**Universidade do Minho**
Escola de Engenharia

Nathalie de Sá Lopes

# The role of *codY* and *pdhA* in *S. epidermidis* biofilms, VBNC cells formation and virulence

abril de 2023

Nathalie de Sá Lopes **The role of codY and pdhA in *S. epidermidis* biofilms, VBNC cells formation and virulence**

UMinho | 2023

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# The role of *codY* and *pdhA* in *S. epidermidis* biofilms, VBNC cells formation and virulence

Tese de Doutoramento
Doutoramento em Engenharia Biomédica

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

abril de 2023

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**Acknowledgements**

A concretização desta longa e muito desafiante jornada da minha vida foi apenas possível devido ao contributo de várias pessoas, a quem endereço agora os meus mais sinceros agradecimentos.

Em primeiro lugar quero agradecer aos meus orientadores que me acompanharam ao longo de todo este percurso. Ao **Doutor Nuno Cerca**, pelo desafio proposto, ao acompanhamento e motivação constante, partilha de conhecimentos e exigência no trabalho, de forma a tornar-me uma pessoa mais dinâmica e autónoma. À **Doutora Ângela França** agradeço toda a disponibilidade e paciência de minuciosamente partilhar todos os seus conhecimentos e métodos, dos debates de ideias e na procura de respostas para todos os problemas que foram surgindo ao longo deste trabalho. Ao **Professor Manuel Vilanova** agradeço pela excelente receção e integração na sua equipa, pela incansável disponibilidade e em horas extraordinárias e por toda a partilha de conhecimentos que existe no mundo da Imunologia.

Agradeço a todos os membros do "grupo NC" com quem tive o privilégio de trabalhar, pelo excelente ambiente de trabalho, pelo apoio, espírito de entreajuda e pela amizade. Um agradecimento em especial à **Vânia**, à **Joana** e ao **Fernando**, por todo o carinho, partilha de conhecimentos, pelos debates de ideias e constante apoio em todos os momentos menos bons. À **Luciana**, à **Ângela Lima**, à **Lúcia** e à **Mafalda**, pelo companheirismo, boa disposição e por todo apoio.

A todos os membros do grupo de **Imunobiologia do I3S**, em particular à **Professora Alexandra Correia** pela disponibilidade e por todos os ensinamentos. Ao **Renato** pela partilha de conhecimentos e pela ajuda que permitiu a realização das tarefas de Imunologia. À **Inês**, à **Joana**, ao **Luís** e à **Claúdia** por toda a ajuda ao longo do meu percurso no I3S.

Um agradecimento muito especial aos meus **Pais** e **Irmão**, por todo carinho, apoio incondicional, pela confiança depositada em mim para concretização deste grande projeto da minha vida. Aos amigos de longa data, em especial à **Marlene**, à **Claúdia** e à **Catarina**, pela amizade, por todo o apoio e pelos bons momentos que passamos juntas. À **Bea**, que apesar de ter entrado nesta aventura numa fase final, teve um grande impacto neste percurso, agradeço pela amizade, pelo constante apoio, pelas nossas aventuras e risadas.

A ti, **Ricardo**, agradeço-te principalmente por todo o amor, amizade e apoio. Pela paciência e compreensão ao longo destes anos juntos. Por acreditares em mim, mais do que eu mesma, dando-me a força e a garra de lutar pelos meus objetivos. Sem ti esta aventura não teria o mesmo significado. Por fim, um agradecimento carinhoso à **Família do Ricardo**, que passou a ser minha família, que de uma forma menos direta, mas não menos importante, tiveram um contributo especial ao longo desta jornada.

This work was supported by the Portuguese Foundation for Science and Technology (FCT) through an individual PhD scholarship (SFRH/BD/136998/2018), further extended by the fellowship COVID/BD/152917/2023, by the funded project PTDC/BIA-MOL/29553/2017, under the scope of COMPETE2020 (POCI-01-0145-FEDER-029553) and by the strategic funding of unit (UIDB/04469/2020).



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**O papel dos genes *codY* e *pdhA* na formação de biofilmes, células VNC e na virulência de *S. epidermidis***


## RESUMO

*Staphylococcus epidermidis*, uma bácteria comensal que habita na pele e mucosas humanas saudáveis, é uma das principais causas de infeções associadas aos cuidados de saúde. A capacidade de *S. epidermidis* formar biofilmes em dispositivos médicos é o seu principal fator de virulência, o que contribui para a redução da eficiência dos antibióticos, evasão à resposta do sistema imunitário do hospedeiro e para a recorrência das infeções. Os biofilmes bacterianos apresentam diferentes subpopulações tal como as células viáveis mas não cultiváveis (VNC), que são conhecidas por surgirem em condições ambientais adversas. CodY, um regulador da transcrição, foi previamente reconhecido como um repressor da expressão de centenas de genes em diferentes espécies bacterianas sob condições desfavoráveis. Ainda, através de uma análise de imunoproteómica, o CodY foi identificado como um potencial fator na indução de células VNC em biofilmes formados por *S. epidermidis*. Considerando estes factos, construímos e caracterizamos uma estirpe mutante, sem o gene *codY*, para determinar o seu papel na indução do estado VNC e noutros fatores de virulência. O primeiro ensaio experimental foi focado na validação de um modelo de indução da formação de células VNC *in vitro* previamente desenvolvido. É importante salientar que também demonstramos a formação de células VNC em populações planctónicas, seguindo o mesmo mecanismo de indução utilizado em biofilmes. Em relação à caracterização da estirpe mutante, esta apresentou uma taxa de crescimento e de capacidade de formação de biofilme reduzidas em comparação com a estirpe selvagem. Sob condições indutoras do estado VNC, foi observado um maior número de células cultiváveis em biofilmes formados pela estirpe mutante. Adicionalmente, avaliamos o impacto da deleção do gene *codY* na capacidade da bactéria sobreviver no sangue e plasma humano, e na sua susceptibilidade a antibióticos. Os nossos dados revelaram que a deleção do gene *codY* tornou a bactéria mais suscetível à rifampicina, mas não foram encontradas diferenças significativas na sobrevivência bacteriana no sangue ou no plasma humano. Contudo, em estudos funcionais com macrófagos humanos, a estirpe mutante mostrou estimular uma resposta pró-inflamatória mais exacerbada em relação à estirpe selvagem. No geral, os nossos dados sugerem um papel importante do gene *codY* na virulência de *S. epidermidis*.



**Palavras-chave:** Biofilmes, *codY*, Células VNC, Macrófagos, *Staphylococcus epidermidis*.

**The role of *codY* and *pdhA* in *S. epidermidis* biofilms, VBNC cells formation and virulence**


## ABSTRACT

*Staphylococcus epidermidis*, a commensal bacteria that inhabits healthy human skin and mucous membranes, is the cause of healthcare-associated infections. The ability of *S. epidermidis* to form biofilm on medical devices is the foremost virulence factor, which contributes to the reduced antibiotics efficiency, evasion from the host immune system response and the recurrence of biofilm-related infections. The bacterial biofilm harbours different subpopulations, such as viable but non-culturable (VBNC) cells, which are known to arise in adverse environmental conditions. CodY, a transcription regulator, was previously found to repress the expression of hundreds of genes in different bacterial species under unfavourable conditions. Additionally, in a previous immunoproteomic analysis, CodY was identified as a potential key player in the induction of VBNC cells in *S. epidermidis* biofilms. Considering these facts, we constructed and characterized a mutant strain lacking the *codY* gene, to determine its role in VBNC state induction and other virulence factors. The first experimental assay was focused on the validation of a previous *in vitro* model used to induce the formation of VBNC cells. Importantly, we also demonstrated the possibility to modulate the formation of VBNC cells in planktonic populations, following the same VBNC induction mechanism used in biofilms. Regarding the characterization of the mutant strain, we found a reduced growth rate and biofilm formation ability in comparison to the wild-type strain. Remarkably, under VBNC-inducing conditions, a higher number of culturable cells was observed in biofilms formed by the *codY*-mutant strain. Additionally, we also evaluated the impact of the deletion of *codY* gene on the bacteria's ability to survive in human blood and plasma, as well as their susceptibility to antibiotics. Our data revealed that the lack of *codY* rendered the bacteria more susceptible to rifampicin, but no significant differences were found in the bacteria's survival in both human blood or plasma. After challenging human macrophages with the *S. epidermidis* strains, a higher pro-inflammatory response was found by the *codY*-mutant strain in comparison to the wild-type strain. Overall, our data suggest an important role of *codY* in *S. epidermidis* virulence.



**Keywords:** Biofilms, *codY*, Macrophages, *Staphylococcus epidermidis*, VBNC cells.

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## List of abbreviations

| | |
|---|---|
| 2-DE | Two-dimensional electrophoresis |
| 6-HAP | 6-N-hydroxyaminopurine |
| Aae | Adhesion protein |
| Aap | Accumulation-associated protein |
| Agr | Accessory gene regulator |
| AIs | Autoinducers |
| AMPs | Antimicrobial peptides |
| ATC | Anhydrotetracyline |
| AtlE | Autolysin protein |
| BAIs | Biofilm-associated infections |
| Bap | Biofilm-accumulation protein |
| BCAAs | Branched-chain amino acids |
| BCFAs | Branched-chain fatty acids |
| Bhp | Biofilm-homologous protein |
| Bp | Base pair |
| $Ca^{2+}$ | Calcium |
| CDM | Chemically defined medium |
| $CDM^{-ILV}$ | CDM lacking isoleucine, leucine and valine |
| $CDM^{-Ile}$ | CDM lacking isoleucine |
| $CDM^{-Leu}$ | CDM lacking leucine |
| $CDM^{-Val}$ | CDM lacking valine |
| cDNA | Complementary DNA |
| CFU | Colony-forming units |
| CIP | Ciprofloxacin |
| CLSM | Confocal laser scanning microscopy |
| $CM_{10}$ | Chloramphenicol at 10 μg/mL |
| COMP | Complementation |
| CONF | Confirmation |
| CoNS | Coagulase-negative staphylococci |
| COX-2 | Cyclooxygenase-2 |

| | |
|---|---|
| cRPMI | Complete RPMI |
| cRPMI$^{-PEN/STREP}$ | cRPMI without PEN/STREP |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DCX | Dicloxacillin |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| eDNA | Extracellular DNA |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| Embp | Extracellular matrix-binding protein |
| Fbe/SdrG | Fibrinogen-binding protein |
| FBS | Fetal bovine serum |
| FW | Forward |
| gDNA | Genomic DNA |
| GlcNAc | N-acetylglucosamine residues |
| GTP | Guanosine triphosphate |
| HAIs | Healthcare-associated infections |
| I | Intermediate |
| *ica* | Intercellular adhesion operon |
| IFN-α | Interferon-alpha |
| IFN-γ | Interferon-gamma |
| IL | Interleukin |
| IND | Induced VBNC state |
| iNOS | Inducible nitric oxide synthase |
| INT | p-iodonitrotetrazolium violet |
| KO | Knockout |
| LDH | Lactate dehydrogenase |
| LipA | Lipoic acid synthetase |
| LPS | Lipopolysaccharides |
| LZD | Linezolid |
| MAC | Membrane attack complex |
| $Mg^{2+}$ | Magnesium |
| $MgCl_2$ | Magnesium chloride |
| MIC | Minimal inhibitory concentration |

| | |
|---|---|
| MOI | Multiplicity of infection |
| mRNA | Messenger ribonucleic acid |
| MRSE | Methicillin-resistant *S. epidermidis* |
| MS | Mass spectrometry |
| MSC | Multiple cloning site |
| MSCRAMMs | Microbial surface components recognizing adhesive matrix molecules |
| NaCl | Sodium chloride |
| NCBI | National Center for Biotechnology Information |
| NRT | No-RT control |
| NTC | No-template control |
| OD | Optical density |
| PAMP | Pathogen-associated molecular pattern |
| PBMC | Peripheral blood mononuclear cell |
| PBP2a | Penicillin-binding protein |
| PCR | Polymerase chain reaction |
| PEN/STREP | Penicillin/streptomycin |
| PI | Propidium iodide |
| PIA | Polysaccharide intercellular adhesin |
| PMA | Phorbol myristate acetate |
| PNAG | Poly-N-Acetylglucosamine |
| PRE | Prevented VBNC state |
| PSC | Peak serum concentrations |
| PSM | Phenol-soluble modulin |
| qPCR | Quantitative PCR |
| QS | Quorum-sensing system |
| R | Resistant |
| RIF | Rifampicin |
| RNA | Ribonucleic acid |
| RNA-Seq | RNA sequencing |
| ROS | Reactive oxygen species |
| Rpf | Resuscitation promoter factor |
| RT | Room temperature |
| RV | Reverse |
| S | Susceptible |

| | |
|---|---|
| SARS-CoV-2 | Severe acute respiratory syndrome coronavirus-2 |
| Sbp | Small basic protein |
| SCCm*ec* | Staphylococcal cassette chromosome mec |
| SdrF | Serine aspartate-binding protein |
| TET | Tetracycline |
| TGF-β | Transforming growth factor-beta |
| TLRs | Toll-like receptors |
| TLR2 | Toll-like receptor 2 |
| TNF-α | Tumor necrosis factor-alpha |
| TSA | Tryptic soy agar |
| $TSA_{CM10}$ | TSA supplemented with 10 μg/mL chloramphenicol |
| TSB | Tryptic soy broth |
| $TSB_{0.4\%G}$ | TSB supplemented with 0.4% glucose |
| $TSB_{0.4\%G+MgCl2}$ | TSB supplemented with 0.4% glucose plus 20 mM of $MgCl_2$ |
| $TSB_{1\%G}$ | TSB supplemented with 1% glucose |
| $TSB_{1\%G+MgCl2}$ | TSB supplemented with 1% glucose plus 20 mM of $MgCl_2$ |
| $TSB_{heparin}$ | TSB supplemented with heparin |
| VAN | Vancomycin |
| VBNC | Viable but non-culturable |
| WGA | Wheat germ agglutinin |
| WT | Wild-type |

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## Scientific outputs

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

### Articles published in peer-reviewed journals

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

### Poster communications at international scientific conferences

Lopes, N, Cerca, N., França, A. "The role of *codY* gene in *S. epidermidis* biofilm formation" in Microbiotec 2021 – Congress of microbiology and biotechnology, Online congress, 23rd to 26th November, 2021.

Lopes N, Cerca N, França A. "*codY* deletion impacts *S. epidermidis* 1457 biomass production and VBNC cells formation "in EuroBioFilms 2022, Mallorca, 31st August to 3rd September.

# CHAPTER 1 – Introduction

**Summary**

This chapter provides an introduction to the significance of the subject of this thesis, including the hypothesis that motivated its development, the research questions raised and the aims proposed, along with an outline describing the main objectives of each chapter.

## 1.1. Background

*Staphylococcus epidermidis* is considered one of the main causes of bloodstream infections associated with the use of indwelling medical devices due to the bacterium's ability to form biofilms on the surface of vascular catheters [1]. Biofilms are composed of cells with different metabolic states, including viable but non-culturable (VBNC) cells [2]. These cells are characterized by low metabolic activity, high tolerance to antibiotics and the loss of culturability on standard culture media [3,4], making the treatment and the diagnosis of *S. epidermidis* biofilm-related infections with current culture-based methods troublesome [5,6]. Given the impact of VBNC cells in the clinical setting, an *in vitro* model capable of modulating the proportion of VBNC cells in *S. epidermidis* biofilms was previously established, allowing the investigation of the mechanisms underlying the emergence of the VBNC state [7]. Upon transcriptomic and proteomic analyses of biofilms formed under VBNC-inducing conditions, alterations in the expression of an array of genes, as well as in the immunoreactivity of a set of proteins were found [8,9], namely in the gene *pdhA* and the protein CodY, linking these molecules to the emergence of VBNC cells in *S. epidermidis* biofilms.

Contrary to PdhA, for which very little information is available in the literature, the function of CodY has been already investigated in several bacterial species. CodY is defined as a global transcription regulator and has been associated with the biofilm phenotype in *Staphylococcus aureus*, *Bacillus cereus* and Group B *Streptococcus* [10–13]. In *S. aureus*, in response to nutrient-limited conditions [14–16], CodY seems to interact and regulate several genes involved in biofilm formation, such as the genes from the *agr* [17] and *ica* loci [14], *hla* [16], in addition to genes involved in cells metabolism [14,15]. However, the role of CodY and PdhA in *S. epidermidis* virulence and pathogenicity is still unclear.

## 1.2. Hypothesis and Research Questions

Based on previous data, we hypothesized that CodY and PdhA have a role in *S. epidermidis* biofilm phenotype and VBNC cell formation, contributing to the persistence and recurrence of *S. epidermidis* infections. To confirm this hypothesis, we initially planned the construction of two mutant strains, one lacking the gene *codY* and another lacking the gene *pdhA,* for posterior investigation of their role. Unfortunately, due to the several technical complications encountered during the deletion of the gene *pdhA*, only the construction of the *codY*-deletion mutant was achieved. As such, our efforts were focused on the study of the role of CodY, specifically addressing the following research questions:

1. Does CodY affect *S. epidermidis* susceptibility to antibiotics?

2. Does CodY have an impact on *S. epidermidis* capacity to form biofilms?
3. Does CodY influences VBNC cell formation in *S. epidermidis* biofilms?
4. Does CodY have a role in *S. epidermidis* evasion from the host immune system response?

Answers to these research questions were expected to elucidate the role of CodY in *S. epidermidis* virulence and its involvement in infection persistence, potentially contributing to paving the way to the development of novel prophylactic and/or therapeutic strategies against *S. epidermidis* biofilm-associated infections.

## 1.3. Aims

To test the hypothesis proposed and to answer the research questions raised, the following goals were delineated:

1. To evaluate, *in vitro,* the ability of *S. epidermidis* strain 1457 to produce VBNC cells;
2. To construct a mutant strain lacking the gene *codY* and the respective complemented strain;
3. To assess the *codY*-mutant susceptibility to antibiotics;
4. To evaluate the ability of the *codY*-mutant to form biofilms and VBNC cells;
5. To characterize the *codY*-mutant capacity to survive in human blood and plasma;
6. To analyse the role of CodY in the interaction of the bacterium with host immune cells.

## 1.4. Thesis outline

The subsequent chapter, **Chapter 2**, presents a literature review of *S. epidermidis* biofilm-related infections and the main virulence factors associated with this bacterium's resistance/tolerance to antibiotics and evasion from the host immune system response. Moreover, it summarizes the current knowledge on VBNC cells, including the existing controversies on the concept of dormancy phenotypes, along with their impact on the susceptibility of *S. epidermidis* cells to antibiotics and the capacity to evade the host immune system response.

**Chapters 3** to **5** are experimental chapters focused on the aims presented above. Each chapter includes a summary, a brief introduction, the materials and methods used, the results obtained and a discussion point.

In **Chapter 3,** the formation of VBNC cells in both planktonic and biofilm cultures of *S. epidermidis* strain 1457 was investigated using a previously established *in vitro* model. Furthermore, the expression of the genes *codY* and *pdhA*, formerly found overexpressed under VBNC-inducing conditions, was evaluated by quantitative PCR.

**Chapter 4** describes the construction of the *S. epidermidis* 1457 strain lacking the gene *codY* and respective complemented strain, which were characterized in terms of growth rate, biofilm formation ability, antibiotic susceptibility and capacity to form VBNC cells.

The interaction of the constructed *S. epidermidis* strains with the host immune system cells is presented in **Chapter 5**, focusing on the inflammatory response and macrophage activation.

At last, **Chapter 6** presents a compilation of the main conclusions of all the experimental chapters, trying to answer the research questions initially raised. The major limitations of this thesis are also detailed. Moreover, suggestions for future work that may improve and reinforce the findings reported in this thesis are also covered in this chapter.

In **Figure 1.1** is represented a schematic diagram showing the outline of this thesis and the relation between the chapters and the aims aforementioned.

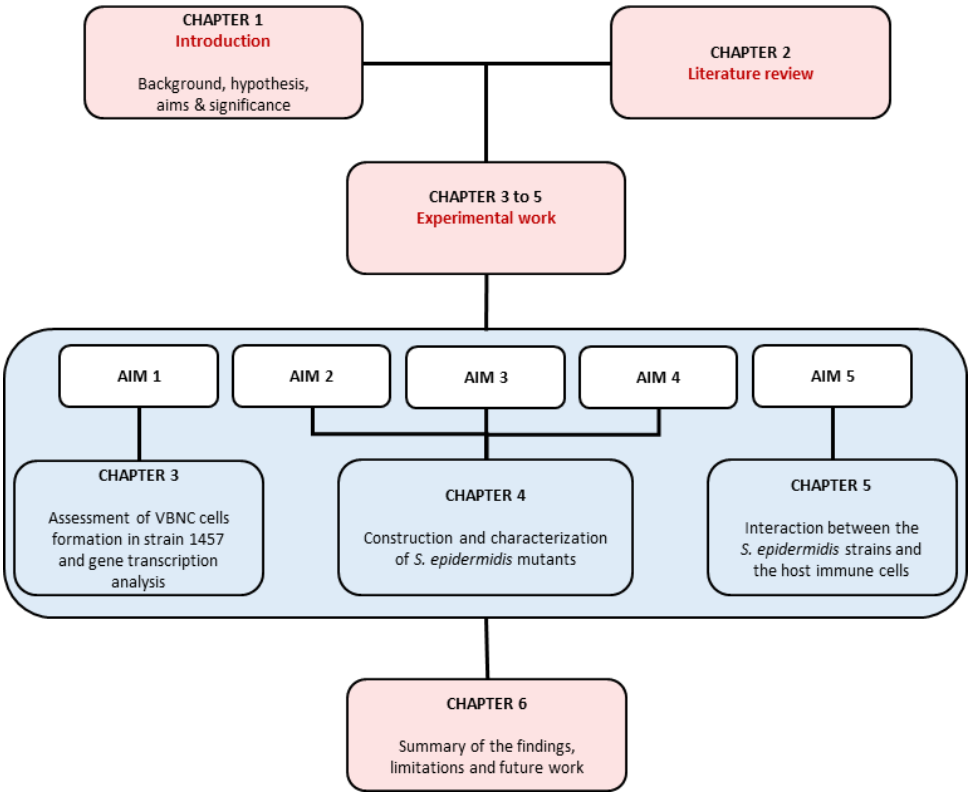


**Figure 1.1** Thesis outline.

## 1.5. References


1. Khatoon, Z., McTiernan, C. D., Suuronen, E. J., Mah, T. F. & Alarcon, E. I. Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention. *Heliyon* **4**, 1–36 (2018).
2. Cerca, F. *et al. Staphylococcus epidermidis* biofilms with higher proportions of dormant bacteria induce a lower activation of murine macrophages. *J. Med. Microbiol.* **60**, 1717–1724 (2011).
3. Li, L., Mendis, N., Trigui, H., Oliver, J. D. & Faucher, S. P. The importance of the viable but non-culturable state in human bacterial pathogens. *Front. Microbiol.* **5**, 1–20 (2014).
4. Zhang, S. *et al.* Induction of *Escherichia coli* into a VBNC state by continuous-flow UVC and subsequent changes in metabolic activity at the single-cell level. *Front. Microbiol.* **9**, 1–11 (2018).
5. Gu, H., Hou, S., Yongyat, C., De Tore, S. & Ren, D. Patterned biofilm formation reveals a mechanism for structural heterogeneity in bacterial biofilms. *Langmuir* **29**, 11145–11153 (2013).
6. Kranjec, C. *et al.* Staphylococcal Biofilms: Challenges and Novel Therapeutic Perspectives. *Antibiotics* **10**, 1–30 (2021).
7. Cerca, F. *et al.* SYBR green as a fluorescent probe to evaluate the biofilm physiological state of *Staphylococcus epidermidis,* using flow cytometry. *Canadian Journal of Microbiology* vol. 57 850–856 (2011).
8. Carvalhais, V. *et al.* Dormancy within Staphylococcus epidermidis biofilms: a transcriptomic analysis by RNA-seq. *Applied Microbiology and Biotechnology* 1–12 (2014) doi:10.1007/s00253-014-5548-3.
9. Carvalhais, V., Cerveira, F., Vilanova, M., Cerca, N. & Vitorino, R. An immunoproteomic approach for characterization of dormancy within *Staphylococcus epidermidis* biofilms. *Mol. Immunol.* **65**, 429–435 (2015).
10. Majerczyk, C. D. *et al. Staphylococcus aureus* CodY negatively regulates virulence gene expression. *J. Bacteriol.* **190**, 2257–2265 (2008).
11. Tu Quoc, P. H. *et al.* Isolation and Characterization of Biofilm Formation-Defective Mutants of *Staphylococcus aureus*. *Infect. Immun.* **75**, 1079–1088 (2007).
12. Pellegrini, A. *et al.* CodY Is a Global Transcriptional Regulator Required for Virulence in Group B *Streptococcus*. *Front. Microbiol.* **13**, 1–15 (2022).
13. Frenzel, E. *et al.* CodY orchestrates the expression of virulence determinants in emetic *Bacillus cereus* by impacting key regulatory circuits. *Mol. Microbiol.* **85**, 67–88 (2012).
14. Waters, N. R. *et al.* A spectrum of CodY activities drives metabolic reorganization and virulence gene expression in Staphylococcus aureus. *Mol. Microbiol.* **101**, (2016).
15. Geiger, T. & Wolz, C. Intersection of the stringent response and the CodY regulon in low GC Gram-positive bacteria. *Int. J. Med. Microbiol.* **304**, 150–155 (2014).
16. Pohl, K. *et al.* CodY in *Staphylococcus aureus*: a Regulatory Link between Metabolism and Virulence Gene Expression. *J. Bacteriol.* **191**, 2953–2963 (2009).
17. Roux, A., Todd, D. A., Velázquez, J. V., Cech, N. B. & Sonenshein, A. L. Cody-Mediated regulation of the *Staphylococcus aureus* Agr System integrates nutritional and population density signals. *J.Bacteriol.* **196**, 1184–1196 (2014).

# CHAPTER 2 – Literature review

**Summary**

This chapter provides a general outline of the clinical relevance of *S. epidermidis* biofilm-associated infections and the bacterium's main virulence factors. Furthermore, this chapter focuses on the dilemma of VBNC cell formation within biofilms.

## 2.1. *Staphylococcus epidermidis* commensal and pathogenic profiles

*S. epidermidis*, a Gram-positive bacterium that belongs to the coagulase-negative Staphylococci (CoNS) group [1], plays a major role in the colonization of healthy human skin and mucosa, being primarily isolated from the nares, axillae and head [2]. Despite its primary role in skin protection, *S. epidermidis* has evolved as one of the most important opportunistic pathogens due to the widespread use of medical devices [3]. The introduction of implantable medical devices represents a major advance in modern medicine, supporting the monitoring of vital functions, administration of drugs to critically ill patients or replace failing organ functions [4]. However, these devices are scaffolds for microorganisms adhesion, resulting in several complications such as the development of Healthcare-Associated Infections (HAIs), which contribute to increased mortality and morbidity rates [5].

### 2.1.1. The role of *S. epidermidis* as a commensal bacterium

As a commensal bacterium, *S. epidermidis* has an important role in maintaining the normal microflora and inhibits the colonization of pathogenic microorganisms by the production of inhibitory molecules such as phenol-soluble modulins (PSMs), which are a family of amphipathic, α-helical peptides with varied biological functions [6,7]. In addition, it was shown that *S. epidermidis* produces an unknown molecule of < 10kDa that engages with the toll-like receptor 2 (TLR2) and promotes a higher antimicrobial peptide expression, enhancing the immune defense against bacterial skin infections [8]. Another molecule produced by *S. epidermidis*, 6-N-hydroxyaminopurine (6-HAP), was shown to suppress the growth of melanomas in a melanoma mouse model [9]. Additionally, the infection of mice with *S. epidermidis* strains producing 6-HAP reduced the incidence of ultraviolet-induced tumors [9]. *S. epidermidis* also seem to eliminate the pathogenic bacteria present in the nasal cavity. This elimination process is achieved through the production of Esp protease which inhibits the colonization of *S. aureus* or by the production of succinic acid that inhibits *Cutibacterium acnes* [10]. Assuming the relevance of the different molecules secreted by *S. epidermidis* to inhibit or eliminate pathogenic bacteria from the nasal cavity, researchers have been questioning if these molecules could also have a role in controlling severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) [10]. Indeed, in a recent study, the transcription of angiotensin-converting enzyme 2 and transmembrane serine protease 2, two receptors implicated in SARS-CoV-2 virus interaction with the human nasal mucosa, was significantly decreased in the nasal epithelium in response to *S. epidermidis* [11]. However, information regarding the protective effect of *S. epidermidis* against SARS-CoV-2 is limited, warranting further research.

### 2.1.2. The role of *S. epidermidis* as a pathogenic bacterium

#### 2.1.2.1. Clinical relevance

Regardless of *S. epidermidis* commensal lifestyle, this species has also been identified as the cause of several types of HAIs [3]. In Europe, CoNS, mainly *S. epidermidis,* are one of the most common isolated bacteria in the context of bloodstream and surgical site infections [12–14]. Bloodstream infections caused by *S. epidermidis* primarily affect immunocompromised patients and preterm neonates [15,16] as they frequently need the prolonged use of intravascular catheters for nutrients and/or drug delivery [17]. Regarding neonatal infections, over the last years, the proportion of late-onset sepsis, which is mainly caused by *S. epidermidis*, remained around 50% in the most developed and developing countries evaluated, with a higher incidence in the Netherlands (78%) [15]. In addition, the diagnosis of infections caused by the bacterium is very challenging. Positive blood cultures are often regarded as the result of blood contamination during sampling collection, hindering the treatment of the infection [18]. For this reason, the search for makers that can differentiate the commensal from the pathogenic profile has been widely explored over the years [18–20].

#### 2.1.2.2. Virulence factors associated with *S. epidermidis*

*S. epidermidis* shares some characteristics with *S. aureus*, however, the mechanisms it engages to survive during infection are less aggressive [21]. Its capacity to switch from a commensal to a pathogenic lifestyle is mostly attributable to its remarkable ability to adhere to foreign material and establish a complex bacterial agglomeration, commonly called biofilm [22]. In 1982, Peters and co-workers found the first evidence of the capacity of *S. epidermidis* to adhere to central venous catheters leading to infection [23]. As such, *S. epidermidis* has been considered an opportunistic pathogen once it breaches the skin surface and enters the bloodstream, adhering to the surface of indwelling medical devices and forming biofilms [24,25]. Interestingly, a high prevalence of biofilm-positive *S. epidermidis* isolates was found in hospitalized patients and outpatients [26], indicating that the high incidence of *S. epidermidis* in HAIs is associated with the biofilm formation ability of this bacterium. There have been several pieces of evidence demonstrating a strong association between *S. epidermidis* biofilm formation and the inefficiency of antimicrobial therapies [27,28] and evasion from the host's immune system [29,30], resulting in the development of persistent and relapsing infections [31,32]. The treatment of these infections often implicates the removal of medical devices [33], leading to an increase in morbidity and, in some cases, mortality rates among affected patients [34].

## 2.2. Biofilm Formation

### 2.2.1. Role of the biofilm mode of growth in microbial communities

In 1999, Costerton *et al.* defined biofilms as "a structured community of microbial cells enclosed in a self-produced polymeric matrix, adherent to an inert or living surface" [35]. However, the definition of biofilm has evolved over the years. Several authors demonstrated the development of biofilm-associated infections (BAIs) without the presence of medical devices or adhered to biological tissue [36–39], being the formation of these biofilms based on bacterial aggregation (free-floating aggregates) rather than adhering to a surface (Fig. 2.1).

Chronic lung infections associated with cystic fibrosis [40] and chronic dermal wounds [41] are examples of BAIs developed by bacteria aggregation. Moreover, biofilms associated with embedded aggregates seem to exhibit similar phenotypes as surface-associated bacteria, such as increased tolerance to antibiotics, evasion of host immune defense and slow growth [38,39].

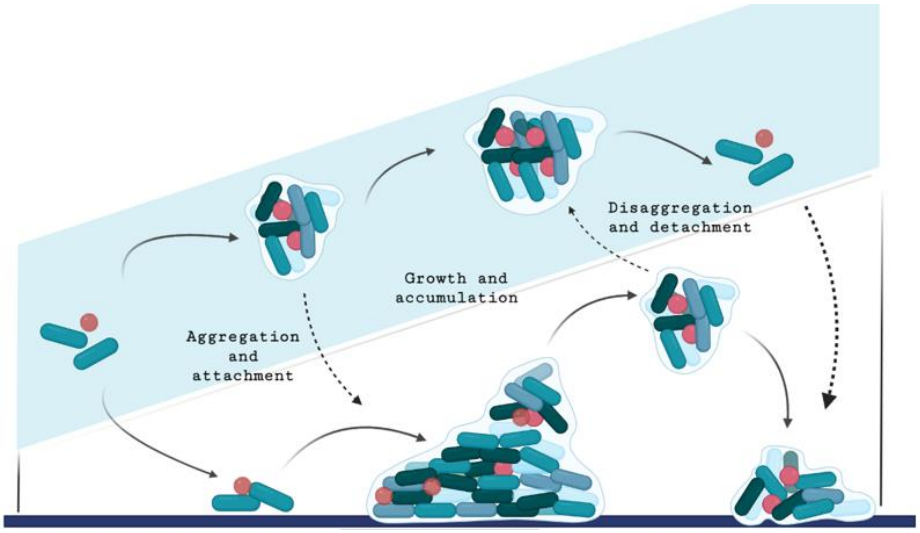


**Figure 2.1** Proposed bacterial biofilm models demonstrating the different biofilm formation scenarios, namely non-surface (highlighted in blue) and surface-associated biofilms. Adapted from [42]; Created in Biorender.com.

However, although the formation of biofilms in some cases is independent of bacterial adhesion (either to biotic or abiotic surfaces), little is known about this process in bacteria other than *Pseudomonas aeruginosa* [38]. In addition, in the case of *S. epidermidis*, the use of indwelling medical devices is a major risk factor to develop infection by this bacterium. Therefore, herein, the formation of biofilms on surfaces, namely on medical devices, is scrutinized.

### 2.2.2. Biofilm formation process and structure

According to different studies, the dynamic process of *S. epidermidis* biofilm formation occurs in three main steps: (i) initial adhesion (to abiotic or biotic surfaces), (ii) accumulation/maturation and (iii) detachment/dispersion (Figure 2.1) [43,44].

#### 2.2.2.1. Initial adhesion

The initial adhesion, to either abiotic (e.g. medical devices) or biotic surfaces (e.g. tissues and/or conditioning film on medical devices), is an important step for biofilm formation by *S. epidermidis* and several adhesion factors are involved in this process [45]. Adhesion to abiotic surfaces is mediated by hydrophobic and electrostatic interactions, Van der Waal's forces, surface tension and temperature [45]. In addition, there are several *S. epidermidis* cell surface proteins involved in initial adhesion, such as autolysin protein (AtlE) [46], adhesion protein (Aae) [47] and teichoic acids [48]. For example, AtlE is a peptidoglycan hydrolase that has important functions in cell separation [49] and its autolytic activity leads to the release of cellular components, such as extracellular DNA (eDNA), which in turn is an important component required for initial adhesion [50,51]. Considering the importance of the initial adhesion in biofilm formation on medical devices, several studies have focused on the development of mechanisms to prevent bacteria initial adhesion. Artini and coworkers demonstrated that the adhesion of *S. epidermidis* strains to abiotic surfaces can be reduced by coating the surfaces with hydrophobins, which are small amphiphilic proteins with the capacity to change the wettability of material surfaces [52]. Additionally, the implantation of silicon in stainless steel surfaces led to a reduced number of attached *S. epidermidis* bacteria and smaller adhesion rates, under static conditions [53].

Moreover, bacterial cells can also adhere to biotic surfaces, which include surfaces coated with proteins derived from host fluids, defined as conditioning film [54]. In this case, the adhesion is mediated by a group of surface proteins so-called Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs), which bind to the host extracellular matrix proteins (collagen, fibrinogen, fibronectin and vitronectin) such as fibrinogen-binding protein (Fbe/SdrG) [55,56], fibronectin-binding protein (Embp) [57,58], serine aspartate-binding protein (SdrF) [59,60] and GehD lipase [61]. AtlE is also involved in adhesion to biotic surfaces since it promotes binding to vitronectin [46,47].

#### 2.2.2.2. Bacterial accumulation and maturation

Bacteria initial adhesion is followed by a series of steps where the replication and recruitment of additional planktonic cells occur [62,63], leading to the development of differentiated layers. At this point, an extracellular matrix composed mainly of extracellular polymeric substances such as polysaccharides, eDNA, proteins and lipids is produced [64] and mediates intercellular adhesion [65].

The Polysaccharide Intercellular Adhesin (PIA), also known as Poly-N-Acetylglucosamine (PNAG), was described as one of the first mechanisms involved in *S. epidermidis* biofilm formation [66]. The chemical structure of the polysaccharide was determined in 1996 by Mack *et al.*, comprising negative charges from O-succinylation and positive charges due to partial de-N-acetylation [67]. The biosynthesis of this molecule is achieved by the proteins encoded in the genes of the *ica* operon (*icaADBC*), where the transferase IcaA and the accessory IcaD membrane proteins are responsible for the synthesis of N-acetylglucosamine residues, (GlcNAc), and the transmembrane protein IcaC for the elongation and transport of the growing polysaccharide chain into the cell surface [68]. Then, the cell surface-located enzyme IcaB partially de-acetylates the GlcNAc residues, introducing positive charges, which are essential for the binding of PNAG to the cell surface [69]. The regulation of expression of *icaADBC* locus is mediated by *icaR* gene located upstream of *icaA* start codon [70].

Meanwhile, it has been demonstrated that some strains lacking *ica* genes can still develop biofilms [71–73], being these defined as *ica*-independent [74]. Therefore, in these cases, other factors such as the accumulation-associated protein (Aap) [75], biofilm-homologous protein (Bhp) [76], extracellular matrix-binding protein (Embp) [77] and small basic protein (Sbp) [78], seem to have an important role in biofilm accumulation. However, it was shown that strains forming more robust biofilms are *ica*-dependent [66,79].

Aap is implicated in *S. epidermidis* biofilm formation as a putative cell wall receptor for PNAG [80,81]. In addition, Aap functions as an intercellular adhesion molecule, leading to biofilm accumulation, having a more prevailing role in *ica*-negative biofilms [75]. The surface protein Bhp, which is a homologue of the biofilm accumulation protein (Bap) from *S. aureus*, seems to promote biofilm accumulation [82]. Nevertheless, information is scarce in the literature, warranting further studies to better understand the role of the Bhp protein in *S. epidermidis* biofilm formation. In regards to Sbp, this protein seems to mostly promote cell aggregation by influencing the intercellular adhesion mediated by PNAG or Aap [78].

During biofilm maturation, bacterial cells begin to agglomerate and divide, establishing a three-dimensional structure with a high complexity and spatial organization [83]. Since bacterial growth depends

on the availability of nutrients, an optimum hydrodynamic flow across the biofilm is necessary to allow the perfusion of nutrients and oxygen and the elimination of metabolic waste [84]. This is achieved by the formation of channels, shaped by the activity of the PSMs, in particular PSMβ, which is produced in higher levels under the biofilm phenotype [85,86]. Biofilms are characterized by having several layers, which are composed of cells with distinct characteristics and behaviors and are remarkably different from planktonic cells [87,88]. Indeed, Rani *et al.* demonstrated that staphylococcal cells within biofilms may show four different physiological states, specifically: (i) aerobic or (ii) anaerobic growing cells, (iii) dormant and (iv) dead cells. Importantly, this heterogeneity seems to contribute to the high antibiotic tolerance rates observed in biofilms [89].

2.2.2.3. Detachment or dispersion

As *S. epidermidis* biofilms mature and reach a certain cell density, their resources become limited and, simultaneously, begin to accumulate toxic compounds [90,91]. To ensure their survival, individual cells or cell clusters detach from the biofilm, eventually leading to the spreading of the infection to other sites [92,93]. Multiple events are required for cell detachment to occur, including the degradation of the extracellular matrix by the action of enzymes or such as proteases and DNases or surfactant-like molecules such as PSMs [94]. In addition, several physiological modifications occur to promote biofilm cells' adaptation to the external conditions [95,96], being this phenomenon strictly mediated through the quorum-sensing system (QS) [97], which produces and secretes signalling molecules named autoinducers (AIs) [98]. These AIs balance, on one hand, the downregulation of genes involved in the previous stages (genes enconding surface proteins) and, on the other hand, the upregulation of genes encoding disruptive factors and other molecules, which will enable bacterial cells to adapt to the new environmental conditions [94].

In Staphylococci two QS systems have been identified and characterized: the Agr (characterized *in vitro* and *in vivo*) [97,99,100] and the LuxS systems (characterized only *in vitro)* [101]. The Agr system regulates de production of multiple proteases and small toxins such as PSMs [102]. PSMs are known to be multifunctional, with major cytolytic properties and the ability to lyse human neutrophils, similar to *S. aureus* PSMs [21,99,103,104]. Among the several classes of PSMs produced by *S. epidermidis* [7,105], PSMβ are produced at higher levels under biofilms conditions and, besides their role in biofilm structuring (when produced in low concentration), they are involved in biofilm dispersion (when produced in elevated concentrations) [24]. Wang *et al.* demonstrated PSMβ role in *S. epidermidis* biofilms both *in vitro* and *in vivo* models [86]. Interestingly, *S. aureus* Agr deficient strains have been frequently isolated in the context of BAIs, suggesting that a non-functional Agr system might be advantageous in certain environments [106]. Therefore,

researchers were prompted to study the role of Agr in biofilms formed by *S. epidermidis* isolated from BAIs. A study performed by Vuong *et al.*, demonstrated that the Agr-mutant constructed using *S. epidermidis* 1457 background showed an increased biofilm formation in comparison to the WT strain, as well as the clinical isolate *S. epidermidis* 047, which is a naturally occurring *agr* mutant [97]. Considering that PSM have a role in biofilm dissemination and their production is strictly regulated by the agr system, the prodution of PSM is arrested in agr mutants, impeding the release of cell clusters from the biofilm [86]. Thus, the higher biofilm formation found in Agr-mutant strains may be associated with dissemination arrest rather than a higher ability to induce biofilm growth [86,107]. Similar findings were also found in *S. aureus* [106]. Therefore, these studies suggest that the loss of the Agr function *in vivo* can enhance the survival of Staphylococcal species, contributing to their persistence.

## 2.3. Metabolic Heterogeneity in Biofilms

Biofilms are characterized by a heterogeneous group of cells with distinct growth rates and with a wide range of physiological states, features that depend on the local environmental conditions [108,109]. Active growing cells are located at the surface of the biofilm, whereas cells with lower metabolic activity are found at deeper biofilm layers, as a result of unfavorable conditions such as nutrient and oxygen deprivation [108,110]. Within these layers, different subpopulations of cells can emerge, presenting altered gene-expression patterns that seem to promote bacterial survival under these conditions [87,111]. More importantly, these physiological and genetic adaptations seem to impair the efficacy of the antimicrobial therapies available [112–115], as well as the ability to endure the host immune system response [116].

### 2.3.1. Dormancy: persisters or VBNC cells?

The emergence of dormant subpopulations and their characterization have raised considerable controversies. Several researchers assume the existence of distinct dormancy phenotypes, while others believe that the dormancy subpopulations are persisters [117,118]. Currently, two distinct dormancy-related phenotypes are accepted: viable but non-culturable (VBNC) [119] and persister cells [120,121]. Persister cells were first detected by Hobby and coworkers, which found a group of *S. aureus* cells (around 1%) that were able to survive upon exposure to penicillin [120]. However, only two years later, Bigger *et al.* demonstrated that these cells were not antibiotic-resistant mutants but instead exhibit a drug-tolerant phenotype, proposing that these cells were persisters [122]. As such, persister cells are defined as a population of cells that lack

genetic traits for antibiotic resistance but arrest their dividing cycle in the presence of high levels of antibiotics, being responsible for the increasing levels of antibiotic tolerance [121,123].

Regarding VBNC cells, these were first detected by Xu *et al.,* where viable *Escherichia coli* and *Vibrio cholerae* cells were found after an extended period in the saltwater microcosm, although they were not able to grow on either selective or non-selective media [119]. Thus, VBNC cells were defined as "viable cells that are no longer capable to be cultured on routine media, due to exposure to environmental stress" (Fig. 2.2) [124].

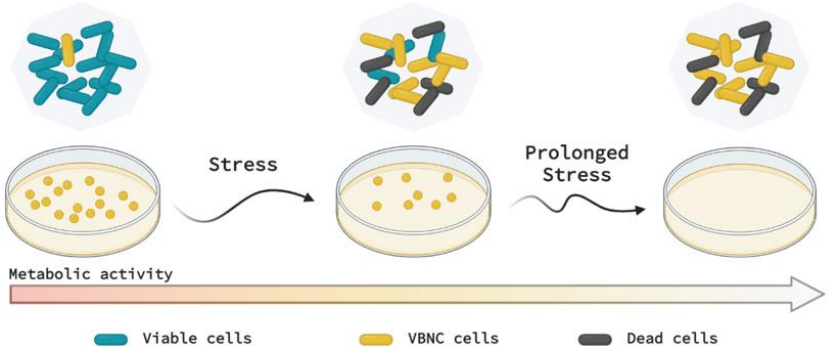


**Figure 2.2** The formation of the VBNC cells after environmental stress. After a stress stimulus viable cells retain their metabolic activity although at significantly lower levels and a small proportion of viable bacteria will enter the VBNC state. Adapted from [125]; Created in Biorender.com.

About 100 bacterial species have been reported to exist in VBNC state at different environmental conditions and habitats, most of them are Gram-negative bacteria [126,127]. Interestingly, both persister and VBNC cells have been associated with chronic BAIs [128–131]. Furthermore, both cell types seem to be induced by more than one stress condition, for example, oxidative and acid stress [132–134]. Ayrapetyan and coworkers have suggested a key feature to distinguish both dormant phenotypes: persister cells can easily be converted and regain growth if the stressor is removed, while VBNC cells cannot be reverted under normal conditions, i.e., in some species, VBNC cells require very specific conditions to resuscitate [135,136]. However, the genetic basis and virulence factors of these dormancy phenotypes are still poorly characterized.

### 2.3.2. Characterization and detection of VBNC cells

VBNC cells have many general characteristics in common with viable cells, despite their non-culturability, including (i) an intact membrane containing undamaged genetic information [127,137,138], (ii) carry out respiration [139,140], (iii) maintain transcription and mRNA production [140], as well as (iv) the uptake and incorporation of amino acids into proteins [141,142]. On the other hand, VBNC cells present some distinct

physiological and molecular characteristics in comparison with culturable cells such as changes in (i) cell morphology [143,144], (ii) cell wall composition [145], (iii) lower metabolic rate [146,147], (iv) modifications of gene expression profile [148–150] and (v) higher tolerance to antibiotics [151,152].

Since VBNC cells do not grow in routine growth media and considering that these cells are an integrant part of biofilms, it is of utmost importance to develop methods capable of detecting VBNC cells. Xu *et al.* first identified VBNC cells discriminating between cells viability and culturability by combining, respectively, immunofluorescence microscopy (to detect viable cells) and colony-forming unit counts (to detect culturable cells) [119]. Based on the same principle, other methods have been developed over the years. The latest method developed for the detection of VBNC cells is the LIVE/DEAD® *Bac*Light™ fluorescence staining method, which indirectly assesses the cell membrane integrity by using two nucleic acid binding fluorescent dyes – SYTO9 and propidium iodide (PI) [153,154]. The combination of LIVE/DEAD staining with flow cytometry analysis has allowed more accurate and expedited results [154–156]. Besides the use of stains, VBNC cells have also been identified through qPCR analysis. Zandri *et al.* detected *S. epidermidis* and *S. aureus* VBNC cells by combining LIVE/DEAD staining with 16S rDNA detection and demonstrated that the majority of biofilm samples studied presented VBNC cells [131]. Another method used to estimate the amount of VBNC cells is based on the activity of an electron transport system, the p-iodonitrotetrazolium violet (INT) assay [157] or the BacLight™ RedoxSensor™ Green [158]. Briefly, the INT competes with oxygen for electro-acceptance and, upon its reduction, it is converted to a formazan complex, which will be accumulated in cells and appear as dark red precipitates under microscope [157].

### 2.3.3. VBNC state induction and resuscitation methods

Induction of VBNC cells has been reported and associated with a variety of both chemical and environmental factors, including nutrient [144,159] and oxygen limitation [134,160], incubation at unfavorable temperature and pH [152,161], variation of osmotic concentrations [148,159], presence of heavy metals [162] and even exposure to white light [163] or UV irradiation [164]. Exposure of *E. coli* and *S. aureus* to ultrasounds also seems to induce bacteria into VBNC state [242]. However, bacteria that enter a VBNC state may regain culturability and this phenomenon is termed resuscitation [165]. Several factors control the resuscitation of VBNC cells and, usually, it is achieved by removing the VBNC-inducing factor [127]. Though, not all species can revert from the VBNC state [127].

It was demonstrated that the emergence of VBNC cells in *S. epidermidis* biofilms can be achieved by supplementing the growth medium with a high percentage of glucose, as a consequence of the

accumulation of acidic compounds that result from the glucose metabolism [116]. On the other hand, by an unknown mechanism, the addition of $Ca^{2+}$ and $Mg^{2+}$ ions reverted the effect, reducing the accumulation of VBNC cells [116]. Lee and coworkers (2019) measured the membrane potential dynamics of *Bacillus subtilis* cells and demonstrated that increasing the $Mg^{2+}$ flux enables the modulation of ribosome function and promotes bacterial transition to a VBNC state [166]. However, the action of this cation in the VBNC state is still very limited.

### 2.3.4. The genetic mechanisms behind VBNC cells formation

VBNC cell formation was previously suggested to be the result of gene regulation by Ravel, back in 1994 [167]. After the discovery of the resuscitation promoter factor (Rpf) that supported *Micrococcus luteus* VBNC cells recovery [168], the mechanisms behind VBNC regulation were further explored. Generally, qPCR and RNA sequencing approaches have been used to evaluate and assess alterations in the gene expression profile of VBNC cells under stress conditions [152,169]. A few studies analyzed the gene expression profile of bacterial populations under VBNC-inducing conditions and identified transcriptional changes mainly associated with the metabolism and oxidative stress [150,170–172]. However, studies addressing the VBNC state of Staphylococcal species frequently associated with BAIs are scarce.

Using high-throughput RNA sequencing, Carvalhais and coworkers characterized the transcriptome of *S. epidermidis* biofilms with a high proportion of VBNC cells and showed significant differences in the expression of 147 genes [150]. Among the 28 upregulated genes, *pdhA* was in the top 10 genes with higher expression values. However, so far, very little is known about the function of this gene. Another study aimed to evaluate the VBNC mechanisms in *S. epidermidis* biofilms, which combined two-dimensional electrophoresis (2-DE) immunoblotting with mass spectrometry (MS), showing that under VBNC-inducing conditions several proteins presented higher reactivity to human serum, namely CodY [149]. CodY has been characterized in *B. subtilis* as a repressor of genes involved in exponential growth and its activity is relieved when cells switch to the stationary phase [173]. As such, in *B. subtilis* CodY controls the expression of more than a hundred genes, mainly associated with nutrient deprivation but also genes encoding transport systems, genetic competence, chemotaxis, motility and sporulation [174–176]. The activation of CodY is mediated by two types of effector molecules: guanosine triphosphate (GTP) and branched-chain amino acids (BCAAs; isoleucine, leucine and valine) [177–179]. Two domains were found in the crystal structure of CodY in *B. subtilis*, a C-terminal domain which seems to be necessary for DNA binding and an N-terminal which contains a GAF domain, responsible to bind to the BCAAs [180]. A *codY* homologous gene was found

in *S. aureus* [181,182] and Majerczyk *et al.* demonstrated that CodY regulates the Agr system, as well as the proteins involved in PNAG synthesis [182]. Interestingly, CodY seems to affect the host immune response by promoting the secretion of cytotoxins that lyse human neutrophils, negatively regulating the SaeRS two-component system, a system that contributes to the expression of a wide range of *S. aureus* virulence factors [183]. However, the role of CodY in the emergence of VBNC cells, including in *S. epidermidis,* is still unknown. Further evaluation of the function of this molecule can provide insights into the mechanisms underlying the emergence of VBNC cells and, consequently, improve the current understanding of how these cells affect the susceptibility to antibiotics and the ability to evade the host immune response.

## 2.4. Antimicrobial resistance and/or tolerance

The treatment of HAIs have been progressively difficult largely due to the failure of current antimicrobial therapies [184]. Antibiotics are mostly positively charged molecules that can present different mechanisms of action such as the inhibition of (i) cell wall [185–187], (ii) protein [188–191], (iii) or nucleic acids biosynthesis [191,192], as well as (iv) the inhibition of metabolic pathways [193]. Cerca *et al.* have demonstrated that independent of the mode of action, CoNS biofilms were less susceptible to antibiotics than planktonic cells, presenting, though, higher tolerance to antibiotics targeting inhibitors of cell wall synthesis [194]. Additionally, the barrier established by the extracellular matrix, along with the physiological conditions of the distinct biofilm layers, are some examples of biofilm-related mechanism of tolerance (Table 2.1) [195,196].

**Table 2.1** Mechanisms presented by biofilms that increases tolerance and/or resistance to antibiotics.

| | |
|---|---|
| **Biofilm structure and matrix** | Biofilm thickness [197]<br>Extracellular DNA [198] |
| **Nutritional and stress conditions** | Oxidative stress response [199]<br>Amino acid limitation [115] |
| **Bacteria physiology** | Small colony variants [200]<br>Dormancy phenotypes [87] |
| **Genetic diversity** | Horizontal gene transfer [201,202]<br>Mutation frequency [203,204] |

Moreover, other aspects may contribute to the increase of antibiotic resistance, namely the overuse of antibiotics administration, the incorrect prescription of antibiotic therapy and the usage of broad-spectrum antibiotics [205,206].

The majority of HAIs originated by Staphylococcal species are caused by methicillin-resistant strains [15]. Methicillin resistance is mediated by the mecA *gen*e inserted on the staphylococcal cassette chromosome

mec (SCCm*ec*), which encodes a penicillin-binding protein (PBP2a) with reduced affinity for all β-lactam antibiotics [207,208]. Several studies have reported a high percentage of *S. epidermidis* clinical strains (around 80%) to be methicillin-resistant (MRSE) [209–211], however, MRSE strains have also been identified in the community although with a lower prevalence (3-18 %) [14,212–214]. Antibiotic therapy against MRSE is challenging, since these strains are frequently resistant to many other antibiotic agents and, depending on the geographical region country, resistance rates to those antibiotics may vary considerably [215]. Among the antibiotics with activity against Gram-positive bacteria, MRSE strains present relatively low resistance to glycopeptides (vancomycin and teicoplanin), becoming one of the last remaining options [216,217]. A prospective study conducted from 2000 to 2011, evaluated the antibiotic susceptibility of eighty-five *S. epidermidis* isolates recovered from blood cultures and observed that the vancomycin minimum inhibitory concentration (MIC) increased over the years (MICs equal or superior to 2 μg/mL increased from 13% to 23%) [218]. The progressive increase of vancomycin MICs raises concerns [218,219], reinforcing the need to find alternative antibiotics for the treatment of infections caused by *S. epidermidis*. Several studies demonstrated that daptomycin and linezolid may be used as an alternative since *S. epidermidis* strains seem to present almost 100% susceptibility to these antibiotics [218,220,221]. Another effective antibiotic used for the treatment of *Staphylococcus* infections is rifampicin [222]. However, rifampicin should be always used in combination with other antimicrobial agents, since it carries a high risk of resistance when used as monotherapy [223–225]. A combination of rifampicin with daptomycin or tigecycline demonstrated high activity against *S. epidermidis* biofilms, validating the efficacy of these antibiotics [226,227].

## 2.5. Immune system response against *S. epidermidis*

Besides the low efficacy presented by the current antibiotic therapies, the mechanisms of defense of the host immune system against *S. epidermidis* biofilms are also inefficient [228]. It is known that the skin is the first line of defense against external bacteria since it functions as a physical barrier and also due to its chemical properties [229]. Therefore, once bacteria cross the skin, the immune system response is triggered [230] to protect the host from pathogenic bacteria or other foreign threats and maintain homeostasis [231]. The immune system has two main lines of defense: (i) innate immunity, an antigen-independent response composed of several defensive barriers including phagocytic cells such as neutrophils, macrophages, and dendritic cells, which does not create immunologic memory and the (ii) adaptive immunity, which is an antigen-specific response mediated by several cells such as B and T lymphocytes that creates an immunologic memory [232].

### 2.5.1. *S. epidermidis* recognition pathways

Staphylococci recognition by the innate immune system is mainly mediated by the complement system and phagocytic cells such as neutrophils and macrophages [233]. Complement proteins are circulating in blood and body fluids, and their primary role is to mark bacteria for phagocytosis, by activating the complement cascade [234]. Complement activation can proceed with three distinct pathways, as illustrated in Fig. 2.3.

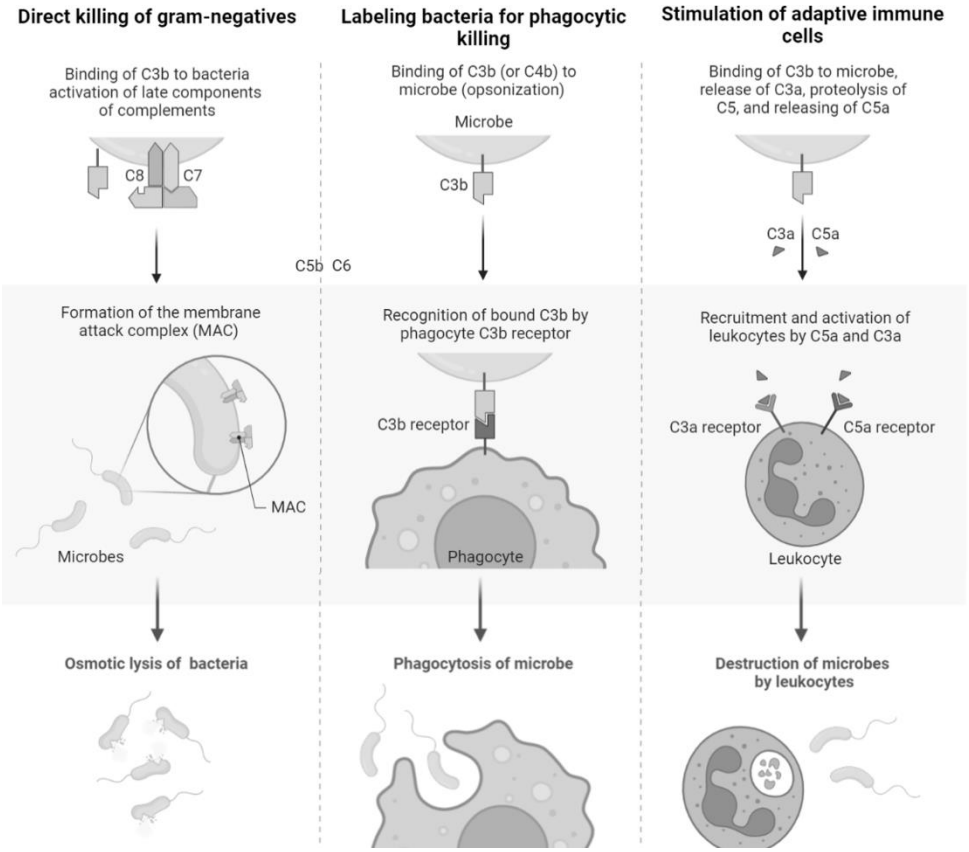


**Figure 2.3** Complement activating pathways. a) Direct killing of Gram-negative bacteria by the formation of a membrane attack complex (MAC), which creates ring-structured pores on bacteria's membrane; b) Gram-positive bacteria are recognized by pathogen-associated molecular patterns (PAMPs) and occurs the formation of convertase enzymes on the bacterial surface (cleavage of protein C3 to generate C3b), which triggers and facilitates phagocytosis by immune cells; c) Labelling of bacteria cells with C3-derived products (chemoattractant C5a) also promotes antigen presenting to B cells and the bridge to activate the adaptive immune response. Adapted from [230,235]; Created in Biorender.com.

Among the three complement activation pathways, the formation of the membrane attack complex (MAC) is insufficient to kill Gram-positive bacteria, perhaps due to their thicker cell wall. However, the mechanism of MAC inhibition in Gram-positive bacteria remains unclear [235]. Furthermore, it was observed that *ica*-dependent biofilms promote a stronger activation of the complement system than *ica*-independent

biofilms [236]. Nevertheless, a study conducted by Kristian and coworkers, demonstrated that *S. epidermidis* can release the C3b from its surface, enhancing its evasion from the action of immune cells [237].

Regarding the recognition by innate immune cells, bacteria possess PAMPs on their surfaces, which are recognized by receptors present on immune cells, specifically toll-like receptors (TLRs) [238,239]. Among the cells of innate immunity, neutrophils are the primary response against *S. epidermidis* [228] and the TLRs present in these cells activate signals to facilitate phagocytosis and neutrophil-mediated killing, promoting the production of reactive oxygen species (ROS; superoxide and hydrogen peroxide) [240], as well as non-oxygen related molecules such as antimicrobial peptides (AMPs) (defensins and cathelicidin) [241] and proteins (inducible nitric oxide synthase, iNOS and cyclooxygenase-2, COX-2) [242–244]. Within the TLRs family, TLR2 seems to play a significant role in *S. epidermidis* recognition [245] by identifying different cell wall-associated molecules, such as lipoproteins [246], lipoteichoic acid [247] and peptidoglycan [248]. Additionally, PNAG [249] and PSMs [250] secreted by *S. epidermidis* seem to stimulate TLR2 recognition. Notwithstanding PNAG and Aap's role in biofilm formation, these molecules have a significant contribution to the evasion from phagocytic cells [69,75]. Indeed, it was shown that *S. epidermidis* cells within the biofilm matrix were more resistant to phagocytic killing mediated by the opsonization of antibodies anti-PNAG [30]. The inhibition of opsonic killing was accomplished by the binding of antibodies to the PNAG released into the matrix, diminishing the binding of the antibody to the PNAG present in the bacterial cell surface [30]. Regarding PSMs, it was shown that the *S. epidermidis* PSMδ has a high potential to lyse human neutrophils, to a similar level as the one presented by the potent *S. aureus* PSMα [21]. PSMδ promotes the release of IL-8, demonstrating *S. epidermidis* pro-inflammatory capacity [21,99]. Recently it was shown that another PSM highly expressed in MRSE isolates, PSM-mec, contributes to bacterial survival and resistance to neutrophil-mediated killing [251].

### 2.5.2. Inflammatory response by cytokines and chemokines

After PAMPs recognition, TLRs stimulate the upregulation of genes involved in inflammatory responses such as genes encoding inflammatory cytokines, chemokines and AMPs (Fig. 2.4) [252]. Cytokines are a group of cell-signalling molecules synthesized by immune cells, that in response to bacterial infections, regulate hematopoiesis and inflammation by binding to cellular receptors, acting on different target cells and affecting the function of other cytokines in a synergistic or antagonistic manner [253]. Therefore, the study of cytokines is important for the characterization of the host immune response to the presence of a certain pathogen, including *S. epidermidis.* The most studied pro-inflammatory cytokines in the context

of bacterial infections are interleukin-1 (IL-1), IL-6, IL-8, IL-12, tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ); while IL-4, IL-10 and transforming growth factor-beta (TGF-β) belong to the most studied anti-inflammatory cytokines group [254].

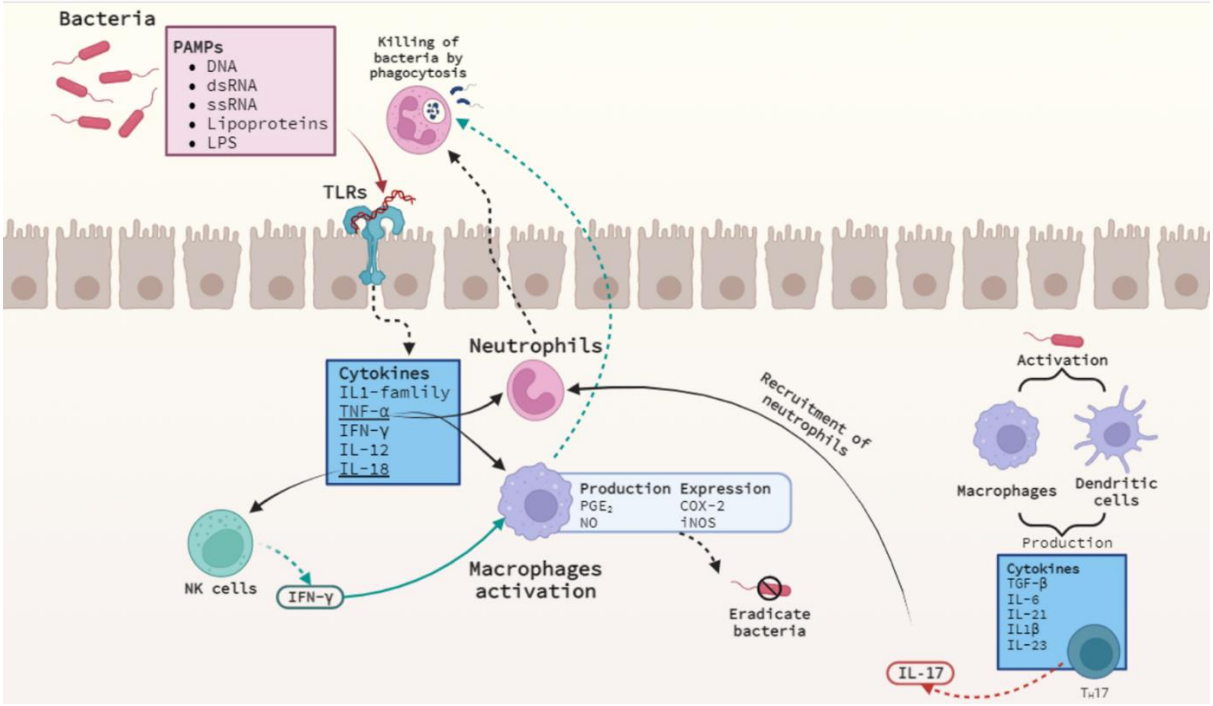


**Figure 2.4** Inflammatory response network in bacterial infections. Adapted from [253]; Created in Biorender.com.

Several *in vitro* studies with peripheral blood mononuclear cells (PBMCs), revealed high secretion of pro-inflammatory cytokines, namely IL-1β, IL-6, TFN-α and interferon-alpha (IFN-α), in the presence of *S. epidermidis* planktonic cells [255,256]. However, the level of the cytokines released was lower than those released against *S. aureus* strains [255]. The stimulation of cytokines in PBMC upon interaction with *S. epidermidis* biofilm cells was also addressed and lower levels of the pro-inflammatory cytokines were detected when compared with planktonic cells, except for IL-8 and IL-13 cytokines [257,258]. Furthermore, *in vivo* studies were also performed, validating *S. epidermidis in vitro* inflammatory responses. Both *ica*-positive and *ica*-negative isogenic strains were tested in a murine model to address PNAG inflammatory potential. Interestingly, the PNAG-producing strain induced higher amounts of IL-10 and IL-6 within the first hours of infection (2h), whereas after 6h these levels were lowered in both strains [259]. França *et al.* also assessed the inflammatory response induced by three *S. epidermidis* bacterial populations (planktonic, biofilm and biofilm-released cells) in a murine model of hematogenous disseminated infection. In the first 2 h of infection, both biofilm and biofilm-released cells induced higher levels of TNF-

α than planktonic cells. In contrast, the levels of TNF-α and IL-6 were significantly reduced after 6 h of infection with biofilm cells when compared with mice infected with planktonic or biofilm-released cells [260].

During *S. epidermidis* infections, the host also releases several chemokines to promote the activation of the immune system response. Strunk *et al.* observed that a planktonic suspension of *S. epidermidis* cells induced the secretion of high levels of CXCL1 and CXCL2 by macrophages [245]. Similar patterns of chemokines were observed in the sera and blood of mice infected with *S. epidermidis* biofilm cells in other studies [259,260]. França *et al.* also detected higher levels of the chemokines CXCL1, CCL3 and CCL4 in the sera of mice infected (2 h) with biofilm-released cells when compared with planktonic or biofilm cells [260]. However, with the course of infection, the levels of these chemokines in the sera of mice infected with planktonic cells increased, reaching closer (CCL4) or even superior levels (CXCL1, CCL2, CCL3) than the ones found in mice infected with biofilm-released or biofilm cells.

### 2.5.3. VBNC cells vs immune system response

Considering the research performed over the years and despite some disparities reported, *S. epidermidis* strains seem to induce lower secretion of pro-inflammatory cytokines, but an increased release of anti-inflammatory cytokines [257]. Importantly, this mechanism of evasion is more evident for biofilm cells, which contributes to their persistence [261]. Another factor contributing to immune response evasion is the biofilm characteristic cell heterogeneity [116]. Bacteria change to a retained state such as the VBNC state, which leads to reduced inflammatory processes and, as a consequence, less recruitment of immune cells [88]. Cerca and coworkers (2014) showed, through the evaluation of pro-inflammatory cytokines production (IL-6, IL-12 and TNF-α), that biofilms with higher proportions of VBNC are less inflammatory [150]. Several bacterial factors have been found to avoid the activity of immune cells during infection [262], but these factors are not associated with the dormancy phenotype. However, studies regarding the capacity of VBNC cells to evade the host immune system's inflammatory response are very limited. Recently, it was demonstrated that the emergence of VBNC cells is critical for *Cronobacter sakazakii* infections, as it enables survival within the macrophages [263]. Therefore, it is important to determine possible mechanisms promoting *S. epidermidis* evasion upon entrance into the VBNC state.

## 2.6. References


1. Becker, K., Heilmann, C. & Peters, G. Coagulase-Negative Staphylococci. *Clin. Microbiol. Rev.* **27**, 870–926 (2014).
2. Brown, M. M. & Horswill, A. R. *Staphylococcus epidermidis*-Skin friend or foe? *PLoS Pathog.* **16**, 1–6 (2020).
3. Lee, E. & Anjum, F. *Staphylococcus epidermidis*. in *StatPearls* (StatPearls Publishing, Treasure Island (FL), 2022).
4. Mack, D. *et al. Staphylococcus epidermidis* in Biomaterial-Associated Infections. in *Biomaterials Associated Infection: Immunological Aspects and Antimicrobial Strategies* (eds. Moriarty, F., Zaat, S. A. J. & Busscher, H. J.) vol. 9781461410 25–56 (Springer-Verlag New York, 2013).
5. Ducel, G., Fabry, J. & Nicolle, L. Prevention of hospital-acquired infections World Health Organization. in (eds. Girad, R. et al.) 72 (2002). doi:10.20982/tqmp.03.2.p043.
6. Cogen, A. L. *et al. Staphylococcus epidermidis* antimicrobial δ-toxin (phenol-soluble modulin-γ) cooperates with host antimicrobial peptides to kill group A Streptococcus. *PLoS One* **5**, 1–7 (2010).
7. Cogen, A. L. *et al.* Selective Antimicrobial Action Is Provided by Phenol-Soluble Modulins Derived from *Staphylococcus epidermidis*, a Normal Resident of the Skin. *J Invest Dermatol* **130**, 192–200 (2010).
8. Lai, Y. *et al.* Activation of TLR2 by a Small Molecule Produced by *Staphylococcus epidermidis* Increases Antimicrobial Defense against Bacterial Skin Infections. *J Invest Dermatol* **130**, 1–20 (2010).
9. Nakatsuji, T. *et al.* A commensal strain of *Staphylococcus epidermidis* protects against skin neoplasia. *Sci. Adv.* **4**, 1–9 (2018).
10. Ortega-Peña, S., Rodríguez-Martínez, S., Cancino-Diaz, M. E. & Cancino-Diaz, J. C. *Staphylococcus epidermidis* Controls Opportunistic Pathogens in the Nose, Could It Help to Regulate SARS-CoV-2 (COVID-19) Infection? *Life* **12**, 1–20 (2022).
11. Ji, J. Y. *et al.* The nasal symbiont *Staphylococcus* species restricts the transcription of SARS-CoV-2 entry factors in human nasal epithelium. *iScience* **24**, 1–13 (2021).
12. Dong, Y., Speer, C. P. & Glaser, K. Beyond sepsis: Staphylococcus epidermidis is an underestimated but significant contributor to neonatal morbidity. *Virulence* **9**, 621–633 (2018).
13. Rogers, K. L., Fey, P. D. & Rupp, M. E. Coagulase-negative staphylococcal infections. *Infect Dis Clin North Am* **23**, 73–98 (2009).
14. Cherifi, S. *et al.* Comparative epidemiology of *Staphylococcus epidermidis* isolates from patients with catheter-related bacteremia and from healthy volunteers. *J. Clin. Microbiol.* **51**, 1541–1547 (2013).
15. Szabó, S. *et al.* An Overview of Healthcare Associated Infections and Their Detection Methods Caused by Pathogen Bacteria in Romania and Europe. *J. Clin. Med.* **11**, 1–29 (2022).
16. Akinkugbe, O., Cooke, F. J. & Pathan, N. Healthcare-Associated bacterial infections in the paediatric ICU. *JAC-Antimicrobial Resist.* **2**, 1–7 (2020).

17. ECDC. Rapid Risk Assessment: Multidrug-resistrant *Staphylococcus epidermidis*. https://ecdc.europa.eu/sites/portal/files/documents/15-10-2018-RRA-Staphylococcus epidermidis%2C Antimicrobial resistance-World_ZCS9CS.pdf (2018).

18. Nuryastuti, T. *Staphylococcus epidermidis*: how to turn from commensal to be a pathogen lifestyle. *J. thee Med. Sci. (Berkala Ilmu Kedokteran)* **50**, 113–127 (2018).

19. Espadinha, D. *et al.* Distinct phenotypic and genomic signatures underlie contrasting pathogenic potential of *Staphylococcus epidermidis* clonal lineages. *Front. Microbiol.* **10**, 1–17 (2019).

20. Brás, S. & França, A. Transcriptome Mining to Identify Molecular Markers for the Diagnosis of *Staphylococcus epidermidis* Bloodstream Infections. *Antibiotics* **11**, 1596 (2022).

21. Cheung, G. Y. C. *et al. Staphylococcus epidermidis* strategies to avoid killing by human neutrophils. *PLoS Pathog.* **6**, 1–10 (2010).

22. Otto, M. *Staphylococcus epidermidis* Pathogenesis. in *Staphylococcus Epidermidis Methods and Protocols* (ed. Fey, P. D.) vol. 19 17–31 (Humana Press, 2014).

23. Peters, G., Locci, R. & Pulverer, G. Adherence and Growth of Coagulase-Negative Staphylococci on Surfaces of Intravenous Catheters. *J. Infect. Dis.* **146**, 479–482 (1982).

24. Otto, M. Staphylococcal Infections: Mechanisms of Biofilm Maturation and Detachment as Critical Determinants of Pathogenicity. *Annu. Rev. Med.* **64**, 1–14 (2013).

25. Widerström, M. Significance of *Staphylococcus epidermidis* in Health Care-Associated Infections , from Contaminant to Clinically Relevant Pathogen : This Is a Wake-Up Call ! *J. Clin. Microbiol.* **54**, 1679–1681 (2016).

26. Jaglic, Z. *et al.* Epidemiology and characterization of *Staphylococcus epidermidis* isolates from humans, raw bovine milk and a dairy plant. *Epidemiol. Infect.* **138**, 772–782 (2010).

27. Asante, J. *et al.* Genomic Analysis of Antibiotic-Resistant *Staphylococcus epidermidis* Isolates From Clinical Sources in the Kwazulu-Natal Province , South Africa. *Front. Microbiol.* **12**, 1–15 (2021).

28. Lopes, N., Freitas, A. I., Ramos, H. & Vasconcelos, C. *S . epidermidis* Isolates from a Tertiary Care Portuguese Hospital Show Very High Antibiotic Non-Susceptible Rates and Significant Ability to Form Biofilms. *Appl. Microbiol.* **1**, 150–161 (2021).

29. de Vor, L. *et al.* Staphylococci evade the innate immune response by disarming neutrophils and forming biofilms. *FEBS Lett.* **594**, 2556–2569 (2020).

30. Cerca, N., Jefferson, K. K., Oliveira, R., Pier, G. B. & Azeredo, J. Comparative antibody-mediated phagocytosis of *Staphylococcus epidermidis* cells grown in a biofilm or in the planktonic state. *Infect. Immun.* **74**, 4849–4855 (2006).

31. Otto, M. Molecular basis of *Staphylococcus epidermidis* infections. *Semin Immunopathol.* **34**, 201–214 (2012).

32. Scherr, T. D., Heim, C. E., Morrison, J. M. & Kielian, T. Hiding in plain sight: Interplay between staphylococcal biofilms and host immunity. *Front. Immunol.* **5**, 1–7 (2014).

33. Agarwal, A. *et al.* Implant Retention or Removal for Management of Surgical Site Infection After Spinal Surgery. *Glob. Spine J.* **10**, 640–646 (2020).

34. Otto, M. *Staphylococcus epidermidis* - the 'accidental' pathogen. *Nat. Rev. Microbiol.* **7**, 555–67 (2009).

35. Costerton, J. W., Stewart, P. S. & Greenberg, E. P. Bacterial biofilms: A common cause of persistent infections. *Science (80-. ).* **284**, 1318–1322 (1999).

36. Burmølle, M. *et al.* Biofilms in chronic infections - A matter of opportunity - Monospecies biofilms in multispecies infections. *FEMS Immunol. Med. Microbiol.* **59**, 324–336 (2010).

37. Hall-Stoodley, L. *et al.* Towards diagnostic guidelines for biofilm-associated infections. *FEMS Immunol. Med. Microbiol.* **65**, 127–145 (2012).

38. Alhede, M. *et al.* Phenotypes of Non-Attached *Pseudomonas aeruginosa* Aggregates Resemble Surface Attached Biofilm. *PLoS One* **6**, 1–12 (2011).

39. Dastgheyb, S. S. *et al.* Staphylococcal persistence due to biofilm formation in synovial fluid containing prophylactic cefazolin. *Antimicrob. Agents Chemother.* **59**, 2122–2128 (2015).

40. Caceres, S. M. *et al.* Enhanced in vitro formation and antibiotic resistance of nonattached *Pseudomonas aeruginosa* aggregates through incorporation of neutrophil products. *Antimicrob. Agents Chemother.* **58**, 6851–6860 (2014).

41. Rosca, A. S., Castro, J., Sousa, L. G. V & Cerca, N. Gardnerella and vaginal health: the truth is out there. *FEMS Microbiol. Rev.* **44**, 73–105 (2020).

42. Sauer, K. *et al.* The biofilm life cycle: expanding the conceptual model of biofilm formation. *Nat. Rev. Microbiol.* **20**, 608–620 (2022).

43. Fey, P. D. & Olson, M. E. Current concepts in biofilm formation of *Staphylococcus epidermidis*. *Future Microbiol.* **5**, 917–933 (2010).

44. Büttner, H., Mack, D. & Rohde, H. Structural basis of *Staphylococcus epidermidis* biofilm formation: Mechanisms and molecular interactions. *Front. Cell. Infect. Microbiol.* **5**, 1–15 (2015).

45. Jiang, Z., Nero, T., Mukherjee, S., Olson, R. & Yan, J. Searching for the Secret of Stickiness : How Biofilms Adhere to Surfaces. *Front. Microbiol.* **12**, 1–19 (2021).

46. Heilmann, C., Hussain, M., Peters, G. & Götz, F. Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface Christine. *Mol. Microbiol.* **24**, 1013–1024 (1997).

47. Heilmann, C. *et al.* Identification and characterization of a novel autolysin (Aae) with adhesive properties from *Staphylococcus epidermidis*. *Microbiology* **149**, 2769–2778 (2003).

48. Schoenfelder, S. M. K. *et al.* Success through diversity - How *Staphylococcus epidermidis* establishes as a nosocomial pathogen. *Int. J. Med. Microbiol.* **300**, 380–386 (2010).

49. Somarathne, R. P. *et al.* Understanding How Staphylococcal Autolysin Domains Interact With Polystyrene Surfaces. *Front. Microbiol.* **12**, 1–13 (2021).

50. Qin, Z. *et al.* Role of autolysin-mediated DNA release in biofilm formation of Staphylococcus epidermidis. *Microbiology* **153**, 2083–2092 (2007).

51. Montanaro, L. *et al.* Extracellular DNA in biofilms. *Int. J. Artif. Organs* **34**, 824–831 (2011).

52. Artini, M. *et al.* Hydrophobin coating prevents *Staphylococcus epidermidis* biofilm formation on different surfaces. *Biofouling* **33**, 601–611 (2017).

53. Gallardo-Moreno, A. M. *et al.* Bacterial adhesion reduction on a biocompatible Si+ ion implanted austenitic stainless steel. *Mater. Sci. Eng. C* **31**, 1567–1576 (2011).

54. Gristina, A. G. Biomaterial- Centered Infection : Microbial Adhesion Versus Tissue Integration. *Science (80-. ).* **237**, 1588–1595 (1987).

55. Hartford, O., O'Brien, L., Schofield, K., Wells, J. & Foster, T. J. The Fbe (SdrG) protein of *Staphylococcus epidermidis* HB promotes bacterial adherence to fibrinoge. *Microbiology* **147**, 2545–2552 (2001).

56. Vitale, C. *et al. Staphylococcus epidermidis* Has Growth Phase Dependent Affinity for Fibrinogen and Resulting Fibrin Clot Elasticity. *Front. Microbiol.* **12**, 1–10 (2021).

57. Williams, R. J., Henderson, B., Sharp, L. J. & Nair, S. P. Identification of a Fibronectin-Binding Protein from *Staphylococcus epidermidis*. *Infect. Immun.* **70**, 6805–6810 (2002).

58. Khan, N. *et al.* The giant staphylococcal protein Embp facilitates colonization of surfaces through Velcro-like attachment to fibrillated fibronectin. *Elife* **11**, 1–22 (2022).

59. Arrecubieta, C., Lee, M. H., Macey, A., Foster, T. J. & Lowy, F. D. SdrF, a *Staphylococcus epidermidis* surface protein, binds type I collagen. *J. Biol. Chem.* **282**, 18767–18776 (2007).

60. Trivedi, S. *et al.* The Surface Protein SdrF Mediates *Staphylococcus epidermidis* Adherence to Keratin. *J. Infect. Dis.* **215**, 1846–1854 (2017).

61. Bowden, M. G. *et al.* Is the GehD lipase from *Staphylococcus epidermidis* a collagen binding adhesin? *J. Biol. Chem.* **277**, 43017–43023 (2002).

62. Stones, D. H. & Krachler, A. M. Against the tide: The role of bacterial Adhesion in host colonization. *Biochem. Soc. Trans.* **44**, 1571–1580 (2016).

63. Trunk, T., S. Khalil, H. & C. Leo, J. Bacterial autoaggregation. *AIMS Microbiol.* **4**, 140–164 (2018).

64. Di Martino, P. Extracellular polymeric substances, a key element in understanding biofilm phenotype. *AIMS Microbiol.* **4**, 274–288 (2018).

65. Karygianni, L., Ren, Z., Koo, H. & Thurnheer, T. Biofilm matrixome: Extracellular components in structured microbial communities. *Trends Microbiol.* **28**, 668–681 (2020).

66. Rohde, H. *et al.* Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. *Biomaterials* **28**, 1711–1720 (2007).

67. Mack, D. *et al.* The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. *J. Bacteriol.* **178**, 175–183 (1996).

68. Gerke, C., Kraft, A., Süßmuth, R., Schweitzer, O. & Götz, F. Characterization of the N-Acetylglucosaminyltransferase activity involved in the biosynthesis of the *Staphylococcus epidermidis* polysaccharide intercellular adhesin. *J. Biol. Chem.* **273**, 18586–18593 (1998).

69. Vuong, C. *et al.* Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. *Cell. Microbiol.* **6**, 269–275 (2004).

70. Conlon, K. M., Humphreys, H. & O'Gara, J. P. icaR encodes a transcriptional repressor involved in environmental regulation of ica operon expression and biofilm formation in *Staphylococcus epidermidis*. *J. Bacteriol.* **184**, 4400–4408 (2002).

71. Hennig, S., Nyunt Wai, S. & Ziebuhr, W. Spontaneous switch to PIA-independent biofilm formation

in an *ica*-positive *Staphylococcus epidermidis* isolate. *Int. J. Med. Microbiol.* **297**, 117–122 (2007).

72. Dice, B. *et al.* Biofilm formation by *ica*-positive and *ica*-negative strains of *Staphylococcus epidermidis* in vitro. *Biofouling* **25**, 367–375 (2009).

73. Qin, Z. *et al.* Formation and properties of in vitro biofilms of *ica*-negative *Staphylococcus epidermidis* clinical isolates. *J. Med. Microbiol.* **56**, 83–93 (2007).

74. Freitas, A. I. *et al.* Comparative analysis between biofilm formation and gene expression in *Staphylococcus epidermidis* isolates. *Future Microbiol.* 415–427 (2018) doi:10.2217/fmb-2017-0140.

75. Rohde, H. *et al.* Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. *Mol. Microbiol.* **55**, 1883–1895 (2005).

76. Tormo, M. Á., Knecht, E., Götz, F., Lasa, I. & Penadés, J. R. Bap-dependent biofilm formation by pathogenic species of *Staphylococcus*: Evidence of horizontal gene transfer? *Microbiology* **151**, 2465–2475 (2005).

77. Christner, M. *et al.* The giant extracellular matrix-binding protein of *Staphylococcus epidermidis* mediates biofilm accumulation and attachment to fibronectin. *Mol. Microbiol.* **75**, 187–207 (2010).

78. Decker, R. *et al.* An 18 kDa scaffold protein is critical for *Staphylococcus epidermidis* biofilm formation. *PLoS Pathog.* **11**, e1004735 (2015).

79. Schommer, N. N. *et al. Staphylococcus epidermidis* uses distinct mechanisms of biofilm formation to interfere with phagocytosis and activation of mouse macrophage-like cells 774A.1. *Infect. Immun.* **79**, 2267–2276 (2011).

80. Hussain, M., Herrmann, M., Von Eiff, C., Perdreau-Remington, F. & Peters, G. A 140-kilodalton extracellular protein is essential for the accumulation of *Staphylococcus epidermidis* strains on surfaces. *Infect. Immun.* **65**, 519–524 (1997).

81. Mack, D. *et al.* Essential functional role of the polysaccharide intercellular adhesin of *Staphylococcus epidermidis* in hemagglutination. *Infect. Immun.* **67**, 1004–1008 (1999).

82. Cucarella, C. *et al.* Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *Microbiology* **183**, 2888–2896 (2001).

83. Guzmán-Soto, I. *et al.* Mimicking biofilm formation and development: Recent progress in *in vitro* and *in vivo* biofilm models. *iScience* **24**, 1–51 (2021).

84. Oliveira, A. & Cunha, M. BACTERIAL BIOFILMS WITH EMPHASIS ON COAGULASE-NEGATIVE STAPHYLOCOCCI. *J. Venom. Anim. Toxins incl. Trop. Dis* **14**, 572–596 (2008).

85. Otto, M. Staphylococcal Biofilms. *Curr Top Microbiol Immunol* **332**, 207–228 (2008).

86. Wang, R. *et al. Staphylococcus epidermidis* surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. *J. Clin. Invest.* **121**, 238–248 (2011).

87. Stewart, P. S. & Franklin, M. J. Physiological heterogeneity in biofilms. *Nat. Rev. Microbiol.* **6**, 199–210 (2008).

88. Yao, Y., Sturdevant, D. E. & Otto, M. Genomewide Analysis of Gene Expression in *Staphylococcus*

*epidermidis* Biofilms: Insights into the Pathophysiology of *S. epidermidis* Biofilms and the Role of Phenol-Soluble Modulins in Formation of Biofilms. *J. Infect. Dis.* **191**, 289–298 (2005).

89. Rani, S. A. *et al.* Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. *J. Bacteriol.* **189**, 4223–4233 (2007).

90. Kaplan, J. B. Biofilm Dispersal: Mechanisms, Clinical Implications, and Potential Therapeutic Uses. *J. Dent. Res.* **89**, 205–218 (2010).

91. McDougald, D., Rice, S. A., Barraud, N., Steinberg, P. D. & Kjelleberg, S. Should we stay or should we go: Mechanisms and ecological consequences for biofilm dispersal. *Nat. Rev. Microbiol.* **10**, 39–50 (2012).

92. Veerachamy, S., Yarlagadda, T., Manivasagam, G. & Yarlagadda, P. K. Bacterial adherence and biofilm formation on medical implants: A review. *Proc. Inst. Mech. Eng. Part H J. Eng. Med.* **228**, 1083–1099 (2014).

93. Petrova, O. E. & Sauer, K. Escaping the biofilm in more than one way: Desorption, detachment or dispersion. *Curr Opin Microbiol.* **30**, 67–78 (2017).

94. Le, K. Y., Dastgheyb, S., Ho, T. V. & Otto, M. Molecular determinants of staphylococcal biofilm dispersal and structuring. *Front. Cell. Infect. Microbiol.* **4**, 1–7 (2014).

95. Boles, B. R. & Horswill, A. R. Staphylococcal biofilm disassembly. *Trends Microbiol.* **19**, 449–455 (2011).

96. Chua, S. L. *et al.* Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles. *Nat. Commun.* **5**, 1–12 (2014).

97. Vuong, C., Gerke, C., Somerville, G. A. A., Fischer, E. R. R. & Otto, M. Quorum-Sensing Control of Biofilm Factors in Staphylococcus epidermidis. *J. Infect. Dis.* **188**, 706–718 (2003).

98. Kong, K. F., Vuong, C. & Otto, M. *Staphylococcus* quorum sensing in biofilm formation and infection. *Int. J. Med. Microbiol.* **296**, 133–139 (2006).

99. Periasamy, S., Chatterjee, S. S., Cheung, G. Y. C. & Otto, M. Phenol-soluble modulins in staphylococci. What are they originally for? *Commun. Integr. Biol.* **5**, 275–277 (2012).

100. Vuong, C. *et al.* Regulated expression of pathogen-associated molecular pattern molecules in *Staphylococcus epidermidis*: quorum-sensing determines pro-inflammatory capacity and production of phenol-soluble modulins. *Cell. Microbiol.* **6**, 753–759 (2004).

101. Xu, L. *et al.* Role of the luxS quorum-sensing system in biofilm formation and virulence of *Staphylococcus epidermidis*. *Infect. Immun.* **74**, 488–496 (2006).

102. Otto, M., O'Mahoney, D. S., Guina, T. & Klebanoff, S. J. Activity of *Staphylococcus epidermidis* Phenol-Soluble Modulin Peptides Expressed in *Staphylococcus carnosus*. *J. Infect. Dis.* **190**, 748–755 (2004).

103. Wang, R. *et al.* Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat. Med.* **13**, 1510–1514 (2007).

104. Yao, Y., Sturdevant, D. E. & Otto, M. Genomewide analysis of gene expression in Staphylococcus epidermidis biofilms: Insights into the pathophysiology of S. epidermidis biofilms and the role of phenol-soluble modulins in formation of biofilms. *J. Infect. Dis.* **191**, 289–298 (2005).

105. Cheung, G. Y. C., Joo, H.-S. S., Chatterjee, S. S. & Otto, M. Phenol-soluble modulins – critical

determinants of staphylococcal virulence. *FEMS Microbiol. Rev.* **38**, 698–719 (2014).

106. Vuong, C., Saenz, H. L., Tz, F. G. & Otto, M. Impact of the agr quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. *J. Infect. Dis.* **182**, 1688–1693 (2000).

107. Otto, M. *Staphylococcus epidermidis* – the "accidental" pathogen. *Nat Rev Microbiol.* **7**, 555–567 (2009).

108. Kirmusaoglu, S. Staphylococcal Biofilms: Pathogenicity, Mechanism and Regulation of Biofilm Formation by Quorum-Sensing System and Antibiotic Resistance Mechanisms of Biofilm-Embedded Microorganisms. in *Microbial Biofilms - Importance and Applications* (eds. Dhanasekaran, D. & Thajuddin, N.) 189–209 (InTech, 2016). doi:10.5772/62943.

109. Veening, J.-W., Smits, W. K. & Kuipers, O. P. Bistability, Epigenetics, and Bet-Hedging in Bacteria. *Annu. Rev. Microbiol.* **62**, 193–210 (2008).

110. Chihara, K., Matsumoto, S., Kagawa, Y. & Tsuneda, S. Mathematical modeling of dormant cell formation in growing biofilm. *Front. Microbiol.* **6**, 1–8 (2015).

111. Hansen, S. K., Rainey, P. B., Haagensen, J. A. J. & Molin, S. Evolution of species interactions in a biofilm community. *Nature* **445**, 533–536 (2007).

112. Levin, B. R. & Rozen, D. E. Non-inherited antibiotic resistance. *Nature* **4**, 556–562 (2006).

113. Gilbert, P., Maira-Litran, T., McBain, A. J., Rickard, A. H. & Whyte, F. W. The Physiology and Collective Recalcitrance of Microbial Biofilm Communities. *Advances in Microbial Physiology* vol. 46 (2002).

114. Olwal, C. O., Ang'Ienda, P. O., Onyango, D. M. & Ochiel, D. O. Susceptibility patterns and the role of extracellular DNA in *Staphylococcus epidermidis* biofilm resistance to physico-chemical stress exposure. *BMC Microbiol.* **18**, 1–13 (2018).

115. Bernier, S. P. *et al.* Starvation, Together with the SOS Response, Mediates High Biofilm-Specific Tolerance to the Fluoroquinolone Ofloxacin. *PLoS Genet.* **9**, 1–14 (2013).

116. Cerca, F. *et al. Staphylococcus epidermidis* biofilms with higher proportions of dormant bacteria induce a lower activation of murine macrophages. *J. Med. Microbiol.* **60**, 1717–1724 (2011).

117. Li, X. *et al.* Methods for characterizing the co-development of biofilm and habitat heterogeneity. *J. Vis. Exp.* **97**, 1–10 (2015).

118. Kim, J. S., Chowdhury, N., Yamasaki, R. & Wood, T. K. Viable but non-culturable and persistence describe the same bacterial stress state. *Environ. Microbiol.* **20**, 2038–2048 (2018).

119. Xu, H. S. *et al.* Survival and Viability of Nonculturable *Escherichia coli* and *Vibrio cholerae* in the Estuarine and Marine Environment. *Microb. Ecol.* **8**, 313–323 (1982).

120. Hobby, G. L., Meyer, K. & Chaffee, E. Observations on the Mechanism of Action of Penicillin. *Exp. Biol. Med.* **50**, 281–285 (1942).

121. Lewis, K. Multidrug tolerance of biofilms and persister cells. *Curr. Top. Microbiol. Immunol.* **322**, 107–131 (2008).

122. Bigger, J. TREATMENT OF STAPHYLOCOCCAL INFECTIONS WITH PENICILLIN BY INTERMITTENT STERILISATION. *Lancet* **244**, 497–500 (1944).

123. Lewis, K. Persister cells and the riddle of biofilm survival. *Biochem.* **70**, 267–274 (2005).

124. Oliver, J. D. The viable but nonculturable state in the human pathogen *Vibrio vulnificus*. *FEMS Microbiol. Lett.* **133**, 203–208 (1995).

125. Pan, H. & Ren, Q. Wake Up! Resuscitation of Viable but Nonculturable Bacteria: Mechanism and Potential Application. *Foods* **12**, 1–15 (2023).

126. Pinto, D., Santos, M. A. & Chambel, L. Thirty years of viable but nonculturable state research: Unsolved molecular mechanisms. *Crit. Rev. Microbiol.* **41**, 61–76 (2013).

127. Li, L., Mendis, N., Trigui, H., Oliver, J. D. & Faucher, S. P. The importance of the viable but non-culturable state in human bacterial pathogens. *Front. Microbiol.* **5**, 1–20 (2014).

128. Spoering, A. L. & Lewis, K. Biofilms and Planktonic Cells of *Pseudomonas aeruginosa* Have Similar Resistance to Killing by Antimicrobials. *J. Bacteriol.* **183**, 6746–6751 (2001).

129. Trevors, J. T. Viable but non-culturable (VBNC) bacteria: Gene expression in planktonic and biofilm cells. *J. Microbiol. Methods* **86**, 266–273 (2011).

130. Costerton, J. W. *et al.* New methods for the detection of orthopedic and other biofilm infections. *FEMS Immunol. Med. Microbiol.* **61**, 133–140 (2011).

131. Zandri, G. *et al.* Detection of viable but non-culturable staphylococci in biofilms from central venous catheters negative on standard microbiological assays. *Clin. Microbiol. Infect.* **18**, E259–E261 (2012).

132. Hong, S. H., Wang, X., O'Connor, H. F., Benedik, M. J. & Wood, T. K. Bacterial persistence increases as environmental fitness decreases. *Microb. Biotechnol.* **5**, 509–522 (2012).

133. Pasquaroli, S. *et al.* Antibiotic pressure can induce the viable but non-culturable state in *Staphylococcus aureus* growing in biofilms. *J. Antimicrob. Chemother.* **68**, 1812–1817 (2013).

134. Kvich, L. *et al.* Oxygen restriction generates difficult-to-culture *P. aeruginosa*. *Front. Microbiol.* **10**, 1–15 (2019).

135. Ayrapetyan, M., Williams, T. C. & Oliver, J. D. Bridging the gap between viable but non-culturable and antibiotic persistent bacteria. *Trends Microbiol.* **23**, 7–13 (2015).

136. Steinert, M., Emödy, L., Amann, R. & Hacker, J. Resuscitation of viable but nonculturable *Legionella pneumophila* philadelphia JR32 by *Acanthamoeba castellanii*. *Appl. Environ. Microbiol.* **63**, 2047–2053 (1997).

137. Pawlowski, D. R. *et al.* Entry of *Yersinia pestis* into the viable but nonculturable state in a low-temperature tap water microcosm. *PLoS One* **6**, 1–9 (2011).

138. Robben, C. *et al.* Induction of the viable but non-culturable state in bacterial pathogens by household cleaners and inorganic salts. *Sci. Rep.* **8**, 1–9 (2018).

139. Besnard, V., Federighi, M., Declerq, E., Jugiau, F. & Cappelier, J.-M. Environmental and physico-chemical factors induce VBNC state in *Listeria monocytogenes*. *Vet. Res.* **33**, 359–370 (2002).

140. Zhang, S. *et al.* Induction of *Escherichia coli* into a VBNC state by continuous-flow UVC and subsequent changes in metabolic activity at the single-cell level. *Front. Microbiol.* **9**, 1–11 (2018).

141. Rahman, I., Shahamat, M., Kirchman, P. A., Russek-Cohen, E. & Colwell, R. R. Methionine uptake and cytopathogenicity of viable but nonculturable *Shigella dysenteriae* type 1. *Appl. Environ. Microbiol.* **60**, 3573–3578 (1994).

142. Alleron, L. *et al.* VBNC *Legionella pneumophila* cells are still able to produce virulence proteins. *Water Res.* **47**, 6606–6617 (2013).

143. Senoh, M. *et al.* Conversion of viable but nonculturable *Vibrio cholerae* to the culturable state by co-culture with eukaryotic cells. *Microbiol. Immunol.* **54**, 502–507 (2010).

144. Cook, K. L. & Bolster, C. H. Survival of *Campylobacter jejuni* and *Escherichia coli* in groundwater during prolonged starvation at low temperatures. *J. Appl. Microbiol.* **103**, 573–583 (2007).

145. Day, A. P. & Oliver, J. D. Changes in membrane fatty acid composition during entry of *Vibrio vulnificus* into the viable but nonculturable state. *J. Microbiol.* **42**, 69–73 (2004).

146. Shleeva, M., Mukamolova, G. V., Young, M., Williams, H. D. & Kaprelyants, A. S. Formation of 'non-culturable' cells of *Mycobacterium smegmatis* in stationary phase in response to growth under suboptimal conditions and their Rpf-mediated resuscitation. *Microbiology* **150**, 1687–1697 (2004).

147. Guo, L. *et al.* Population and single cell metabolic activity of UV-induced VBNC bacteria determined by CTC-FCM and D2O-labeled Raman spectroscopy. *Environ. Int.* **130**, 1–10 (2019).

148. Asakura, H. *et al.* Differential expression of the outer membrane protein W (OmpW) stress response in enterohemorrhagic *Escherichia coli* O157:H7 corresponds to the viable but non-culturable state. *Res. Microbiol.* **159**, 709–717 (2008).

149. Carvalhais, V., Cerveira, F., Vilanova, M., Cerca, N. & Vitorino, R. An immunoproteomic approach for characterization of dormancy within *Staphylococcus epidermidis* biofilms. *Mol. Immunol.* **65**, 429–435 (2015).

150. Carvalhais, V. *et al.* Dormancy within *Staphylococcus epidermidis* biofilms: a transcriptomic analysis by RNA-seq. *Appl. Microbiol. Biotechnol.* **98**, 2585–2596 (2014).

151. Oliver, J. D. Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol. Rev.* **34**, 415–425 (2010).

152. Nowakowska, J. & Oliver, J. D. Resistance to environmental stresses by *Vibrio vulnificus* in the viable but nonculturable state. *FEMS Microbiol. Ecol.* **84**, 213–222 (2013).

153. Cunningham, E., Byrne, C. O. & Oliver, J. D. Effect of weak acids on Listeria monocytogenes survival: Evidence for a viable but nonculturable state in response to low pH. *Food Control* **20**, 1141–1144 (2009).

154. Berney, M., Hammes, F., Bosshard, F., Weilenmann, H. U. & Egli, T. Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight kit in combination with flow cytometry. *Appl. Environ. Microbiol.* **73**, 3283–3290 (2007).

155. Cerca, F. *et al.* SYBR green as a fluorescent probe to evaluate the biofilm physiological state of *Staphylococcus epidermidis,* using flow cytometry. *Canadian Journal of Microbiology* vol. 57 850–856 (2011).

156. Allegra, S. *et al.* Use of Flow Cytometry To Monitor Legionella Viability. *Appl. Environ. Microbiol.* **74**, 7813–7816 (2008).

157. Zimmermann, R., Iturriaga, R. & Becker-birck, J. Bacteria and the Number Thereof Involved in Respiration. *Appl. Environ. Microbiol.* **36**, 926–935 (1978).

158. Lahtinen, S. J. *et al.* Degradation of 16S rRNA and attributes of viability of viable but nonculturable

probiotic bacteria. *Lett. Appl. Microbiol.* **46**, 693–698 (2008).

159. Li, Y. *et al.* Study on the Viable but Non-culturable (VBNC) State Formation of *Staphylococcus aureus* and Its Control in Food System. *Front. Microbiol.* **11**, 1–9 (2020).

160. Wu, B., Liang, W. & Kan, B. Growth phase, oxygen, temperature, and starvation affect the development of viable but non-culturable state of *Vibrio cholerae*. *Front. Microbiol.* **7**, 1–9 (2016).

161. Wei, C. & Zhao, X. Induction of Viable but Nonculturable *Escherichia coli* O157:H7 by low temperature and its resuscitation. *Front. Microbiol.* **9**, 1–9 (2018).

162. Grey, B. & Steck, T. R. Concentrations of Copper Thought To Be Toxic to Escherichia coli Can Induce the Viable but Nonculturable Condition. *Appl. Environ. Microbiol.* **67**, 5325–5327 (2001).

163. Orruño, M., Parada, C., Kaberdin, V. R. & Arana, I. The effect of visible light on cell envelope subproteome during vibrio harveyi survival at 20◦ c in seawater. *Microorganisms* **9**, 1–15 (2021).

164. Zhang, S., Ye, C., Lin, H., Lv, L. & Yu, X. UV disinfection induces a VBNC state in *Escherichia coli* and *Pseudomonas aeruginosa*. *Environ. Sci. Technol.* **49**, 1721–1728 (2015).

165. Roszak, D. B., Grimes, D. J. & Colwell, R. R. Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. *Can. J. Microbiol.* **30**, 334–338 (1984).

166. Lee, D. yeon D. *et al.* Magnesium Flux Modulates Ribosomes to Increase Bacterial Survival. *Cell* **177**, 352–360 (2019).

167. Ravel, J., Hill, R. T. & Colwell, R. R. Isolation of a *Vibrio cholerae* transposon-mutant with an altered viable but nonculturable response. *FEMS Microbiol. Lett.* **120**, 57–62 (1994).

168. Mukamolova, G. V., Kaprelyants, A. S., Young, D. I., Young, M. & Kell, D. B. A bacterial cytokine. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 8916–8921 (1998).

169. Pasquaroli, S. *et al.* Role of daptomycin in the induction and persistence of the viable but non-culturable state of *Staphylococcus aureus* biofilms. *Pathogens* **3**, 759–768 (2014).

170. Keren, I., Minami, S., Rubin, E. & Lewis, K. Characterization and transcriptome analysis of *Mycobacterium tuberculosis* persisters. *MBio* **2**, 3–12 (2011).

171. Postnikova, O. A., Shao, J., Mock, N. M., Baker, C. J. & Nemchinov, L. G. Gene expression profiling in viable but nonculturable (VBNC) cells of *Pseudomonas syringae* pv. *syringae*. *Front. Microbiol.* **6**, 1–14 (2015).

172. Gaio, V., Lopes, N., Cerca, N. & França, A. *codY* and *pdhA* Expression Is Induced in *Staphylococcus epidermidis* Biofilm and Planktonic Populations With Higher Proportions of Viable but Non-Culturable Cells. *Front. Cell. Infect. Microbiol.* **11**, 1–8 (2021).

173. Joseph, P., Ratnayake-Lecamwasam, M. & Sonenshein, A. L. A region of *Bacillus subtilis* CodY protein required for interaction with DNA. *J. Bacteriol.* **187**, 4127–4139 (2005).

174. Fisher, S. H., Rohrer, K. & Ferson, A. E. Role of CodY in regulation of the *Bacillus subtilis hut* operon. *J. Bacteriol.* **178**, 3779–3784 (1996).

175. Barbieri, G. *et al.* CodY regulates expression of the *Bacillus subtilis* extracellular proteases Vpr and Mpr. *J. Bacteriol.* **197**, 1423–1432 (2015).

176. Serror, P. & Sonenshein, A. L. CodY is required for nutritional repression of *Bacillus subtilis* genetic competence. *J. Bacteriol.* **178**, 5910–5915 (1996).

177. Shivers, R. P. & Sonenshein, A. L. Activation of the *Bacillus subtilis* global regulator CodY by direct interaction with branched-chain amino acids. *Mol. Microbiol.* **53**, 599–611 (2004).

178. Ratnayake-Lecamwasam, M., Serror, P., Wong, K. W. & Sonenshein, A. L. *Bacillus subtilis* CodY represses early-stationary-phase genes by sensing GTP levels. *Genes Dev.* **15**, 1093–1103 (2001).

179. Levdikov, V. M. *et al.* Structure of the branched-chain Amino Acid and GTP-sensing global regulator, Cody, from Bacillus subtilis. *J. Biol. Chem.* **292**, 2714–2728 (2017).

180. Levdikov, V. M., Blagova, E., Joseph, P., Sonenshein, A. L. & Wilkinson, A. J. The structure of CodY, a GTP- and isoleucine-responsive regulator of stationary phase and virulence in gram-positive bacteria. *J. Biol. Chem.* **281**, 11366–11373 (2006).

181. Majerczyk, C. D. *et al.* Direct targets of CodY in *Staphylococcus aureus*. *J. Bacteriol.* **192**, 2861–2877 (2010).

182. Majerczyk, C. D. *et al. Staphylococcus aureus* CodY negatively regulates virulence gene expression. *J. Bacteriol.* **190**, 2257–2265 (2008).

183. Mlynek, K. D. *et al.* Nutritional regulation of the Sae two-component system by CodY in *Staphylococcus aureus*. *J. Bacteriol.* **200**, 1–16 (2018).

184. Herman-Bausier, P. & Dufrêne, Y. F. Force matters in hospital-acquired infections. *Science (80-. ).* **359**, 1464–1465 (2018).

185. Sarkar, P., Yarlagadda, V., Ghosh, C. & Haldar, J. A review on cell wall synthesis inhibitors with an emphasis on glycopeptide antibiotics. *Medchemcomm* **8**, 516–533 (2017).

186. Reynolds, P. E. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *Eur. J. Clin. Microbiol. Infect. Dis.* **8**, 943–950 (1989).

187. Goldman, R. C., Baizman, E. R., Longley, C. B. & Branstrom, A. A. Chlorobiphenyl-desleucyl-vancomycin inhibits the transglycosylation process required for peptidoglycan synthesis in bacteria in the absence of dipeptide binding. *FEMS Microbiol. Lett.* **183**, 209–214 (2000).

188. Siibak, T. *et al.* Erythromycin- and chloramphenicol-induced ribosomal assembly defects are secondary effects of protein synthesis inhibition. *Antimicrob. Agents Chemother.* **53**, 563–571 (2009).

189. Azeh, I. *et al.* Protein synthesis inhibiting clindamycin improves outcome in a mouse model of *Staphylococcus aureus* sepsis compared with the cell wall active ceftriaxone. *Crit. Care Med.* **30**, 1560–1564 (2002).

190. Fines, M. & Leclercq, R. Activity of linezolid against Gram-positive cocci possessing genes conferring resistance to protein synthesis inhibitors. *J. Antimicrob. Chemother.* **45**, 797–802 (2000).

191. Yilancioglu, K. Antimicrobial drug interactions: Systematic evaluation of protein and nucleic acid synthesis inhibitors. *Antibiotics* **8**, 1–8 (2019).

192. Campbell, E. A. *et al.* Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell* **104**, 901–912 (2001).

193. Griffith, E. C. *et al.* The structural and functional basis for recurring sulfa drug resistance mutations in *Staphylococcus aureus* dihydropteroate synthase. *Front. Microbiol.* **9**, 1–16 (2018).

194. Cerca, N. *et al.* Comparative assessment of antibiotic susceptibility of coagulase- negative staphylococci in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry. *J Antimicrob Chemother* **56**, 331–336 (2005).

195. Dufour, D., Leung, V. & Lévesque, C. M. Bacterial biofilm: structure, function, and antimicrobial resistance. *Endod. Top.* **22**, 2–16 (2012).

196. Francolini, I. & Donelli, G. Prevention and control of biofilm-based medical-device-related infections. *FEMS Immunol. Med. Microbiol.* **59**, 227–238 (2010).

197. Singh, R., Sahore, S., Kaur, P., Rani, A. & Ray, P. Penetration barrier contributes to bacterial biofilm-associated resistance against only select antibiotics, and exhibits genus-, strain- and antibiotic-specific differences. *Pathog. Dis.* **74**, 1–6 (2016).

198. Doroshenko, N. *et al.* Extracellular DNA impedes the transport of vancomycin in *Staphylococcus epidermidis* biofilms preexposed to subinhibitory concentrations of vancomycin. *Antimicrob. Agents Chemother.* **58**, 7273–7282 (2014).

199. Jensen, P. *et al.* Formation of hydroxyl radicals contributes to the bactericidal activity of ciprofloxacin against *Pseudomonas aeruginosa* biofilms. *Pathog. Dis.* **70**, 440–443 (2014).

200. Proctor, R. A. *et al.* Small colony variants: A pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat. Rev. Microbiol.* **4**, 295–305 (2006).

201. Król, J. E. *et al.* Invasion of *E. coli* biofilms by antibiotic resistance plasmids. *Plasmid* **70**, 110–119 (2013).

202. Savage, V. J., Chopra, I. & O'Neill, A. J. *Staphylococcus aureus* biofilms promote horizontal transfer of antibiotic resistance. *Antimicrob. Agents Chemother.* **57**, 1968–1970 (2013).

203. Woodford, N. & Ellington, M. J. The emergence of antibiotic resistance by mutation. *Clin. Microbiol. Infect.* **13**, 5–18 (2007).

204. Schaaff, F., Reipert, A. & Bierbaum, G. An elevated mutation frequency favors development of vancomycin resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **46**, 3540–3548 (2002).

205. Otto M. VIRULENCE FACTORS OF THE COAGULASE-NEGATIVE STAPHYLOCOCCI. *Front. Biosci.* **9**, 841–863 (2004).

206. Ventola, C. L. Antibiotic Resistance Crisis. *Int. J. Med. Dev. Ctries.* **40**, 561–564 (2019).

207. Pinho, M. G., De Lencastre, H. & Tomasz, A. An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 10886–10891 (2001).

208. Ziebuhr, W. *et al.* Nosocomial infections by *Staplylococcus epidermidis*: how a commensal bacterium turns into a pathogen. *Int. J. Antimicrob. Agents* **28**, 14–20 (2006).

209. Farrell, D. J., Mendes, R. E., Ross, J. E., Sader, H. S. & Jones, R. N. LEADER program results for 2009: An activity and spectrum analysis of linezolid using 6,414 clinical isolates from 56 medical centers in the United States. *Antimicrob. Agents Chemother.* **55**, 3684–3690 (2011).

210. Marques De Mattos, E. *et al.* Isolation of methicillin-resistant coagulase-negative staphylococci from patients undergoing continuous ambulatory peritoneal dialysis (CAPD) and comparison of different molecular techniques for discriminating isolates of *Staphylococcus epidermidis*. *Diagn.*

*Microbiol. Infect. Dis.* **45**, 13–22 (2003).

211. Altayb, H. N. *et al.* Whole-Genome Sequence of Multidrug-Resistant Methicillin-Resistant *Staphylococcus epidermidis* Carrying Biofilm-Associated Genes and a Unique Composite of SCCmec. *Antibiotics* **11**, 1–15 (2022).

212. Rolo, J., de Lencastre, H. & Miragaia, M. Strategies of adaptation of *Staphylococcus epidermidis* to hospital and community: Amplification and diversification of SCCmec. *J. Antimicrob. Chemother.* **67**, 1333–1341 (2012).

213. Fariña, N., Samudio, M., Carpinelli, L., Nentwich, M. M. & de Kaspar, H. M. Methicillin resistance and biofilm production of *Staphylococcus epidermidis* isolates from infectious and normal flora conjunctiva. *Int. Ophthalmol.* **37**, 819–825 (2017).

214. Watanabe, K. *et al.* Methicillin-resistant *Staphylococcus epidermidis* is part of the skin flora on the hands of both healthy individuals and hospital workers. *Biol. Pharm. Bull.* **39**, 1868–1875 (2016).

215. Carbon, C. MRSA and MRSE: Is there an answer. *Clin. Microbiol. Infect.* **6**, 17–22 (2000).

216. Biavasco, F., Vignaroli, C. & Varaldo, P. E. Glycopeptide resistance in coagulase-negative staphylococci. *Eur. J. Clin. Microbiol. Infect. Dis.* **19**, 403–417 (2000).

217. Ahlstrand, E., Svensson, K., Persson, L., Tidefelt, U. & Söderquist, B. Glycopeptide resistance in coagulase-negative staphylococci isolated in blood cultures from patients with hematological malignancies during three decades. *Eur. J. Clin. Microbiol. Infect. Dis.* **30**, 1349–1354 (2011).

218. Pinheiro, L. *et al.* Susceptibility Profile of *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* Isolated from Blood Cultures to Vancomycin and Novel Antimicrobial Drugs over a Period of 12 Years. *Microb. Drug Resist.* **22**, 283–293 (2016).

219. Natoli, S. *et al.* Characterization of coagulase-negative staphylococcal isolates from blood with reduced susceptibility to glycopeptides and therapeutic options. *BMC Infect. Dis.* **9**, 1–8 (2009).

220. Hamad, T., Hellmark, B., Nilsdotter-Augustinsson, Å. & Söderquist, B. Antibiotic susceptibility among *Staphylococcus epidermidis* isolated from prosthetic joint infections, with focus on doxycycline. *APMIS* **123**, 1055–1060 (2015).

221. Fu, X. *et al.* Treatment of intracranial infection caused by methicillin-resistant *Staphylococcus epidermidis* with linezolid following poor outcome of vancomycin therapy: A case report and literature review. *Infect. Drug Resist.* **14**, 2533–2542 (2021).

222. Zheng, Z. & Stewart, P. S. Penetration of rifampin through *Staphylococcus epidermidis* biofilms. *Antimicrob. Agents Chemother.* **46**, 900–903 (2002).

223. Stavrakis, A. I. *et al.* Combination prophylactic therapy with rifampin increases efficacy against an experimental *Staphylococcus epidermidis* subcutaneous implant-related infection. *Antimicrob. Agents Chemother.* **58**, 2377–2386 (2014).

224. Yan, Q., Karau, M. J. & Raval, Y. S. Evaluation of Oritavancin Combinations with Rifampin , Associated Methicillin-Resistant *Staphylococcus aureus* Biofilms. *Antimicrob. Agents Chemother.* **62**, e00943-18 (2018).

225. Cirioni, O. *et al.* Daptomycin and rifampin alone and in combination prevent vascular graft biofilm formation and emergence of antibiotic resistance in a subcutaneous rat pouch model of staphylococcal infection. *Eur. J. Vasc. Endovasc. Surg.* **40**, 817–822 (2010).

226. Szczuka, E. & Kaznowski, A. Antimicrobial activity of tigecycline alone or in combination with rifampin against *Staphylococcus epidermidis* in biofilm. *Folia Microbiol. (Praha).* **59**, 283–288 (2014).

227. Leite, B. *et al.* In vitro activity of daptomycin, linezolid and rifampicin on *Staphylococcus epidermidis* biofilms. *Curr. Microbiol.* **63**, 313–317 (2011).

228. Le, K. Y., Park, M. D. & Otto, M. Immune Evasion Mechanisms of *Staphylococcus epidermidis* Biofilm Infection. *Front. Microbiol.* **9**, 1–8 (2018).

229. Elias, P. M. Stratum corneum defensive functions: An integrated view. *J. Invest. Dermatol.* **125**, 183–200 (2005).

230. Heesterbeek, D. A. C., Angelier, M. L., Harrison, R. A. & Rooijakkers, S. H. M. Complement and Bacterial Infections: From Molecular Mechanisms to Therapeutic Applications. *J. Innate Immun.* **10**, 455–464 (2018).

231. Marques, R. E., Marques, P. E., Guabiraba, R. & Teixeira, M. M. Exploring the homeostatic and sensory roles of the immune system. *Front. Immunol.* **7**, 1–7 (2016).

232. Hand, T. W. The Role of the Microbiota in Shaping Infectious Immunity. *Trends Immunol.* **37**, 647–658 (2016).

233. Foster, T. J. Immune evasion by Staphylococci. *Nat. Rev. Microbiol.* **3**, 948–958 (2005).

234. Lambris, J. D., Ricklin, D. & Geisbrecht, B. V. Complement evasion by human pathogens. *Nat. Rev. Microbiol.* **6**, 132–142 (2008).

235. Laarman, A., Milder, F., Van Strijp, J. & Rooijakkers, S. Complement inhibition by gram-positive pathogens: Molecular mechanisms and therapeutic implications. *J. Mol. Med.* **88**, 115–120 (2010).

236. Fredheim, E. G. A. *et al. Staphylococcus epidermidis* is polysaccharide intercellular adhesin activates complement. *FEMS Immunol. Med. Microbiol.* **63**, 269–280 (2011).

237. Kristian, S. A. *et al.* Biofilm Formation Induces C3a Release and Protects *Staphylococcus epidermidis* from IgG and Complement Deposition and from Neutrophil-Dependent Killing. *J. Infect. Dis.* **197**, 1028–1035 (2008).

238. Kawasaki, T. & Kawai, T. Toll-like receptor signaling pathways. *Front. Immunol.* **5**, 1–8 (2014).

239. Akira, S. & Hemmi, H. Recognition of pathogen-associated molecular patterns by TLR family. *Immunol. Lett.* **85**, 85–95 (2003).

240. Winterbourn, C. C., Kettle, A. J. & Hampton, M. B. Reactive Oxygen Species and Neutrophil Function. *Annu. Rev. Biochem.* **85**, 765–792 (2016).

241. Nagaoka, I., Niyonsaba, F., Tsutsumi-Ishii, Y., Tamura, H. & Hirata, M. Evaluation of the effect of human β-defensins on neutrophil apoptosis. *Int. Immunol.* **20**, 543–553 (2008).

242. Lu, T., Porter, A. R., Kennedy, A. D., Kobayashi, S. D. & DeLeo, F. R. Phagocytosis and killing of *Staphylococcus aureus* by human neutrophils. *J. Innate Immun.* **6**, 639–649 (2014).

243. Guerra, F. E., Borgogna, T. R., Patel, D. M., Sward, E. W. & Voyich, J. M. Epic immune battles of history: Neutrophils vs. *Staphylococcus aureus*. *Front. Cell. Infect. Microbiol.* **7**, 1–19 (2017).

244. Deng, S. *et al.* Over-expression of Toll-like receptor 2 up-regulates heme oxygenase-1 expression

and decreases oxidative injury in dairy goats. *J. Anim. Sci. Biotechnol.* **8**, 2–11 (2017).

245. Strunk, T. *et al.* TLR2 mediates recognition of live *Staphylococcus epidermidis* and clearance of bacteremia. *PLoS One* **5**, 2–9 (2010).

246. Tawaratsumida, K. *et al.* Characterization of N-terminal structure of TLR2-activating lipoprotein in *Staphylococcus aureus*. *J. Biol. Chem.* **284**, 9147–9152 (2009).

247. Ip, W. K. E., Takahashi, K., Moore, K. J., Stuart, L. M. & Ezekowitz, R. A. B. Mannose-binding lectin enhances Toll-like receptors 2 and 6 signaling from the phagosome. *J. Exp. Med.* **205**, 169–181 (2008).

248. Müller-Anstett, M. A. *et al.* Staphylococcal peptidoglycan co-localizes with Nod2 and TLR2 and activates innate immune response via both receptors in primary murine keratinocytes. *PLoS One* **5**, 1–13 (2010).

249. Stevens, N. T. *et al. Staphylococcus epidermidis* polysaccharide intercellular adhesin induces IL-8 expression in human astrocytes via a mechanism involving TLR2. *Cell. Microbiol.* **11**, 421–432 (2009).

250. Hajjar, A. M. *et al.* Cutting Edge: Functional Interactions Between Toll-Like Receptor (TLR) 2 and TLR1 or TLR6 in Response to Phenol-Soluble Modulin. *J. Immunol.* **166**, 15–19 (2001).

251. Qin, L. *et al.* Toxin Mediates Sepsis Caused by Methicillin-Resistant *Staphylococcus epidermidis*. *PLoS Pathog.* **13**, 1–16 (2017).

252. Takeuchi, O. & Akira, S. Pattern Recognition Receptors and Inflammation. *Cell* **140**, 805–820 (2010).

253. Muñoz-Carrillo, J. L. *et al.* Cytokine Profiling Plays a Crucial Role in Activating Immune System to Clear Infectious Pathogens. in *Immune Response Activation and Immunomodulation* (eds. Tyagi, R. K. & Bisen, P. S.) 1–30 (2019). doi:10.5772/intechopen.80843.

254. L. Ferreira, V., H.L. Borba, H., de F. Bonetti, A., P. Leonart, L. & Pontarolo, R. Cytokines and Interferons: Types and Functions. in *Autoantibodies and Cytokines* 65–88 (2019). doi:10.5772/intechopen.74550.

255. Megyeri, K., Mándi, Y., Degré, M. & Rosztóczy, I. Induction of cytokine production by different staphylococcal strains. *Cytokine* **19**, 206–212 (2002).

256. Strunk, T. *et al.* Responsiveness of human monocytes to the commensal bacterium *Staphylococcus epidermidis* develops late in gestation. *Pediatr. Res.* **72**, 10–18 (2012).

257. Spiliopoulou, A. I. *et al.* Bacterial adhesion, intracellular survival and cytokine induction upon stimulation of mononuclear cells with planktonic or biofilm phase *Staphylococcus epidermidis*. *FEMS Microbiol. Lett.* **330**, 56–65 (2012).

258. Kaya, E. *et al.* Planktonic and biofilm-associated *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* elicit differential human peripheral blood cell responses. *Microorganisms* **9**, (2021).

259. Ferreirinha, P. *et al.* Poly-N-Acetylglucosamine Production by *Staphylococcus epidermidis* Cells Increases Their In Vivo Proinflammatory Effect Pedro. *Infect. Immun.* **84**, 2933–2943 (2016).

260. França, A. *et al. Staphylococcus epidermidis* Biofilm-Released Cells Induce a Prompt and More Marked *In vivo* Inflammatory-Type Response than Planktonic or Biofilm Cells. *Front. Immunol.* **7**, 1–12 (2016).

261. Brescó, M. S. *et al.* Pathogenic mechanisms and host interactions in *Staphylococcus epidermidis* device-related infection. *Front. Microbiol.* **8**, 1–24 (2017).

262. Reddick, L. E. & Alto, N. M. Bacteria Fighting Back : How Pathogens Target and Subvert the Host Innate Immune System. *Mol. Cell* **54**, 321–328 (2014).

263. Zhou, A. *et al.* Survival of viable but nonculturable *Cronobacter sakazakii* in macrophages contributes to infections. *Microb. Pathog.* **158**, 1–9 (2021).

# CHAPTER 3 – Characterization of *codY* and *pdhA* genes transcription under VBNC state

**Summary**

Under stressful environmental conditions or nutrient limitations, VBNC cells can arise in *S. epidermidis* biofilms. Bacteria in this phenotypic state can regulate the expression of a significant number of genes as a strategy to survive. It was previously shown that *codY* and *pdhA* genes transcription was increased in *S. epidermidis* biofilm cells under VBNC-inducing conditions. To further investigate the potential involvement of these genes in the VBNC state emergence in biofilms, their transcription was confirmed by quantitative PCR, in several *S. epidermidis* strains. Additionally, the induction of the VBNC state and *codY* and *pdhA* gene expression levels were also assessed in planktonic populations. Our data confirmed that transcription of both genes was upregulated in biofilms with a higher proportion of VBNC cells. Furthermore, we demonstrated that planktonic cells may also enter a VBNC state and that *codY* and *pdhA* expression was also upregulated under VBNC-inducing conditions. Interestingly, both genes presented higher expression levels in planktonic than in biofilms cultures. Overall, our findings indicate that *codY* and *pdhA* are involved in the formation of VBNC cells, constituting good candidates for future studies addressing the role of the VBNC state in *S. epidermidis* virulence.

Part of the work presented in this chapter was published in *Front. Cell. Infect. Microbiol. (2021) 11:771666*.

## 3.1. Brief introduction

The presence of cells with distinct metabolic states within biofilms, namely VBNC cells, has been associated with biofilms' ability to tolerate antibiotics and, thus, linked with the difficulty of eradicating biofilm-associated infections[1]. Ever since the identification of VBNC cells, several studies have investigated their cell wall and membrane composition, structure, metabolic activity, as well as their gene expression profile under different stress conditions to better understand their role in pathogenesis [2–5]. Through the use of magnesium and high concentrations of glucose, it was possible to modulate the proportion of VBNC cells in *S. epidermidis* biofilms [6], enabling the establishment of an *in vitro* model for the study of VBNC cells. Using this model, it was observed that under VBNC conditions, the transcription levels of several genes, mainly involved in processes such as oxidation-reduction, ribosome activity, glucose and pyruvate metabolism, were altered [7]. Among those genes, *codY* and *pdhA* showed a higher expression under VBNC-inducing conditions, highlighting these genes for further studies [7,8]. As such, our goal was to investigate the role of CodY and PdhA in the emergence of VBNC cells and their involvement in *S. epidermidis* virulence potential.

To do so, the construction of mutant strains for the target genes is essential. However, *S. epidermidis* is highly refractory to mutagenesis with only a few strains being amenable to genetic manipulations, such as strain 1457. Since it was previously observed that the modulation of VBNC cells in *S. epidermidis* is strain-dependent [9] it was first necessary to determine if the VBNC state could be induced in strain 1457. Therefore, herein, we confirmed the applicability of the VBNC state induction *in vitro* model to modulate the proportion of VBNC cells in *S. epidermidis* strain 1457, as well as the transcription of *codY* and *pdhA.*

## 3.2. Material and methods

### 3.2.1. Strains and growth conditions

*S. epidermidis* strain 1457, isolated from venous catheter-associated infection and *S. epidermidis* strain 9142, isolated from a blood culture, were used in this study [10]. Additionally, the commensal isolate COM040A (recovered from a skin sample of a healthy volunteer) [11] and the clinical isolate PT11004 (recovered from bloodstream infection) [12] were also used. For each experiment, *S. epidermidis* overnight cultures were prepared from the glycerol stocks and grown in Tryptic Soy Broth (TSB) at 37 °C with shaking (120 rpm). For the experiments described below, overnight cultures were adjusted to an optical

density at 640 nm ($OD_{640nm}$) of 0.25 ± 0.05, which corresponds ≈ to 2 × $10^8$ colony-forming units/mL (CFU/mL) [13], and then used directly or diluted to obtain the appropriate concentration of cells.

### 3.2.2. Biofilm formation

Biofilm formation was performed in 24-well polystyrene plates (Orange Scientific, Braine-l' Alleud, Belgium) as previously described [6,7]. Briefly, 10 µL of the suspension with ≈ 2 × $10^8$ CFU/mL was inoculated in 1 mL TSB (dilution 1:100) supplemented with either 0.4% of glucose ($TSB_{0.4\%G}$) or with 0.4% glucose plus 20 mM of $MgCl_2$ ($TSB_{0.4\%G+MgCl2}$). Plates were then incubated at 37 °C, 120 rpm for 24 h. Afterwards, media was carefully removed, and fresh TSB supplemented with either 1% of glucose ($TSB_{1\%G}$) or 1% of glucose plus 20 mM of $MgCl_2$ ($TSB_{1\%G+MgCl2}$) was added to induce or prevent, respectively, VBNC cells formation. The plates were incubated under the same temperature and agitation conditions for an additional 24 h. Thereafter, the biofilm bulk fluid was carefully removed and biofilms were washed twice with 1 mL of 0.9% sodium chloride (NaCl; VWR, Alfragide, Portugal). Lastly, biofilm cells were scrapped and suspended with 1 mL of 0.9% of NaCl for further analysis.

### 3.2.3. Planktonic growth

Two types of planktonic cultures were investigated in this study: (i) 24 h and (ii) 48 h old cultures. For 24 h old cultures, a suspension with ≈ 2 × $10^8$ CFU/mL was diluted 1:100 in $TSB_{1\%G}$ or $TSB_{1\%G+MgCl2}$, transferred into a 10 mL Erlenmeyer and incubated at 37 °C at 120 rpm for up to 24 h. In the case of 48 h old planktonic cultures, to mimic biofilm formation conditions, a suspension with ≈ 2 × $10^8$ CFU/mL was diluted 1:100 in $TSB_{0.4\%G}$ or $TSB_{0.4\%G+MgCl2}$ and incubated at 37 °C and 120 rpm for 24 h. Thereafter, cells were centrifuged at 16000 × *g* for 10 min at 4 °C, the spent medium replaced with $TSB_{1\%G}$ or $TSB_{1\%G+MgCl2}$ and the cells were incubated for a further 24 h under the same temperature and agitation conditions.

### 3.2.4. Biofilm and planktonic cultures quantification by biomass assessement

Before quantification, both biofilm and planktonic cells were sonicated for 10 s at 33% of amplitude (Ultrasonic Processor Model CP750, Cole-Parmer, Illinois, U.S.A.), to dissociate cell clusters. Of note, the sonication cycle used has no significant effect on cell viability as previously determined by CFU counting and propidium iodide incorporation [13,14]. Biomass quantification was measured by determining the $OD_{640\ nm}$.

### 3.2.5. Biofilm and planktonic cultures quantification by culturability assessement

As described in the previous section, bacterial cultures were sonicated to dissociate cells clusters before quantification. The number of culturable cells in both biofilms and planktonic cultures was determined, at the same time, by CFU counting. In brief, serial dilutions of biofilm and planktonic cells were made in 0.9% NaCl and then, 5 µL of each dilution was spread on TSA plates (TSB supplemented with 1.5% (w/v) agar (VWR). Plates were incubated at 37 °C for at least 16 h before CFU counting.

### 3.2.6. Gene expression analysis

#### 3.2.6.1. RNA extraction

The RNA extraction protocol used was previously optimized for *S. epidermidis* by our group [15]. The protocol combines mechanical lysis with glass beads with a silica membrane-based RNA isolation (ExtractMe RNA Bacteria & Yeast Kit, Blirt S.A., Poland). Previously to RNA isolation, biofilms were washed, scraped from the bottom of the plate and suspended in 1 mL of 0.9% of NaCl. Three independent biofilms were pooled together to reduce variability [16]. One mL of both biofilm and planktonic cultures was then collected and immediately centrifuged at 16000 ×*g* for 10 min at 4 °C. Once centrifuged, the supernatant was discarded, and the bacterial pellet was suspended in 600 µL of RYBL Buffer (lysis solution). The suspension was transferred into 2 mL safe lock tubes containing 0.5 g of acid-washed silica beads (150–212 mm) (Sigma-Aldrich, USA) and the cells were lysed, in a BeadBug 6 Microtube Homogenizer (Scientific, NJ, USA), for 35 s at 4350 rpm. Subsequently, samples were immediately placed on ice for 5 min. The cell disruption and cooling steps were repeated three times more. Afterwards, samples were centrifuged at 16000 ×*g* for 1 min at 4 °C, the supernatants were transferred into 2 mL DNase/RNase-free tubes (avoiding the aspiration of glass beads) and mixed with an equal volume of 70% ethanol. The subsequent steps were performed according to the manufacturer's instructions.

#### 3.2.6.2. DNase Treatment

Genomic DNA was degraded by adding, to each RNA sample, 5 µL of DNase buffer and 2 µL of DNase I. To induce enzyme activity, samples were then incubated at 37 °C for 30 min. DNase I was inactivated by incubating the sample at 65 °C for 10 min, after adding 5 µL of 50 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0). All reagents were purchased from Thermo Fisher Scientific Inc, MA, USA.

#### 3.2.6.3. RNA quantification and integrity

The analysis of total RNA concentration and purity was determined in a NanoDrop One (Thermo Scientific, USA). RNA purity was assessed by analysing the absorbance ratios $A_{260/A280}$ and $A_{260/A230}$ to confirm potential protein and chemical contamination (phenol, chaotropic salts, etc), respectively. For each RNA sample, duplicate measurements were performed. RNA integrity was assessed through the visualization of the 23S/16S ribosomal RNA band pattern in a 1% agarose gel stained with GreenSafe Premium DNA staining (NZYTech, Portugal) and carried out in an electrophoresis system (Cleaver scientific, Warwickshire, UK) for 60 min (±20 min) at 80 V. Bands were visualized using ChemiDoc™ XRS+ (Bio-Rad, CA, USA). RNA samples were stored at -20 °C until further use.

#### 3.2.6.4. cDNA synthesis

Complementary DNA (cDNA) synthesis was performed using the enzyme RevertAid H minus Reverse Transcriptase (Thermo Fisher Scientific Inc.) and following the manufacturer's instructions. The total RNA concentration was adjusted to 250 ng and reverse transcribed, in a 10 µL reaction, using random primers as a priming strategy. A sample reaction with all reagents but lacking the reverse transcriptase enzyme (no-RT control, NRT) was prepared to determine the level of genomic DNA contamination.

#### 3.2.6.5. Quantitative PCR

Gene expression was determined by quantitative PCR (qPCR). Briefly, a 10 µL reaction containing 2 µL diluted cDNA or NRT control (1:400), 5 µL of Xpert Fast SYBR Mastermix (GRiSP, Lda., Porto, Portugal), 0.5 µL of each primer (forward and reverse; final concentration of 0.5 µM/reaction), and 2 µL of nuclease-free water was prepared. qPCR run was performed in a CFX96 (BioRad, CA, USA), with the following cycling parameters: 95 °C for 2 min, and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. A no-template control (NTC) for each primer mixture was included to assess reagent contamination. Melt curve analysis was performed at the end to ensure the absence of unspecific products and primer dimers. Additionally, reaction efficiency was assessed at 60 °C by performing 10-fold dilution series of the cDNA samples and determined from the slope of a standard curve. Primers were designed with the support of Primer3 software using *S. epidermidis* RP62A (for 16S rRNA primers) or 1457 complete genomes as a template (NCBI accession no. CP000029.1 and CP020463.1, respectively) (Table 3.1.). The specificity of the sequences of the designed primer was evaluated by BLASTn.

**Table 3.1** List of primers used in the qPCR analysis.

| Target gene | Forward sequence (5'-3') | Reverse sequence (3'-5') | Product size (bp) | Efficiency (%) |
|---|---|---|---|---|
| *16S rRNA* | AATCTTGACATCCTCTGACC | AGAGTGCCCAACTTAATGAT | 165 | 100.0 |
| *gyrB* | GCATTTGGTACGGGTATTGG | CATCAACATCGGCATCAGTC | 88 | 93.0 |
| *codY* | TTCGCCACCACCTAAAATTGG | AGAAGATCGACATATACCGCGTGA | 166 | 88.3 |
| *pdhA* | GCTTCTTGTCCTGCTGTTGGTG | TTGGATGAAGACGGAAATGTCG | 173 | 99.4 |

Gene expression data were normalized to the expression of two reference genes (*16S* rRNA and *gyrB*), by calculating the geometric mean of the cycle threshold of both reference genes [17] and using a variation of the LivaK method (Eq. (1)), where *E* stands for the reaction efficiency experimentally determined and Cq for cycle quantification. Data are represented as the ratio of $TSB_{1\%G}$ /$TSB_{1\%G+MgCl_2}$, i.e., ratio induced/prevented [18].

Eq. (1) $$E^{\Delta Ct} = E^{\text{geometric mean } Ct_{16S/gyrB} - \text{Ct}_{\text{target gene}}}$$

### 3.2.7. Statistical analysis

Statistical significance was determined by using GraphPad Prism version 7. A $p < 0.05$ was considered statistically significant. At least three independent experiments for each assay were performed.

## 3.3. Results

### 3.3.1. High glucose concentration induces VBNC cells formation in *S. epidermidis* strain 1457

*S. epidermidis* strain 1457 was grown under VBNC-preventing or -inducing conditions, using the *in vitro* model previously established by our group. The total biomass, as well as the total number of culturable cells, were assessed to estimate the percentage of VBNC cells. To evaluate the level of VBNC cell formation in strain 1457, strains 9142, COM040A and PT11004, which are known to form high amounts of VBNC cells under these conditions [7,9], were also included.

As shown in Table 3.2, reduced cell culturability was observed for all strains tested under VBNC-inducing conditions. However, the loss of culturability in strain 1457 was less marked than in the other tested strains (≈29% versus ≈ 2 to 12%). Nevertheless, a 0.5 $Log_{10}$ reduction in culturability (corresponding to ≈ a 32% loss of culturability) was previously considered a significant reduction [9]. Regarding the biofilm total

biomass, the supplementation of the medium with either 1% glucose or 1% glucose plus $MgCl_2$, did not seem to influence the total biomass of the tested strains, presenting ratio values near 100%.

**Table 3.2** Percentage of culturability (CFU/mL) and total biomass ($OD_{640nm}$) ratio of *S. epidermidis* biofilms formed by different strains. Biofilms were grown under VBNC-inducing or -preventing conditions for 48 h. Results represent the mean ± SEM of six independent experiments. IND, induced; PRE, prevented.

| | | ***S. epidermidis* strains** | | | |
|---|---|---|---|---|---|
| | | **1457** | **9142** | **COM040A** | **PT11004** |
| **Ratio IND/PRE (%)** | **Cell Culturability (CFU/mL)** | 29.35 ±4.45 | 7.02 ±1.57 *** | 2.17 ±0.65 **** | 11.66 ±4.15 ** |
| | **Total Biomass ($OD_{640nm}$)** | 113.44 ±5.69 | 120.86 ±6.94 | 89.26 ±6.85 | 108.80 ±8.84 |

Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ , 1457 vs. reference strains (9142, COM040A, PT11004).

### 3.3.2. VBNC state-inducing conditions influence gene expression in biofilms

VBNC cell induction in *S. epidermidis* changes the gene expression profile of several genes. Indeed, it was found, in an RNA-Seq analysis, that the expression of the genes *codY* and *pdhA* was upregulated upon the induction of VBNC cell formation [7,8]. Despite RNA-Seq's ability to quantify gene expression, depending on the technical conditions used such as depth, it is important to validate the results obtained.

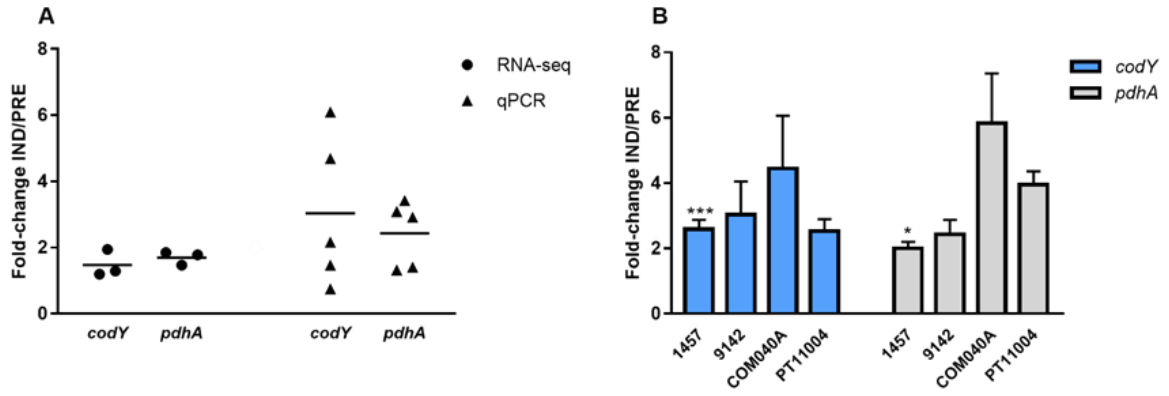


**Figure 3.1** Fold-change expression of the genes *codY* and *pdhA* in 48 h old *S. epidermidis* biofilms. **(A)** Validation of RNA-seq data obtained in *S. epidermidis* 9142 (ratio induced/prevented of Reads Per Kilobase Million values) by qPCR. Each point represents the fold-change of the individual assays, and the horizontal lines are the mean of at least three independent experiments. **(B)** Fold-change expression of *codY* and *pdhA* genes in 48 h old biofilms formed by the different *S. epidermidis* strains. The results are displayed as the mean + SEM of at least three experiments. Statistical significance was analysed using the unpaired t-test with Welch's correction (two-tailed). ** $p<0.05$, *** $p<0.001$*. IND, induced; PRE, prevented.

Hence, since qPCR is considered the gold standard for gene expression quantification, the expression of both genes was verified by qPCR in strain 9142, which was used for the RNA-Seq study referred to above (Fig. 3.1a), strain 1457, our target for mutagenesis studies and the control strains COM040A and PT11004 (Fig. 3.1b).

The qPCR results validated the previous RNA-Seq data, demonstrating an upregulation of both genes in *S. epidermidis* 9142 biofilms grown under VBNC-inducing conditions, despite the higher variability observed in qPCR assays. More importantly, the upregulation of the genes *codY* and *pdhA* in biofilms formed by strain 1457 grown under VBNC-inducing conditions was confirmed.

### 3.3.3. *S. epidermidis* planktonic cells can enter into a VBNC state

Considering that VBNC cell modulation in planktonic cultures has not yet been determined in *S. epidermidis*, we aimed to evaluate if the VBNC model established in biofilms could also be used to modulate the amount of VBNC cells in planktonic populations.

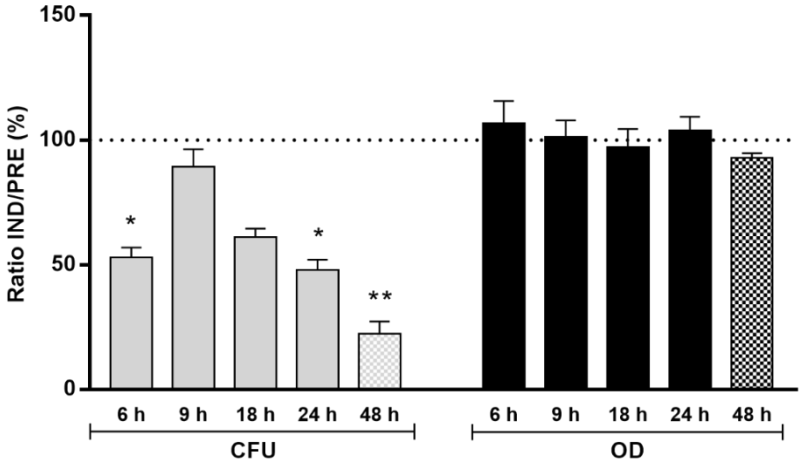


**Figure 3.2** Culturability (CFU/mL) and biomass quantification ($OD_{640nm}$) of *S. epidermidis* 1457 planktonic suspensions grown under VBNC conditions. Solid bars represent the growth of planktonic cells directly in $TSB_{1\ \%G}$/$TSB_{1\%G\ +\ MgCl2}$ for up to 24 h. Bars with patterns represent 48 h old planktonic cells, grown with $TSB_{0.4\%G}$/$TSB_{0.4\%G\ +\ MgCl2}$ in the first 24 h and $TSB_{1\ \%G}$/$TSB_{1\%G\ +\ MgCl2}$ in the next 24 h. The results are displayed as the mean + SEM of at least six independent experiments. Statistical analysis was performed between inducing and preventing VBNC conditions for each timepoint, both for CFU and OD data, using the unpaired t-test (two-tailed). ** p<0.05, ** p<0.01*. IND, induced; PRE, prevented.

As shown in Figure 3.2, *S. epidermidis* 1457 planktonic populations grown directly with 1% of glucose for 24 h, showed significant levels of VBNC cells at 6 h and 24 h, with a higher percentage of VBNC cells found at 24 h of growth (≈ 52%). Interestingly, after 9 h of growth, the culturability of bacteria grown under VBNC conditions was higher (only ≈ 10% reduction in culturability) (Fig. 3.2). However, since VBNC

modulation was achieved by supplementing the medium directly with 1% of glucose at beginning of the incubation, these conditions did not allow us to directly compare these results with the ones obtained with biofilm cultures. Therefore, we have also assessed the proportion of VBNC cells in planktonic populations grown under biofilm-forming conditions, i.e., 24 h of growth with $TSB_{0.4\%G}$ or $TSB_{0.4\%G+MgCl2}$ followed by an additional 24 h with $TSB_{1\%G}$ or $TSB_{1\%G+MgCl2}$. With this model of growth, a higher proportion of VBNC cells were detected in planktonic cultures, reaching values similar to the ones found in biofilms (≈ 79% in planktonic cultures vs ≈ 70% in biofilms).

### 3.3.4. The expression of *codY* and *pdhA* genes is also upregulated at VBNC conditions in planktonic populations

Since the applicability of the VBNC state induction model in *S. epidermidis* 1457 planktonic cultures was validated, we assessed the expression of our target genes in planktonic cultures as well.

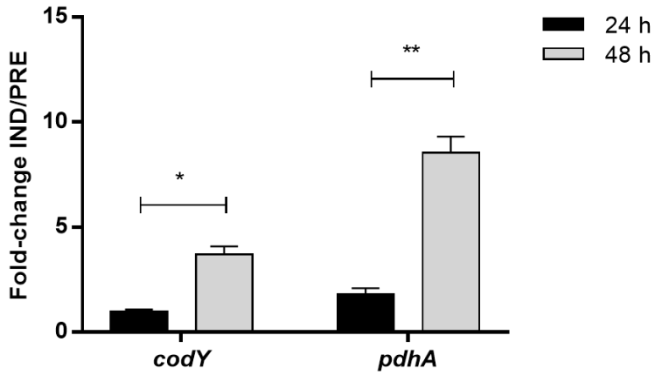


**Figure 3.3** Expression of the genes *codY* and *pdhA* in 24 and 48 h old planktonic populations. Data is represented as the fold-change between VBNC-inducing and -preventing conditions. The results are displayed as the mean + SEM of at least three experiments. Statistical significance was analysed using the unpaired t-test with Welch's correction (two-tailed). *** p<0.05, ** p<0.01*. IND, induced; PRE, prevented.

The expression of both *codY* and *pdhA* genes is upregulated under VBNC-inducing conditions as shown in Figure 3.3. Interestingly, by growing planktonic cells under biofilm-forming conditions (48 h cultures), a higher fold-change in *codY* and *pdhA* gene expression was obtained, resulting in a significantly higher induced/prevented ratio in comparison to biofilm data.

## 3.4. Discussion

The development of VBNC cells in biofilms has a great impact on antibiotics effectiveness and, subsequently, on the recurrence of biofilm-associated infections, as discussed in chapter 2. *S. epidermidis* can adopt this physiological state when under unfavorable conditions by switching to a more controlled and lower metabolic activity [19]. This alteration has been demonstrated to be primarily associated with modifications in gene expression [7,20]. For this reason, transcriptomic profiling of VBNC cells is extremely important to identify the genetic determinants underlying the development of the VBNC state. Based on a previous transcriptomic and immunoproteomic analysis, we have focused our study on the role of the genes *codY* and *pdhA* in VBNC cells formation in *S. epidermidis,* particularly in strain 1457*,* which is amenable to genetic manipulations and, thus, can be used for later mutagenesis studies.

The alteration in the expression profile of the gene *codY* under nutrient limitation has already been observed in other bacterial species, such as *B. subtilis* and *S. aureus* [21,22], but not in association with the VBNC state. Since the entrance of bacteria into a VBNC state can promote physiological and metabolic changes, and considering that *codY* expression has been associated with the growth under unfavorable conditions [23,24], this could explain the upregulation of *codY* observed under VBNC-inducing conditions. Concerning the gene *pdhA*, so far, limited information is available regarding its function and role in the emergence of the VBNC state. However, a recent study in *S. aureus* demonstrated that *pdhA* is associated with the levels of branched-chain fatty acids (BCFAs) which, in turn, are responsible for membrane composition and fluidity. Furthermore, it was demonstrated that the lack of *pdhA* gene compromised membrane integrity by increasing membrane fluidity. Additionally, alterations in BCFAs composition of the cell membrane appeared to significantly impact the growth of an *S. aureus* strain lacking *pdhA* gene, as well as the strain sensitivity to cell wall-inhibiting (vancomycin) and membrane-acting (daptomycin) antibiotics [25].

Our results demonstrated that the proportion of VBNC cells can be modulated in strain 1457, with 48 h old planktonic cells showing levels of VBNC cells similar to that found in biofilms. With this in mind, the analysis of the expression of the genes *codY* and *pdhA*, which are putatively involved in the VBNC cells formation in biofilms, was also performed in planktonic cultures. Noteworthy, *codY* and *pdhA* genes were also upregulated in planktonic cultures under VBNC-inducing conditions, suggesting that these genes play a role in VBNC cell formation in planktonic cells as well.

Collectively, the results reported in this study suggest that CodY and PdhA have a role in VBNC state emergence and targeting these genes may be a potential strategy to better understand the mechanisms behind VBNC state development in *S. epidermidis*.

## 3.5. References


1. Bernier, S. P. *et al.* Starvation, Together with the SOS Response, Mediates High Biofilm-Specific Tolerance to the Fluoroquinolone Ofloxacin. *PLoS Genet.* **9**, 1–14 (2013).

2. Trudeau, K., Dang Vu, K., Shareck, F. & Lacroix, M. Capillary electrophoresis separation of protein composition of γ-irradiated food pathogens *Listeria monocytogenes* and *Staphylococcus aureus*. *PLoS One* **7**, 1–9 (2012).

3. Heim, S. *et al.* The Viable but Nonculturable State and Starvation Are Different Stress Responses of Enterococcus faecalis, as Determined by Proteome Analysis. *J. Bacteriol.* **184**, 6739–6745 (2002).

4. Guo, L. *et al.* Population and single cell metabolic activity of UV-induced VBNC bacteria determined by CTC-FCM and D2O-labeled Raman spectroscopy. *Environ. Int.* **130**, 1–10 (2019).

5. Signoretto, C., Lleò, M. D. M., Tafi, M. C. & Canepari, P. Cell wall chemical composition of Enterococcus faecalis in the viable but nonculturable state. *Appl. Environ. Microbiol.* **66**, 1953–1959 (2000).

6. Cerca, F. *et al. Staphylococcus epidermidis* biofilms with higher proportions of dormant bacteria induce a lower activation of murine macrophages. *J. Med. Microbiol.* **60**, 1717–1724 (2011).

7. Carvalhais, V. *et al.* Dormancy within *Staphylococcus epidermidis* biofilms: A transcriptomic analysis by RNA-seq. *Appl. Microbiol. Biotechnol.* **98**, 2585–2596 (2014).

8. Carvalhais, V., Cerveira, F., Vilanova, M., Cerca, N. & Vitorino, R. An immunoproteomic approach for characterization of dormancy within *Staphylococcus epidermidis* biofilms. *Mol. Immunol.* **65**, 429–435 (2015).

9. Carvalhais, V. *et al.* Tetracycline and rifampicin induced a viable but nonculturable state in Staphylococcus epidermidis biofilms. *Future Microbiol.* **13**, 27–36 (2018).

10. Mack, D., Siemssen, N. & Laufs, R. Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: Evidence for functional relation to intercellular adhesion. *Infect. Immun.* **60**, 2048–2057 (1992).

11. Oliveira, F. & Cerca, N. Antibiotic resistance and biofilm formation ability among coagulase-negative staphylococci in healthy individuals from portugal. *J. Antibiot. (Tokyo).* **66**, 739–741 (2013).

12. Freitas, A. I. *et al.* Comparative analysis between biofilm formation and gene expression in *Staphylococcus epidermidis* isolates. *Future Microbiol.* 415–427 (2018) doi:10.2217/fmb-2017-0140.

13. Freitas, A. I., Vasconcelos, C., Vilanova, M. & Cerca, N. Optimization of an automatic counting system for the quantification of *Staphylococcus epidermidis* cells in biofilms. *J. Basic Microbiol.* **54**, 750–757 (2014).

14. Cerca, F. *et al. SYBR green as a fluorescent probe to evaluate the biofilm physiological state of* Staphylococcus epidermidis *, using flow cytometry. Canadian Journal of Microbiology* vol. 57 850–856 (2011).

15. França, A., Freitas, A. I., Henriques, A. F. & Cerca, N. Optimizing a qPCR gene expression quantification assay for *S. epidermidis* biofilms: A comparison between commercial kits and a customized protocol. *PLoS One* (2012) doi:10.1371/journal.pone.0037480.

16. Sousa, C., França, A. & Cerca, N. Assessing and reducing sources of gene expression variability in Staphylococcus epidermidis biofilms. *Biotechniques* **57**, 295–301 (2014).

17. Vandesompele, J. *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, (2002).

18. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* **25**, 402–408 (2001).

19. Ramamurthy, T., Ghosh, A., Pazhani, G. P. & Shinoda, S. Current perspectives on viable but non-culturable (VBNC) pathogenic bacteria. *Front. Public Heal.* **2**, 1–9 (2014).

20. Postnikova, O. A., Shao, J., Mock, N. M., Baker, C. J. & Nemchinov, L. G. Gene expression profiling in viable but nonculturable (VBNC) cells of *Pseudomonas syringae* pv. *syringae*. *Front. Microbiol.* **6**, 1–14 (2015).

21. Levdikov, V. M. *et al.* Structure of the branched-chain Amino Acid and GTP-sensing global regulator, cody, from *Bacillus subtilis*. *J. Biol. Chem.* **292**, 2714–2728 (2017).

22. Audretsch, C., Gratani, F., Wolz, C. & Dandekar, T. Modeling of stringent-response reflects nutrient stress induced growth impairment and essential amino acids in different *Staphylococcus aureus* mutants. *Sci. Rep.* **11**, 1–15 (2021).

23. Barbieri, G. *et al.* CodY regulates expression of the *Bacillus subtilis* extracellular proteases Vpr and Mpr. *J. Bacteriol.* **197**, 1423–1432 (2015).

24. Waters, N. R. *et al.* A spectrum of CodY activities drives metabolic reorganization and virulence gene expression in Staphylococcus aureus. *Mol. Microbiol.* **101**, (2016).

25. Singh, V. K. *et al.* Roles of pyruvate dehydrogenase and branched-chain a-keto acid dehydrogenase in branched-chain membrane fatty acid levels and associated functions in staphylococcus Aureus. *J. Med. Microbiol.* **67**, (2018).

# CHAPTER 4 – *In vitro* characterization of *S. epidermidis codY*-mutants and the role of CodY in the emergence of VBNC cells

**Summary**

According to the gene expression analysis of the *S. epidermidis* strain 1457, shown in **Chapter 3**, *codY* and *pdhA* genes seem to have a role in the development of VBNC cells in both planktonic and biofilm modes of growth. To confirm the involvement of those genes in the emergence of the VBNC state in *S. epidermidis*, the construction and characterization of *codY* and *pdhA* deletion mutants are necessary. However, due to several difficulties encountered during the construction of a strain lacking the gene *pdhA*, only *codY* deletion mutant was constructed. The cells lacking the gene *codY* were characterized in terms of their growth profile, biofilm formation ability, antibiotic susceptibility and survival in human blood and plasma. Additionally, the role of *codY* in the emergence of VBNC cells was also assessed. Our results demonstrated that the deletion of the gene *codY* had a negative impact on bacterial growth and biofilm formation ability, and resulted in increased susceptibility to rifampicin. Furthermore, we found evidence that the gene *codY* seems to be involved in the formation of VBNC cells. Taken together, these data suggest that *codY* contributes to *S. epidermidis* 1457 virulence.

## 4.1. Brief introduction

Genetic manipulation in bacteria enables the disruption or deletion of genes of interest such as the ones encoding virulence factors. These alterations allow the evaluation of the impact of those genes on bacteria physiology and virulence [1]. Staphylococcal species, however, are often refractory to genetic manipulation due to their strong restriction-modification barrier, which hinders the transformation of plasmids with foreign DNA [2]. These restriction-modification systems are responsible for the recognition and restriction of foreign DNA and are classified into four types based on their homology and associated functions [3]. Essentially, they comprise a restriction endonuclease that recognizes and cuts the foreign DNA sequences, and a methyltransferase which transfers methyl groups to the same specific DNA sequence, to ensure the correct discrimination between the self and nonself DNA [4]. Considering these restriction barriers, several studies involving genetic manipulation in *S. epidermidis* have been performed in strain 1457 [5,6], since it is the most amenable to genetic manipulation, allows the use of multiple resistant markers [7] and has been widely used for biofilm-related research [8,9]. Moreover, the complete genome of *S. epidermidis* strain 1457 has already been sequenced and is publicly available [7]. Considering the advantages of this strain, the deletion of the gene *codY* was performed in this background to further investigate its role in biofilm formation and the emergence of VBNC cells, as well as its impact on antibiotic action and survival in human blood and plasma.

## 4.2. Material and methods

### 4.2.1. Growth media

Bacterial strains were cultured in either TSB, a chemically defined medium (CDM) or in a CDM lacking: (i) Isoleucine ($CDM^{-Ile}$), (ii) Leucine ($CDM^{-Leu}$), (iii) Valine ($CDM^{-Val}$) or (iv) the three Branched-Chain Amino Acids (BCAA, $CDM^{-ILV}$). The CDM was prepared following a recipe previously published [10], with some minor changes. CDM formulation requires the preparation, separately, of several groups of compounds, as described in table 4.1.

In brief, the components of groups 1 and 2 were dissolved in 700 mL and 100 mL of water, respectively. Groups 3, 4 and 5 were made as concentrated solutions as follows: groups 3 and 5 (100× concentrated) were dissolved in 80 mL; while group 4 (5× concentrated) was dissolved in 200 mL. Except for group 1, which was filter sterilized with a 0.22 µm PES filter, the rest of the groups were autoclaved at 121°C for

15 min. The total volume of each stock solution of groups 1, 2 and 4 was mixed with 10 mL of the stock solution of groups 3 and 5, to reach the final volume of 1 L.

**Table 4.1** Composition of the CDM medium. G, group.

| | Components | mg/L | | Components | mg/L |
|---|---|---|---|---|---|
| | $Na_2HPO_4 \cdot 2H_2O$ | 10000 | G2 | Glucose[1] | 10000 |
| | $KH_2PO_4$ | 3000 | | $MgSO_4 * 7H_20$ | 500 |
| | L-Aspartic acid | 150 | G3 | Biotin | 0.1 |
| | L- Alanine | 100 | | Nicotinic acid | 2 |
| | L- Arginine | 100 | | D-Pantothenic acid, Ca | 2 |
| | L-Cystine | 50 | | Pyridoxal | 4 |
| | Glycine | 100 | | Pyridoxamine dihydro | 4 |
| | L-Glutamic acid | 150 | | Riboflavin | 2 |
| | L-Histidine | 100 | | Thiamin hydrochloride | 2 |
| G1 | L-Isoleucine | 150 | G4 | Adenine sulphate | 20 |
| | L-Lysine | 100 | | Guanine hydrochloride | 20 |
| | L-Leucine | 150 | G5 | $CaCl_2 * 6H_20$ | 10 |
| | L-Methionine | 100 | | $MnSO_4$ | 5 |
| | L-Phenylalanine | 100 | | $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ | 6 |
| | L-Proline | 150 | | | |
| | L-Serine | 100 | | | |
| | L-Threonine | 150 | | | |
| | L-Tryptophan | 100 | | | |
| | L-Tyrosine | 100 | | | |
| | L-Valine | 150 | | | |

[1] – corresponds to 1% of glucose in the CDM medium

### 4.2.2. Strains, plasmids and growth conditions

The list of bacterial strains and plasmids used for mutant and complemented strains construction is presented in table 4.2. Bacteria were grown overnight (≈ 16 h) in TSB at 37 °C with agitation at 120 rpm. For plasmid propagation, bacteria were grown under 250 rpm of agitation at 37 °C. When appropriate, antibiotics were added to the medium for the selection of the constructions: 10 μg/mL of chloramphenicol ($CM_{10}$; Sigma-Aldrich, Darmstadt, Germany) were used for the selection of both *E. coli* and *S. epidermidis* clones, and the selection of *S. epidermidis* mutants after allelic replacement procedure was accomplished with 1 μg/mL of Anhydrotetracyline hydrochloride ($ATC_1$; Sigma-Alrich). For *E. coli* selection during the complementation process, 100 μg/mL of ampicillin was added to the medium, while for plasmid maintenance in *S. epidermidis* the medium was supplemented with $CM_{10}$.

### 4.2.3. Construction of mutant and complementary strains

Gene deletion was achieved following a protocol previously described [11]. Briefly, the upstream and downstream regions of the target gene (≈ 1 kb each) were amplified by PCR using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc., MA, USA). The amplicons were further ligated through an overlap extension PCR. The resultant fragment was inserted into the polylinker region of the temperature-sensitive shuttle vector pIMAY, previously cut open with SmaI (NEB, Ipswich, MA, USA), and the ligation performed with T4 DNA ligase enzyme (NEB). The knockout plasmid was then transformed by chemical transformation into *E. coli* strain DC10B for propagation. Clones were selected with $TSA_{CM10}$ plates and tested for the presence of the correct plasmid. The plasmid of the selected clones was then sequenced to ensure that the PCR fragments did not harbor any mutation. Sequencing services were outsourced to Eurofins Genomics (Ebersberg, Germany).

**Table 4.2** Description of bacterial strains and plasmids used in this study.

| Strain or Plasmid | Relevant Characteristics | Reference |
|---|---|---|
| ***Strains*** | | |
| *E. coli* DC10B | *Δdcm* in *E. coli* K-12 (DH10B strain) background, *dam+* | [11] |
| *S. epidermidis* 1457 | WT strain isolated from a central venous catheter infection; *icaADBC+*, *aap+*, *embp+*, strong biofilm formation | [13] |
| *S. epidermidis* 1457*ΔcodY* | Mutant carrying a deletion of the entire *codY* gene (*ΔcodY*) | This study |
| *S. epidermidis* 1457*ΔcodY::codY* | 1457*ΔcodY* carrying plasmid pRB473 with gene *codY*; $CM_{10}^R$ | This study |
| ***Plasmids*** | | |
| *pIMAY* | Shuttle vector for *E. coli* and staphylococci | pIMC5 with tetracycline; inducible *secY* antisense from pKOR1; $CM_{10}^R$ [11] |
| *pRB473* | | Constitutive gene expression in staphylococci via *vegII* promoter [12] |

$CM_{10}^R$, resistant to chloramphenicol.

After confirmation, the knockout plasmid was transformed by electroporation into *S. epidermidis* strain 1457, using 2 mm gap cuvettes (Thermo Fisher Scientific Inc.), following previously optimized conditions (2100 V, 25 μF and 100 Ω). The clones were selected with $TSA_{CM10}$ plates and to confirm that they harbored the plasmids with the required inserts, the plasmids were purified using QIAGEN plasmid midi kit and tested by PCR using plasmid pIMAY screening primers, IM151 and IM152 [11]. Of note, to isolate plasmids from *S. epidermidis*, 25 U of lysostaphin (Sigma-Aldrich) were added to the resuspension buffer

provided with the kit, and the cell suspension was incubated for ± 30 min at 37 °C to lyse the cells. Subsequently, to accomplish gene deletion, an allelic replacement procedure was performed [2], selecting the clones without the target gene by counter-selection with $ATC_1$. The deletion was confirmed by PCR, using a set of primers designed outside the sequence used for the knockout construction, and by direct sequencing of the respective amplicons.

To determine, later on, whether the mutant phenotype was derived from the deletion of the gene under study, the sequence of *codY* was introduced in the mutant strain using the plasmid pRB473 [12]. For that, the sequences of the target gene and its promoter region were amplified by PCR, using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc.), and inserted into the polylinker region of the plasmid pRB473, previously cut open with SmaI (NEB).

The complementation plasmid was tested by PCR using the screening primers pRB473_HIND and pRB473_ECO [12] and then transformed, by electroporation, into the previously constructed *S. epidermidis codY*-mutant strain. The complementation was validated by assessing the expression of *codY* by qPCR, using the protocol described in section 3.2.6. Primers used for the mutant and complemented strains construction were designed with the support of Snapgene software and using the 1457 genome as a template, available at National Center for Biotechnology Information (NCBI; accession no.: NZ_CP020463). The specificity of the primers was evaluated through BLASTn. The list of primers used is shown in table 4.3.

### 4.2.4. Analysis of bacterial growth over time

In a 250 mL Erlenmeyer, overnight cultures of 1457 and 1457Δ*codY* strains, grown in TSB, were previously adjusted to an $OD_{640nm}$ = 0.25 and diluted down 10× in either TSB, CDM or CDM lacking all ($CDM^{-ILV}$) or each BCAAs ($CDM^{-Iso}$, $CDM^{-Leu,}$ $CDM^{-Val}$). Cultures were then incubated at 37 °C, 120 rpm, for 24 h. At T0, 2, 4, 6, 7, 8, 9, 10, 11, 12 and 24 h of growth, bacteria were sonicated at 33% of amplitude for 10 s, to disrupt cell clusters, and 1 mL of the sample was collected for $OD_{640nm}$ measurements to assess cell growth. When appropriate, concentrated samples were diluted in TSB or CDM for accurate measurements.

### 4.2.5. Biofilm formation and quantification

Biofilm formation was investigated under two different conditions. To evaluate the role of *codY* in *S. epidermidis* biofilm formation capacity, biofilms were grown in either $TSB_{0.4\%G}$ or CDM for up to 48 h, at 37

°C and 120 rpm, with medium replacement at 24 h. On the other hand, to assess the role of *codY* in the formation of VBNC cells, the VBNC-inducing model described in section 3.2.2, from Chapter 3, was used. In both cases, total biomass was estimated by $OD_{640nm}$ at 24 and 48 h upon incubation, as detailed in section 3.2.4, chapter 3.

**Table 4.3** List of primers used for mutant and complemented strains construction.

| Designation | Sequence (5'-3') | Product size (bp) | Purpose |
|---|---|---|---|
| P1_FW *codY* | CTTGTTCAGATTCTTCTTTGTCATCTAA | 1060 | Amplification of the upstream region of the gene *codY* |
| P2_RV *codY* | AATATGCGAATAATGAATGGACTGATTT | | |
| P3_FW* *codY* | tcattattcgcatattTGCACTTAAATCTTTATTTGT TGAGATTGAT | 1072 | Amplification of the downstream region of the gene *codY* |
| P4_RV *codY* | CGTTTAGTAAAAGACGAGAAAAAGTCATT | | |
| P1_FW *pdhA* | CTTGTCCTCCTAAGATTTACGTATTTAAATT | 1066 | Amplification of the upstream region of the gene *pdhA* |
| P2_RV *pdhA* | CTTAAAAGTGAACTCAAAAGAGACGAAG | | |
| P3_FW* *pdhA* | ttcacttttaagCCTTAATTGTATCACAAATAAAA ATCTCTGT | 1044 | Amplification of the upstream region of the gene *pdhA* |
| P4_RV *pdhA* | ATGTTTCTTTATCATTGTTAGAAAGTGGG | | |
| P1_CONF | TTATCACCCATTTAAACTATCTCTTAGTTTG | 3133 (WT) 2277 (1457Δ*codY*) | Screening for gene deletion |
| P4_CONF | ACCATTTATTAAAGTTGAGGCAACTAAATTT A | | |
| P6_COMP_FW§ | atat**ggatcc**CCTCTAACCATTGTATAGTGCG A | 1027 | Gene transcription quantification |
| P5_COMP_RV§ | atat**gtcgac**CCGAATGCTGTTGTGGATATTA C | | |
| IM151 | TACATGTCAAGAATAAACTGCCAAAGC | 2399 (Knockout plasmid) | Screening for knockout plasmid [11] |
| IM152 | AATACCTGTGACGGAAGATCACTTCG | | |
| pRB473_HIND | CCAGTAATGACCTCAGAACTCC | 1227 (plasmid plus *codY* gene) | Screening for complementation plasmid [12] |
| pRB473_ECO | CCCCAGGCGTTTAAGGGC | | |

* P3 forward primer harbors a tail with the reverse complement of the first 16 bp of P2 primer (represented with lowercase letters) to further promote the overlap of the PCR products from the up and downstream flanking regions.

§ In bold, restriction enzyme sequences; BamHI in P6 and SalI in P5. Before each restriction enzyme recognition sequence is a nonsense sequence (underlined) to promote restriction enzyme binding.

Abbreviations: Bp, base pair; COMP, complementation; CONF, confirmation; FW, forward; KO, knockout; MSC, multiple cloning site; RV, reverse; WT, wild type.

### 4.2.6. Cell's culturability

The number of culturable cells, in planktonic or biofilm cultures, was determined by CFU counting, as described in section 3.2.5, chapter 3. Cell culturability was determined simultaneously with biomass quantification.

### 4.2.7. Confocal laser scanning microscopy (CLSM) analysis

To analyse the structure of the biofilms formed by all *S. epidermidis* strains, 24 h old biofilms were prepared in $TSB_{0.4\%G}$, as described above, but Nunc™ thermanox™ coverslips (Thermo Fisher Scientific Inc.) were placed inside the wells of the 24-well plate before the addition of bacteria. Thereafter, biofilms were washed twice with 1 mL of 0.9% of NaCl and the cover slides were carefully transferred onto a glass slide. In the dark, biofilms were stained with a combination of DAPI (4′,6-diamidino-2-phenylindole), which binds to nucleic acids, and wheat germ agglutinin (WGA-TRITC) that binds to polysaccharides. To evaluate cell viability, biofilms were also stained using a LIVE/DEAD™ *Bac*Light™ Bacterial Viability Kit, which uses SYTO™ 9 that binds to nucleic acids, and propidium iodide (PI) which also binds nucleic acids but can only enter cells with compromised membranes. Furthermore, to calibrate the confocal lasers, a control with only dead cells was used. For that, biofilms were incubated with methanol for 15 min at RT. Then, methanol was removed, biofilms were washed with 0.9% of NaCl and stained, in the dark, with LIVE/DEAD stains. In all staining procedures, the excess of stain was removed, and the biofilms were analysed with an Olympus™ FluoView FV1000, confocal scanning laser microscope (CLSM, Olympus, Lisbon, Portugal). Biofilms were observed using a 10× and a 40× water-immersion objective, and images of different regions, in each biofilm, were acquired with 640 × 640 resolution. The CLSM images were analysed using the FV10-ASW 4.2 Viewer Software (Olympus). Two independent biofilms of each strain were analysed for DAPI/WGA and LIVE/DEAD stain combinations.

### 4.2.8. Antibiotic susceptibility tests

#### 4.2.8.1. Determination of the minimal inhibitory concentration (MIC)

The MIC values of the selected antibiotics (all purchased from Sigma-Aldrich) were determined by the microdilution method using TSB. Importantly, *Staphylococcus aureus* ATCC 29213 was used as quality control. Vancomycin (VAN) and linezolid (LZD) concentrations tested ranged between 0.125 – 32 mg/L, tetracycline (TET) and ciprofloxacin (CIP) between 0.06 – 8 mg/L, dicloxacillin (DCX) between 0.0020 – 1 mg/L and rifampicin (RIF) between 0.0010 – 0.125 mg/L. Subsequently, 100 µL of bacterial cultures (≈ $10^6$ CFU/mL) were inoculated into 100 µL of TSB media supplemented with antibiotics (obtaining a final concentration of ≈ $5\times10^5$ CFU/mL). Bacteria without antibiotics and medium without bacteria were used as positive and negative controls, respectively. The plates were then incubated at 37 °C for ≈ 24 h. MICs were defined as the lowest concentration of the antibiotic that visually inhibited bacterial growth. Moreover, the $OD_{620nm}$ was read to aid in the identification of the MIC value for each antibiotic.

4.2.8.2. Determination of the bacteria susceptibility to antibiotics using peak serum concentrations (PSC)

Following MIC determination, bacteria susceptibility to PSC of the selected antibiotics was assessed. Briefly, in 14 mL glass tubes, 6.3 mL of TSB was inoculated with 0.7 mL of a suspension with ≈ $2 \times 10^8$ CFU/mL and, then, each antibiotic was added at their PSC. The tubes were incubated for up to 6 h at 37 °C, 120 rpm and protected from the light. Bacteria without antibiotics and medium without bacteria were used as positive and negative controls, respectively. One mL of suspension was collected upon 2, 4 and 6 h of incubation and centrifuged at 16000 ×*g* for 10 min at 4 °C. Supernatants were removed, and the pellets were washed twice with 0.9% of NaCl. Then, pellets were suspended in 1 mL of the same saline solution and sonicated as described earlier. To assess bacteria culturability, serial 10-fold dilutions were performed in 0.9% NaCl and 5 µL of each dilution was plated onto TSA or $TSA_{CM10}$ plates. The plates were incubated at 37 °C and CFUs count was performed after ≈ 16 h of incubation. The number of CFU/mL was log-transformed ($log_{10}$) and then the values of each timepoint (T2, T4 and T6 h) were subtracted to T0 h.

### 4.2.9. Interaction with human blood and plasma

The survival of *S. epidermidis* strains in human blood and plasma was assessed by following a preestablished human blood *ex vivo* model [14,15]. Human blood was collected from healthy volunteers, under a protocol approved by the University of Minho Institutional Review Board [SECVS 002/2014 (ADENDA)], which is in strict accordance with the Declaration of Helsinki and Oviedo Convention. Importantly, all donors gave written informed consent to have blood taken. The blood was drawn into tubes spray-coated with lithium heparin and maintained in agitation (80 rpm at RT), while plasma collection was performed by centrifugation of whole blood at 4400 ×*g* for 20 min at 4 °C. Afterwards, 50 µL of bacterial cultures (≈ $10^6$ CFU/mL) was inoculated into 450 µL of either $TSB_{heparin}$ (as control), human plasma or whole blood. Of note, in the case of the complemented strain, $CM_{10}$ was added to ensure plasmid replication. Samples were incubated at 37 °C at 80 rpm for up to 4 h. After 2 and 4 h, 50 µL of each sample was collected and serial 10-fold dilutions were performed and plated onto TSA or $TSA_{CM10}$ plates. The number of culturable cells was assessed after ≈ 16 h of plate incubation at 37 °C. Due to donor-to-donor variability, six independent assays were performed, collecting blood from 3 males and 3 females.

### 4.2.10. Statistical analysis

Statistical differences were evaluated using GraphPad Prism version 7 (CA, USA) and were determined using either Two-way ANOVA or One-way ANOVA with Tukey's or Šídák's multiple comparisons tests. A $p<0.05$ was considered statistically significant. At least three independent experiments were performed for each assay detailed above, except for the evaluation of bacterial growth over time in TSB, where two experiments were performed.

## 4.3. Results

### 4.3.1. Construction of *S. epidermidis codY*-mutant and complemented strains

Despite several attempts, the amplification of the flanking regions of the *pdhA* gene was problematic, obtaining a low concentration of PCR product, which further influenced a successful ligation of both flanking regions through the overlap extension PCR. To increase the PCR yield, we (i) designed new primers with a longer overlap region, (ii) added DMSO to the PCR master mix, and (iii) increased the number of PCR cycles, as well as the PCR extension time. Additionally, a different high-fidelity DNA polymerase was used, which increased the amplification efficiency but only for *codY* flanking regions (Fig. 4.1). Hence, after several unsuccessful attempts, we decided only to proceed with the deletion of the gene *codY* from *S. epidermidis* 1457 genome.

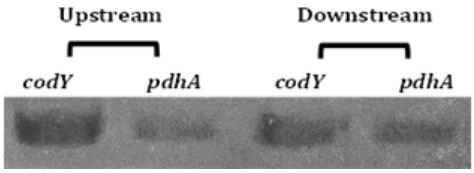


**Figure 4.1** Agarose gel electrophoresis of the PCR amplification of the flanking regions of the target genes.

As can be seen in Figure 4.2, the *codY* gene was successfully deleted in the two clones tested, as determined by the reduction of approximately 856 bp in the size of the amplicons (Fig. 4.2A), whcih correspond to the size of the gene *codY.* From the two obtained mutants, clone 2 was randomly selected for further studies. Through gene expression analysis (Fig. 4.2B), we further confirmed the gene deletion by the absence of *codY* transcripts, as well as the successful complementation of the mutant strain, as demonstrated by the restoration of *codY* gene expression. Of note, as observed in other gene deletion studies [16,17], the complemented strain showed significantly higher expression of the gene *codY* compared

to the WT strain. *S. epidermidis* 1457 *codY* mutant and complemented strains are henceforward referred to as 1457Δ*codY* and 1457Δ*codY::codY*, respectively.

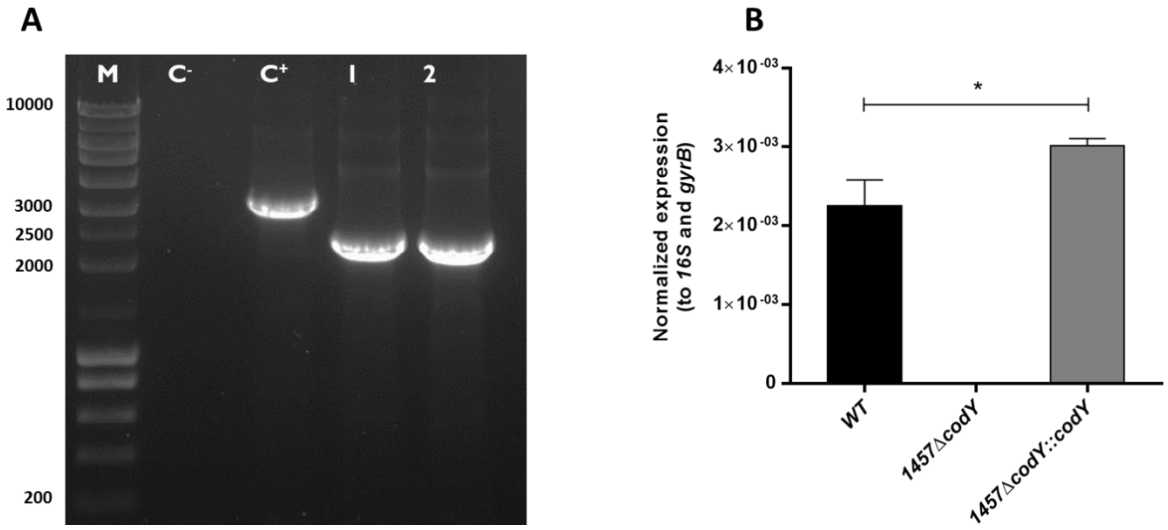


**Figure 4.2** Validation of 1457Δ*codY* and 1457Δ*codY::codY* constructions. **(A)** Agarose gel electrophoresis of a PCR performed using the genomic DNA of the selected clones as a template. Lane M – marker size (10000 bp DNA ladder); Lane $C^-$ – no template control; Lane $C^+$ – positive control (gDNA from *S. epidermidis* 1457 WT); Lane 1 to 2 – Clones obtained after allelic replacement. Expected sizes: WT, ≈ 3133 bp; 1457Δ*codY*, ≈ 2277 bp. **(B)** The transcription level of the gene *codY*, normalized to both *16S* and *gyrB*, in 24 h planktonic cultures. The results are represented as the mean + SEM of at least three independent experiments. Statistical significance was analysed by one-way ANOVA, using Tukey's multiple comparisons tests. * *p<0.05;* WT – Wild-type; 1457Δ*codY* – *codY* deletion mutant; 1457Δ*codY::codY* – complemented strain.

### 4.3.2. The deletion of the gene *codY* seems to influence *S. epidermidis* growth rate

Several studies performed in other bacterial species, namely *S. aureus* and *B. subtilis*, have suggested that CodY plays a role during the transition from the exponential to stationary growth phase [18–20]. Furthermore, CodY has been linked with nutrients limitations and its activation seem to be dependent on the levels of BCAAs availability [18]. As such, considering these reports, the growth profile of the constructs in a complex medium (TSB), in a CDM and a CDM lacking all of the BCAAs ($CDM^{-ILV}$) was evaluated over time (Fig. 4.3).

When growing in a complex medium, the absence of the target gene seems to negatively influence the bacterium growth profile over time, showing a consistently lower optical density when compared with the WT strain (Fig. 4.3A), although the difference was not statistically significant.

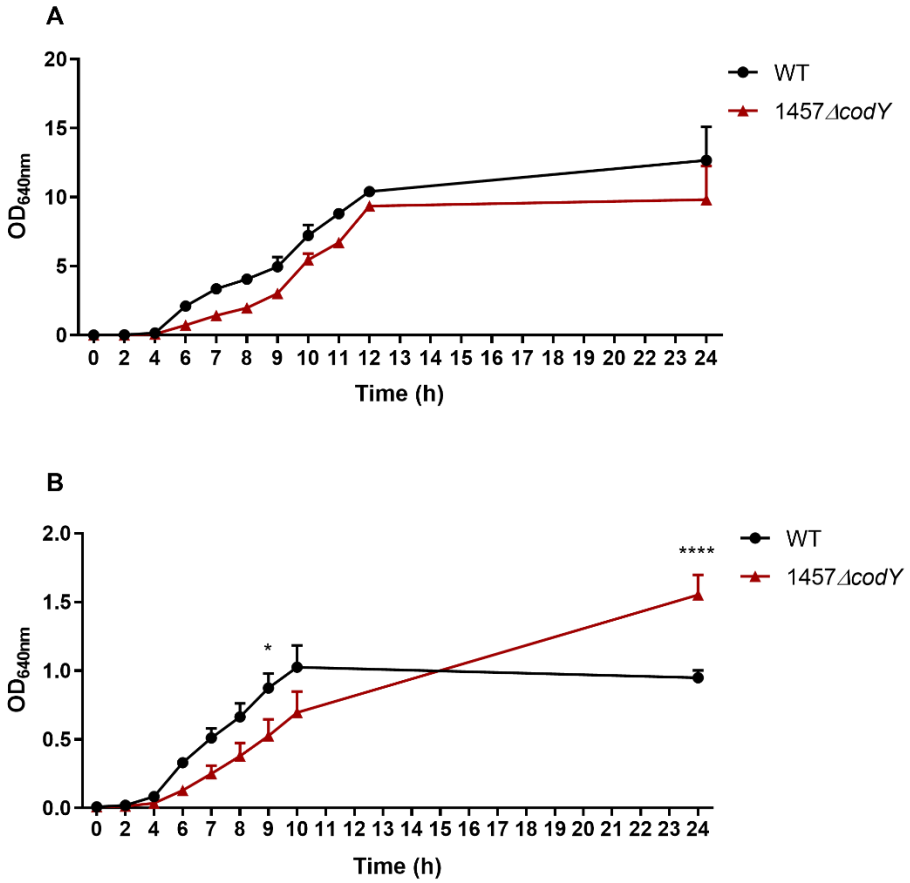


**Figure 4.3** Growth curve of *S. epidermidis* 1457 strains evaluated by $OD_{640nm}$. Strains were grown planktonically for 24 h in TSB **(A)** and CDM **(B)**. The data are represented as the mean +SEM of two **(A)** or four **(B)** independent experiments. Statistical significance was analysed by one-way ANOVA, using Šídák's multiple comparisons tests. * *p<0.05;* **** *p<0.0001.* WT – Wild-type; 1457*ΔcodY* – *codY* deletion mutant.

In the case of CDM, the growth level for both WT and mutant strains was markedly lower, though, as shown in Figure 4.3B, in the first 10 h, a similar profile was observed in both TSB and CDM. However, when looking at the endpoint of the experiment (24 h), it is possible to observe that the mutant strain was able to reach a significantly higher OD than the WT strain. In the case of $CDM^{-ILV}$, none of the strains was able to grow. Hence, to pinpoint which amino acid was essential for *S. epidermidis* growth, we evaluated the growth ability of all strains in the absence of each amino acid (Fig. 4.4).

Interestingly, we observed that *S. epidermidis* cannot grow in the absence of valine, justifying the growth arrest observed in $CDM^{-ILV}$. By evaluating the bacterium growth profile in the absence of the other BCAAs, we observed that 1457*ΔcodY* cultures presented a higher $OD_{640nm}$ (Fig. 4.4A), which is in accordance with the trend shown before in Figure 4.3B.

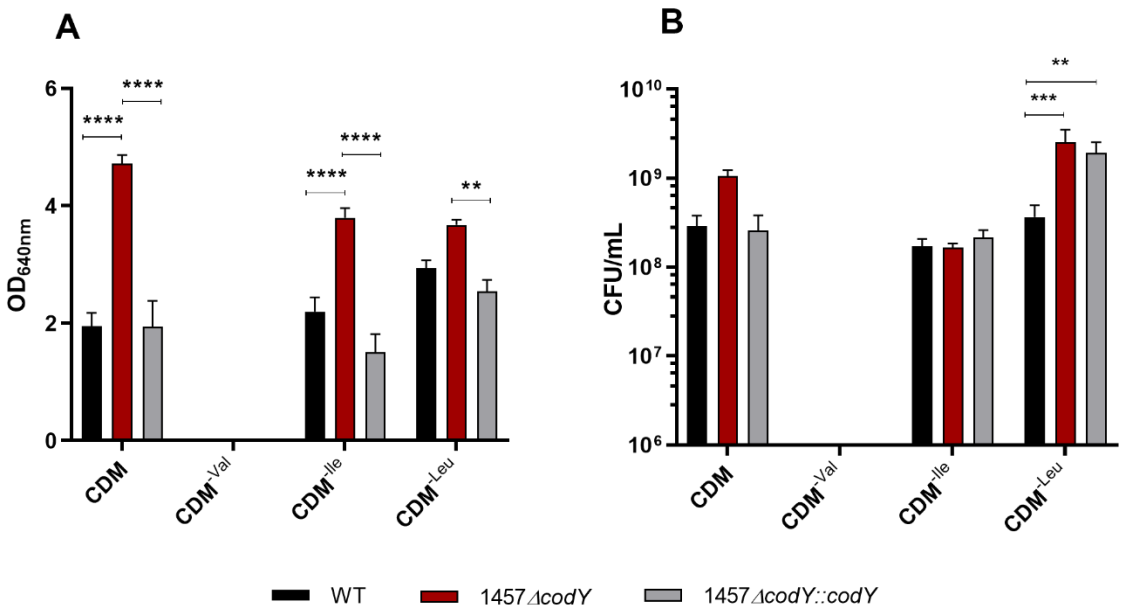


**Figure 4.4** Analysis of *S. epidermidis* planktonic cultures grown for 24 h in CDM and CDM without valine ($CDM^{-Val}$), isoleucine ($CDM^{-Ile}$) or leucine ($CDM^{-Leu}$). **(A)** Quantification of the total amount of cells was evaluated by $OD_{640nm}$ and **(B)** culturability by CFUs count. The results are represented as the mean + SEM of at least three independent experiments. Statistical analysis was performed with two-way ANOVA with Tukey's multiple comparisons tests. ** p< 0.05, ** p<0.01, *** p<0.001, **** p<0.0001.* WT – Wild-type; 1457*ΔcodY* – *codY* deletion mutant; 1457*ΔcodY::codY* – complemented strain.

In contrast, when analyzing the number of CFU/mL, the higher OD values obtained in the mutant did not always translate into higher CFU/mL (Fig. 4.4B). In the absence of isoleucine, despite the differences in OD values, all *S. epidermidis* strains showed a similar number of CFU/mL and in the absence of leucine, the culturability of complemented strain was slightly higher than 1457*ΔcodY* strain, despite presenting a reduced $OD_{640nm}$.

### 4.3.3. *codY*-mutant revealed a higher susceptibility to rifampicin

The antimicrobial activity of six antibiotics was evaluated against WT and 1457*ΔcodY* strains. Our results demonstrated no differences between the WT and mutant strains (Table 4.4).

Independently of the antibiotic mechanism of action, both strains were susceptible to most of the antibiotics tested, with the exception of ciprofloxacin, where strains revealed an intermediate susceptibility.

**Table 4.4.** Determination of the MIC values and interpretation of WT and 1457*ΔcodY* strains susceptibility to the six antibiotics tested.

| Strains | Antibiotic | | | Clinical Breakpoints[1] | | Susceptibility |
|---|---|---|---|---|---|---|
| | Mechanism of Action | | MICs range tested (mg/L) | S ≤ | R > | |
| WT 1457*ΔcodY* | Cell wall synthesis inhibitors | VAN | 2 – 4 | 4 | 4 | S |
| | | DCX | 0.0625 – 0.125 | 0.25 | 0.5 | S |
| | Nucleic acids synthesis inhibitors | CIP | 0.5 – 1 | 0.001 | 1 | I |
| | | RIF | 0.008 – 0.0325 | 0.06 | 0.06 | S |
| | Protein synthesis inhibitors | TET | 0.5 | 1 | 2 | S |
| | | LZD | 2 – 4 | 4 | 4 | S |

[1] Clinical breakpoints (mg/L) presented are available in EUCAST standards from antimicrobial susceptibility tests. S – Susceptible, I – Intermediate, R – resistant, VAN – vancomycin, DCX – dicloxacillin, CIP – ciprofloxacin, RIF – rifampicin, TET – tetracycline and LZD – Linezolid.

Additionally, the susceptibility of the *S. epidermidis* strains to the antibiotics under test was also evaluated over time at their PSC (Fig. 4.5), which is the maximum concentration of an antibiotic in the blood after a dose is given in a period before the next dose [21,22]. Overall, by adding the antibiotics to the medium, the number of CFU/mL was reduced considerably, except in the presence of protein synthesis inhibitors (linezolid and tetracycline), which seemed to inhibit bacterial growth, keeping the bacteria concentration at the same level at the beginning of the incubation (T0 h) (Fig. 4.5E). Importantly, statistically significant differences between the mutant and the WT or complemented strains, were only found with rifampicin (Fig. 4.5D). Nucleic acid synthesis inhibitors (ciprofloxacin and rifampicin) were more efficient in killing bacteria than protein synthesis inhibitors. Regarding cell wall synthesis inhibitors (vancomycin and dicloxacillin), the action of the antibiotics appeared to be only effective at the first hours (T2 h), since we obtained a similar number of CFU/mL in the later time points (Fig. 4.5A and B).

### 4.3.4. The lack of *codY* does not influence bacterial survival in human blood or plasma

Human blood is composed of several components that are involved in the clearance of invading microorganisms such as phagocytic cells, antimicrobial peptides and proteins from the complement system. Since CodY seems to regulate genes encoding molecules involved in the cell wall and membrane biogenesis [23], we wondered if the deletion of *codY* could render the bacterium more susceptible to soluble immune factors. The contribution of *codY* to bacterial survival in the host was inferred by exposing *S.*

*epidermidis* strains to human blood and plasma. A control with TSB supplemented with heparin ($TSB_{heparin}$) was performed to evaluate the possible side effects of the anticoagulant used during blood collection.

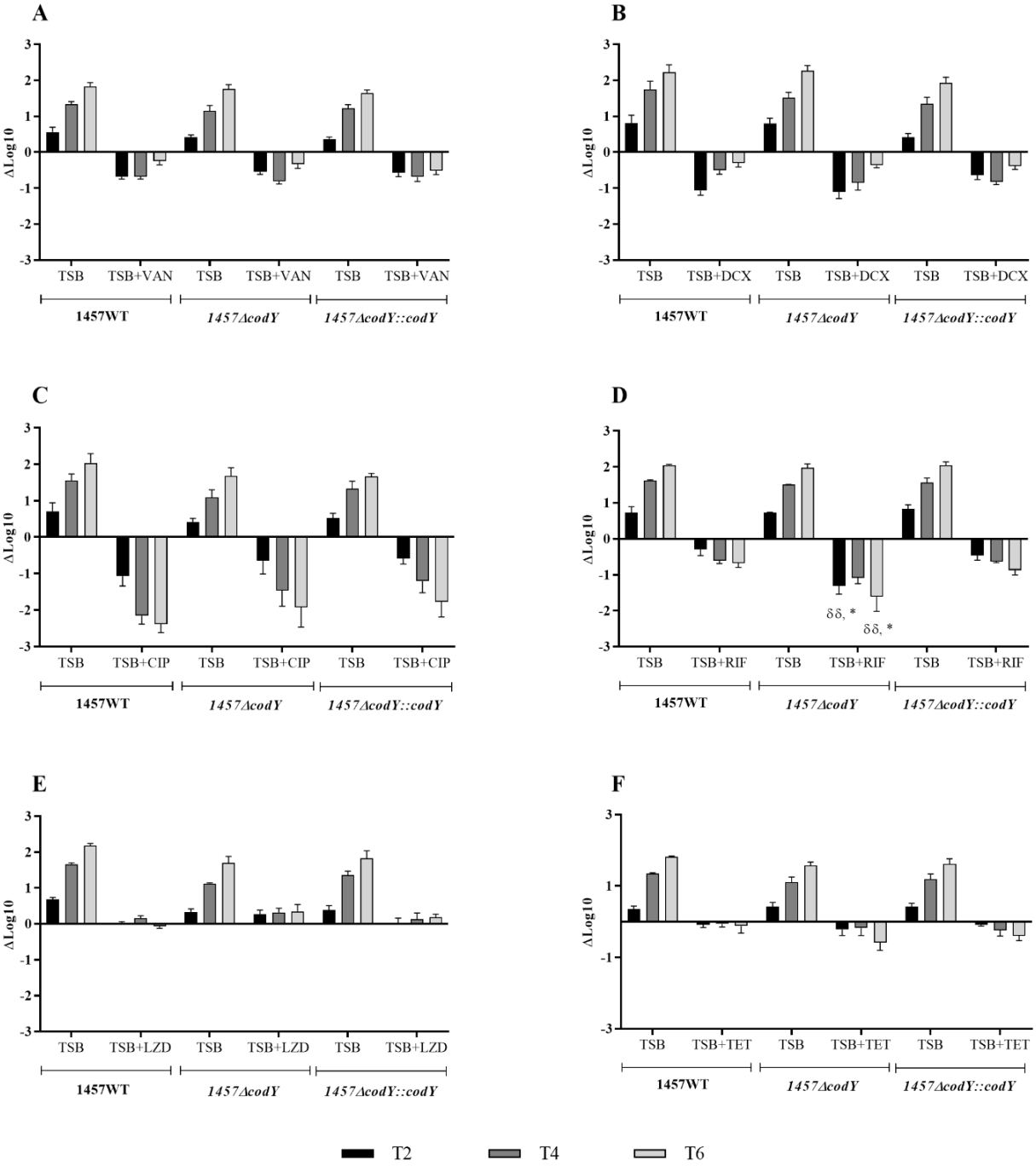


**Figure 4.5** Variation of $log_{10}$ CFU/mL reduction of *S. epidermidis* strains upon 2, 4 and 6 h of incubation with **(A)** Vancomycin (VAN, 40 mg/L), **(B)** Dicloxacillin (DCX, 59 mg/L), **(C)** Ciprofloxacin (CIP, 45 mg/L), **(D)** Rifampicin (RIF, 10 mg/L), **(E)** Linezolid (LZD, 18 mg/L) and **(F)** Tetracycline (TET, 16 mg/L), at the respective PSC. Data is represented by the mean + SEM of at least three independent assays. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons tests. $^{\delta\delta}$ *p<0.01*, 1457*ΔcodY vs.* WT. * *p<0.05*, 1457*ΔcodY vs.* 1457*ΔcodY::codY*. WT – Wild-type; 1457*ΔcodY* – *codY* deletion mutant; 1457*ΔcodY::codY* – complemented strain.

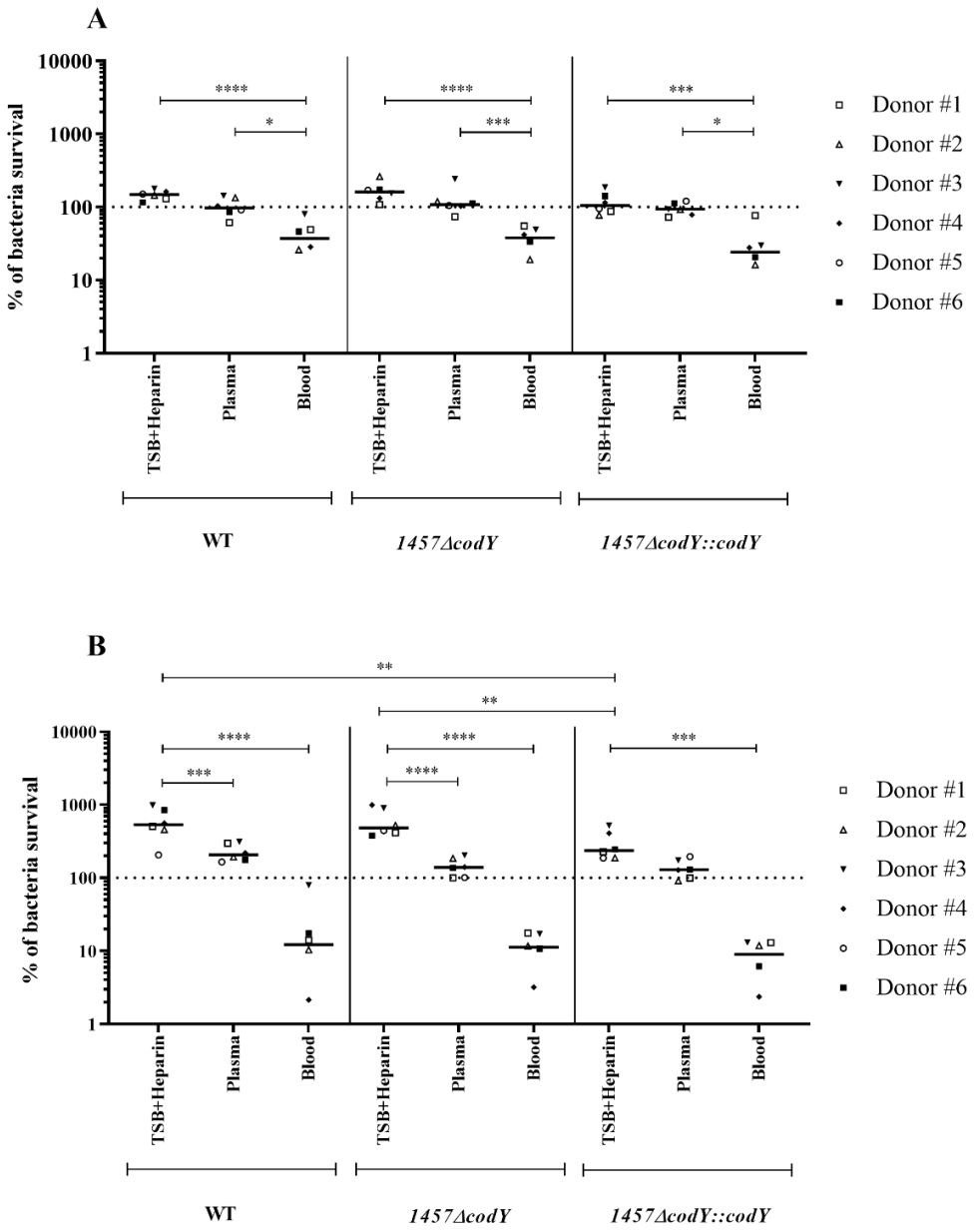


**Figure 4.6** *S. epidermidis* strains survival after **(A)** 2 and **(B)** 4 h of incubation with $TSB_{heparin}$, human plasma or blood. Blood was collected from three female (open symbols) and three male donors (full symbols). Data are presented as percentage of survival in relation to T0 h and the horizontal solid lines represent the median of six independent assays. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons tests. ** p< 0.05, ** p<0.01, *** p<0.001, **** p<0.0001.* WT – Wild-type; 1457*ΔcodY* – *codY* deletion mutant; 1457*ΔcodY::codY* – complemented strain.

As shown in Fig. 4.6, the deletion of the gene *codY* does not seem to provide any advantage for *S. epidermidis* to survive in human blood or plasma. Despite some growth inhibition, all strains were able to survive in the presence of human plasma and statistical differences with the control $TSB_{heparin}$ were only

found after 4 h of incubation. On the other hand, the incubation with human blood significantly reduced the survival of all strains in comparison to the control $TSB_{heparin}$, at both incubation points.

### 4.3.5. CodY global regulator may have a role during biofilm formation

In other bacterial species, CodY seems to regulate the expression of genes that codify proteins that are involved in biofilm accumulation and maturation. Yet, its role seems to be species-dependent, since in certain studies the deletion of the gene *codY* appears to reduce biofilm biomass [24,25], while in others it increases it [26,27]. As such, we evaluated the biofilm formation ability of 1457*ΔcodY* (Fig. 4.7).

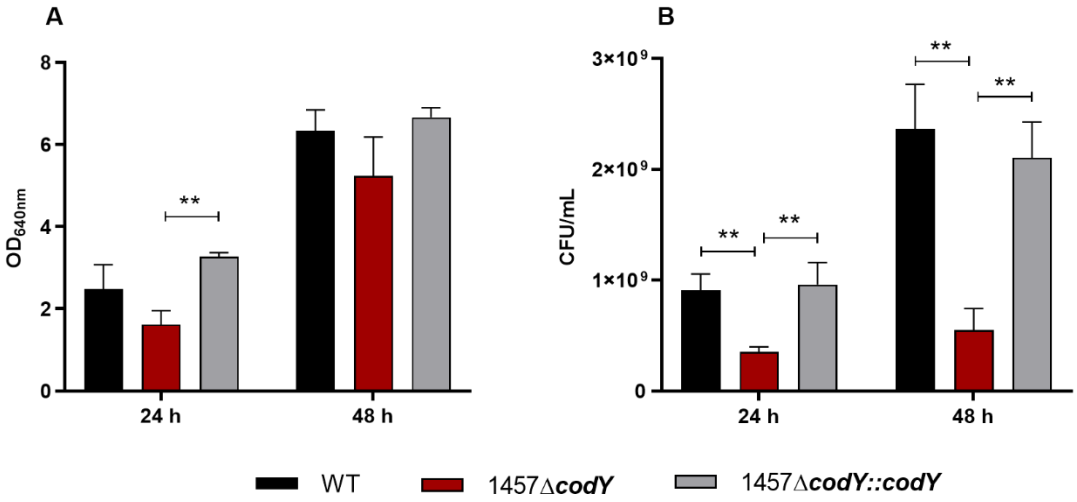


**Figure 4.7** *S. epidermidis* biofilms grown in $TSB_{0.4\%G}$ for up to 48 h. **(A)** Total biofilm biomass was evaluated by $OD_{640nm}$ and **(B)** culturability was assessed by CFUs. The results are represented as the mean + SEM of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons tests. *** p<0.01*. WT – Wild-type; 1457*ΔcodY* – *codY* deletion mutant; 1457*ΔcodY::codY* – complemented strain.

The 24 h old biofilms formed by 1457*ΔcodY* presented lower biomass, as well as a significantly less number of CFU/mL when compared with both the WT and the complemented strains. However, after 48 h of growth, biofilm biomass among the strains levelled (Fig. 4.7A). Interestingly, 1457*ΔcodY* biofilms maintained a significantly lower number of CFU/mL, in contrast to the increase observed for both the WT and complemented strains (Fig. 4.7B). The biofilm formation ability of all the *S. epidermidis* strains was also evaluated in CDM, for 24 h, and similar results were obtained in terms of biomass production and the number of CFU/mL, as can be seen in Fig. 4.8.

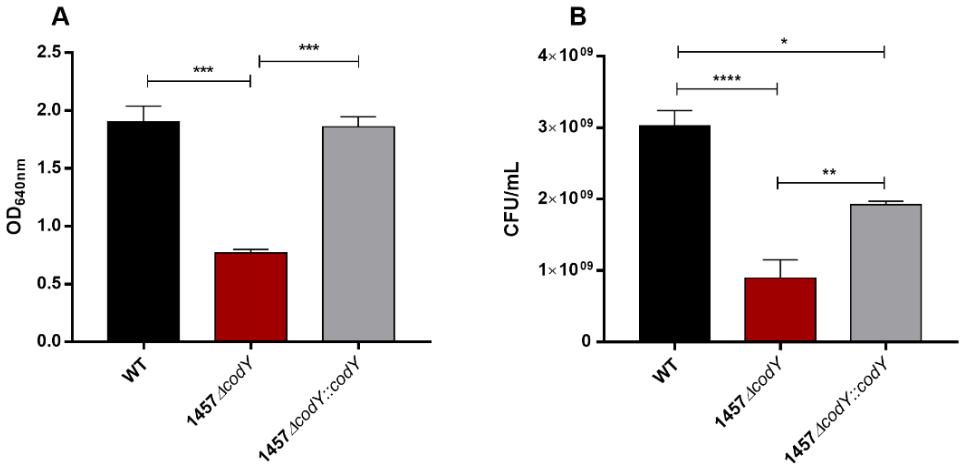


**Figure 4.8** *S. epidermidis* biofilms grown in CDM for 24 h. **(A)** Total biofilm biomass evaluated by $OD_{640nm}$ and **(B)** culturability assessed by CFUs count. The results are represented as the mean + SEM of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons tests. * *p<0.05,* ** *p<0.01,* *** *p<0.001,* **** *p<0.0001*. WT – Wild-type; 1457*ΔcodY* – *codY* deletion mutant; 1457*ΔcodY::codY* – complemented strain.

Considering the significant reduction in the biomass and number of CFU/mL observed in the *codY* mutant, we further explored the biofilm structure and cell viability through CLSM (Fig. 4.9). To do so, two sets of stains were used: (i) a combination of DAPI/WGA for structural analysis and (ii) SYTO9/PI (LIVE/DEAD staining) to evaluate cells viability and to help to substantiate CFU results.

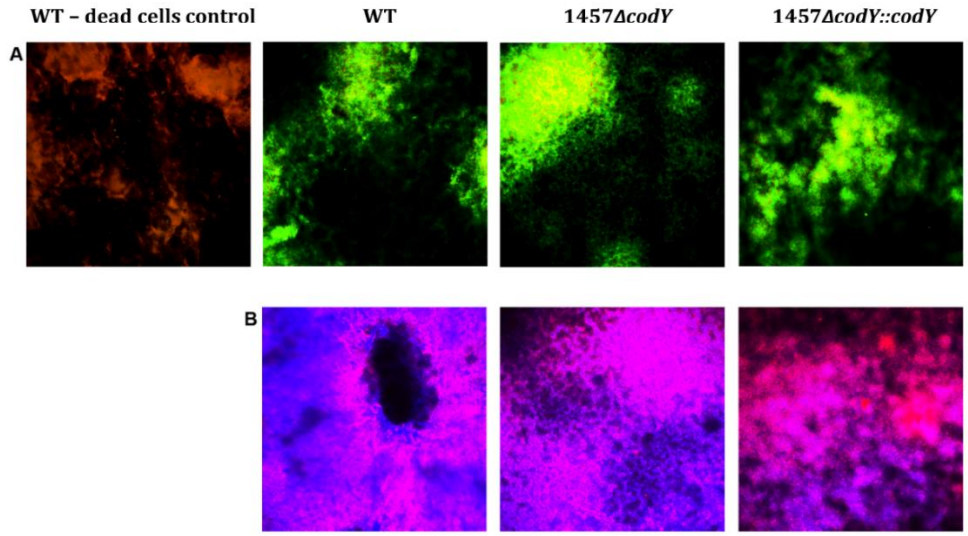


**Figure 4.9** Representative CLSM images of 24 h old biofilms formed by *S. epidermidis* strains grown in $TSB_{0.4\%G}$. **(A)** Analysis of the viability of the *S. epidermidis* cells using LIVE/DEAD staining; viable cells are shown in green (SYTO9) and dead cells in red (PI). **(B)** Analysis of polysaccharides distribution; cells are shown in blue (DAPI) and PNAG in red (WGA). WT – Wild-type; 1457*ΔcodY* – *codY* deletion mutant; 1457*ΔcodY::codY* – complemented strain.

As shown in Figure 4.9A, some damaged cells (represented in yellow) were observed in the strain 1457*ΔcodY*, with a seemingly minor representation in 1457*ΔcodY::codY* and WT strain. However, using this qualitative analysis we were not able to detect marked differences among the strains. In addition, no major structural differences were observed, suggesting that the deletion of the gene *codY* does not seem to have a noticeable impact on the biofilm structure or in the formation of cell clusters, at least in 24 h old biofilms.

### 4.3.6. *codY*-mutant may have a role in the emergence of VBNC cells

Considering the hypothesis of the role of the gene *codY* in VBNC cell formation presented in chapter 3, we decided to evaluate the level of VBNC cells in biofilms formed by the *codY*-mutant (Fig. 4.10).

The supplementation of the medium with either $TSB_{1\%G}$ or $TSB_{1\%G+MgCl2}$, did not seem to influence the total biofilm biomass of the mutant strain, presenting a ratio value near 100%, as the WT and complemented strains (Fig. 4.10A). In contrast, analysing the number of CFU/mL, we found a higher IND/PRE ratio, i.e., less number of VBNC cells in the biofilms formed by the 1457*ΔcodY* strain (Fig. 4.10B). Additionally, both ratios of the biofilm biomass and the number CFU/mL of the WT strain are in accordance with the ratios presented in table 3.2, chapter 3. Taking into account these results, *codY* seems to be involved in the emergence of VBNC cells in biofilms formed by *S. epidermidis* strain 1457.

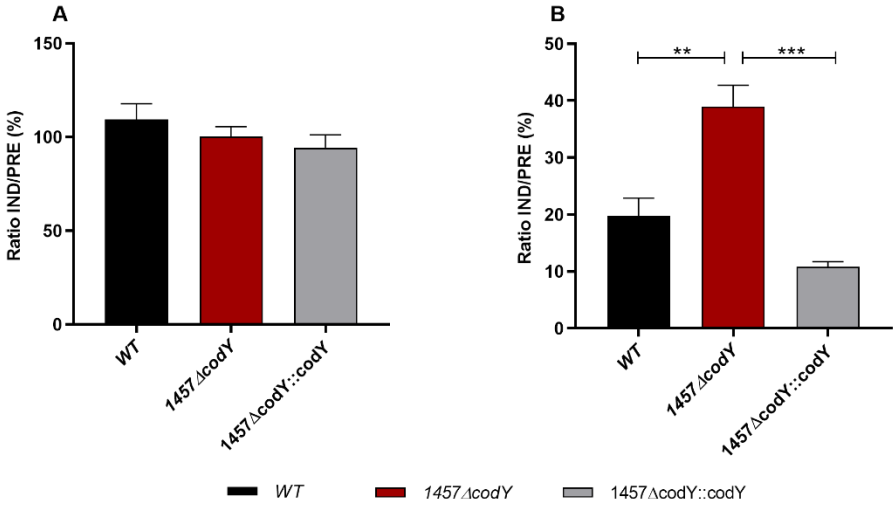


**Figure 4.10** *S. epidermidis* biofilms grown under VBNC-inducing conditions for 48 h. Ratio induced/prevented (IND/PREV) regarding **(A)** total biomass as evaluated by $OD_{640nm}$ and **(B)** culturability assessed by CFUs count. The results represent the mean + SEM of at least four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons tests. ** *p<0.01,* *** *p<0.001*. WT – Wild-type; 1457*ΔcodY* – *codY* deletion mutant; 1457*ΔcodY::codY* – complemented strain.

## 4.4. Discussion

The construction of *S. epidermidis* mutants is known to be a time-consuming process due to the low efficiency of the transformation process [11]. In addition to the known complications associated with *S. epidermidis* transformation, we encountered difficulties related to the knockout plasmid suspension after extraction from the *S. epidermidis* clones. After several failed attempts, we have changed the electro-transformation procedure, specifically, the width/size of the gap of the cuvettes used. In the protocol optimized by Monk et al., they used 1 mm gap cuvettes [11]. However, we thought that by increasing the gap size of the cuvettes, from 1 to 2 mm, we would avoid possible cell viability issues and stresses related to the high field strength obtained in 1 mm gap [28], thereby reducing the potential plasmid damage or stress and improving plasmid suspension. Using 2 mm cuvettes, we were able to suspend the plasmid pellets recovered and confirm the selection of the right clone.

After the successful construction of the *codY*-mutant and complemented strains, we began their characterization. The first observed difference between strains was the smaller size of the colonies formed by 1457*ΔcodY*, which was also observed in other *codY* mutant bacteria, namely *S. aureus* and *Streptococcus spp.* [26,29]. Since CodY seems to be involved in the regulation of the transcription of genes encoding proteins participating in the metabolism and biosynthesis of cell envelopes [23,30], we hypothesized that the lack of *codY* gene may impact the formation of the cell membrane and, somehow, delay the bacteria's growth, explaining the smaller size of the colonies formed.

In addition, we found a reduction in the 1457*ΔcodY* strain capacity to grow in TSB, similar to what was observed in other bacteria [19,26,31]. Since it is known that CodY hinders the transcription of genes associated with nutrient limitation, we further explored the growth ability of the mutant strain in a CDM. Following the same trend observed with TSB, the 1457*ΔcodY* strain showed a lower growth capacity in the first 10 to 12 h (Fig. 4.3B), which was also demonstrated in *S. aureus* and *Streptococcus spp* lacking CodY [32,33]. Interestingly, from 12 to 24 h, the mutant strain continued to grow while the WT presented a stationary profile. To the best of our knowledge, no studies have investigated the growth profile of *codY*-mutants after 12 hours of growth and, thus, we cannot correlate the results obtained with the literature. Taking into consideration the involvement of *codY* in the transition from an exponential to a stationary phase, we hypothesized that the lack of *codY* gene enables the bacteria to actively grow, and restrains the transition into the stationary phase. Of note, our CDM contains a high concentration of glucose (1%), which may have induced the entrance of bacteria into a VBNC state, resulting in fewer numbers of CFU/mL. Therefore, an additional analysis of the number of total viable cells would help to understand if the reduced

number of CFU/mL observed in CDM in comparison with TSB media is related to the emergence of VBNC cells or the different components and concentrations of our CDM. Considering that bacteria may require different amino acids to survive and to promote their virulence factors, depending on their genetic makeup and the availability of nutrients in the surrounding environment [34], we decided to analyse which of the BCAAs were essential for *S. epidermidis* growth. According to our results, valine was indispensable for *S. epidermidis* growth. Considering that it was shown that valine was also required for the growth of *S. aureus* Newman and UAMS-1, it is probable that this amino acid is essential for the growth of bacteria of the genus *Staphylococcus* [32]. Interestingly, some studies reported that CodY seems to regulate the amino acids required for *S. aureus* USA300 and HG001 strains growth [35,36] and that isoleucine is a major signal for CodY regulation in *S. aureus*. Nevertheless, accordingly to our results, the absence of isoleucine or leucine did not impair *S. epidermidis* growth, suggesting that these amino acids may not be directly associated with *codY* regulation in *S. epidermidis* strain 1457. Taking into account the results obtained, and the role of CodY in coping with stressful conditions such as nutrient limitation [35], it would be interesting to evaluate the growth profile of all strains in a minimal medium. Perhaps the influence of the deletion of the gene *codY* in *S. epidermidis* growth could be more significant. Regarding antibiotics susceptibility, the deletion of the gene *codY* rendered bacteria more susceptible to rifampicin, a nucleic acid synthesis inhibitor. Rifampicin is active against slow-growing staphylococcal species involved in biofilm-associated infections [37,38]. Therefore, the increased susceptibility to rifampicin may be associated with the changes in the bacteria metabolism or cell membrane biosynthesis due to the lack of CodY regulation.

Concerning the survival of *S. epidermidis* strains in both whole human blood and plasma, we concluded that the deletion of *codY* gene did not affect its survival capacity. Moreover, these results have shown that the potential alterations in the cell membrane due to the lack of the gene *codY* did not render the bacteria more susceptible to antimicrobial peptides or complement proteins present in human plasma.

Since CodY regulates bacterial growth under stressful conditions and taking into consideration that biofilm formation is considered a bacterial response to stressful conditions [39], we investigated the biofilm formation ability of the constructed strains. Similarly to what was observed in other species [24,25], our data revealed a significant reduction in the capacity of the mutant to form biofilms. Lastly, we have shown, for the first time, that the gene *codY* seems to regulate the induction of bacteria into a VBNC state, as *codY* absence has resulted in a higher number of culturable cells.

Overall, our results suggest that the gene *codY* may be involved in *S. epidermidis* 1457 growth and biofilm formation, as well as in the formation of VBNC cells. However, the mechanisms mediating these

alterations remain unknown. As such, in the future, efforts should be made to identify the genes regulated by CodY so that the impact on growth, biofilm formation ability and formation of VBNC cells could be better elucidated and strategies targeting these processes delineated.

## 4.5. References


1. Falkow, S. Molecular Koch's postulates applied to microbial pathogenicity. *Rev. Infect. Dis.* **10**, 274–276 (1988).
2. Monk, I. R. Genetic manipulation of Staphylococci—breaking through the barrier. *Front. Cell. Infect. Microbiol.* **2**, 1–9 (2012).
3. Costa, S. K., Donegan, N. P., Corvaglia, A. R., François, P. & Cheung, A. L. Bypassing the restriction system to improve transformation of Staphylococcus epidermidis. *J. Bacteriol.* **199**, 1–14 (2017).
4. Vasu, K. & Nagaraja, V. Diverse Functions of Restriction-Modification Systems in Addition to Cellular Defense. *Microbiol. Mol. Biol. Rev.* **77**, 53–72 (2013).
5. Cogen, A. L. *et al. Staphylococcus epidermidis* antimicrobial δ-toxin (phenol-soluble modulin-γ) cooperates with host antimicrobial peptides to kill group A Streptococcus. *PLoS One* **5**, 1–7 (2010).
6. Wang, R. *et al. Staphylococcus epidermidis* surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. *J. Clin. Invest.* **121**, 238–248 (2011).
7. Galac, M. R. *et al.* Complete Genome Sequence of Staphylococcus epidermidis 1457. *Am. Soc. Microbiol.* **5**, 15–17 (2017).
8. Rohde, H. *et al.* Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. *Mol. Microbiol.* **55**, 1883–1895 (2005).
9. Schommer, N. N. *et al. Staphylococcus epidermidis* uses distinct mechanisms of biofilm formation to interfere with phagocytosis and activation of mouse macrophage-like cells 774A.1. *Infect. Immun.* **79**, 2267–2276 (2011).
10. Hussain, M., Hastings, J. G. M. & White, P. J. A chemically defined medium for slime production by coagulase-negative staphylococci. *J. Med. Microbiol.* **34**, 143–147 (1991).
11. Monk, I. R., Shah, I. M. & Xu, M. Transforming the Untransformable: Application of Direct Transformation To Manipulate Genetically Staphylococcus aureus and Staphylococcus epidermidis. *MBio* **3**, 1–11 (2012).
12. Brückner, R. A series of shuttle vectors for Bacillus subtilis and Escherichia coli. *Gene* **122**, 187–192 (1992).
13. Mack, D., Siemssen, N. & Laufs, R. Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: Evidence for functional relation to intercellular adhesion. *Infect. Immun.* **60**, 2048–2057 (1992).
14. França, A. *et al.* Alterations in the *Staphylococcus epidermidis* biofilm transcriptome following interaction with whole human blood. *Pathog. Dis.* **70**, 444–448 (2014).
15. França, A. & Cerca, N. Plasma is the main regulator of *Staphylococcus epidermidis* biofilms virulence genes transcription in human blood. *Pathog. Dis.* **74**, 1–5 (2016).
16. Vinué, L. & Hooper, D. C. Rsp activates expression of the Cnt system in *Staphylococcus aureus*. *BMC Microbiol.* **20**, 1–8 (2020).

17. Yang, X.-Y., Li, N., Xu, J.-Y., Sun, X. & He, Q.-Y. Lipoprotein SPD_1609 of Streptococcus pneumoniae Promotes Adherence and Invasion to Epithelial Cells Contributing to Bacterial Virulence. *Front. Microbiol.* **10**, 1–11 (2019).

18. Majerczyk, C. D. *et al.* Direct targets of CodY in *Staphylococcus aureus*. *J. Bacteriol.* **192**, 2861–2877 (2010).

19. Waters, N. R. *et al.* A spectrum of CodY activities drives metabolic reorganization and virulence gene expression in Staphylococcus aureus. *Mol. Microbiol.* **101**, (2016).

20. Joseph, P., Ratnayake-Lecamwasam, M. & Sonenshein, A. L. A region of *Bacillus subtilis* CodY protein required for interaction with DNA. *J. Bacteriol.* **187**, 4127–4139 (2005).

21. Urso, R., Blardi, P. & Giorgi, G. A short introduction to pharmacokinetics. *Eur. Rev. Med. Pharmacol. Sci.* **6**, 33–44 (2002).

22. Fleming, W. W. Mechanism of Drug Action. in *Modern Pharmacology with Clinical Applications* (eds. Craig, C. R. & Stitzel, R. E.) vol. 58 337–337 (Lippincott Williams and Wilkins, 2004).

23. Pellegrini, A. *et al.* CodY Is a Global Transcriptional Regulator Required for Virulence in Group B *Streptococcus*. *Front. Microbiol.* **13**, 1–15 (2022).

24. McDowell, E. J., Callegari, E. A., Malke, H. & Chaussee, M. S. CodY-mediated regulation of *Streptococcus pyogenes* exoproteins. *BMC Microbiol.* **12**, 1–8 (2012).

25. Hsueh, Y. H., Somers, E. B. & Wong, A. C. L. Characterization of the *codY* gene and its influence on biofilm formation in *Bacillus cereus*. *Arch. Microbiol.* **189**, 557–568 (2008).

26. Majerczyk, C. D. *et al. Staphylococcus aureus* CodY negatively regulates virulence gene expression. *J. Bacteriol.* **190**, 2257–2265 (2008).

27. Rom, J. S. *et al.* Impact of *Staphylococcus aureus* regulatory mutations that modulate biofilm formation in the USA300 strain LAC on virulence in a murine bacteremia model. *Virulence* **8**, 1776–1790 (2017).

28. Campillo-Davo, D. *et al.* The Ins and Outs of Messenger RNA Electroporation for Physical Gene Delivery in Immune Cell-Based Therapy. *Pharmaceuticals* **13**, 1–23 (2021).

29. Geng, J. *et al.* Impact of growth pH and glucose concentrations on the CodY regulatory network in Streptococcus salivarius. *BMC Genomics* **19**, 1–16 (2018).

30. Geng, J. *et al.* Identification of a Putative CodY Regulon in the Gram-Negative *Phylum Synergistetes*. *Int. J. Mol. Sci.* **23**, (2022).

31. Roux, A., Todd, D. A., Velázquez, J. V., Cech, N. B. & Sonenshein, A. L. Cody-Mediated regulation of the *Staphylococcus aureus* Agr System integrates nutritional and population density signals. *J. Bacteriol.* **196**, 1184–1196 (2014).

32. Pohl, K. *et al.* CodY in *Staphylococcus aureus*: a Regulatory Link between Metabolism and Virulence Gene Expression. *J. Bacteriol.* **191**, 2953–2963 (2009).

33. Feng, L. *et al.* The CodY regulator is essential for virulence in Streptococcus suis serotype 2. *Sci. Reports 2016 61* **6**, 1–15 (2016).

34. Liu, Y. K., Kuo, H. C., Lai, C. H. & Chou, C. C. Single amino acid utilization for bacterial categorization. *Sci. Reports 2020 101* **10**, 1–12 (2020).

35. Audretsch, C., Gratani, F., Wolz, C. & Dandekar, T. Modeling of stringent-response reflects nutrient stress induced growth impairment and essential amino acids in different *Staphylococcus aureus* mutants. *Sci. Rep.* **11**, 1–15 (2021).

36. Kaiser, J. C. *et al.* Repression of branched-chain amino acid synthesis in Staphylococcus aureus is mediated by isoleucine via CodY, and by a leucine-rich attenuator peptide. *PLOS Genet.* **14**, e1007159 (2018).

37. Zimmerli, W., Widmer, A. F., Blatter, M., Frei, R. & Ochsner, P. E. Role of rifampin for treatment of orthopedic implant-related staphylococcal infections: a randomized controlled trial. Foreign-Body Infection (FBI) Study Group. *JAMA* **279**, 1537–1541 (1998).

38. Wi, Y. M. *et al.* Rifampicin resistance in *Staphylococcus epidermidis*: molecular characterisation and fitness cost of rpoB mutations. *Int. J. Antimicrob. Agents* **51**, 670–677 (2018).

39. de la Fuente-Núñez, C., Reffuveille, F., Fernández, L. & Hancock, R. E. Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. *Curr. Opin. Microbiol.* 580–589 (2013) doi:10.1016/j.mib.2013.06.013.

# CHAPTER 5 – Interaction between *S. epidermidis* strains and human macrophages

**Summary**

CodY has formerly been shown to regulate several virulence-associated genes in bacteria and may indirectly mediate the shift between commensal and pathogenic lifestyles. Indeed, in pathogenic bacteria such as *Listeria monocytogenes* and *Streptococcus suis*, *codY*-mutant strains displayed attenuated virulence *in vitro* when used to challenge macrophages. Herein, THP-1 cell line-derived macrophages were infected with the *S. epidermidis* 1457 WT and the constructed strains to investigate whether the *codY* gene can impact the activation of innate immune cells. To do so, the production of both pro- and anti-inflammatory cytokines by the challenged THP-1-derived macrophages was evaluated, as well as the bacterial survival within these phagocytes. Overall, our results show that THP-1-derived macrophages responded with higher cytokine production to *S. epidermidis* 1457 bacteria lacking the gene *codY.*

## 5.1. Brief introduction

*S. epidermidis* host invasion commonly stimulates a strong inflammatory response, activating and recruiting phagocytes such as neutrophils and macrophages to the infection site [1]. However, *S. epidermidis* cells can escape the host immune response due to their ability to form biofilms and produce immune evasion molecules, which impede killing by the phagocytes [2]. Considering that this bacterium lives as a commensal but can also switch to an opportunistic pathogen [3], the identification of *S. epidermidis* features triggering the activation of the host's innate immune system is of high importance.

Insights on the mechanism used by *S. epidermidis* to evade the host immune response are still scarce, and little is known regarding the contribution of CodY in regulating the production of evasion molecules. Until now, the role of CodY as a regulator of virulence genes involved in the evasion of the host immune response has been only addressed in other pathogenic bacteria [4–6]. In *L. monocytogenes*, PrfA, a major activator of the bacteria's virulence, is activated upon the interaction with host immune cells, which, in turn, is directly activated by CodY. A mutation in the gene *codY* reduced *prfA* transcription and attenuated bacterial virulence both *in vitro* (cultured macrophages) and *in vivo* (mouse model) [6]. In *S. aureus*, CodY was shown to repress the levels of expression of genes codifying proteins mediating the host-pathogen interaction, disturbing the immune response and further reverting the repression of enzymes that promote the death of host immune cells [4].

Herein, human monocytic THP-1 cells were differentiated into macrophages, as they are considered professional phagocytic cells that have a fundamental role in the innate immune defense against bacterial infections [7]. Macrophages can be differently polarized in response to specific stimuli and signals, activating distinct functional properties, such as M1 and M2 phenotype macrophages [8]. These M1- and M2-macrophages mainly differ in the cytokine's secretion; while M1-macrophages present a more pro-inflammatory profile, with robust phagocytic and cytotoxic capacity, M2-macrophages present an anti-inflammatory profile, promoting cell proliferation and tissue repair [9].

In this chapter, the impact of abrogating *codY* expression in the interaction of *S. epidermidis* with macrophage cells was assessed, focusing on the levels of induced cytokine production and bacterial survival upon internalization by macrophages.

## 5.2. Material and methods

### 5.2.1. Strains and growth conditions

The *S. epidermidis* strains used herein are described in table 4.2 from section 4.2.3, chapter 4. The strains were grown in TSB at 37 °C, 120 rpm. For the complemented strain, $CM_{10}$ was added to the medium.

### 5.2.2. THP-1 cell culture and macrophage differentiation

The human monocytic cell line derived from peripheral blood from an acute monocytic leukemia patient (ATCC TIB-202), termed THP-1, was used in this study. THP-1 cells were cultured in RPMI 1640 medium (VWR) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Biowest, Riverside, MO, USA), 4 mM L-glutamine (Sigma-Aldrich), 10 mM HEPES solution (Sigma-Aldrich), 1% (v/v) penicillin/streptomycin (PEN/STREP, Sigma-Aldrich) and 0.05 mM β-mercaptoethanol (Sigma-Aldrich), as from now on designated as complete RPMI (cRPMI). Cultures were maintained in T-25 flasks at 37 °C in a humified atmosphere containing 5% of $CO_2$ and subcultured up to a maximum of 10 passages. For macrophage differentiation, THP-1 cells were seeded in flat bottom 96-well plates, at the concentration $1 \times 10^5$ cells per well, in cRPMI medium supplemented with 50 ng/mL of phorbol myristate acetate (PMA) and incubated at 37 °C, 5% of $CO_2$ for 24 h. Afterwards, the medium was removed and replaced with warm cRPMI without PMA and maintained in the same conditions for 24 h before infection.

### 5.2.3. Infection of macrophages with *S. epidermidis* strains

The differentiated macrophages were infected with live or fixed *S. epidermidis* cells for the analysis of cytokine production and phagocytosis. Briefly, 1 mL of bacterial culture grown overnight was collected and centrifuged at 16000 *×g*, for 10 mins at 4 °C. The pellet was washed twice with 1 mL of PBS and resuspended in 1 mL of the same solution. For bacteria fixation, after the washing step, cells were resuspended in 1 mL of methanol and incubated for 15 mins on ice. Then, bacterial suspensions were centrifugated, washed twice and resuspended in 1 mL of PBS.

The number of live and fixed cells was determined by flow cytometry (BD Accuri™ C6 Plus), using SYTO™ BC (1:10000, Thermo Fisher Scientific Inc.) and PI (5 µg/mL, Sigma-Aldrich) staining. Thereafter, for live bacteria infections, a multiplicity of infection (MOI) of 1 bacteria to 1 macrophage (MOI 1:1) and 1:3 were prepared. In the case of fixed cells, an MOI of 10:1 was used. Subsequently, bacterial strains adjusted to the selected MOI, in cRPMI without PEN/STREP ($cRPMI^{-PEN/STREP}$), were incubated with the differentiated

macrophages at 37 °C, 5% of $CO_2$ for 24 h. Additionally, cells with cRPMI$^{-PEN/STREP}$ only (with and without $CM_{10}$) and with lipopolysaccharides (LPS, from *E. coli* O127:B8), were used as negative and positive controls, respectively. After the incubation period, the plates were centrifuged at 300 ×*g* for 10 mins and the supernatants were collected for further analysis. For the measurement of cytokine secretion, supernatants were stored at - 80 °C until further use.

### 5.2.4. Assessment of *S. epidermidis* strains growth under macrophage infection conditions

The growth of the *S. epidermidis* WT, mutant and complemented strains under macrophage infection conditions were assessed. For the complemented strain, the medium was supplemented with $CM_{10}$. Overnight grown bacterial cultures of each strain were adjusted to $2.5\times10^6$ cells/mL and inoculated in cRPMI$^{-PEN/STREP}$ culture medium in flat bottom 96-well plates and incubated at 37 °C, 5% of $CO_2$ for 24 h. Then, the bacterial suspension was collected, washed twice with 1 mL of PBS and resuspend in 1 mL of the same solution. Serial dilutions in PBS were performed and plated on TSA or $TSA_{CM10}$ for CFU counts.

### 5.2.5. Cytotoxicity assays

The viability of the THP-1-derived macrophages upon incubation with *S. epidermidis* cells was assessed using the lactate dehydrogenase (LDH) cytotoxicity assay kit (Invitrogen), following the manufacturer's instructions. Briefly, 45 min before the end-point established for the *in vitro* infection of the THP-1 cells, 20 µL of a lysis buffer was added to the plate in a well without bacteria, to be used as a positive control. The plates were centrifuged at 300 ×*g*, for 10 min, and 25 µL of the supernatants were transferred into a new 96-well plate. Then, 25 µL of the reaction mixture was added to the supernatants and the plate was incubated for 30 mins at room temperature and protected from light. After the incubation period, 25 µL of the stop solution was added and the absorbance was measured at $OD_{490nm}$ and $OD_{680nm}$. To calculate cells viability, the $OD_{680nm}$ was subtracted from the $OD_{490nm}$ values, and then from the resultant values the following formula (Eq. (2)) was applied:

Eq. (2)
$$\frac{(Mean\ OD_{samples} - Mean\ OD_{negative\ control})}{(Mean\ OD_{positive\ control} - Mean\ OD_{negative\ control})} \times 100\%$$

### 5.2.6. Gentamicin protection assay

The internalization of bacteria by the THP-1-derived macrophages was assessed through a gentamicin protection assay. Infection of macrophage cells with *S. epidermidis* was performed as described in section

5.2.3, using an MOI of 10:1 and 5:1. Then, the plates were centrifuged at 300 ×*g* for 10 min, to allow syncronization and internalization of bacteria, and incubated at 37 °C, 5% of $CO_2$ for 30 min. After the internalization period, the supernatants were removed, the cells suspended in serum-free cRPMI supplemented with 50 μg/mL of gentamicin (Biowest) and let to incubate for 60 min to eliminate extracellular bacteria. Thereafter, culture supernatants were discarded and THP-1 cells were washed twice with 1 mL of PBS to remove any trace of gentamicin. Macrophages were lysed with PBS containing 0.1% of saponin (Sigma-Aldrich) and the lysates were diluted in PBS and plated on TSA or $TSA_{CM10}$ for CFU counts.

### 5.2.7. Quantification of cytokine production

The secretion of pro-inflammatory (IL-6, IL-8, Tumor Necrosis Factor-alpha, TNF-α) and anti-inflammatory (IL-10) cytokines by THP-1-derived macrophages upon infection with *S. epidermidis* cells was evaluated by enzyme-linked immunosorbent assay (ELISA), for each cytokine, using DuoSet® ELISA (R&D system, USA) kits. Cytokine levels in culture supernatants were measured and calculated by following the manufacturer's instructions.

### 5.2.8. Statistical analysis

Statistical significance was determined by using GraphPad Prism version 7. Statistical differences were determined using one-way ANOVA with either Tukey's or Kruskal-Wallis multiple comparisons test. A $p< 0.05$ was considered significant. At least three independent experiments were performed in all assays described above.

## 5.3. Results

### 5.3.1. *S. epidermidis* growth under macrophage infection conditions

To ensure that the results obtained after challenging the macrophages with the bacteria were due to the deletion of the gene *codY* and not because of the different growth conditions, the growth of all the *S. epidermidis* strains was evaluated under macrophages growth conditions (using $cRPMI^{-PEN/STREP}$), no agitation and 5% $CO_2$. As shown in Fig. 5.1, no significant alterations were found among the strains in the number of CFU recovered after 24 h of growth, indicating that the strains have a similar ability to grow under these conditions. As such, any differences detected on the functional assays performed were, thus, considered to be the result of the deletion of the gene *codY.*

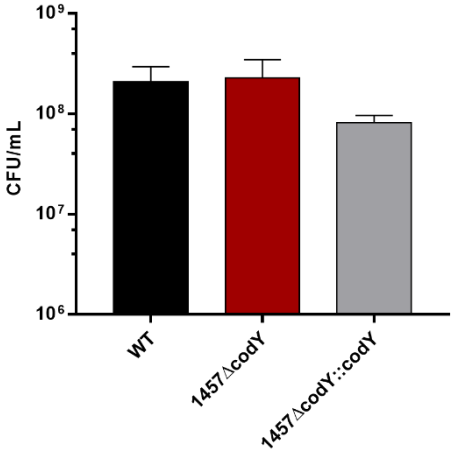


**Figure 5.1** *S. epidermidis* culturability assessed by CFUs count after 24 h of growth. The results represent the mean + SEM of at least three independent experiments. WT – Wild-type; 1457*ΔcodY* – *codY* deletion mutant; 1457*ΔcodY::codY* – complemented strain.

### 5.3.2. *codY*-mutant revealed a higher pro-inflammatory response

As referred to before, macrophages can polarize into different subtypes, namely M1- and M2-like macrophages. As such, considering the different roles of each macrophage type, we determined the levels of the pro-inflammatory cytokines IL-6, IL-8 and TNF-α (expressed by M1) and anti-inflammatory cytokine IL-10 (expressed by M2) in culture supernatants of both types of macrophages upon being challenged with *S. epidermidis* strains. At first, to ensure that the bacterial concentrations selected were not affecting the viability of the THP-1-derived macrophages, an analysis of LDH release was performed at MOI 1:1 and 1:3 (Fig. 5.2).

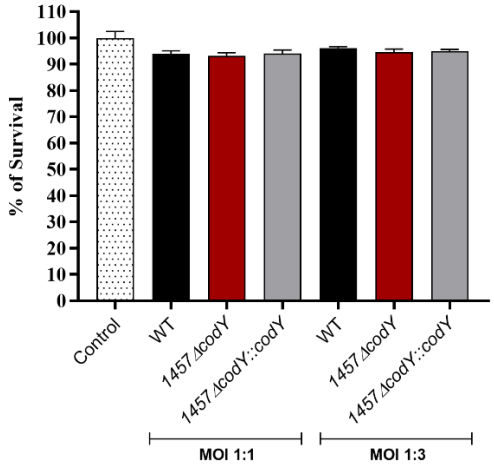


**Figure 5.2** Survival of macrophage cells as assessed by LDH release assay after infection with the indicated *S. epidermidis* strains at MOI 1:1 and MOI 1:3 (bacteria: macrophages). The control group corresponds to macrophages without bacterial infection. WT – Wild-type; 1457*ΔcodY* – *codY* deletion mutant; 1457*ΔcodY::codY* – complemented strain.

As expected, a higher quantity of dead macrophage cells was found in MOI 1:1 when compared to MOI 1:3. Nevertheless, the percentage of survival was between 93 to 96%, presenting no significant cell death under the two tested conditions. Hence, none of the concentrations of *S. epidermidis* strains was significantly harmful to the THP-1-derived macrophages.

By evaluating the levels of cytokines secreted into the culture supernatants, it is evident that strain 1457*ΔcodY* induced a higher production of cytokines in comparison to both WT and complemented strains (Fig. 5.3).

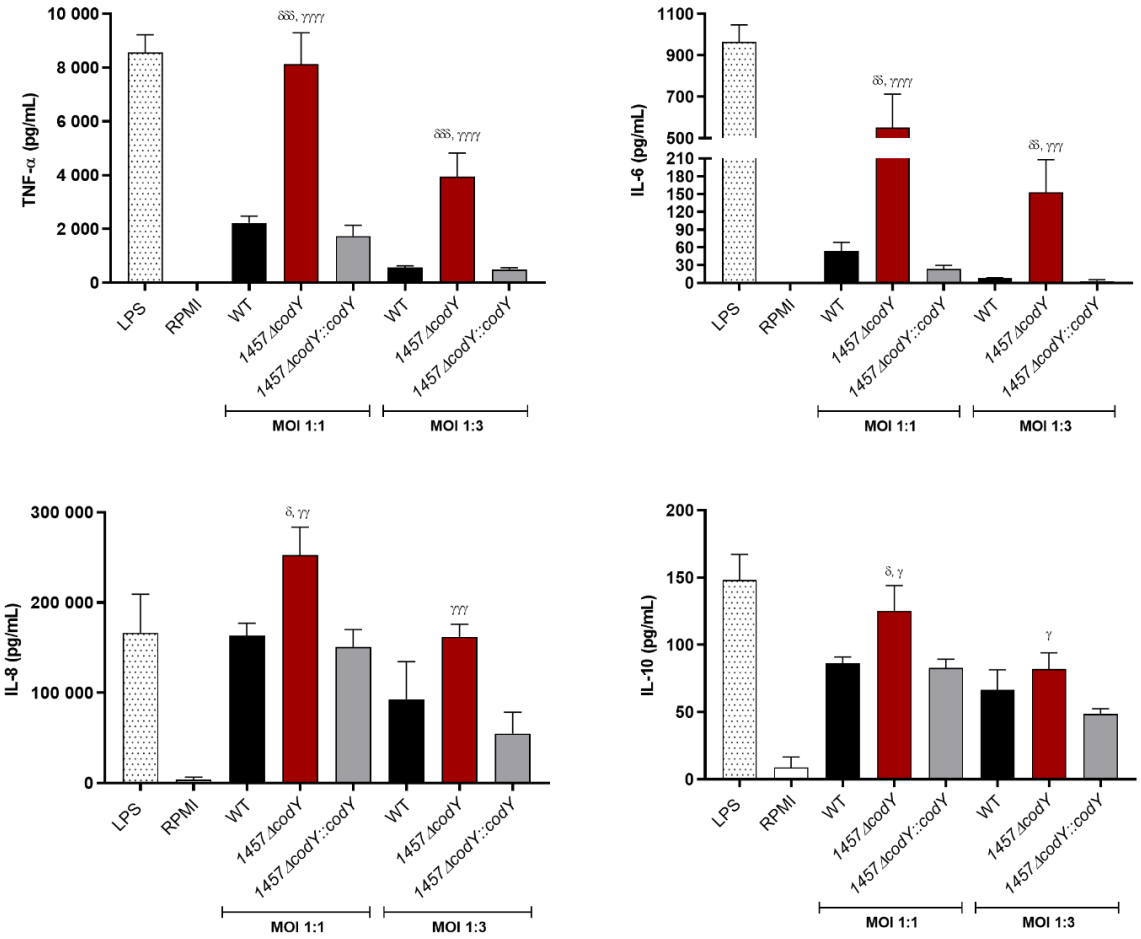


**Figure 5.3** Levels of pro- (IL-6, TNF-α and IL-8) and anti-inflammatory (IL-10) cytokines detected in the supernatant of THP-1 cells infected with *S. epidermidis* strains for 24 h. The results represent the mean + SEM of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Kruskal-Wallis multiple comparisons test. $^{\delta}$ 1457*ΔcodY* vs. WT ($^{\delta}$ *p<0.05,* $^{\delta\delta}$ *p<0.01,* $^{\delta\delta\delta}$ *p<0.001*). $^{\gamma}$ 1457*ΔcodY* vs. 1457*ΔcodY::codY* ($^{\gamma}$ *p<0.05,* $^{\gamma\gamma\gamma}$ *p<0.001,* $^{\gamma\gamma\gamma\gamma}$ *p<0.0001*). WT – Wild-type; 1457*ΔcodY* – *codY* deletion mutant; 1457*ΔcodY::codY* – complemented strain.

Additionally, a higher stimulation in comparison to the control was found in pro-inflammatory cytokines IL-8 and TNF-α, with the last reaching similar levels and IL-8 surpassing the levels found in the positive

control (LPS). Of note, in all cytokines studied, the higher the MOI tested, the higher the production of cytokines. In general, analyzing the production of cytokines upon infection with *S. epidermidis* strains, the pro-inflammatory cytokine IL-8 was the most abundantly produced cytokine, while the IL-6 was the one produced in lower amounts (except for the mutant strain).

To better understand the high cytokine production induced by the 1457*ΔcodY* strain, the stimulatory effect on THP-1-derived macrophages challenged with methanol-fixed *S. epidermidis* cells was also assessed. Considering that no cell division occurs after bacteria fixation with methanol, a higher MOI (10:1) was used to potentiate the stimulatory effect and facilitate the comparison with the results obtained with live bacteria. As performed with live cells, macrophage cell viability in an MOI of 10:1 was first determined through the LDH release assay (Fig. 5.4).

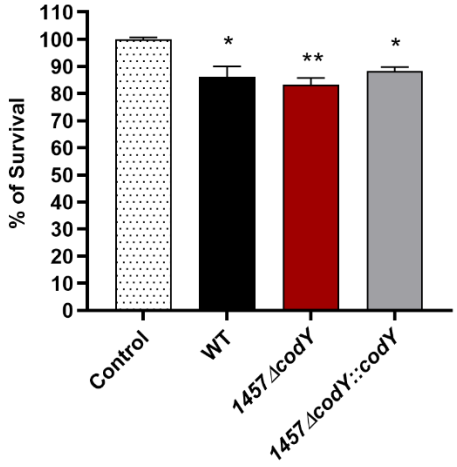


**Figure 5.4** Survival of macrophage cells as assessed by LDH release assay after infection with methanol-fixed *S. epidermidis* cells at MOI 10:1 (bacteria: macrophages). The control represents macrophages without bacterial infection. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons tests. ** p<0.05, ** p<0.01*. WT – Wild-type; 1457*ΔcodY* – *codY* deletion mutant; 1457*ΔcodY::codY* – complemented strain.

After the challenge with *S. epidermidis* fixed cells at MOI 10:1, a reduction in cell viability was found, with the percentage of survival ranging from 83 to 88% (Fig. 5.4), showing significant differences in comparison to the control. However, the percentage of survival is still within the range that we consider acceptable (above 80%) and similar among all the *S. epidermidis* strains tested.

In regard to the production of cytokines upon infection, both pro-inflammatory cytokines studied showed the same tendency as with the live bacteria (Fig. 5.5).

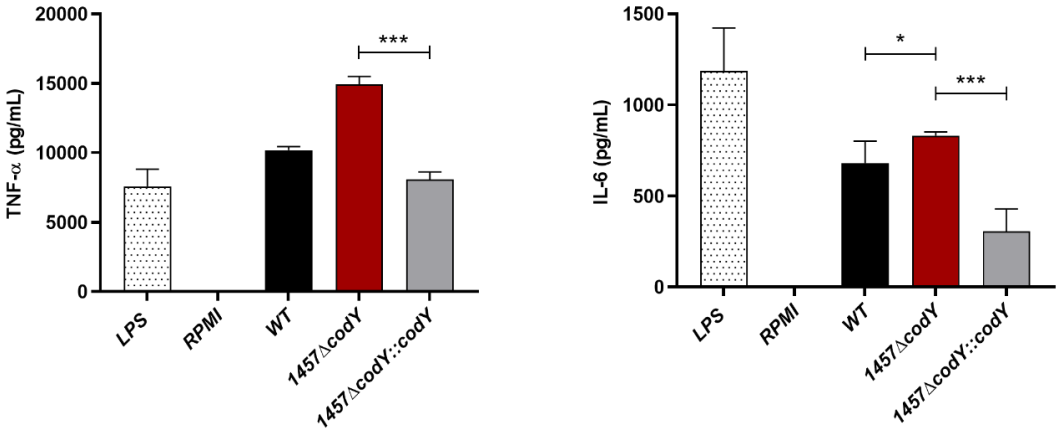


**Fig. 5.5** Levels of pro-inflammatory cytokines TNF-α and IL-6 detected in the supernatant of THP-1 cells infected with methanol-fixed *S. epidermidis* strains for 24 h. The results represent the mean + SEM of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Kruskal-Wallis multiple comparisons test. ** p<0.05, *** p<0.001*. WT – Wild-type; 1457*ΔcodY* – *codY* deletion mutant; 1457*ΔcodY::codY* – complemented strain.

### 5.3.3. The lack of *codY* does not influence bacterial internalization by the macrophages

Phagocytosis and subsequent intracellular elimination of pathogenic bacteria by macrophages are important processes to control infection [10]. Although several bacteria have been found to survive within macrophages [11,12], the ability of *S. epidermidis* to survive within phagocytic cells is not well known. Hence, a gentamicin protection assay was carried out to assess the extent of phagocytosis, as well as the ability of *S. epidermidis* cells to survive within THP-1 macrophage cells (Fig. 5.6).

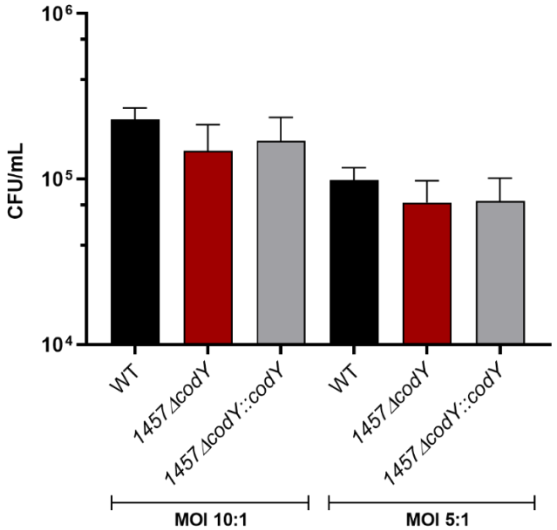


**Figure 5.6** Number of *S. epidermidis* cells internalized by THP-1-derived macrophages. Macrophages were infected at an MOI of 10:1 and 5:1. The results represent the mean + SEM of at least three independent experiments. WT – Wild-type; 1457*ΔcodY* – *codY* deletion mutant; 1457*ΔcodY::codY* – complemented strain.

The THP-1-derived macrophages were able to internalize both the WT and constructed strains, with no differences in the number of CFU/mL recovered. Thus, these data indicated that the recognition of this bacterium by the macrophages was not affected by the deletion of the gene *codY*. Furthermore, the observed higher stimulation of the pro-inflammatory cytokines seems not to be associated with macrophage internalization.

## 5.4. Discussion

Although *S. epidermidis* may behave as an opportunistic pathogen, the knowledge concerning the interaction of this bacterium with the cells of the innate immune system is still to be fully explored. As we described in chapter 4, the expression of the gene *codY* was associated with a higher capacity to form biofilms and with the emergence of VBNC cells. Herein, we aimed to assess how abrogating *codY* expression interfered with the interaction of *S. epidermidis* and human macrophage cells. Firstly, we determined that the conditions used for the challenge of THP-1-derived macrophages with the *S. epidermidis* strains were adequate for bacteria growth and that did not induce immune cell death. This was necessary to guarantee that the subsequently detected effects were the result of the interaction of the bacteria with macrophages and not by any stress or impairment caused to the bacterial or immune cells.

In literature, *S. epidermidis* strains have been reported to elicit pro-inflammatory responses, resulting in high cytokine stimulation [19,29], nevertheless, the pro-inflammatory capacity of *S. epidermidis* may differ between strains [29]. Several *in vitro* studies addressing the interaction of *S. epidermidis* planktonic bacteria and immune cells have revealed high secretion of pro-inflammatory cytokines [13–15]. For instance, Zwicker *et al.* found a marked increase in both IL-6 and TNF-α secretion by THP-1-derived macrophages upon the infection with *S. epidermidis* strain ATCC 35984 (also known as RP62A) [15]. Similarly, when evaluating the stimulation of human peripheral blood mononuclear cells with *S. epidermidis* strain 9142 planktonic cells, a high production of IL-6, IL-8 and TFN-α [13,14,16] was detected. *In vivo* studies have also been performed and have confirmed the results obtained *in vitro*. In that line, França *et al.* evaluated the pro-inflammatory response elicited *in vivo* in a murine model, 2, 6 and 14 h upon an infectious challenge with *S. epidermidis* planktonic cell populations [17]. The stimulation-induced high levels of both IL-6 and TFN-α, peaking at 6 h of post-challenge [17].

Herein, we have demonstrated a similar stimulation upon macrophage infection with *S. epidermidis* strains, with the mutant strain inducing a higher pro-inflammatory response. Thus, the deletion of the

*codY* gene significantly enhanced the secretion of the pro-inflammatory cytokines IL-6, IL-8 and TNF-α by THP-1-derived macrophages challenged with this bacterium. Although IL-8 was the cytokine produced in higher amounts, IL-6 and TNF-α showed more marked differences when comparing the mutant with WT or complemented strains.

Focusing on the role of VBNC cells in the response of the host immune system, a study realized by Cerca *et al*. demonstrated that *S. epidermidis* strain 9142 under VBNC-inducing conditions stimulated a lower IL-6 and TNF-α production by murine bone-marrow-derived macrophages as compared with the bacteria grown in TSB or VBNC-preventing conditions [18]. The higher pro-inflammatory response induced by *S. epidermidis* bacteria lacking *codY* would be in line with a possible association of this gene with the emergence of VBNC cells. However, additional studies would be necessary to elucidate this argument.

Regarding the production of counter-inflammatory cytokines, in a recent study, the stimulation of neonatal umbilical cord blood with *S. epidermidis* isolates obtained from clinical sepsis patients induced higher levels of IL-10 than colonization isolates [19]. In addition, the stimulatory effect on IL-10 production appeared lower than the one observed for pro-inflammatory cytokine IL-6 [19]. Kaya *et al*., found higher levels of IL-10 when infecting human peripheral blood mononuclear cells with planktonic than with biofilm *S. epidermidis* cells [20]. These studies are in agreement with our results, since all tested *S. epidermidis* strains stimulated the secretion of IL-10, following the same trend as pro-inflammatory cytokines, i.e., we observed a higher stimulation when challenging the macrophage cells with the mutant strain. Moreover, IL-6 has been associated with host protection against *S. epidermidis* [21], but also with deleterious effects resulting from disseminated infection with this pathogen [22]. Therefore, a balanced cytokine production appears necessary to contain *S. epidermidis* infection and simultaneously avoid the deleterious effect of excessive inflammation. The counter-inflammatory role of IL-10 may contribute to this regulation as previously shown in murine models of *S. epidermidis* infection [23,24]. The concomitant production of TNF-α, IL-6 and IL-10 induced in the host by *S. epidermidis* has been previously shown and correlated with bacterial-caused diseases [25]. In newborns, lower production of these cytokines correlated with a higher risk of developing late-onset sepsis [25].

Cytokine production induced by *S. epidermidis* may be elicited by the recognition of bacterial soluble or cell-wall components [24,26]. Since our results demonstrated a similar type of response with both live and methanol-fixed *S. epidermidis* cells, this suggests that the higher cytokine stimulation observed in the mutant strain is associated with the recognition of the bacterial structural components, rather than with secreted factors or cell proliferation. Thus, this indicates the deletion of the gene *codY* could indirectly

change the cell wall structure, by the regulation of proteins involved in cell membrane biosynthesis, in a way that facilitates bacterial recognition by the innate immune cells. In *S. aureus* infection, for instance, the activation of macrophages and consequent phagocytosis requires the recognition of lipoproteins [27], thus, the deletion of the lipoic acid synthetase (LipA) suppressed macrophage activation [28]. Therefore, we hypothesized that the deletion of the gene *codY* could have influenced the activation of macrophages and, consequently, the phagocytosis process. For this reason, the phagocytic capacity of the THP-1-derived macrophages was assessed upon incubation of macrophages with all *S. epidermidis* strains. Interestingly, no differences were found among the tested strains, revealing that the deletion of the gene had no impact on the internalization by macrophages.

Overall, our results show that the deletion of the gene *codY* results in a stronger cytokine response, indicating that *codY* seems to be involved in *S. epidermidis* immune evasion.

## 5.5. References


1. Zheng, Y., He, L., Asiamah, T. K. & Otto, M. Colonization of medical devices by staphylococci. *Environ. Microbiol.* **20**, 3141–3153 (2018).
2. Cheung, G. Y. C. *et al. Staphylococcus epidermidis* strategies to avoid killing by human neutrophils. *PLoS Pathog.* **6**, 1–10 (2010).
3. Otto, M. *Staphylococcus epidermidis* - the 'accidental' pathogen. *Nat. Rev. Microbiol.* **7**, 555–67 (2009).
4. Waters, N. R. *et al.* A spectrum of CodY activities drives metabolic reorganization and virulence gene expression in Staphylococcus aureus. *Mol. Microbiol.* **101**, (2016).
5. Feng, L. *et al.* The CodY regulator is essential for virulence in Streptococcus suis serotype 2. *Sci. Reports 2016 61* **6**, 1–15 (2016).
6. Lobel, L. *et al.* The metabolic regulator CodY links *L. monocytogenes* metabolism to virulence by directly activating the virulence regulatory gene, prfA. *Mol. Microbiol.* **95**, 624–644 (2015).
7. Gasteiger, G. *et al.* Cellular Innate Immunity: An Old Game with New Players. *J. Innate Immun.* **9**, 111–125 (2017).
8. Martinez, F. O. & Gordon, S. The M1 and M2 paradigm of macrophage activation: Time for reassessment. *F1000Prime Rep.* **6**, 1–13 (2014).
9. Italiani, P. & Boraschi, D. From monocytes to M1/M2 macrophages: Phenotypical vs. functional differentiation. *Front. Immunol.* **5**, 1–22 (2014).
10. Pidwill, G. R., Gibson, J. F., Cole, J., Renshaw, S. A. & Foster, S. J. The Role of Macrophages in *Staphylococcus aureus* Infection. *Front. Immunol.* **11**, 1–30 (2021).
11. Cano, V. *et al.* </i>Klebsiella pneumoniae</i> survives within macrophages by avoiding delivery to lysosomes. *Cell. Microbiol.* **17**, 1537–1560 (2015).
12. Tranchemontagne, Z. R., Camire, R. B., O'Donnell, V. J., Baugh, J. & Burkholder, K. M. *Staphylococcus aureus* strain USA300 perturbs acquisition of lysosomal enzymes and requires phagosomal acidification for survival inside macrophages. *Infect. Immun.* **84**, 241–253 (2015).
13. Megyeri, K., Mándi, Y., Degré, M. & Rosztóczy, I. Induction of cytokine production by different staphylococcal strains. *Cytokine* **19**, 206–212 (2002).
14. Strunk, T. *et al.* Responsiveness of human monocytes to the commensal bacterium *Staphylococcus epidermidis* develops late in gestation. *Pediatr. Res.* **72**, 10–18 (2012).
15. Zwicker, P. *et al.* In vitro response of THP-1 derived macrophages to antimicrobially effective PHMB-coated Ti6Al4V alloy implant material with and without contamination with *S. epidermidis* and *P. aeruginosa*. *Biomater. Res.* **26**, 1–13 (2022).
16. Strunk, T. *et al.* TLR2 mediates recognition of live *Staphylococcus epidermidis* and clearance of bacteremia. *PLoS One* **5**, 2–9 (2010).
17. França, A. *et al. Staphylococcus epidermidis* Biofilm-Released Cells Induce a Prompt and More Marked *In vivo* Inflammatory-Type Response than Planktonic or Biofilm Cells. *Front. Immunol.* **7**, 1–12 (2016).
18. Cerca, F. *et al. Staphylococcus epidermidis* biofilms with higher proportions of dormant bacteria

induce a lower activation of murine macrophages. *J. Med. Microbiol.* **60**, 1717–1724 (2011).

19. Tröger, B. *et al.* Modulation of *S. epidermidis*-induced innate immune responses in neonatal whole blood. *J. Microbiol. Immunol. Infect.* **53**, 240–249 (2020).

20. Kaya, E. *et al.* Planktonic and biofilm-associated *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* elicit differential human peripheral blood cell responses. *Microorganisms* **9**, (2021).

21. Dhanagovind, P. T., Kujur, P. K., Swain, R. K. & Banerjee, S. IL-6 Signaling Protects Zebrafish Larvae during *Staphylococcus epidermidis* Infection in a Bath Immersion Model. *J. Immunol.* **207**, 2129–2142 (2021).

22. Bi, D. *et al. Staphylococcus epidermidis* bacteremia induces brain injury in neonatal mice via toll-like receptor 2-dependent and -independent pathways. *J. Infect. Dis.* **212**, 1480–1490 (2015).

23. Ferreirinha, P. *et al.* Poly-N-Acetylglucosamine Production by *Staphylococcus epidermidis* Cells Increases Their In Vivo Proinflammatory Effect Pedro. *Infect. Immun.* **84**, 2933–2943 (2016).

24. Volz, T. *et al.* Induction of IL-10-balanced immune profiles following exposure to LTA from *Staphylococcus epidermidis*. *Exp. Dermatol.* **27**, 318–326 (2018).

25. Strunk, T. *et al.* Impaired Cytokine Responses to Live *Staphylococcus epidermidis* in Preterm Infants Precede Gram-positive, Late-onset Sepsis. *Clin. Infect. Dis.* **72**, 271–278 (2021).

26. Lai, Y. *et al.* Activation of TLR2 by a Small Molecule Produced by *Staphylococcus epidermidis* Increases Antimicrobial Defense against Bacterial Skin Infections. *J Invest Dermatol* **130**, 1–20 (2010).

27. Wardenburg, J. B., Williams, W. A. & Missiakas, D. Host defenses against *Staphylococcus aureus* infection require recognition of bacterial lipoproteins. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 13831–13836 (2006).

28. Grayczyk, J. P., Cameron J. Harvey, Laczkovich, I. & III, F. A. A Lipoylated Metabolic Protein Released by *Staphylococcus aureus* Suppresses Macrophage Activation. *Cell Host Microbe* **22**, 678-687.e9 (2017).

29. Dong, Y., Glaser, K., Schlegel, N., Claus, H. & Speer, C. P. An underestimated pathogen: *Staphylococcus epidermidis* induces pro-inflammatory responses in human alveolar epithelial cells. *Cytokine* **123**, 154761 (2019).

# CHAPTER 6 – Major conclusions and perspectives

**Summary**

This chapter describes the main conclusions, major findings and limitations found throughout this study. Moreover, suggestions are presented for future directions.

## 6.1. Main conclusions

The main goal of this study was to elucidate the role of the gene *codY* in the pathogenic profile of *S. epidermidis*, as well as its role in the formation of VBNC cells. We demonstrated that the deletion of *codY* from the *S. epidermidis* genome influenced the bacterium growth profile and capacity for biofilm formation in different growth media. Moreover, the infection of THP-1-derived macrophages with all the *S. epidermidis* strains allowed us to show that the absence of the gene *codY* promoted a higher production of pro-inflammatory cytokines by the *S. epidermidis*-challenged macrophages. At last, we have shown that *codY* has a role in the formation of VBNC cells in *S. epidermidis* biofilms and, for the first time, we demonstrated that planktonic populations can also enter into a VBNC state, as previously described for biofilm cultures.

## 6.2. Answers to the main research questions raised

Through the progression of this work, it was possible to answer the questions raised at the beginning of this study, as described below.

1. **Does the deletion of the *codY* gene affect *S. epidermidis* susceptibility to antibiotics?**

Due to the current problem related to bacterial resistance and tolerance to antibiotics, we intended to understand if the deletion of the gene *codY* from the genome of the *S. epidermidis* strain 1457 could affect the susceptibility of the planktonic populations to the antibiotics tested. We found no differences between the WT and the constructed strains, except for rifampicin, to which the mutant strain was significantly more susceptible. Overall, our results indicated a minor role of *codY* in this bacterium susceptibility to antibiotics.

2. **Does the deletion of the *codY* gene have an impact on the capacity of *S. epidermidis* to form biofilms?**

In studies performed on other bacterial species, CodY seems to have a contradictory role in biofilm formation. Whereas in some bacterial species, the deletion of *codY* seems to enhance biofilm formation, in other studies it reduces it. For this reason, we aimed to evaluate the impact of the deletion of the gene *codY* in *S. epidermidis* biofilm formation capacity. Our data revealed a negative impact on the ability of *S. epidermidis* strain 1457 to form biofilms, both at 24 and 48 h of incubation and with both TSB and CDM media, confirming that *codY* has an important role in *S. epidermidis* biofilm formation.

### 3. Does *codY* influences VBNC cell formation in *S. epidermidis* biofilms?

To substantiate the potential role of CodY in the emergence of VBNC cells in *S. epidermidis* biofilms, we evaluated the profile of the *codY*-mutant strain grown under VBNC-inducing conditions. Our findings demonstrated marked differences between the WT and the mutant strain, indicating an important role of *codY* in the modulation of VBNC cells in the biofilms formed by strain 1457.

### 4. Does *codY* have a role in *S. epidermidis* evasion from the host immune system?

To evaluate whether abrogation of *codY* gene expression affected the response to *S. epidermidis* by host innate immune cells, the activation of macrophages derived from THP-1 cells was assessed. The secretion of the chemokine IL-8 promotes a pro-inflammatory response by inducing the activation and recruitment of neutrophils, which are important cells in the clearance of extracellular pathogens. According to our data, the presence of CodY leads to lower secretion of IL-8 by *S. epidermidis*-challenged macrophages. This indicates that in an *in vivo* scenario this would translate into lower recruitment of neutrophils to the infection site, contributing to the evasion of *S. epidermidis* from the immune system response. Indeed, the interaction of macrophage cells with the *codY*-lacking strain led to a higher level of both pro- and anti-inflammatory cytokines, indicating that CodY has a bacterial protective effect. Yet, the mechanism underlying this activation/protection remains to be elucidated.

## 6.3. Major limitations and perspectives

With this work, we have contributed to the elucidation of the role of the gene *codY* in *S. epidermidis* virulence. However, some limitations were also found, which restricted the breadth of our findings, as pointed out below.

The first limitation of this work was that we have only used one *S. epidermidis* strain (strain 1457). Hence, while we have described the role of *codY* in strain 1457, it is not possible to claim that the observed effect is widespread in different clinical isolates of *S. epidermidis*. As such, to achieve a better knowledge of the function of the *codY* gene in *S. epidermidis*, mutants in other strains whould be constructed and characterized. However, the construction of *S. epidermidis* mutants in strains other than 1457 remains challenging due to the low efficiency of the transformation process.

Moreover, biofilm formation and the emergence of VBNC cells are responsible for the increase in bacterial tolerance/resistance to antibiotics. Therefore, since *codY* seems to have an important role in these two factors, an analysis of the susceptibility of the WT and the constructed strains to the selected antibiotics

should be made in biofilms populations and under VBNC conditions, as well as, with a broader variety of antibiotics.

Lastly, the analysis of the interaction of the *S. epidermidis* strains with the host immune cells was only focused on THP-1-derived macrophages, limiting the characterization of the role of the gene *codY* in *S. epidermidis* virulence. Thus, it would be worth evaluating the interaction of these strains with other key immune cells, namely neutrophils and dendritic cells, as well as primary human blood cells. Furthermore, the use of *in vivo* mouse models would help to better characterize the role of *codY* in *S. epidermidis* pathogenicity. Regarding the adaptive immune response, less information is available concerning *S. epidermidis* infections, however, an evaluation of antibody production by *S. epidermidis*-specific B cells would elucidate the efficiency of these antibodies to bind and neutralize the *S. epidermidis* strains.

Nevertheless, the mechanism by which the mutant strain elicits a more marked immune response in comparison to the WT strain was not established and, thus, remains to be clarified. It would be interesting to evaluate the cytokine stimulation after *S. epidermidis* strains interaction with cell lines that have TLRs blocked/knockout, to elucidate by which receptor the recognition of the bacteria is achieved and whether the mutant strain has a higher affinity for a given receptor than the WT strain.

Despite the relevant findings observed in this study, several new questions were raised. For instance, since CodY is a repressor of hundreds of genes that codify proteins involved in biofilm formation, it would be interesting to compare the transcriptome of the WT with the mutant to uncover the genes that are regulated by *codY*. Also, since CodY has been described as a global transcription regulator of several virulent genes in situations of nutrient limitation, the characterization of the *codY*-mutant in minimal media would also be useful to better understand the mutant strain behaviour under those growth conditions.