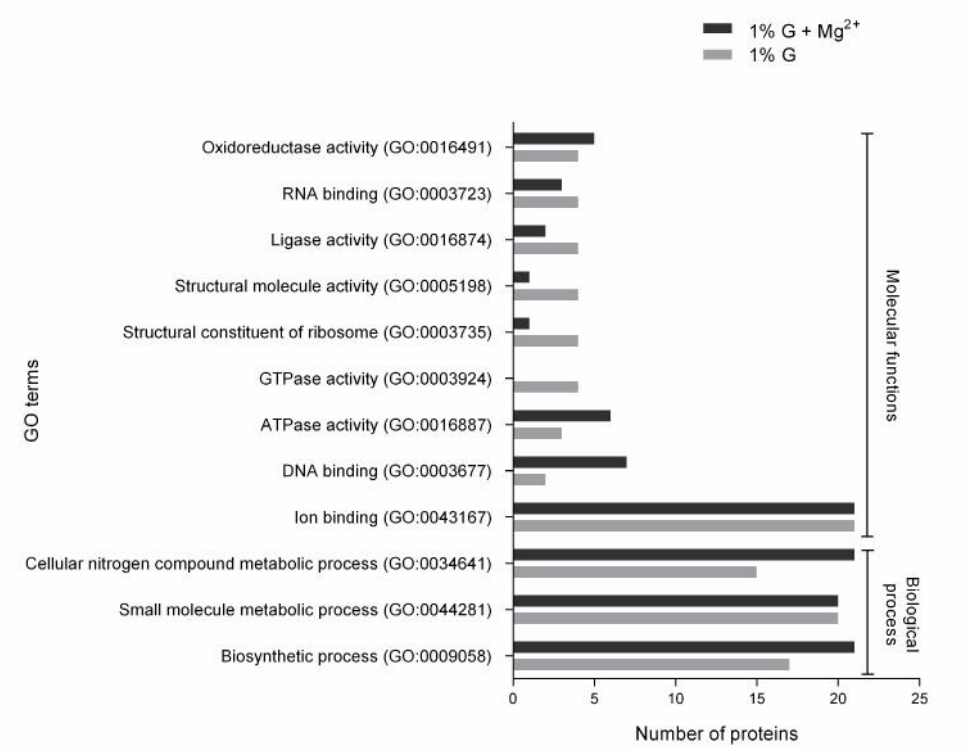

Figure 4.3.: The three most prevalent biological processes and molecular functions terms in proteins only found in *S. epidermidis* biofilms in prevented (1%G + Mg^{2+}) or induced (1%G) dormancy. Data obtained by label-free methodology.

Main differences were found in molecular function annotations. Herein, GTPase activity (GO:0003924), structural constituent of ribosome (GO:0003735) and structural molecule activity (GO:0005198) were categories predominant in induced dormancy. Conversely, DNA binding (GO:0003677) (including AddA, Rex, GreA, LexA, GrlA, PhoP and PcrA proteins) and ATPase activity (GO:0016887) (including AddA, TagH, GrlA, Rho, PcrA and MetN1 proteins) were predominant in *S. epidermidis* biofilm with prevented dormancy.

In order to analyze relative quantitation of label-free data, emPAI was compared amongst biological replicates. The reproducibility of data collected from the two biological replicates showed a Pearson's correlation indicating good reliability of emPAI data, with a correlation higher than 0.810 in both conditions. Thus, we used emPAI average for further analysis.

4.3.2. Label-based data analyses

iTRAQ provide the possibility to quantitatively examine a large number of proteins for differential expression [470,475]. First, samples were labeled, pooled, fractionated by HPLC and then, separated by liquid chromatography and analyzed by tandem mass spectrometry (MS/MS). Using this approach, a total of 409 proteins were identified. Among them, 408 proteins had a calculated ratio value. Herein, a total of 29 proteins were differentially expressed, wherein 16 proteins were overexpressed and 13 proteins were underexpressed in *S. epidermidis* biofilms with prevented dormancy (Table 4.1). The two most represented biological processes, molecular functions and KEGG pathways in differently expressed proteins were detailed in Figure 4.4.

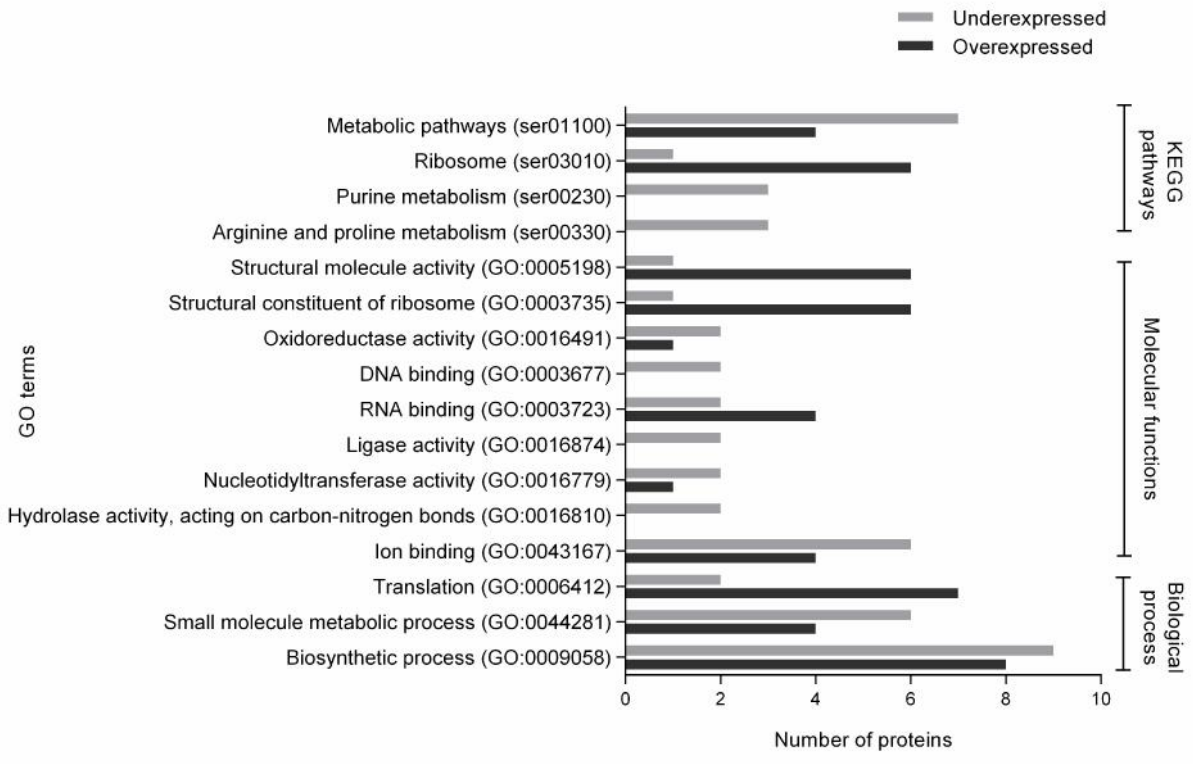

Figure 4.4.: The two most prevalent biological processes, molecular functions and KEGG pathways in statistically significant overexpressed and underexpressed proteins from *S. epidermidis* biofilms with prevented dormancy. Data obtained by label-based methodology.

Table 4.1.: Significantly expressed proteins in iTRAQ**.**

Protein	Accession number	Protein function	Peptides (95%)	Ratio (Mg^{2+}/1%G)
ArgF	Q5HKJ4	Ornithine carbamoyltransferase	20	0.4848
SERP1299	Q5HNH0	UPF0478 protein SERP1299	6	0.5015
SERP1140	Q5HNX4	UPF0365 protein SERP1140	10	0.5811
BetA	Q5HL11	Choline dehydrogenase	7	0.6084
ArcA	Q5HKU2	Arginine deiminase	11	0.6399
GuaA	Q5HRX1	GMP synthase [glutamine-hydrolyzing]	5	0.696
Atl	Q5HQB9	Bifunctional autolysin	12	0.7037
RplT	Q5HNM5	50S ribosomal protein L20	7	0.711
GlnA	Q5HPN2	Glutamine synthetase	8	0.7219
RpoC	Q5HRK9	DNA-directed RNA polymerase subunit beta	22	0.7579
EF-Tu	Q5HRK4	Elongation factor Tu	21	0.7643
SERP2115	Q5HL72	Pyruvate oxidase	2	0.7771
RpoB	Q5HRL0	DNA-directed RNA polymerase subunit beta	18	0.7773
SERP1777	Q5HM52	Iron compound ABC transporter, iron compound-binding protein	4	1.3045
GpmI	Q5HQV1	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	20	1.4027
Pgk	Q5HQV3	Phosphoglycerate kinase	17	1.4537
RpmI	Q5HNM4	50S ribosomal protein L35	4	1.4606
RplX	Q5HM10	50S ribosomal protein L24	3	1.4924
Efp	Q5HP20	Elongation factor P	4	1.5269
GroS	Q5HMZ0	10 kDa chaperonin	7	1.5296
GlmU	Q5HRQ6	Bifunctional protein GlmU	1	1.596
RplL	Q5HRL2	50S ribosomal protein L7/L12	7	1.6098
PdhC	Q5HQ74	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	13	1.7096
RpsR	Q5HRZ3	30S ribosomal protein S18	4	1.8413
SERP1917	Q5HLR6	Oxidoreductase, short chain dehydrogenase/reductase family	5	1.9109
RplY	Q5HRQ4	50S ribosomal protein L25	2	2.0165
RpmB	Q5HPW7	50S ribosomal protein L28	8	2.0485
EbpS	Q5HP65	Probable elastin-binding protein EbpS	3	2.3042
SERP0665	Q5HQ90	Uncharacterized protein	1	3.0005

Additionally to ion binding, GO terms related to catalytic activity, such as hydrolase (GO:0016810) (Atl and ArcA proteins), ligase (GO:0016874) (GlnA and GuaA proteins) and nucleotidyltransferase (GO:0016779) (RpoC and RpoB proteins) activity were associated with induced dormancy, since these GO terms were underexpressed. Purine (ser00230), arginine and proline (ser00330) metabolism were the most prevalent pathways in underexpressed proteins in biofilms with prevented dormancy. Conversely, molecular functions, such as structural constituent of ribosome (GO:0003735), structural molecule activity (GO:0005198) (including RpmI, RplL, RpmB, RplX, RplY and RpsR proteins) and RNA binding (GO:0003723) were associated with proteins more expressed in *S. epidermidis* biofilm with prevented dormancy. Moreover, ribosome synthesis pathway (ser03010, including RpmI, RplL, RpmB, RplX, RplY and RpsR proteins) was the most prevalent KEGG pathway in overexpressed proteins, which shows an involvement in translation process.

To elucidate the behavior of proteins which may be considered as Staphylococci virulence factors [1,64], we analyzed label-based data and list here their iTRAQ ratio: LipA (ratio = 1.13), ClpP (ratio = 0.96), ClpC (ratio = 0.96), ClpB (ratio = 0.93), PSMβ1 (ratio = 0.52), SitC (ratio = 0.80), AtlE (ratio = 0.70, $p < 0.05$), TagD (ratio = 0.50), DltC (ratio = 0.45) and EbpS (ratio = 2.3, $p < 0.05$). However, only AtlE (an autolysin, also involved in the attachment of *S. epidermidis* to abiotic surfaces [79]) and EbpS (primary attachment [476]) had significant differences between induced and prevented dormancy. Although, these proteins did not present the same expression tendency. Interestingly, PSMβ1 (a non-cytolytic modulin involved in *in vitro* detachment [89]) had a ratio of 0.52 but without statistical significance. Although differences were not statistically significant in the majority of proteins, virulence proteins seemed to be more expressed in biofilms with induced dormancy.

Search Tool for Interactions of Chemicals (STITCH v.4) allows the creation of a protein-chemical interaction network based on experimental data, databases, text mining and predicted interactions [477]. Since the growth model previously described includes addition of Mg^{2+} to obtain *S. epidermidis* biofilms with prevented dormancy [138], STITCH resource was used to predict interactions of

magnesium with unique proteins in prevented dormancy (identified by label-free) and overexpressed proteins (identified by label-based methodology) (accessed at 10.12.2014). Additionally, we applied the Markov Cluster Algorithm (MCL) to identify which proteins generate a cluster with magnesium (Figure 4.5). Five of these proteins present nucleic acid binding function and three of them are localized in cytoplasmic membrane.

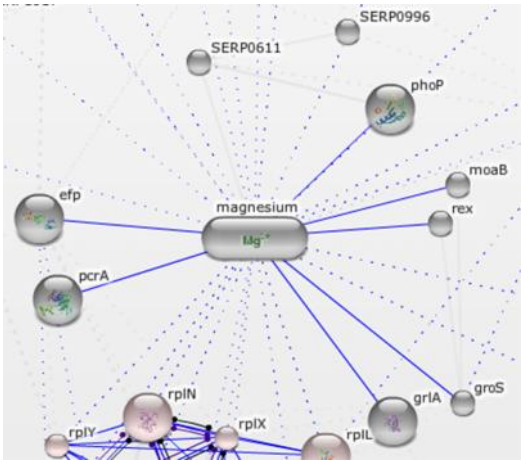

Figure 4.5.: Cluster of magnesium-protein interactions generated and predicted by STITCH (v.4). PhoP - alkaline phosphatase synthesis transcriptional regulatory protein PhoP; Efp - elongation factor P; PcrA - ATP-dependent DNA helicase PcrA; MoaB - molybdenum cofactor biosynthesis protein B; GroS - 10 kDa chaperonin; GrlA - DNA topoisomerase 4 subunit A; Rex - redox-sensing transcriptional repressor Rex; SERP0996 - probable CtpA-like serine protease; SERP0611 - serine protease HtrA-like;

4.3.3. Protein susceptibility to carbonylation

Oxidative modification of proteins can assume many forms such as nitrotyrosination, carbonylation, methionine, sulfhydryl oxidation, etc. [478]. From those, protein carbonyl content is widely used as a marker for oxidative stress, since is considered to be a major form of protein oxidation [479]. Previously, we compared the transcriptome of *S. epidermidis* biofilms with prevented and induced dormancy, which suggested that oxidation-reduction molecular function was increased in *S. epidermidis* biofilms with induced dormancy (Chapter 2). Following this, we evaluated protein oxidation in *S. epidermidis* biofilms with prevented and induced dormancy, namely protein carbonylation. A comparison of the 2-DE immunoblots reveals a different carbonylated pattern between prevented and induced dormancy (Figure 4.6).

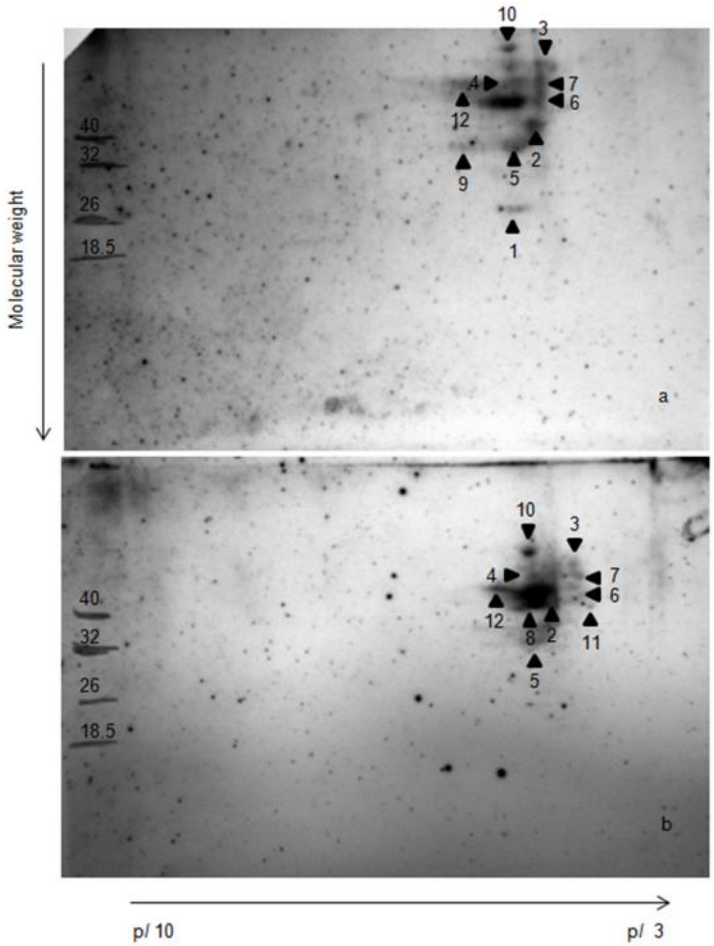

Figure 4.6.: Carbonylated proteins in induced (a) and prevented (b) biofilm dormancy. 1- TpiA (triosephosphate isomerase); 2- EF-Tu (elongation factor Tu); 3- DnaK (chaperone protein DnaK); 4- GpmI (2,3-bisphosphoglycerate-independent phosphoglycerate mutase); 5- Fda (fructose-bisphosphate aldolase class 1); 6- AhpF (alkyl hydroperoxide reductase subunit F); 7- GroEL (60 kDa chaperonin); 8- Pgk (phosphoglycerate kinase); 9- PfkA (ATP-dependent 6-phosphofructokinase) 10- FusA (elongation factor G); 11- RpsA (30S ribosomal protein S1); 12- GuaB (inosine-5'-monophosphate dehydrogenase).

Protein oxidation assessed by protein carbonyl content was mainly localized in the region around p*I* of 5, with a molecular weight varying from 27 to 77 KDa. From the most susceptible proteins to oxidation identified in both conditions showed that TpiA and PfkA proteins seems to be associated to biofilm dormancy, whereas RpsA and Pgk were more reactive to DNP in *S. epidermidis* biofilms with prevented dormancy. A few more proteins presented the same oxidation susceptibility among the two tested conditions.

4.4. Discussion

Adaptation to local environment conditions contribute to physiological heterogeneity of bacteria within biofilms [215]. A subpopulation of dormant bacteria may be formed due to such unfavorable environment [215]. With a purpose of characterize dormancy within biofilms, a global scale analysis of transcriptome and proteome may be achieved with integration of high-throughput techniques and quantitative proteomics. Recently, we assessed transcriptome profile of *S. epidermidis* with prevented and induced dormancy. However, similarly to other bacteria studies [433], comparison among *S. epidermidis* proteins and transcripts showed a modest correlation (Chapter 3). Currently, quantitative proteomics is still the bottleneck of comparative approaches, since the numbers of identified proteins are most of the times behind the expected numbers [480]. Herein, two different proteomic approaches were used to quantify proteins of *S. epidermidis* biofilms, since iTRAQ methodology do not allow to identify unique protein in each condition, and it is used to validate protein expression data.

Based on label-free method, GO annotation strongly suggests the involvement of GTPase activity and ion binding functions in dormancy within *S. epidermidis* biofilms (Figure 4.2), since Mg^{2+} was used to avoid dormancy entry [138]. Furthermore, proteins only found in dormant condition had a high prevalence of GTPase activity (Figure 4.3), which is in accordance with hydrolase activity of underexpressed proteins, found in label-based approach. GTPase enzymes are responsible for GTP hydrolysis and may be involved in several biological functions, from cell cycle regulation, tRNA modification, energy metabolism, stringent response and stress response, among others [481]. Furthermore, in a nutrient limitation environment, GTP is converted by RelA (GTP pyrophosphokinase) to (p)ppGpp [481,482]. RelA is a ribosome-associated (p)ppGpp synthetase and a hydrolase, which is activated in response to amino-acid starvation [483,484]. Interestingly, RelA was among the uniquely expressed proteins in *S. epidermidis* biofilms with induced dormancy was RelA protein. Since in deeper biofilm layers bacteria have limited access to nutrients, this may increase their levels of (p)ppGpp [318], leading to higher expression of RelA protein. In the opposite, prevented dormancy condition presented enhancement of DNA binding and ATPase activity molecular functions, which is related to the

higher number of metabolic active cells. Nowadays, it is assumed that TA modules are involved in dormant persister cells formation [232]. Currently, TA systems are assigned to five classes (I–V) according to their genetic structure and regulation [351,485]. Increased expression of type I TA modules, such as TisB and HokE in *E. coli*, has ATP synthesis cellular process as a target [485]. Although not extensively studied, a type I TA module was already described in *S. aureus* [354]. Additionally, the existence of other type I TA modules in *S. aureus* was predicted by bioinformatics tools, but their biological activities were not confirmed [486]. The characterization of dormancy was also assessed by iTRAQ methodology as cross-check validation of protein expression data, which showed main differences in molecular functions, equivalently to label-free analysis (Figure 4.4).

Proteomic analyses of dormant cells are limited [250]. Previous proteomic studies found a relation among GroEL (60 KDa chaperonin), DnaK (chaperone protein DnaK), AtpD (ATP synthase subunit beta) and Eno (enolase) proteins with VBNC bacteria, since their expression were lower [343]. Also, a decreased expression of AhpF protein (alkyl hydroperoxide reductase subunit F), an oxidation-responsive factor, were previously found in VBNC [487]. Our quantitative results showed that these proteins were decreased in dormancy, however, without statistically significant difference. Additionally, EF-Tu protein (Elongation factor Tu) was also associated with VBNC, but results are not consistent among studies [250]. Interestingly, here, EF-Tu showed a significant increased expression in dormancy. Similarly to transcriptomic profile of dormancy, overexpressed proteins were related to an enrichment of ribosome synthesis pathway and structural constituent of ribosome molecular function (Chapter 2). However, by a proteomic approach, we did not find a significant underexpression of proteins involved in oxidation-reduction, pyruvate metabolism and acetyl-CoA metabolic processes. Nevertheless, we recently showed that correlation among transcripts and proteins were not strong in *S. epidermidis* biofilms (Chapter 3). Thus, underexpressed proteins were mainly associated with purine and, arginine and proline KEGG pathways. Ion binding and catalytic activity were the main molecular functions associated with underexpressed proteins in *S. epidermidis* biofilms with prevented dormancy (Figure 4.4). Ligase, nucleotidyltransferase and hydrolase activity combined with DNA and RNA binding, may suggest that proteins less expressed

in *S. epidermidis* biofilms with prevented dormancy, are involved in transference of nucleotidyl groups and amino acid metabolism. Moreover, hydrolase and transferase protein classes have large number of cysteines, which are more susceptible to oxidation [488,489]. *Staphylococcus* spp. use cysteine oxidation to respond to and overcome reactive oxygen species found in the host environment [490,491]. Oxidative stress is described as a factor that contributes to persister cells increase [341]. However, Leszczynska *et al.* found that persister cells frequency was not correlated with the oxidation of proteins [492]. Interestingly, here we found that dormancy within *S. epidermidis* biofilms affected the pattern of oxidized proteins (Figure 4.6). Among the carbonylated proteins, a few were related to oxidoreductase activity (such as GuaB and AhpF), with RNA binding (RpsA, EF-Tu and FusA proteins) and others were involved in ATP binding (such as PfkA, Pgk, GroEL and DnaK). Additionally, proteins involved in response to stress, DnaK and AhpF, were found carbonylated. Interestingly, among the carbonylated proteins, only EF-Tu, GpmI and Pgk showed significant differences in protein expression between prevented and induced dormancy. Our results suggest that Pgk protein is more active in biofilms with prevented dormancy, but may be more susceptible to oxidation and may suffer biological modifications. However, future work in protein oxidation exploration may help to unravel if oxidation susceptibility may contribute as a factor that leads to dormancy.

It is known that magnesium is involved in several biological processes in bacteria, such as genomic stability, membrane stabilization and cofactor in several enzymatic reactions [305]. Consistent with this fact, we found that proteins which formed a closer cluster with magnesium, presented mainly nucleic acid binding function (Figure 4.5). Interestingly, cytoplasmic membrane proteins were also found associated with magnesium action. Dunne *et al.* were the first to demonstrate the magnesium action over *S. epidermidis* adhesion [281]. Earlier, Cutinelli and Galdiero aimed to describe the binding capacity of Mg^{2+}, among other ions, to *S. aureus* cell wall [291]. They proposed that high pH values increased the amount of Mg^{2+} bound to cell wall. Since it was shown that pH does not vary between the two growth conditions [406], this statement cannot explain the Mg^{2+} binding in the present model. Similarly, Mg^{2+} affinity to *Bacillus subtilis* cell wall was influenced by the presence of functional groups in the peptidoglycan matrix

[493]. In addition, Piddington *et al.* demonstrated that Mg^{2+} is essential to *M. tuberculosis* growth in acidic conditions and it could not be replaced by other divalent cations [409]. Also, a possible influence of Mg^{2+} in protein structure and physical properties was also described in *M. tuberculosis* [410]. Recently, it was shown that catheters coated with magnesium fluoride nanoparticles presented a significantly reduction of *S. aureus* and *E. coli* colonization [494]. However, the Mg^{2+} effect in biofilm maturation and its role on dormancy remains poorly understood [302,409].

To the best of our knowledge, the present study represents the first attempt to determine protein expression differences associated to dormancy within biofilms. For the first time, quantitative proteomics was performed in order to assess dormancy within *S. epidermidis* biofilms. Label-free and label-based methods afforded similar and relevant biological data of dormancy physiological state. Overexpressed proteins were mainly related to ribosome synthesis pathways. Conversely, based on the biofilm dormancy model, ion binding and catalytic activity molecular functions seem to be related to the absence of magnesium enrichment, which contribute to dormancy entrance. It should also be emphasized that the output of the different quantitative strategies here employed, contributed to a further exploration of dormant physiological condition.

CHAPTER 5.

AN IMMUNOPROTEOMIC APPROACH FOR CHARACTERIZATION OF DORMANCY WITHIN *S. EPIDERMIDIS* BIOFILMS

AN IMMUNOPROTEOMIC APPROACH FOR CHARACTERIZATION OF DORMANCY WITHIN *S. EPIDERMIDIS* BIOFILMS

Summary

Physiological heterogeneity of biofilms may influence host immune response and sensitivity to antibiotics. The aim of this study was to identify immunogenic proteins of *S. epidermidis* biofilms associated with dormancy mechanism, by using 2-DE immunoblotting and MS. A total of 19 bacterial proteins, recognized by human serum samples, were identified. These proteins were mainly involved in small molecule metabolic biological processes. Catalytic activity and ion binding were the most representative molecular functions. CodY and GpmA proteins were more reactive to sera when biofilm dormancy was induced, while FtnA and ClpP were more reactive when dormancy was prevented. This is the first work that identifies differences in immunoreactive proteins within bacterial biofilms with induced or prevented dormancy. Considering the importance of dormancy within biofilms, further evaluation of these proteins can provide insights into the mechanisms related to dormancy and help to improve current understanding on how dormancy affects the host immune response.

5.1. Introduction

Nowadays, proteomic approaches are contributing to elucidate the immunological response to microorganisms [495]. Immunoproteomics allows the identification of immunogenic and immunoreactive proteins that may participate in host-pathogen interactions and in host immune response [174,496,497]. Furthermore, immunoproteome analysis improves the understanding of pathogenesis and unravel novel therapeutics targets based on the repertoire of immunogens [498]. Thus, plasma is one of the most relevant environmental factors in indwelling medical devices-related infections [499]. A few aspects of immune reaction to *S. epidermidis* infections were already elucidated [140,142,143,156,175,500]. By using serum from rabbits immunized with live *S. epidermidis*, or serum proteins eluted from the surface of bacteria grown in rabbit serum reactive against bacterial cell-surface extracts, immunogenic and serum binding proteins were identified by Western blotting [142]. Sellman *et al.* found 5 antigenic components candidates for the development of *S. epidermidis* vaccine, namely, acetyl-coenzyme A acetyltransferase (YqiL), Na^+/H^+ antiporter (SE1873), lipoate ligase (SE0360), cysteine synthase (CysK), and alanine dehydrogenase (Ald). Also, Pourmand *et al.* identified autolysin AtlE, lipase GehD and surface protein ScaB antigenic components with therapeutic potential since they had opsonic activity *in vitro* [175].

To determine the immunoproteomic pattern of *S. epidermidis* biofilms with prevented and induced dormancy, we resolved whole cell lysate by 2-DE and performed immunoblotting with human sera. We then identified the immunoreactive protein spots by MALDI-TOF/TOF. With this work we intend to define the reactive protein repertoire of *S. epidermidis* biofilms with different proportions of dormant bacteria to human serum and contribute to decipher the host immune differences to dormancy.

5.2. Materials and methods

5.2.1. Growth conditions

Growth culture condition was performed as previously described [138]. *S. epidermidis* strain 9142 (isolated from blood culture [501]) was used to establish biofilms with induced and prevented dormancy. Briefly, one colony of *S. epidermidis* was inoculated in TSB (LiofilChem) and incubated at 37 ºC in an orbital shaker at 120 rpm for 18 h. The overnight culture was adjusted to an optical density at 640 nm of 0.250 (±0.05) and 10 µL of the suspension was transferred into a 24-well plate (Orange Scientific) containing 1 mL of TSB supplemented with 0.4% glucose (v/v) (Fisher Scientific) or TSB 0.4%G enriched with 20 mM magnesium chloride ($MgCl_2$) (Merck). The culture plates were then incubated at 37 ºC in an orbital shaker at 120 rpm for 24 h. After this period, the culture medium was removed and replaced by fresh TSB supplemented with 1% glucose (v/v) or TSB 1%G containing 20 mM $MgCl_2$ (1%G + Mg^{2+}). Biofilms were then allowed to grow in the same conditions for an additional 24 h. Next, biofilm culture medium was removed and biofilms were washed twice with PBS. Then, bacteria within the biofilms were resuspended in 1 mL of PBS. As previously described, biofilm dormancy was determined using the spread plate method in TSA (LiofilChem) through calculation of the number of CFU/mL in each biofilm growth condition [138]. A reduction of about one log difference is typically expected in similarly grown biofilms without Mg^{2+} [138].

5.2.2. Preparation of protein extracts

Total protein extraction was performed from multiple biofilm replicates, as described in Chapter 4.

5.2.3. Two-dimensional electrophoresis (2-DE)

A total of 80 µg of protein was resuspended in rehydration sample buffer (8 M urea, 2 M thiourea, 1% CHAPS, 12 mM DTT, 0.5% IPG buffer). Then, IPG 3–10 non-linear strips, 7 cm, (Immobiline™ pH Gradient, GE Healthcare) were in-gel rehydrated overnight for the first dimension isoelectric focusing (IEF), performed on a horizontal Ettan™ IPGPhor (Amersham Biosciences). Isoelectric separation

was performed using the following focusing program: 12 h at 50 mW (rehydration), 1 h at 150 V (gradient), 1 h at 500 V (gradient), 1 h at 1000 V (gradient) and 90 min at 5000 V ("step-and-hold"). After IEF, IPG strips were equilibrated with equilibration buffer (2% (w/v) SDS, 6 M urea, 30% glycerol, 0.05 M Tris–HCl pH 8.8 and 20 mg/ml DTT) for 30 min at room temperature. Strips were then placed on the top of a 12% SDS-PAGE gel for the second dimension separation and ran at a constant voltage. Gels were stained with colloidal Coomassie G-250 or gels were transferred onto a nitrocellulose membrane. Proteins were blotted on a nitrocellulose membrane (Whatman®, Protan) in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3 and 20% methanol) during 2 h at 200 mA. Stained gels were analyzed by using Melanie analysis software v.7.0 (GeneBio). Protein separations by 2-DE was carried out three independent times. The signal intensities of proteins spots were compared among both conditions and scored by fold-change.

5.2.4. Immunoblotting

Sera samples were obtained from three volunteers after informed consent was obtained. The experiment was approved by the Ethics Committee of *Instituto Ciências Biomédicas Abel Salazar* (document number 081/2014).

Nitrocellulose membranes were blocked in 5% BSA (Sigma-Aldrich) in TBS-T (Tris-Buffered Saline-Tween 20) for 2 h. Then, membranes were incubated with human serum (1:200) for 2 h at room temperature. Following this incubation, membranes were washed with TBS-T for 10 min. Membrane washing step was repeated three times. Membranes were incubated with a secondary anti-human Imunoglobulin G (IgG, A0170, Sigma-Aldrich) (1:1000) during 1 h at room temperature. After washing the membranes, immunodetection was performed with enhanced chemiluminescence ECL (Amersham Pharmacia Biotech) according to the manufacturer's procedure and the images were recorded using X-ray films (GE Healthcare). The films and the gels were scanned in Molecular Imager Gel Doc XR+System (Bio-Rad) and analyzed with QuantityOne software (v. 4.6.9 Bio-Rad).

5.2.5. In-gel protein digestion and protein identification by mass spectrometry

Reactive protein spots were selected and manually excised from stained gels. An *in-gel* digestion of gel spots, with trypsin TPCK treated (ABSciex), was performed. Briefly, peptides extraction was made with 10% formic acid/ACN. Dried peptides were dissolved in 5% ACN (VWR), 0.1% formic acid (Fluka Analytical, Sigma-Aldrich) and 0.1% TFA (Sigma-Aldrich). Peptides were directly deposited onto 384-well MALDI plates (Applied Biosystems) with α -CHCA matrix solution. α-CHCA matrix solution was prepared by diluting 2.5 mg/mL of α-CHCA in ACN 70%/TFA 0.3%. Experiments were carried out in technical duplicates. Mass spectra were obtained on a matrix assisted laser desorption/ionization–time of flight MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems) in the positive ion reflector mode and obtained in the mass range from 700-4500 Da with 900 laser shots. A fragmentation voltage of 2 kV was used throughout the automated runs. The spectra were processed and analyzed by the Global Protein Server (GPS) Workstation (Applied Biosystems). Searches were performed against the SwissProt (release date 06.02.2013) protein database for *Firmicutes*. The database search parameters were: mass tolerance of 40 ppm for precursor ions and 0.4 Da for fragment ions; trypsin digestion with two missed cleavages. Protein identity was accepted at the 95% confidence level.

5.2.6. Bioinformatic analyses

GO [385] and KEGG pathway [386] analysis were performed to determine the function of identified proteins, using STRING (version 9.1) (at 28.11.2014) [387]. Cellular localization of proteins was predicted or identified by PSORTb program v.3.0.2 [396] and by Cello v.2.5 [502]. Immunoreactive proteins were query in ABCPred to predict B cells epitopes *in silico* [503].

5.3. Results and discussion

Biofilm formation is considered the major virulence factor of *S. epidermidis* [5]. It has been described that bacteria antigen profile is affected by the mode of bacterial growth [504], such as planktonic or biofilm cells, which may have potentially meaningful implications in host recognition and consequent immune response.

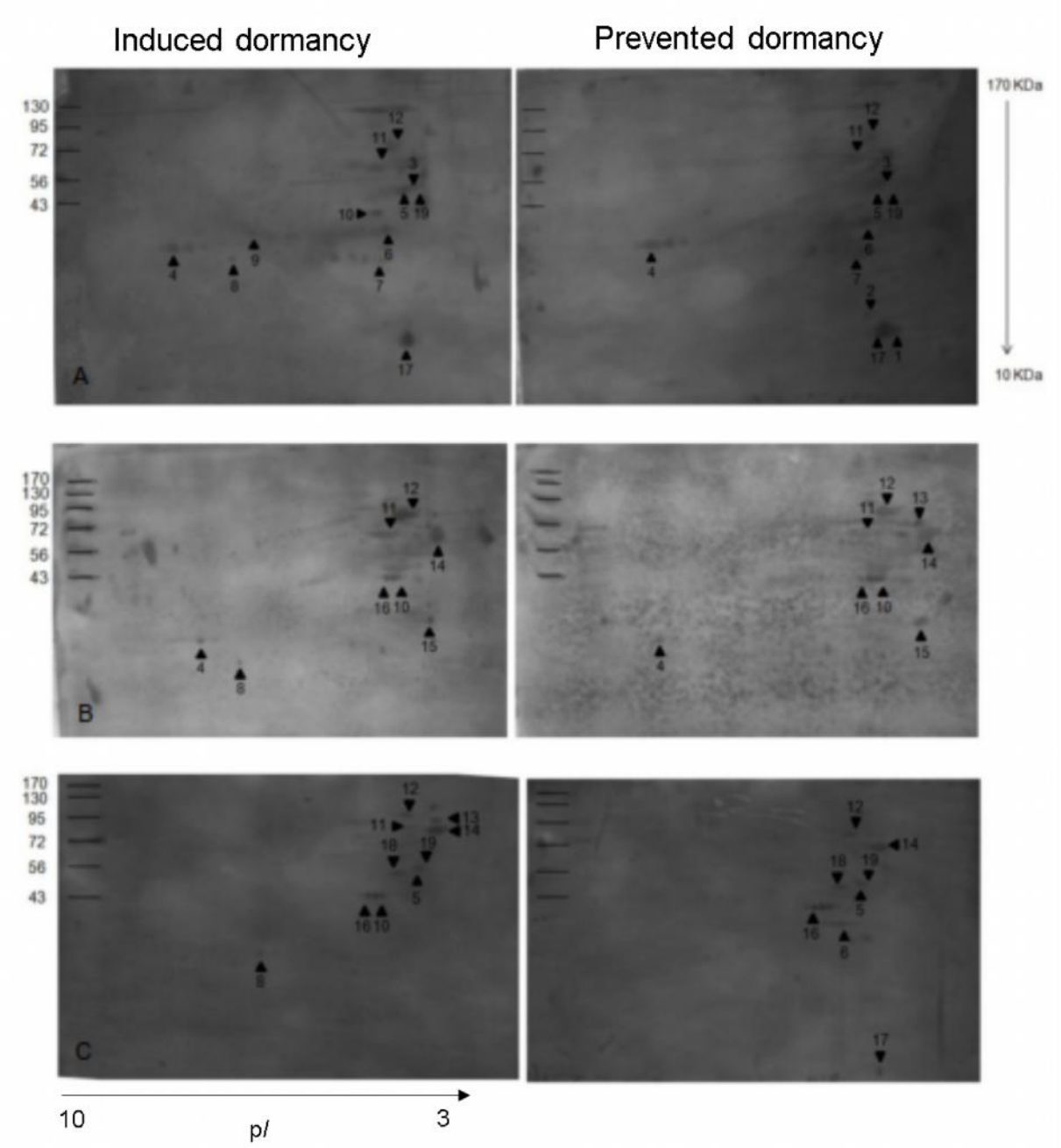

Figure 5.1.: Immunoblotting profile of whole proteins of *S. epidermidis* biofilms with induced and prevented dormancy. The immunoblotting analysis was performed with different human sera. A) donor 1, B) donor 2, C) donor 3. Protein spot identification is mentioned in Table 5.1.

Here, to identify the repertoire of immunoreactive proteins in dormant condition, we characterized the immunoproteome of *S. epidermidis* biofilms with induced and prevented dormancy, using human serum as a probe. Analysis of the 2-DE separation of whole biofilm cell protein extract from both conditions showed more than 120 protein spots in induced and prevented dormancy. A 2.0 fold-change protein intensity was set as a significant threshold between the two conditions. Apparently, up to 5 spots in each experiment had a fold-change higher than 2.0. The employment of 2-DE methodology in immunoproteomic studies is a powerful tool to identify antigens when combined with Western blotting [495]. Interestingly, a distinct immunoreactive protein profile of *S. epidermidis* biofilms with prevented and induced dormancy was found in all tested sera (Figure 5.1). Our results showed a total of 19 immunoreactive protein spots identified by MALDI-TOF/TOF (Table 5.1). Most of the immunoreactive proteins spots were located in the pH range of 4–5 and in the molecular weight range of 20–40 KDa (Table 5.1).

Only GpmI and FusA proteins were reactive to all tested sera, independently of biofilm dormancy. Both are related to ion binding molecular function, but no more evident connections are known, since GpmI is associated with glycolysis and FusA with protein biosynthesis. On the other hand, GpmA protein seemed to be more immunoreactive in induced dormancy, in all tested sera. Other proteins, such as Pgk, TpiA and Ald, were recognized by only one serum sample. The reactive pattern diversity found among sera samples may be due to differences on immune response of donors or previous exposure to *S. epidermidis*, since it is a commensal microorganism of skin and mucosae [1]. Interestingly, the immunoblot pattern included a set of reactive proteins which seemed to be only immunoreactive in biofilms with induced dormancy, such as CodY and GpmA. Conversely, FtnA and ClpP proteins were reactive in biofilms with prevented dormancy condition. Interestingly, CodY is a GTP-binding protein which responds to GTP and senses nutrient availability, controlling the expression of genes involved in the biosynthesis and transport of amino acids in several Gram-positive species [482], such as the regulation of TCA cycle activity [505]. Typically, CodY is repressed during rapid growth and induced when cells experience nutrient deprivation [482], which may be related with dormancy.

Table 5.1.: Immunoreactive proteins identified by 2-DE-MALDI-TOF/TOF.

Spot	Protein	Acession Number	Protein name	MW (KDa)	p*I*	Function	PSORTb localization	Cello localization	Number of epitopes
1	FtnA	Q5HN41	Ferritin	19.58	4.55	Iron-storage protein	Cytoplasmic	Cytoplasmic	7
2	ClpP	Q5HQW0	ATP-dependent Clp protease proteolytic subunit	21.38	5	Cleaves peptides in various proteins in a process that requires ATP hydrolysis. Has a chymotrypsin-like activity. Plays a major role in the degradation of misfolded proteins	Cytoplasmic	Cytoplasmic	7
3	Pgk	Q5HQV3	Phosphoglycerate kinase	42.74	4.76	Catalyzes the transference of a phosphate group from 3-phospho-D-glycerate to ADP	Cytoplasmic	Cytoplasmic	14
4	SsaA	Q5HLV2	Staphylococcal secretory antigen SsaA	27.91	8.4	Not known; immunogenic protein expressed during sepsis and particularly during episodes of infective endocarditis	Extracellular	Extracellular	16
5	EF-Tu	Q5HRK4	Elongation factor Tu	43.16	4.7	This protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis	Cytoplasmic	Cytoplasmic	22
6	Fda	Q5HL21	Fructose-bisphosphate aldolase class 1	32.99	4.89	Glycolytic enzyme that catalyses D-fructose 1,6-bisphosphate into glycerone phosphate and D-glyceraldehyde 3-phosphate	Unknown	Cytoplasmic	8
7	TpiA	Q5HQV2	Triosephosphate isomerase	27.37	4.9	Catalyses the interconversion of D-glyceraldehyde 3-phosphate and glycerone phosphate	Cytoplasmic	Cytoplasmic	13

8	GpmA	Q5HLI0	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	26.7	6.46	Catalyzes the interconversion of 2-phosphoglycerate and 3-phosphoglycerate	Cytoplasmic	Cytoplasmic	10
9	CodY	Q5HPT7	GTP-sensing transcriptional pleiotropic repressor CodY	28.75	5.61	It is a GTP-binding protein that senses the intracellular GTP concentration as an indicator of nutritional limitations. At low GTP concentration it no longer binds GTP and stop to act as a transcriptional repressor	Cytoplasmic	Cytoplasmic	15
10	Ldh	Q5HL31	L-lactate dehydrogenase	34.1	4.93	Catalyzes the reduction of pyruvate into lactate	Cytoplasmic	Cytoplasmic	9
11	GpmI	Q5HQV1	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	56.36	4.8	Catalyzes the interconversion of 2-phosphoglycerate and 3-phosphoglycerate	Cytoplasmic	Cytoplasmic	18
12	FusA	Q5HRK5	Elongation factor G	76.88	4.8	This protein promotes the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome	Cytoplasmic	Cytoplasmic	34
13	DnaK	Q5HNW6	Chaperone protein DnaK	66.15	4.57	Acts as a chaperone	Cytoplasmic	Cytoplasmic	28
14	GroEL	Q5HMZ1	60 kDa chaperonin	57.75	4.59	Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions	Cytoplasmic	Cytoplasmic	26

15	ButA	Q5HKG6	Diacetyl reductase [(S)-acetoin forming]	27.91	4.66	Catalyzes the irreversible reduction of 2,3-butanediol to (S)-acetoin in the presence of NADH	Cytoplasmic	Cytoplasmic	12
16	PfkA	Q5HNK6	6-phosphofructokinase	34.88	5.34	Catalyzes the reaction of D-fructose 6-phosphate into D-fructose 1,6-bisphosphate	Cytoplasmic	Cytoplasmic	12
17	Asp23	Q5HM47	Alkaline shock protein 23	19	4.92	May play a key role in alkaline pH tolerance	Unknown	Cytoplasmic	7
18	Ald	Q5HNJ6	Alanine dehydrogenase	40.2	5.03	May play a role in cell wall synthesis as L-alanine is an important constituent of the peptidoglycan layer	Cytoplasmic	Cytoplasmic	14
19	RpsA	Q5HP69	30S ribosomal protein S1	43.37	4.46	Binds mRNA; thus facilitating recognition of the initiation point. It is needed to translate mRNA with a short Shine-Dalgarno (SD) purine-rich sequence	Cytoplasmic	Cytoplasmic	15

Despite no direct involvement with dormancy has been previously described [319], genes encoding proteins here identified, have been linked to particular circumstances in biofilms. For example, the expression of *ftnA* was affected by the presence of iron and manganese ions in the biofilm growth conditions [506]. Also, the deletion of *clpP* was associated with a reduced ability to form *S. epidermidis* biofilms and with reduced virulence in a rat model of biofilm-associated infection [507]. Interestingly, among the immunoreactive proteins, 3 had previously been reported to be immunoreactive with sera from rabbits infected with live *S. epidermidis* [142], namely EF-Tu, Fda and Ald. Additionally, surface proteins are known to be crucial determinants for host colonization [508], such as SsaA, which is a well-known immunogenic protein [509]. Similarly, in *Chlamydia trachomatis*, proteins like DnaK, EF-Tu, GroEL and RpsA were also immunoreactive [510]. Since EF-Tu, DnaK and GroEL are highly conserved genes [511], they are frequently found as immunogens in several species [510,512-514]. Additionally, EF-Tu, DnaK and GroEL are among the proteins here identified, with a higher number of antigenic determinants to B-cell [515] (Table 5.1), which may be the reason for been highly reactive.

The three most representative classes of the identified proteins for biological processes, molecular functions and KEGG pathways are shown in Figure 5.2. The immunogenic proteins represented a broad range of biological functions, including small molecule metabolic process (GO:0044281), catabolic processes (GO:0009056), carbohydrate metabolic processes (GO:0005975), generation of precursor metabolites and energy (GO:0006091) and biosynthetic processes (GO: 0009058). These proteins are mainly involved in metabolic pathways, such as glycolysis/gluconeogenesis, microbial metabolism in diverse environments, biosynthesis of secondary metabolites, fructose and mannose metabolism and methane metabolism. The main molecular functions found were related to catalytic activity (GO:0003824), ion binding (GO:0043167) and oxidoreductase activity (GO:0016491). Bioinformatic analysis were used to predict subcellular localization of proteins. Results showed one protein with extracellular localization. Remaining proteins were predicted to have cytoplasmic localization (Table 5.1). These results suggest that ion binding function may influence the host immune response, since

previously, we identified this class of proteins with altered expression between *S. epidermidis* biofilms with induced and prevented dormancy (Chapter 4).

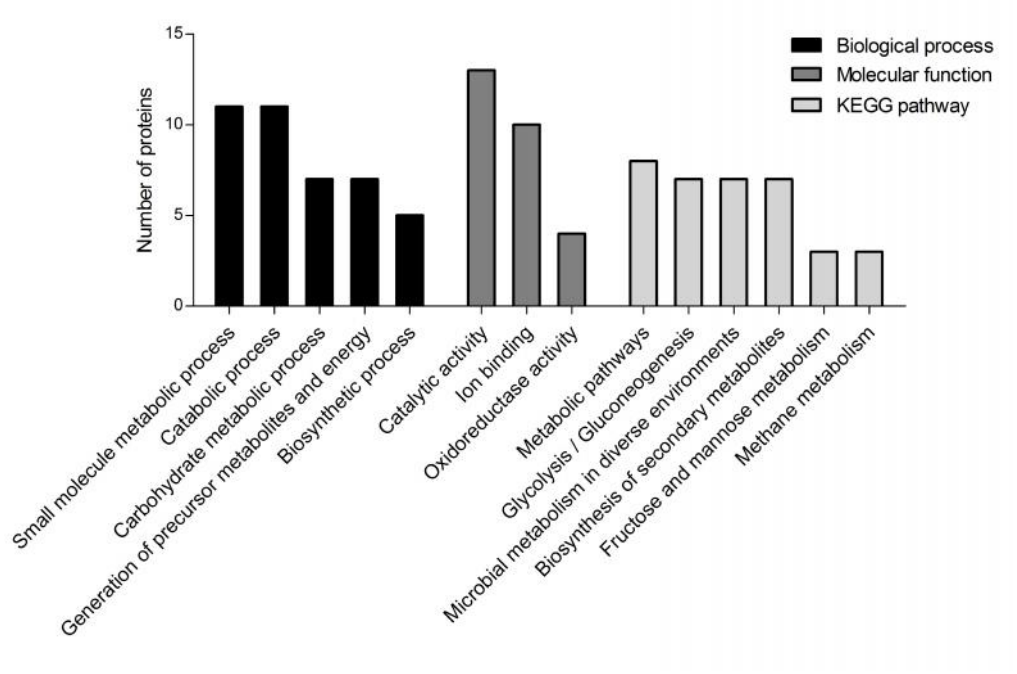

Figure 5.2.: The most representative GO terms of biological processes, molecular functions and KEGG pathways of immunoreactive proteins.

5.4. Conclusion

Taken together, our results showed that proteins from *S. epidermidis* biofilms with prevented and induced dormancy had different reactivity to human serum, providing the first evidences of dormancy impact in the human-bacteria immune interaction. Nevertheless, differences in the reactivity pattern were mainly observed in intracellular proteins, which can present difficult access to immune system. Despite individual host factors, we found differences in the immunoreactive protein pattern between *S. epidermidis* biofilms with different proportion of dormant bacteria. The immunoreactive proteins made part of a diverse group of proteins, ranging from proteins with proteolysis activity (ClpP), through proteins involved in iron transport (FtnA), proteins associated with glycolysis (GpmA) or proteins with transcription regulation biological function (CodY). In general, it is most likely that immunoreactive proteins are involved in

small molecule metabolic processes or catabolic process, with capacity to interact with ion or charged atoms and catalytic activity.

Characterization of dormancy within biofilms using immunoproteomics provided new insights into the protein expression that may determine *S. epidermidis* contact with the host. Moreover, these proteins are promising candidates as biofilm markers allowing the discrimination of physiological condition displayed by biofilm bacteria and will be worth to consider in further studies.

CHAPTER 6.

INFLUENCE OF DORMANCY IN BIOFILM TOLERANCE TO THE HOST IMMUNE SYSTEM AND ANTIMICROBIAL THERAPY

INFLUENCE OF DORMANCY IN BIOFILM TOLERANCE TO THE HOST IMMUNE SYSTEM AND ANTIMICROBIAL THERAPY

Summary

The host response and biofilm susceptibility to antibiotic therapy are influenced by bacteria physiological conditions as dormant bacteria contribute to the recalcitrance of infections. In this work, we tested if different clinical and commensal strains were able to go into a dormant state. Secondly, the susceptibility of biofilm cells with prevented and induced dormancy to human whole blood and antibiotics was assessed in a few isolates. Our preliminary results suggest that human blood did not affect differently the survival of bacteria from *S. epidermidis* biofilms with prevented and induced dormancy. However, it was observed that rifampicin and tetracycline induced a viable but-not culturable state in biofilm cells. Additionally, rifampicin was able to diffuse and kill bacteria within a mature biofilm. To achieve this conclusion, it was crucial to distinctly assess the culturability and viability of bacteria upon contact with the antibiotics. This highlight the importance of using suitable methodologies to assess antimicrobial susceptibility to avoid misleading interpretations.

6.1. Introduction

Metabolic activity heterogeneity of cells within biofilms contribute to distinct antibiotics susceptibility [101], tolerance to host immune response [184] and relapsing infections [39].

Whole human blood has been used as an *ex vivo* model for studying the pathogenesis of *S. epidermidis* [136,137,139,143]. Interestingly, it has been shown that a significant percentage of cells within a *S. epidermidis* biofilm could survive in whole human blood [143]. Nevertheless, the interaction between dormant bacteria and human blood has not been studied so far.

Currently, it is accepted that bacterial metabolism influences the action of many classes of antibiotics [516]. Most antibiotics act over active targets, killing growing bacteria [1,131,217-221,517]. Despite its use as therapeutic agents, a few antibiotics are known as important environmental factors that lead to persister cells induction [201,231,239,518]. As referred in the general introduction of this thesis, both persisters and dormant cells are recognized as highly tolerant to antibiotics [124]. The discovery of new compounds or peptides displaying activity against *S. epidermidis* biofilms has recently brought new hope to future successful treatment of biofilms [171-173,519,520]. Most important, novel strategies to combat persister cells are been pursued [115,521-526], since those survive even to high doses of antibiotics [131,217,248,249].

Hence, based on the *in vitro* model to induce dormancy in *S. epidermidis* biofilms, previously described by Cerca *et al.* [138], in the present work we intended: (a) to determine if the dormancy model previously developed could be consistent using a wide collection of clinical and commensal *S. epidermidis* isolates; (b) to assess the survival rate of *S. epidermidis* biofilm cells with induced and prevented dormancy when challenged *ex vivo* with human blood; (c) to assess the effect of different classes of antibiotics on *S. epidermidis* biofilms with induced and prevented dormancy. These goals were accomplished by employing methodologies to quantify culturability and viability, such as CFU counting and flow cytometry, respectively [284].

6.2. Material and methods

6.2.1. Bacterial strain collection and biofilm formation

Clinical *S. epidermidis* strains were collected at Hospital Geral de Santo António (Centro Hospitalar do Porto, EPE) and commensal isolates were isolated from healthy donors from the region of Minho, as previously referred by Freitas AI [527] and Oliveira and Cerca [528]. All volunteers gave written informed consent. This study was approved by the Ethics Subcommission for Health and Life Sciences (process SECVS 002/2013) of the University of Minho, Portugal and, the Ethics Committee Board of Hospital de Santo António, Porto Hospital Centre, Portugal (Reference 015/09: 014-DEFI/014-CES). A total of 18 clinical (PT13003, PT11001, PT11002, PT11003, PT11004, PT12002, PT12003, PT12006, PT12007, PT12013, PT12016, PT12019, PT12021, PT12022, PT12025, PT13001, PT12018, PT12020) and 24 commensal (COM020A, COM022A, COM031A, COM034A1, COM034A2, COM035A, COM036A, COM037A1, COM038A1, COM045A, COM040A, COM042A, COM001B, COM057, COM058A, COM049A, COM063B, COM002A, COM024A, COM023A, COM004A, COM003A, COM005A, COM010B) *S. epidermidis* strains were included in this study.

Bacterial growth was performed as previously described [138]. *S. epidermidis* strains were used to establish biofilms with induced (1%G) and prevented (1%G + Mg^{2+}) dormancy. Briefly, one colony of a given *S. epidermidis* strain was inoculated in TSB (LiofilChem) and incubated at 37 ºC in an orbital shaker at 80 rpm for 18 h. The overnight culture was adjusted to an optical density at 640 nm of 0.250 (±0.05) and 10 µL of the suspension was transferred into a 24-well plate (ThermoScientific) containing 1 mL of TSB supplemented with 0.4% glucose (v/v) (Merck) or TSB 0.4%G enriched with 20 mM magnesium chloride ($MgCl_2$) (Merck). The culture plates were then incubated at 37 ºC in an orbital shaker at 80 rpm for 24 h. After this period, the culture medium was removed and replaced by fresh TSB supplemented with 1% glucose (v/v) or TSB 1%G containing 20 mM $MgCl_2$ (1%G + Mg^{2+}). Biofilms were then allowed to grow in the same conditions for an additional 24 h. Finally, biofilm culture medium was removed from each well and washed twice with PBS. Before plating the serial dilutions of biofilm bacteria suspension in TSA, the number of viable cells was determined by flow cytometry

(as described below) and normalized to the same concentration of viable bacteria between the two conditions.

6.2.2. Susceptibility to human blood

An *ex vivo* model was used to assess the susceptibility of *S. epidermidis* cells from biofilms with prevented and induced dormancy to human blood. Whole venous blood from different healthy donors was collected into heparin tubes by using a vacuum system (approved by the Ethics Committee of *Instituto de Ciências Biomédicas Abel Salazar* (document number 081/2014)) (BD Vacuteiner®, Becton, Dickinson and Company), since heparin will not deplete ions that could be essential for bacterial growth [529]. After biofilm formation, biofilm cells were resuspended in 1 mL of PBS and the number of viable cells was determined by flow cytometry, as referred below. In a 2 mL eppendorf, 1×10^7 bacteria were added to 1 mL of blood, and incubated at 37 ºC, at 80 rpm. At every 30 min, until 120 min, an aliquot of each condition was collected and sonicated during 15 s at 18W (W185D model, MSE). The number of culturable cells was determined by performing 10-fold serial dilutions of the biofilm suspension and plating the dilutions on TSA plates. Plates were then incubated for 20 h at 37 °C. Simultaneously, the number of viable cells was determined by flow cytometry. To determine the reduction of viable or culturable cells in each timepoint, the Δ*log* was calculated by subtracting the $\log_{10}$ bacteria/mL recovered after blood incubation to the $\log_{10}$ bacteria/mL recovered at the untreated bacteria (control) of the same timepoint. Experiments were carried out 1 to 2 times, with a pool of 4 biofilms and with blood from healthy volunteers.

6.2.3. Quantification of viable cells by flow cytometry

The number of viable cells were determined by using flow cytometry, as previously described [284]. Briefly, 30 µL of cell suspension (after sonication) was added to 270 µL of PBS containing 5 µg/mL of PI (Sigma-Aldrich) and SYBR® Safe (Invitrogen) diluted at 1:5000. Then, 3 µL of fluorescent counting beads (Invitrogen) were added to allow bacteria quantification in flow cytometer (COULTER® EPICS® XL™ Flow Cytometer, Beckman Coulter). Data were

acquired using Expo 32 ADC XL 4 Color. SYBR fluorescence was detected on the FL1 channel, while PI fluorescence was detected on the FL3 channel.

6.2.4. Antibiotic susceptibility of in vitro biofilms

Biofilms were prepared as described above. Biofilm culture medium was removed from each well containing the biofilm, and replaced by 1 mL of TSB or 1 mL of TSB with one of the studied antibiotics at the Peak Serum concentration (40, 16 and 10 µg/mL for vancomycin, tetracycline and rifampicin, respectively) [99]. Then, biofilms were incubated at 37 ºC in an orbital shaker at 80 rpm. Every 2 h, until 8 h, biofilm culture medium was removed and biofilms were washed twice with PBS. Then, biofilms were resuspended in 1 mL of PBS and washed twice with PBS, followed by sonication during 15 s at 18W. The number of culturable cells was determined by plating 10-fold serial dilutions of biofilm suspension on TSA plates, which were incubated for 24 h at 37 °C. Simultaneously, the number of viable cells was determined by flow cytometry. To determine the antibiotic efficacy, the reduction of viable or culturable cells in each timepoint (Δ*log*) was calculated by subtracting the $\log_{10}$ bacteria/mL recovered after antibiotic incubation to the $\log_{10}$ bacteria/mL recovered in untreated biofilm (control). This experiment was repeated 3 to 4 times, including two biological replicates in each experiment.

6.2.5. Confocal laser scanning microscopy (CLSM) of biofilms upon contact with antibiotics

S. epidermidis biofilms formed by strain 9142 were grown as described above, with minor modifications: the biofilms were formed on 13 mm of diameter sterile plastic coverslips (Thermanox, Nunc) that were previously added to the 24-well culture plates. Biofilms with induced and prevented dormancy were exposed to tetracycline, rifampicin and vancomycin peak serum concentrations during 6 h, at 37 ºC and 80 rpm. Then, biofilms were washed twice with PBS. Biofilm cell viability was determined with Live/Dead BacLight Bacterial Viability kit (Molecular Probes), following the manufacturer's instructions. Biofilms were incubated with the staining for 15 min at room temperature, which was carefully removed by PBS. A positive control, without incubation with antibiotics was analyzed at the same time. A

negative control was included, by incubating the biofilm with 96% ethanol for 2 h, as previously described [179]. Images were acquired in Confocal Scanning Laser Microscope Olympus BX61, model FluoView1000. Biofilms were observed using a 40× water-immersion objective, in at least three different regions of two independent surfaces and images were acquired with 640 × 640 resolution. This experiment was repeated twice.

6.2.6. Statistical analysis

All graphs were generated using GraphPad Prism Software v.6 (GraphPad Software). Normality was tested with Shapiro-Wilk test. When normality test was passed, unpaired t-test was performed, assuming that both populations have the same standard deviation. When populations did not pass the normality test, Mann-Whitney was performed. A *p* value of less than 0.05 was considered statistically significant.

6.3. Results

6.3.1. Biofilm dormancy in clinical and commensal S. epidermidis isolates

Clinical and commensal isolates were used to establish *in vitro S. epidermidis* biofilms. In order to determine if these strains were able to respond in accordance with the model previously reported in which dormancy was induced in *S. epidermidis* biofilms [138], the number of viable and culturable cells were determined. Viable cells ($SYBR^+PI^-$) within a biofilm was determined and adjusted by flow cytometry. Then, culturable bacteria were determined by spread plate method. The number of culturable cells/mL is shown in Figure 6.1A.

As shown in Figure 6.1A, overall, the number of culturable cells in commensal and clinical *S. epidermidis* isolates resulted in lower number of CFU/mL in biofilms grown in dormant condition (1%G), indicating that Mg^{2+} prevented the bacterial entrance in dormancy. These results indicate that the dormancy effect was observed in both groups of isolates. However, the same degree of dormancy was not observed in all isolates (Figure 6.1B). Of note, 4 clinical (22.2%) and 6

commensal (25%) isolates presented less than 0.5 Log difference between induced and prevented dormancy.

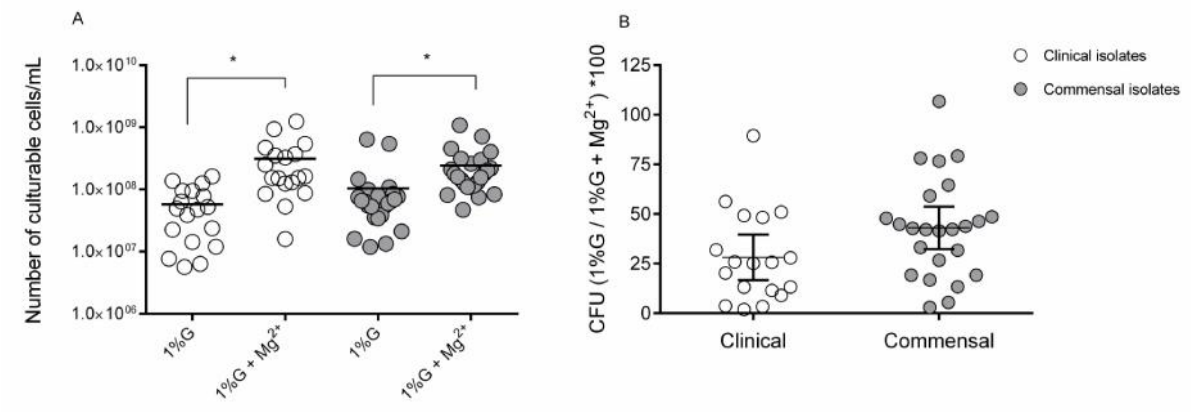

Figure 6.1.: (A) Number of culturable cells/mL of clinical and commensal *S. epidermidis* isolates in TSA plates. Each symbol corresponds to the mean of the CFUs performed in duplicate. Horizontal bars represent the mean of the indicated group. Comparison between induced (1%G) and prevented (1%G + Mg^{2+}) dormancy in each group of strains was performed by using Wilcoxon test. *$p < 0.05$; (B) Ratio between the number of culturable cells/mL in 1%G and 1%G + Mg^{2+}, in percentage. Horizontal bars represent the mean with 95% of confidence interval.

6.3.2. Biofilm cells susceptibility to human blood

Using an *ex vivo* model, we investigated the survival of *S. epidermidis* biofilm cells upon contact with human blood. For that, we randomly selected 2 commensal and 2 clinical isolates and, incubate the same number of viable bacteria obtained from biofilms with prevented or induced dormancy with whole human blood. The model strain 9142 was also included in this study. Following incubation at different timepoints, we determined the number of culturable cells by CFUs counting (Figure 6.2).

We observed a reduction of *S. epidermidis* culturability up to 120 min, after exposure to human blood, with no apparent distinction between commensal and clinical isolates. Since this experiment was performed a limited number of times, we cannot draw major conclusions regarding how dormancy affects survival of biofilm cells exposed to human blood. However, based on these preliminary results, we can suggest that no major differences were observed between induced and prevented dormancy, and after 60 min of incubation both conditions presented a similar decrease in bacteria culturability.

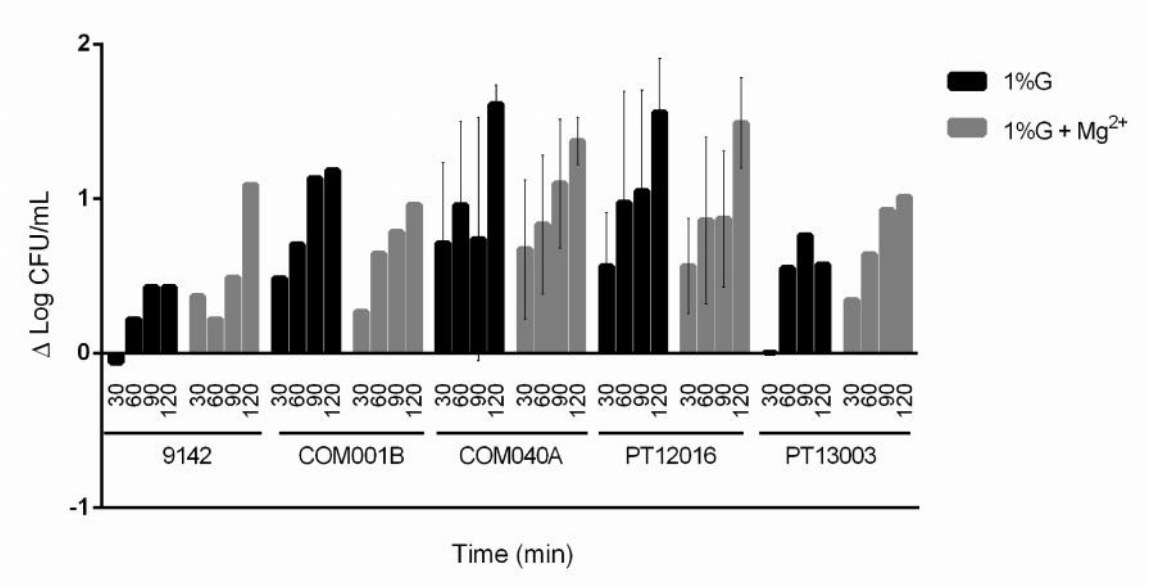

Figure 6.2.: Mean fold reduction of cellular culturability after incubation with whole human blood. The y-axis indicates the difference between the strain without incubation with blood and the strain upon contact with blood, in induced dormancy (1%G) and prevented dormancy (1%G + Mg^{2+}). Vertical bars represent standard deviation of isolates with two independent experiments. The remaining isolates were tested only once and do not have standard deviation.

6.3.3. Biofilm susceptibility to antibiotics

To assess the effect of dormancy in biofilms susceptibility to antibiotics, we selected different antibiotic classes, such as the glycopeptide vancomycin (cell-wall biosynthesis inhibitor), the rifamycin rifampicin (transcription inhibitor) and tetracycline (protein synthesis inhibitor) [530]. The individual effect of these antibiotics against intact *S. epidermidis* biofilms from different clinical and commensal isolates was assessed. First, we determined the effect of antibiotics over time, using the strain 9142 and up to 8 h of incubation. Figure 6.3A, 6.33C and 6.3E show the effect of rifampicin, vancomycin and tetracycline over culturability of *S. epidermidis* biofilms formed with strain 9142.

As observed, rifampicin was the most effective antibiotic acting on intact biofilms and presented an increased action over time. Additionally, a reduction in culturability was also observed when tetracycline was used, although with a lower effect when compared to rifampicin. Vancomycin had no effect in cell culturability, even after 8 h of interaction. Interestingly, significantly differences in culturability reduction were detected in prevented and induced dormancy, when rifampicin and tetracycline were tested.

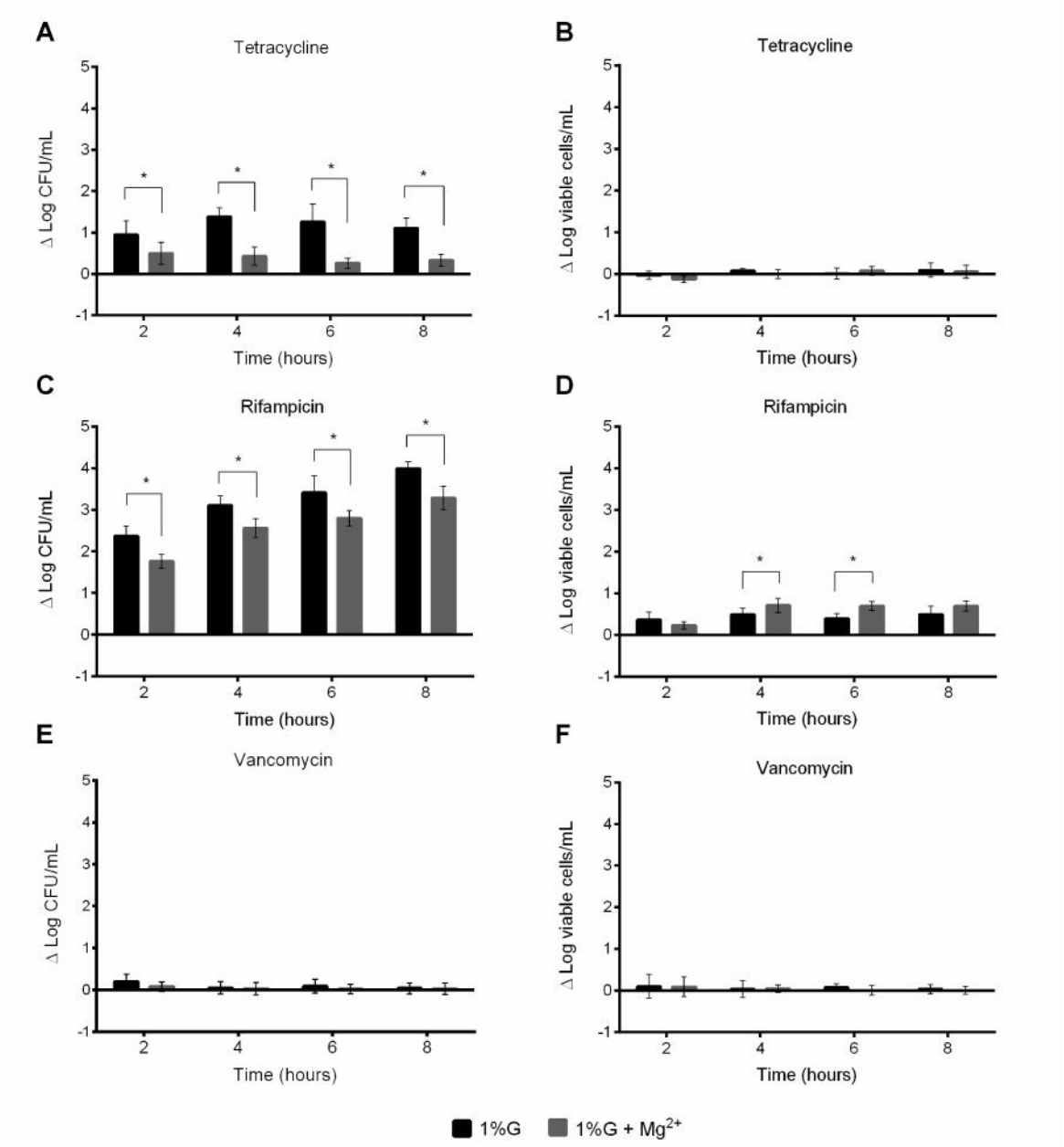

Figure 6.3.: Mean fold reduction of cellular culturability (A, C, E) and of cellular viability (B, D, F) after incubation with antibiotics. The y-axis indicates the difference between the *S. epidermidis* strain 9142 without incubation with antibiotics and the strain upon contact with them, in induced dormancy (1%G) and prevented dormancy (1%G + Mg^{2+}). This experiment was repeated four times with independent replicates. Vertical bars represent standard deviation. * Indicates significant differences when 1%G was compared to 1%G + Mg^{2+}, with $p < 0.05$.

In order to clarify if the observed effect on culturability was caused by death or dormancy, SYBR/PI staining was used to quantify viable cells by flow cytometry (Figure 6.3B, 6.3D, 6.3F). These results demonstrated that the only antibiotic which reduced *S. epidermidis* biofilm cell viability was rifampicin. However cell viability reduction was significantly lower than culturability reduction. Biofilms with

prevented dormancy showed a higher decrease in viability when treated with rifampicin, which increased until 4 h. Interestingly, tetracycline treatment did not lead to a reduction of viability. In agreement with culturability data, vancomycin did not affect viability.

To confirm these results, the same *S. epidermidis* isolates tested in the human blood experiments, were used. Biofilms were formed with clinical and commensal *S. epidermidis* isolates and placed in contact with the antibiotics for 6 h. The reduction of bacterial cell culturability is shown in Figure 6.4. Similar to what was observed for strain 9142, rifampicin was the most effective antibiotic in reducing bacterial cell culturability and vancomycin had a low effect over bacterial culturability, in all strains tested. Interestingly, tetracycline had an increased effect in biofilms with induced dormancy. However, the variable effect of rifampicin in biofilms with induced or prevented dormancy, seems to be strain dependent, since it led to a higher reduction in culturability of induced dormancy biofilms of PT13003 in opposite to COM040A and PT12016 isolates. Additionally, an analysis of the effect on viable cell counting revealed that similarly to strain 9142, it was only observed a reduction of viability when rifampicin was tested (Figure 6.4B, 6.4D, 6.4F). Herein, significant differences were found in COM001B and PT12016 isolates, where there was a higher reduction in viability of biofilms with prevented dormancy (Figure 6.4D).

6.3.4. Effect of antibiotics on biofilm viability and structure

The effect of antibiotics over the biofilm structure and viability of *S. epidermidis* was assessed by CLSM, using the model strain 9142 (Figure 6.5). CLSM analysis confirmed our previous observations: among all tested antibiotics, rifampicin was the only one that significantly changed structure and cell viability. Importantly, CLSM images demonstrated that rifampicin was able to diffuse and kill bacteria even in the deeper biofilm layers (Figure 6.5C and 6.5D). In the opposite, biofilms treated with vancomycin (Figure 6.5E and 6.5F) and tetracycline (Figure 6.5G and 6.5H) contained mainly viable bacteria.

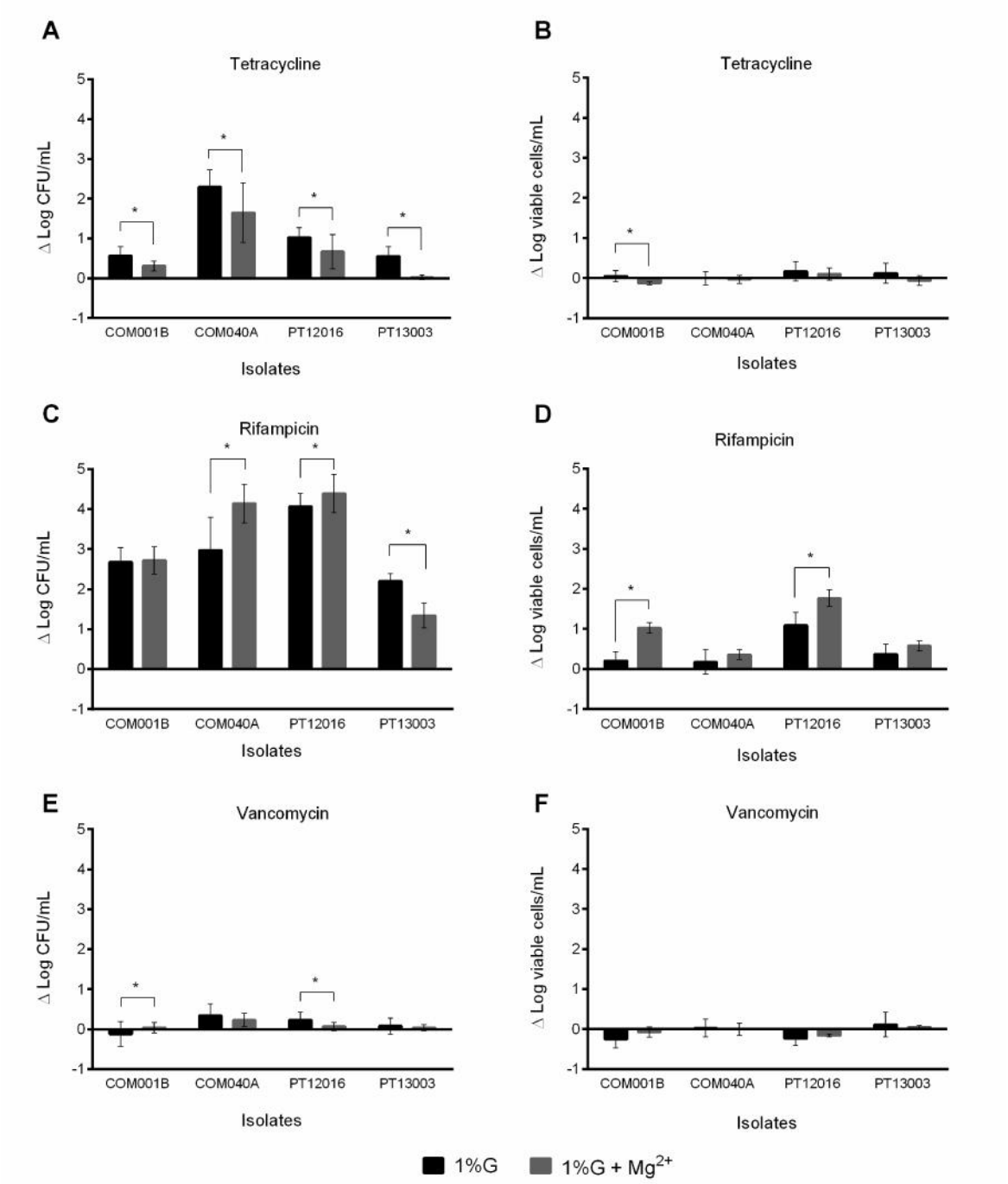

Figure 6.4.: Mean fold reduction of cellular culturability (A, C, E) and of cellular viability (B, D, F) after 6 hours of incubation with antibiotics of different commensal and clinical isolates. The y-axis indicates the difference between the *S. epidermidis* strain without incubation with antibiotics and the strain upon contact with them, in induced dormancy (1%G) and prevented dormancy (1%G + Mg^{2+}). The experiment was repeated at least three times with two independent replicates. Vertical bars represent standard deviation. * Indicates significant differences when 1%G was compared to 1%G + Mg^{2+} with $p < 0.05$.

6.4. Discussion

It is estimated that biofilm-related infections represent between 65% (to CDC) to 80% (to NIH) of all human infections [42]. Between 30% and 50% of catheter-related bacteremia are caused by coagulase-negative Staphylococci [16,530]. The interest on clinical and commensal isolates ability to form biofilm and its relevance to clinical infections is not recent [418,419,531-536]. From genome to proteome, deep analyses have been carried out in order to find the trigger between commensalism and pathogenicity of *S. epidermidis*. Using an *in vitro* model to induce and prevent dormancy within *S. epidermidis* biofilms, we first tested if clinical and commensal strains were able to enter into a dormant state (Figure 6.1). Our results confirmed that dormancy modulation is widespread in different *S. epidermidis* isolates obtained from different sources. These results suggest that this is indeed a relevant mechanism of *S. epidermidis* biofilm physiology, not exclusive to clinical strains. However, culturable differences between biofilms with induced and prevented dormancy tend to be lower in commensal isolates, when compared to the tested clinical isolates. Furthermore, we were interested in assessing how dormancy would influence the biofilm survival when exposed to human blood. Interestingly, despite a reduction of culturability of bacteria upon blood interaction, we did not found significant differences between clinical and commensal isolates, neither between induced and prevented dormancy (Figure 6.2). However, due the low availability of blood donors, this experiment was performed a limited number of times. Furthermore, taken in consideration donor-to-donor variability inherent in human blood, conclusions from these experiments need to be considered carefully.

Contrary to the *ex vivo* blood experiments, we found important differences in antibiotics action in biofilms with induced and prevented dormancy. It is known that biofilm tolerance to antibiotics may be associated with the existence of subpopulations of bacteria with distinct metabolic activity that consequently tolerate antibiotics and persist [222,403,516,537,538]. As could be expected [179,539-541], vancomycin had a limited action against mature biofilms (Figure 6.3E, 6.3F, 6.4E, 6.4F, 6.5E, 6.5F). Interestingly, vancomycin is the recommended treatment for methicillin-resistant *S. epidermidis* (MRSE) infections, despite the

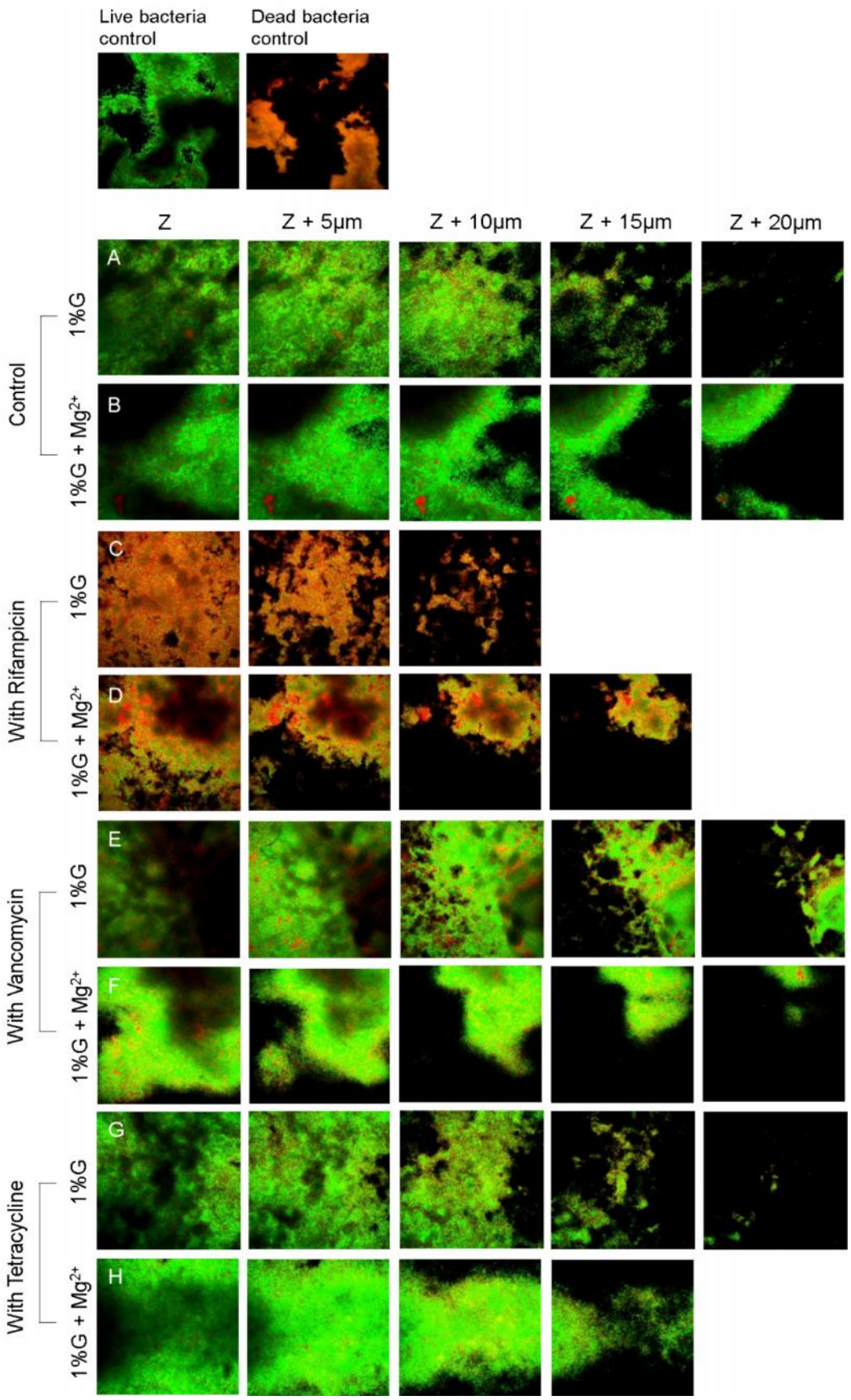

Figure 6.5.: Effect of rifampicin, vancomycin and tetracycline, after 6 hours of incubation, on biofilm structure formed by strain 9142 with induced (1%G) and prevented (1%G + Mg^{2+}) dormancy.

emergence of strains with reduced susceptibility to vancomycin [542]. Contrary, rifampicin and tetracycline led to a reduction of bacteria culturability. However, when the number of viable cells was assessed, only rifampicin led to a reduction of cell viability over time. These results were further confirmed by CLSM (Figure 6.5). Most importantly, the reduction in viability upon incubation with rifampicin (~1 Log) was not as drastically as observed for culturability (~2 - 4 Log). These results underline that rifampicin is not primarily leading to cell death, but inducing bacteria to go into a viable but non-culturable state in a higher extent. This observation was further confirmed by testing further strains of *S. epidermidis*. On the other hand, our data clearly suggests that tetracycline is leading biofilms to enter into a viable but non-culturable state instead of causing bacterial death, since it was not observed a reduction of bacteria viability (Figure 6.3A, 6.3B, Figure 6.4A, 6.4B and Figure 6.5B, 6.5H). Flemming *et al.* have shown that biofilms treated with tetracycline contained a low metabolic activity despite a significant levels of live cells was detected by CLSM [173], which may suggest that indeed, tetracycline was inducing a physiological state characterized by VBNC bacteria. Interestingly, Pasquaroli *et al.* also demonstrated that the environmental antibiotic stress was able to induce a VBNC state in *S. aureus* biofilms [201]. Curiously they demonstrated, by epifluorescence microscopy and flow cytometry after Live/Dead staining, that vancomycin promotes the emergence of persistent VBNC. Additionally, their resuscitation studies showed that persistent VBNC forms recover full metabolic activity and culturability in supplemented fresh medium.

Due to the clinical relevance of dormant bacteria within biofilms, an appropriate assay should be employed to detect this sub-population of bacteria and to assess the exact effect of antibiotics against bacteria. Most of the times, CFUs and XTT (tetrazolium salt) assay have been used as an indicator of viability, despite limited capacity to detect viable but non-growing bacteria. For instance, it is easily found in the literature that rifampicin lead to a high reduction of culturability in *S. epidermidis* biofilms [99,104,179,541,543,544]. However, in most cases, the reduction in cell culturability has been directly associated to cell death. Furthermore, methods to assess the metabolic activity reduction, also lack clear evidence that the reduction in metabolism is, in fact, resultant of cell death [99,173,545]. Therefore, a direct correlation between culturability, metabolic

activity and viability is scarce [251]. More recently, techniques involving Live/Dead fluorescence probes were introduced, being microscopy and flow cytometry [138,201,244,284,546-548] used to determine culture viability. In parallel, microscopy may be used to demonstrate the damage caused by the antibiotic action. For instance, a flow-cell associated with a confocal laser scanning microscopy were used to simulate the changing antibiotic concentrations in humans associated with intravenous dosing on *P. aeruginosa* biofilms [549].

In conclusion, this study emphasizes the importance of assessing the effect of antibiotics using appropriate methodologies that allow distinguishing culturable and viable cells. This point represents an important drawback in published studies that address antimicrobial effect as a measurement of cellular culturability which can raise many doubts about the real effect of antimicrobial drugs. Importantly, even when not detectable, dormant cells may have significant clinical implications. First, since dormant bacteria are not detected by traditional culture methods, misleading interpretations about the infection status may affect the success of therapeutic options [243]. Secondly, there is a possibility of dormant bacteria revert the phenotype into metabolic active cells [550]. Lastly, despite the lower inflammatory properties of dormant bacteria [138,296] they can still elicit an inflammatory process that may affect the patient's quality of life.

CHAPTER 7.

FINAL REMARKS

7.1. Final remarks

Due to the clinical consequences of the presence of dormant bacteria within a biofilm, an extended characterization of dormancy may contribute to underline mechanisms of this specific microbial phenotype. More information on dormant physiology will enable the development of strategies to obtain a successful treatment.

Most probably, the mechanisms involving dormant cells formation are dependent on multiple genes or pathways. This lack of knowledge depends mostly on the fact that, direct analysis of dormant bacteria on a single cell level is not yet feasible. Additionally, the existence of dormant bacteria within a biofilm was demonstrated *in vitro*, but despite all evidences, it has been difficult to demonstrate their presence *in vivo* [178,243]. It is of general knowledge that *in vitro* experiments represent an important limitation, since *in vitro* models respond differently as compared with *in vivo* biofilms [176-178]. Yet, efforts have been made to develop biofilm models which include biofilm properties such as, complexity and cellular heterogeneity [551].

Thus, we undertook a global characterization of *in vitro S. epidermidis* biofilms with prevented and induced dormancy employing an integrated analysis of different "*omics*". Transcriptomic, proteomic and immunoproteomic profile analysis were conducted. Additionally, we were also interested in assessing the relevance of dormancy in terms of clinical aspects, such as the ability of different isolates to trigger dormancy and their interaction with human blood and antibiotics.

I. Which are the main transcriptomic and proteomic features of biofilm dormancy?

Taken in consideration the whole transcriptome and proteome profile analysis of *S. epidermidis* biofilms with prevented and induced dormancy, the main features that characterizes dormancy within *S. epidermidis* biofilms were draw (Figure 7.1).

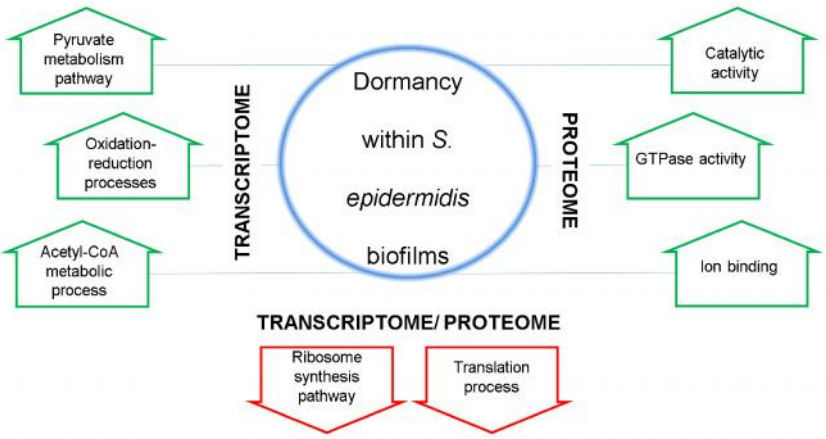

Figure 7.1.: Main aspects of dormancy within *S. epidermidis* biofilms accomplished by transcriptomic and proteomic approaches.

Although not surprisingly, translation process and ribosome synthesis pathways were found decreased in dormancy. Curiously, pyruvate metabolic pathway was enhanced in dormancy in comparison with biofilms with prevented dormancy. Pyruvate is the product of glycolysis pathway and it is known that in the aerobic conditions in the presence of glucose, there is an increase expression of glycolytic enzymes. However, the role of pyruvate metabolic pathway in dormancy was not further investigated. Additionally, proteins with GTPase activity seems to be increase in a magnesium limited environment. Given the high number of transcripts and proteins of *S. epidermidis*, the comparison of the transcriptome and proteome is extremely complex and may lead to misinterpretations. More specifically, proteins which could play a role in the dormancy process may be less frequent and in lower concentration [552]. Nevertheless, it is worth noting that the global changes found in biofilms with induced dormancy refer to the bulk of biofilm cells and not only to dormant bacteria.

II. Is there a correlation between transcriptomic and proteomic data?

Despite the correlation between transcriptomic and proteomic data from *S. epidermidis* biofilms was shown to be low, it was possible to identify common features between both approaches (Figure 7.1). These were expected results since the correlation between transcriptome and proteome expression has been assumed to be low in bacteria [432]. Nevertheless, comparative analysis in

biofilms may be neglecting the importance of post-translational modifications (PTMs) since it was reported that: (a) phosphorylation seems to play a key role in biofilm development steps [553]; (b) an increase in the number of Ser/Thr-phosphorylated proteins seems to be important in nutrient-induced bacteria dispersion [554]; (c) and glycosylation may be a key signaling for host-bacteria interaction correlated with bacteria pathogenicity [555].

III. Are there any differences between immunoproteomic profile of induced and prevented biofilm dormancy?

Since the bacterial biofilm colonization is a complex interplay between the bacteria, the host and the biomaterial, the interest on how the biofilm interact with the host could reveal important aspects associated to the immune evasion. The most remarkable feature of immunoproteomic profile between both conditions was the observation that CodY protein could be more reactive to human sera when dormancy was induced, and ClpP protein when dormancy was prevented. Nevertheless, the expression of CodY is increased when cells experience nutrient deprivation, suggesting that magnesium is an important nutrient in *in vitro S. epidermidis* biofilms. More importantly, ClpP protein cleaves peptides in the presence of ATP and magnesium and it is associated with virulence of a biofilm-associated infection. In fact, *S. epidermidis* biofilms with prevented dormancy were previously linked to a higher inflammatory profile [138,296].

IV. What's the clinical relevance of dormancy?

Lastly, dormancy was observed in the majority of clinical and commensal strains of our bacteria collection (n = 43). It is worth to note that the survival of bacteria from biofilms with prevented and induced dormancy did not seem to be distinctly affected upon contact with whole human blood. The most interesting new finding was related to the effect of antibiotics, such as tetracycline and rifampicin, against *S. epidermidis* biofilms. Curiously, both antibiotics are inducing biofilm cells to enter in a VBNC state. In order to achieve these new findings, it was determinant to assess the culturabilty and viability of bacteria upon contact with antimicrobials.

7.2. Future perspectives

The described work highlighted several aspects regarding *S. epidermidis* biofilms with induced and prevented dormancy. However, several questions remain open and may be taken in consideration in the near future.

First, as mentioned above, we would like to identify the contribution of ClpP and CodY to the dormant mechanism and understand if they constitute important markers related to dormancy and inflammatory properties of a biofilm infection. Furthermore, all proteins found reactive to human sera should be considered for additional studies since they are promising candidates as biofilm markers and also, as potential preventive or therapeutic targets.

Second, antibiotics caused a decrease of culturable cells without reduction of viability. However, we can not distinguish these cells of being persister or VBNC or dormant bacteria. Therefore, further experiments are needed to overcome this limitation, despite the difficulty to differentiate between the resuscitation of VBNC cells and the normal growth of residual culturable cells in the remaining sample.

Third, bacteria behavior is influenced by the interaction with the host. In order to minimize this disadvantage in our future investigations, we should include a host component that contributes to mimic the host conditions. However, heterogeneity among individual donors' will imply a higher number of independent experiments to be performed.

Fourth, it would be of upmost importance to identify the role of magnesium during the development of our model, in *in vivo* conditions. Recently, implants with magnesium were tested for *in vitro* and *in vivo* antibacterial properties against *S. aureus* [556,557]. These studies revealed that the magnesium implant reduced bacterial adhesion, prevented the implant contamination and led to a faster new bone tissues formation, indicating that the degradation of magnesium could also promote the osteogenic process with good biocompatibility.

Lastly, detection and analysis of post-translational modifications, such as phosphorylation and glycosylation, may be considered to understand bacterial pathogenicity.

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