Universidade do Minho
Escola de Engenharia

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Ecological interactions in *Pseudomonas aeruginosa – Staphylococcus aureus* mixed biofilms: deciphering co-infection in cystic fibrosis airways

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Professora Doutora Maria Olívia Pereira
E do
Doutor Nuno Cerca

agosto de 2022

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"Disse a flor para o pequeno príncipe: é preciso que eu suporte duas ou três larvas se quiser conhecer as borboletas"

Antoine de Saint-Exupéry, em O Principezinho

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"Tudo é considerado impossível, até acontecer."

Nelson Mandela

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Interações ecológica em biofilmes mistos de *P. aeruginosa–S. aureus:* decifrando o papel da co-infeção em ambiente de fibrose cística

Resumo

A fibrose cística (FC) é uma doença caracterizada por elevadas taxas de morbidade e mortalidade associadas a infeções microbianas pulmonares. O estudo das interações que se estabelecem entre *P. aeruginosa* e *S. aureus* é particularmente importante, uma vez que estas duas bactérias são clinicamente as mais relevantes nas infeções associadas a FC. Este projeto de doutoramento teve como objetivo principal compreender o papel das interações que se estabelecem entre estas bactérias quando em biofilmes polimicrobianos e perceber o seu contributo para a virulência e persistências de infeções em FC. Através de uma abordagem *in silico*, os dados de interação entre *P. aeruginosa* e *S. aureus* foram curados e sistematicamente organizados numa nova base de dados, a *Inter-Species CrossTalk Database* (www.ceb.uminho.pt/ISCTD). A análise das redes reconstruídas permitiu identificar entidades chave envolvidas na regulação das interações e, por integração com a base de dados PCQuorum, reconhecer potenciais alvos terapêuticos e possíveis moléculas inibidoras.

Os estudos experimentais demostraram que, em biofilmes duplos, *P. aeruginosa* foi a espécie dominante e induziu *S. aureus* para um estado viável mas não cultivável (VBNC), promovendo assim a sua coexistência. Pela análise do transcriptoma identificou-se um aumento da expressão de genes de virulência de *S. aureus,* sugerindo que o estado VBNC pode, em parte, justificar a patogenicidade de *S. aureus* e, assim, influenciar o resultado das estratégias terapêuticas das infeções associados a FC. A coexistência das bactérias nos consórcios polimicrobianos e a diferenciação de *S. aureus* para o estado VBNC continuou a manifestar-se após tratamento antimicrobiano, tendo-se observado, ainda, um aumento da tolerância dos biofilmes duplos, sugerindo que as espécies interagem sinergisticamente. Nos biofilmes duplos *P. aeruginosa* induziu ainda a formação de *small colony variants* por *S. aureus,* ainda que numa prevalência baixa, não sendo claro o papel deste fenótipo na resposta do consórcio aos antibióticos. O uso de um modelo *in vivo-like*, desenvolvido e otimizado para desenvolver coinfecção em células epiteliais, permitiu constatar que, na presença destas, as bactérias continuaram a coexistir, passando, contudo, *S. aureus* a predominar no consórcio. Verificou-se, ainda, que a sobrevivência intracelular aumentou significativamente quando ambas as bactérias co-infetaram o hospedeiro, e que o efeito citotóxico nas células epiteliais foi menor.

Os dados resultantes deste projeto de doutoramento mostraram que *P. aeruginosa* e *S. aureus* estabelecem uma interação de coexistência não competitiva, com benefício de ambas, mesmo quando os consórcios foram expostos a antibióticos e co-infetaram as células epiteliais, revelando uma nova faceta nas interações ecológicas estabelecidas entre estes microrganismos, tradicionalmente considerados competidores. Estes dados evidenciam, de forma clara, o papel das interações estabelecidas entre espécies na virulência e persistência de bactérias, e de como estas podem afetar a progressão e o resultado terapêutico da infeção.

Ecological interactions in *Pseudomonas aeruginosa – Staphylococcus aureus* mixed biofilms: deciphering co-infection in cystic fibrosis airways

Abstract

Cystic Fibrosis (CF) disease is characterized by high rates of morbidity and mortality caused mostly by pulmonary infections. Of particular interest is the interaction established between *P. aeruginosa* and *S. aureus*, the two most prominent bacteria in the CF lung. As such, the main aim of this PhD thesis was to decipher the ecological interactions of these pathogens and appraise how they contribute to virulence and persistence traits in the context of polymicrobial biofilm infections.

Firstly, by using an *in silico* approach, experimental data on the molecular basis of *P. aeruginosa - S. aureus* interactions were systematically organized in the new Inter-Species CrossTalk Database (www.ceb.uminho.pt/ISCTD). Reconstructed networks revealed key entities regulating *P. aeruginosa* and *S. aureus* interactions and disclosed important information on potential therapeutic targets and their respective reported inhibitors when integrated with the PCQuorum Database.

Experimental data demonstrated that, in dual-species biofilms, *P. aeruginosa* dominated the consortia and triggered the switching of *S. aureus* to a viable but non-cultivable (VBNC) state promoting their co-existence. The transcriptomic analysis revealed an increased expression of key genes associated with *S. aureus* virulence, suggesting that the phenotypic switching to VBNC state might account for *S. aureus* pathogenicity and, in turn, influence the clinical outcome of the infection. Additionally, *P. aeruginosa – S. aureus* co-existence led to an increased tolerance of the consortia towards antibiotics, with *S. aureus* preserving its VBNC phenotype, indicating that both bacteria can even interact synergistically. Under dual-species biofilms with *P. aeruginosa, S. aureus* also diversifies into small colony variants, however its role in the response to antimicrobial treatment was unclear. The use of an *in vivo*-like model, engineered to develop co-infection on epithelial cells, allowed to notice that both species continue co-existing, though *S. aureus* became the dominant bacteria in the consortia. Furthermore, the intracellular survival of both bacteria significantly increased during co-infection, as compared to mono-infection, and, surprisingly, the cytotoxic effect of both species on host seemed to be delayed.

Together, this PhD project pointed out a non-competitive co-existence of *P. aeruginosa* and *S. aureus* in a CF environment, in which both bacteria had mutual benefits even when challenged by antibiotics or in the presence of host cells, disclosing new ecological facets of interaction between these two species traditionally thought of as competitors. These new findings illustrate how inter-species interactions play a key role in bacterial virulence and persistence, which may subsequently rule infection progression and outcome.

Keywords: *P. aeruginosa – S. aureus* biofilms, viable but not culturable cells, bacteria-bacteria-host interactions, cystic-fibrosis *in vitro* models.

Table of contents

List of abbreviations

3-oxo-C12-HSL - 3-oxododecanoyl-L-homoserine lactone

AEC - Airway epithelial cells

AI - auto-inducers

AIP - autoinducing peptide

ASL - airway surface liquid

BRC - biofilm released cells

C4-HSL - N-butanoyl homoserine lactone

CF – cystic fibrosis

CFTR – CF conductance regulator gene

CFU - colony forming units

CI - Competitive Index

CIP – Ciprofloxacin

CLSI - Clinical and Laboratory Standards Institute

CV - crystal violet

DAPI - 4',6-diamidino-2-phenylindole

DMEM - Dulbecco modified eagle medium

eDNA - extracellular DNA

EM - Epifluorescence microscopy

ENaC - epithelial sodium channel

EPS - extracellular polymeric substances

EUCAST - European Committee on Antimicrobial Susceptibility Testing

FBS - fetal bovine serum

FC – Flow cytometry

FITC – fluorescein isothiocyanate

GlcNAc - N-acetyl glucosamine

HQNO - 2-heptyl-4-hydroxyquinoline N-oxide

LDH - Lactase dehydrogenase

MALDI-TOF MS - matrix-assisted laser desorption ionization time-of-flight mass spectrometry

MCC - mucociliary clearance

MDR - multidrug resistant

MHB - Mueller Hinton Broth

MIC - Minimum Inhibitory Concentration

MRSA - methicillin-resistant *S. aureus*

MSA - Mannitol Salt Agar

ND – Not determined

OD - optical density

PAMP - pathogen-associated molecular pattern molecules

PBS - phosphate-buffered saline

PCL - periciliary liquid layer

PCR – polymerase chain reaction

PI – Propidium iodide

PIA - Pseudomonas isolation agar

PQS - Integrated Quorum Sensing Signal

PRR - pattern recognition receptors

PSC - peak serum concentration

PSM - phenol-soluble modulin

PVL - Panton-Valentine leukocidin

qPCR – Quantitative PCR

QS – Quorum sensing

R - Resistant

RAP - RNAIII-activating peptide

RAP - RNAIII-activating peptide

RIR - Relative Increase Ratio

ROS - reactive oxygen species

S – Susceptible

Sae - *S. aureus* exoprotein

SAK – staphylokinase

SarA - staphylococcal accessory regulator

SCV - small colony variants

SD - standard deviation

SEM - Scanning electron microscopy

SigB - staphylococcal alternative sigma factor B

SpA - Staphylococcal protein A

TSB - tryptic soy broth

TSST - toxic shock syndrome toxin

VAC – Vancomycin

VBNC - viable but non-cultivable

WGA – Wheat Germ Agglutinin

WHO - World Health Organization

WMT - wild morphotype

List of figures

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 6

List of tables

List of publications

A significant proportion of the work described in this thesis has been published elsewhere.

Papers in peer-reviewed journals

Magalhães AP, Grainha T, Sousa AM, França A, Cerca N, Pereira MO (2021) Viable but non-cultivable state: a strategy for *Staphylococcus aureus* survivable in dual-species biofilms with *Pseudomonas aeruginosa*? Environmental Microbiology 23(9), 5639-5649 (doi: 10.1111/1462-2920.15734)

Magalhães AP, Jorge P, Pereira MO (2019) *Pseudomonas aeruginosa* and *Staphylococcus aureus* communication in biofilm infections: insights through network and database construction. Critical Reviews in Microbiology, 45(5-6), 712-728 (doi: 10.1080/1040841X.2019.1700209)

Oral communication at international scientific conferences

Magalhães AP, Grainha T, Sousa AM, França A, Cerca N, Pereira MO (2019) Viable but non-culturable state: a strategy for *Staphylococcus aureus* survivable in dual-species biofilms with *Pseudomonas aeruginosa.* EUROBIOFILMS 2019 - 6th European Congress on Biofilms. No. 164, Glasgow, Scotland, Sep 03-06, 67, 2019

Poster communication at international scientific conferences

Jorge P*, **Magalhães, AP*;** Pereira MO (2019) Interactions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in biofilm-related infections: insights through network reconstruction and creation of a new online database. EUROBIOFILMS 2019. Glasgow, Scotland,03-07 September, 2019. *Equally contributing authors

Magalhães AP, Pereira MO, Cerca N (2017) Revealing the dynamics of polymicrobial infections: Update on the qPCR as a promising tool for the quantification of bacterial jungles. Microbiotec'17 Congress of Microbiology and Biotechnology Porto, Portugal, Dec 7-9, 2017.

Magalhães AP, Sousa AM, Lopes SP, Pereira MO (2017). *S. aureus* modulates *P. aeruginosa* small-colony variants formation. EUROBIOFILMS 2017 - 5th European Congress on Microbial Biofilms. No. 004, Amesterdam, Netherlands, 19-22 September,2017.

CHAPTER 1

Introduction

This chapter introduces the research questions and hypothesis that led to the development of this thesis. A brief background is provided, as well as the significance and outline of the thesis.

1.1 Background

Cystic fibrosis (CF) patients suffer from severe lung infections responsible for progressive pulmonary function decline, ultimately leading to respiratory failure and death [1]. The lungs of CF patients are a recognized example of a rich and complex microbial ecosystem shared by a large diversity of microorganisms that can interact between them, affecting the disease progression and compromising the efficacy of the treatments [2]. *Pseudomonas aeruginosa* is one of the most prevalent bacteria within CF lungs, being recognized as the most relevant pathogenic agent responsible for poor clinical outcomes due to the difficulty of eradicating it even using aggressive and long antibiotic treatments [3, 4]. *Staphylococcus aureus* is also a predominant pathogens in CF lungs and is detected simultaneously with *P. aeruginosa* in at least a third of CF patients [5–7]. The establishment of co-infections by *P. aeruginosa* and *S. aureus* likely promotes their interaction. Indeed, until recently, the anti-staphylococcal behaviour of *P. aeruginosa* was the only interaction observed between the two species and was thus extensively reported [8]. However, Baldan *et al.* [10] firstly observed that during the development of CF chronic infections a non-competitive interaction could be established between *P. aeruginosa* and *S. aureus*, calling into question the established antagonistic model [9]. The co-existence of both species is therefore an important issue as it impacts how to effectively and efficiently treat CF infections. Also, co-infections are particularly complex and have more severe effects on lung function and poorer clinical prognosis than mono-infections [10–12]. In addition, chronic infections allow bacteria to adopt a biofilm lifestyle that confers adaptive resistance, where bacteria have been shown to be significantly more tolerant to antimicrobials and host immune responses than planktonic cells [13–15]. So, it is not surprising that bacteria within polymicrobial biofilms can benefit from each other through inter-species interactions, with certainly consequences on antibiotic resistance and microbial pathogenesis [16, 17]. Antimicrobial resistance is of emerging concern worldwide, with many bacteria developing resistance to multiple or all classes of antibiotics [18]. The Centers for Disease Control and Prevention has listed multidrug resistant *P. aeruginosa* and methicillin- resistant *S. aureus* (MRSA) as serious threats to human health [19]. Hence, there is a high demand for new strategies to tackle antimicrobial resistance. Thus, understanding how *P. aeruginosa* and *S. aureus* interact within a polymicrobial infection is of utmost importance to design more effective methods to combat antibiotic tolerance.

This study was conducted to determine the ecological interactions established between *P. aeruginosa* and *S. aureus*, and to appraise how their interplay can contribute to virulence and persistence traits of the polymicrobial consortia. This PhD project attempts to advance our understanding on the complex interactions established between *P. aeruginosa* and *S. aureus* that can hinder CF treatment strategies.

1.2 Research questions

The following questions will be addressed throughout this thesis:

1. What is the current knowledge regarding inter-species interactions occurring between *P. aeruginosa* and *S. aureus* and how it can help to identify promising antimicrobial targets?

2. Can *P. aeruginosa* and *S. aureus* increase its virulence features when they are growing as dual-species biofilms?

3. What happens to *P. aeruginosa* - *S. aureus* dual-species biofilms upon exposure to antibiotic therapy?

4. What happens to *P. aeruginosa* and *S. aureus* interactions when growing under *in vivo*-like conditions?

Answers to these questions are expected to improve the current knowledge on *P. aeruginosa* - *S. aureus* pathogenesis, which ultimately may contribute to the design of improved treatment strategies against CF-related infections.

1.3 Hypothesis and aims

1.3.1 Hypothesis

While *P. aeruginosa* is recognized as the leading cause of lung function decline, the significance of *S. aureus* in the course of CF disease is still being debated. This investigation tested the following hypothesis:

Inter-species interactions promote phenotypic and transcriptomic adaptations that provide enhanced virulence and persistence traits of *P. aeruginosa* and *S. aureus* co-infections.

1.3.2 Aims

To better understand the mechanisms underlying *P. aeruginosa and S. aureus* interactions, this PhD project aimed at deciphering the ecological interaction occurring between these pathogens

and appraising how it contributes to virulence and persistence traits in the context of polymicrobial biofilm infections. To achieve this major goal, the following specific aims were addressed.

Aim 1: To reconstruct the *in silico* inter-species communication network between *P. aeruginosa* and *S. aureus* in biofilm co-infections.

a) To collect and analyse experimental information on *P. aeruginosa* and *S. aureus* interactions retrieved from the scientific literature.

b) To construct a publically available database containing all the gathered information about *P. aeruginosa* and *S. aureus* interactions.

Aim 2: To characterize *P. aeruginosa* and *S. aureus* dual-species biofilms.

a) To analyse the biofilm formation ability of different *P. aeruginosa* and *S. aureus* strains under single- and dual-species consortia.

b) To compare the biofilm-forming capacity of both bacteria when growing as single- and dual-species biofilms.

c) To quantify the bacterial populations of dual-species biofilms by colony-forming units counting and by flow cytometry.

d) To identify the ecological interactions occurring between *P. aeruginosa* and *S. aureus* through competitive and relative increase ratio indexes determination.

e) To analyse the expression of *P. aeruginosa* and *S. aureus* virulence-related genes as dual-species biofilm communities.

Aim 3: To characterize *P. aeruginosa* and *S. aureus* dual-species biofilms under exposure to antibiotics of clinical relevance in CF infections.

a) To evaluate the antimicrobial susceptibility of *P. aeruginosa* and *S. aureus* to ciprofloxacin and vancomycin.

b) To quantify the bacterial populations of dual-species biofilms after exposure to ciprofloxacin and vancomycin treatments by colony-forming units counting and by flow cytometry.

c) To assess the impact of morphological diversification on the dual-species biofilms pathogenicity.

Aim 4: To evaluate the *in vitro* initial adhesion of dual-species biofilms to lung epithelial cells and their cytotoxicity effects on an epithelial monolayer.

a) To compare the biofilm-forming capacity of *P. aeruginosa* and *S. aureus* developed as single- and dual-species biofilms in plastic plate *versus* epithelial monolayer.

b) To assess the initial adhesion and internalization of single- and dual-species biofilms to a monolayer of epithelial cells.

c) To evaluate the ability of single- and dual-species biofilms to persist intracellularly in bronchial epithelial cells and to analyse their cytotoxic effects.

1.4 Significance

The airways of CF patients are colonized by multiple species, whereas *P. aeruginosa* and *S. aureus* are the most prevalent and problematic pathogens [20, 21]. Despite their seemingly sequential appearance, both pathogens remain highly prevalent through all stages of the lives of CF patients [22]. While *P. aeruginosa* is recognized as the leading cause of lung function decline [23, 24], the contribution of *S. aureus* to infection progression remains controversial [5, 25]. Yet, *P. aeruginosa* and *S. aureus* co-infection is associated with increased morbidity and mortality [11, 26, 27] and *in vivo* models indicate that co-infection increases disease severity and antimicrobial resistance [28–32]. Thus, it is essential unveiling the circumstances allowing *P. aeruginosa* and *S. aureus* co-existence, and how inter-species interactions rule the behaviour of the individual species, the activity of the polymicrobial community, and the relationship between host and bacteria, which are still mostly unknown. It is anticipated that this knowledge will represent a significant advancement towards the characterization of the ecological interactions and virulence factors that contribute to the recurrence of *P. aeruginosa* - *S.* aureus co-infection. Furthermore, this PhD project will be able to disclose new insights into the interactions emerged between of these pathogens and to gain knowledge helping to more comprehensively understand how those interactions contribute to the enhanced virulence and persistence traits of CF infections.. Together, these findings may be an important contribution to assist the design of novel and successful strategies to treat CF polymicrobial infections.

1.5 Thesis outline

Following this introductory chapter, this thesis presents a literature review (**Chapter 2**) that provides a general outline of the major aspects regarding the pathophysiology and microbiology of CF lung infections. Furthermore, important information on *P. aeruginosa* and *S. aureus* virulence factors will be also addressed, carefully emphasizing the biofilm development as a key step on infection establishment. Lastly, special emphasis will be also given to the inter-species interactions occurring between *P. aeruginosa* and *S. aureus* in the context of CF infections.

Chapter 3 presents the bioinformatics resources and the *in silico* methods used for the retrieval and analysis of experimental information on the molecular basis of *P. aeruginosa* and *S. aureus* interactions from the scientific literature, and the creation of a public database.

All experimental data obtained are shown throughout **Chapters 4** to **6.** Each of the experimental chapters stands alone, presenting a summary, brief introduction, materials and methods, results and discussion.

Chapter 4 is focused on the examination of the phenotypic characteristics and ecological interactions of biofilms formed by different strains of *P. aeruginosa* and *S. aureus*. Single- and dual-species biofilms were grown over time and biofilm formation and transcription of virulence related-genes were addressed.

Chapter 5 addresses the effect of antibiotic therapy on *P. aeruginosa* and *S. aureus* biofilm composition and tolerance. The single- and dual-species biofilms underwent analysis for antimicrobial susceptibility profile, biofilm formation and morphological diversification.

Chapter 6 shows the impact of the host cells in driving the inter-species interactions stablished between P. aeruginosa and S. aureus by evaluating biofilm formation ability, bacterial adhesion, bacterial invasion, and the ability of both species to persist within host cells and cause cell damage, using a *in vivo*-like conditions model.

Finally, **Chapter 7** presents a summary of the research findings, major outcomes, the significance of the findings, limitations and future directions.

A schematic diagram showing the general layout of this thesis and the relationship among different chapters is presented in **Figure 1.1**.

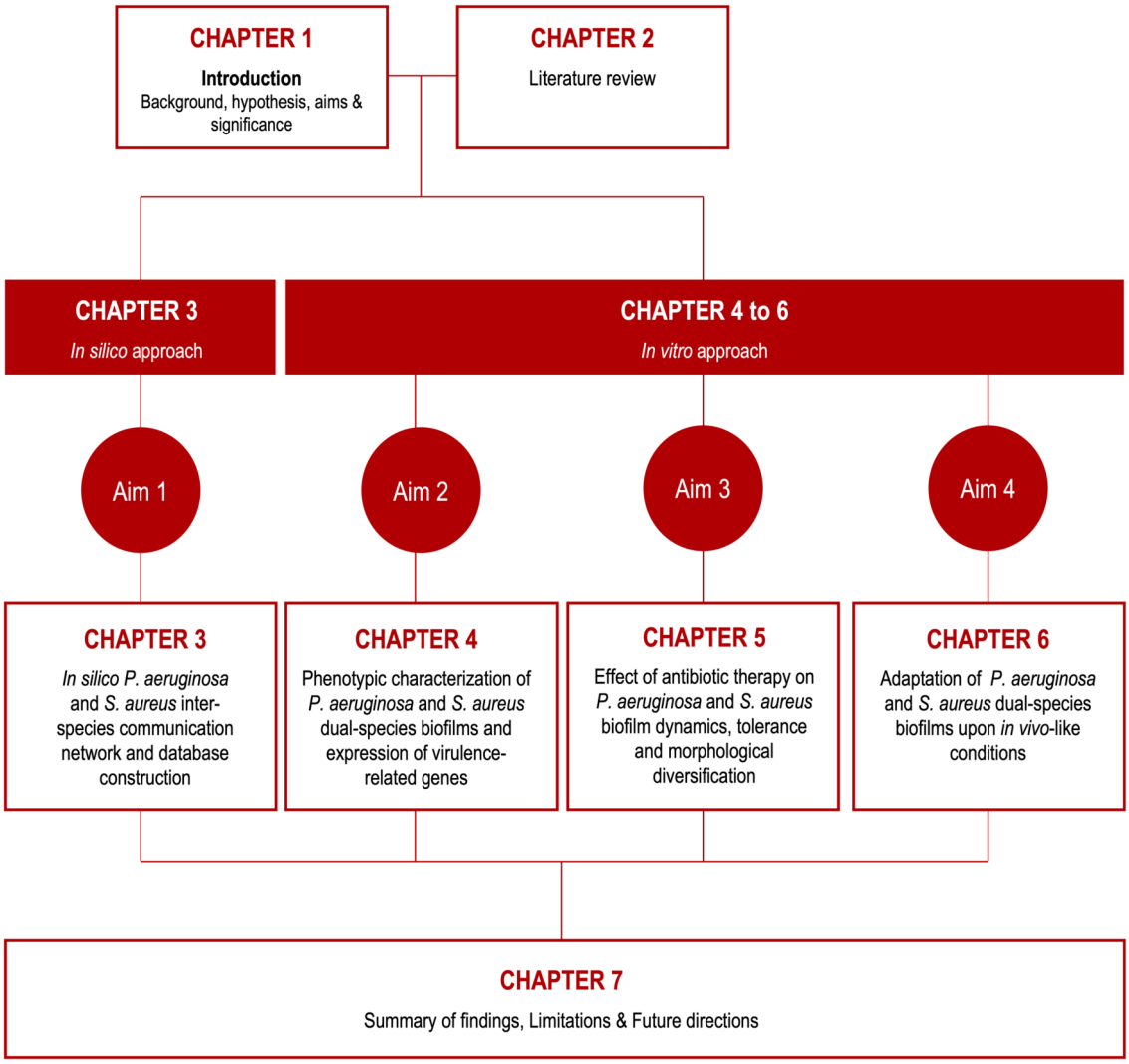

Figure 1.1. Thesis outline.

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CHAPTER 2

Literature Review

This chapter provides a general outline of the major aspects regarding the pathophysiology and microbiology of CF lung diseases, carefully emphasizing the biofilm development as a key step on infection establishment. Relevant information on *P. aeruginosa* and *S. aureus* virulence factors is also addressed. Lastly, a special attention will be given to the inter-species interactions occurring between *P. aeruginosa* and *S. aureus* in the context of CF lung infections.

2.1 Cystic Fibrosis lung disease

Cystic fibrosis (CF) is the most common life-threatening genetic inherited disorder among Caucasians. When CF was first described in 1938 [1], the predicted survival age of patients was only 6 months [2]. During the past decades, median age survival has increased progressively and it is now more than 40 years in developed countries [3]. This improvement in life expectancy has been achieved by understanding the importance of augmenting airway clearance, aggressively treating infection, and correcting nutrition deficits [4]. Mutations in the CF conductance regulator (CFTR) gene, which is located on chromosome 7, alter transepithelial fluid and electrolyte movement throughout the body and, therefore, the function of many organs [5]. More than 2000 variants in the CFTR gene have been identified, many of which have been associated with disease causation [6]. The most prevalent of those mutations (ΔF508) is the deletion of three nucleotides at the position 508 of the CFTR protein sequence [7]. Despite being a multi-system disorder causing several complications on the human body, the CFTR depletion or malfunction has significant impact on lungs due to the severe respiratory symptoms that patients suffer and the high mortality rate associated [8, 9].

2.1.1 Pathophysiology

The CFTR protein acts as a channel for the chloride and sodium ions transport across the cell membranes [5, 10]. Therefore, a dysfunctional CFTR protein leads to the absence or a decreased chloride secretion, resulting in an intracellular accumulation of those ions and, ultimately, to the depletion of chloride, sodium and water from the airway lumen. This causes abnormal thick and viscous secretions and impairs mucociliary clearance in CF airways [11–13] (**Figure 2.1**).

Typically, the airway epithelial cells have a thin and hydrated mucus layer, located on top of the periciliary liquid layer (PCL), that enables an efficient mucociliary clearance [14–16]. In CF, the airway epithelium absorbs sodium (Na^+) and chloride (Cl^-) ions and water from the lumen, depletes the PCL and slow down or even stop the mucus transport. The persistent mucus hyper secretion leads to the production of mucus plaques and plugs, hence increasing the mucus layer on the epithelial cells and generating steep oxygen gradients, with zones ranging from aerobic (generally located at the top) and microaerobic and/or even completely anaerobic (located in the deeper layers) [14–16].

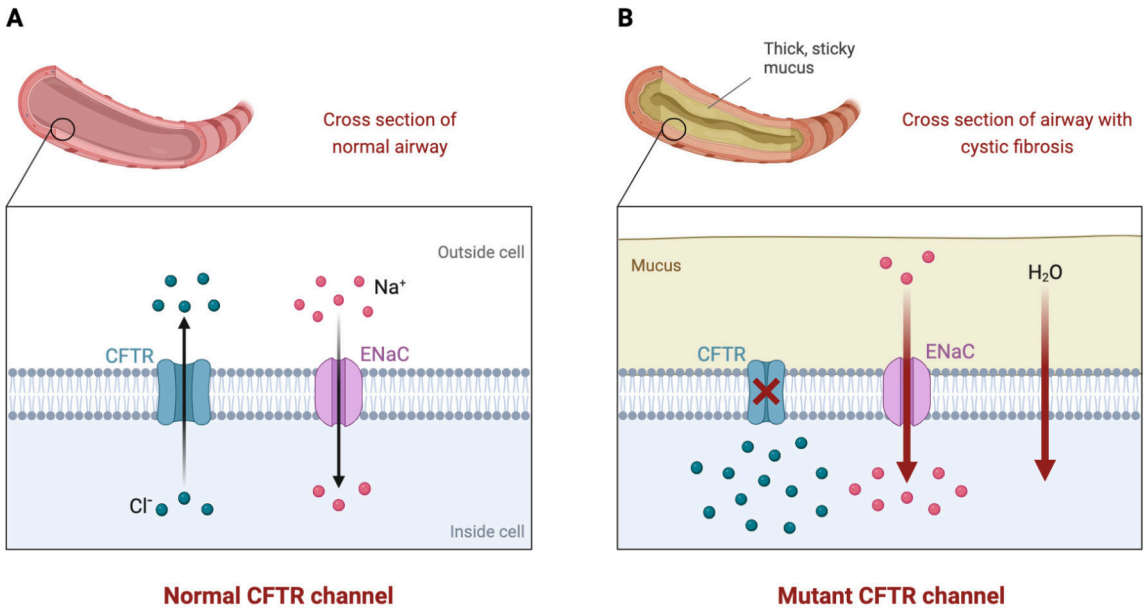


Figure 2.1. Ion transport in the CF airway. In healthy people **(A)**, a well-balanced epithelial sodium (Na^+) absorption and secretion of chloride anions hydrates airway surfaces and promotes efficient mucociliary transport. In the CF airway **(B)**, the defective CF transmembrane regulator (CFTR) channel and increased activity of the epithelial sodium channel (ENaC) lead to dehydrated mucus, failed mucus clearance, and mucus adhesion to airway surfaces. Adapted from Turcios *et al.* [17]. Created with BioRender.com.

The accumulated sputum, rich in nutrients, and the optimal temperature and humidity makes CF lungs a propitious environment for microbial colonization and growth [5, 10]. It is well established that the greatest contributor to CF patients morbidity and/or mortality is the failure of lung function that generally occurs in older patients, caused by recurrent cycles of infections and inflammation. Indeed, the persistent bronchial inflammation and chronic bacterial infections lead to progressive airway and lung damage, respiratory failure and eventually death [18–20]. Accordingly, the study of CF resident microbiome becomes one of the central topics in CF disease management.

2.1.2 Airway inflammation and host response

Inflammation is a transient, self-limiting physiological response to injury or infection [21]. However, in CF lung disease, airway inflammation is exaggerated and sustained, playing a central role in disease pathogenesis [22]. Impaired mucociliary clearance, airway infection, and abnormal host immune responses all contribute to the dysregulated hyperinflammatory state in CF patients [23]. Although initially just a few studies highlighted the importance of the CFTR in the regulation of

immune cell function, there is now more evidence showing the relevance of the CFTR expression in different cells of the immune system [21, 24–29]

Airway epithelial cells (AEC) are the initial checkpoints in the defence against pathogens as they are able to detect pathogen-associated molecular pattern molecules (PAMP) via a broad range of pattern recognition receptors (PRR) [28–30].The sensing of pathogen and release of inflammatory mediators are orchestrated to promote neutrophil recruitment and rapid pathogen clearance [31]. However, in CFTR-deficient AEC, innate immune responses are intrinsically defective, resulting in altered pathogen interactions and immune cell communication [32, 33], which usually leads to abnormal production of pro-inflammatory cytokines [34] and decreased levels of anti-inflammatory cytokines [35, 36]. CF airway inflammation is dominated by excessive neutrophils recruitment, which produce reactive oxygen species (ROS) [37], neutrophil extracellular traps [38], proteases such as neutrophil elastase [39, 40], and pro-inflammatory mediators [41], thereby propagating a cycle of progressive tissue damage, immune cell recruitment, and inflammation.

Although epithelial cells and neutrophils contribute to the pathogenesis of CF, macrophages are usually among the first cells encountering inhaled pathogens and have also been shown to exhibit abnormal function in CF [42]. Exaggerated inflammatory is not the only altered pathways in macrophages harbouring CFTR mutations [43]. It has been reported that such macrophages display increased apoptosis and impaired bacterial killing, associated with decreased phagocytic capacity [44].

The ineffectiveness of the immune response allows the establishment of a relentless cycle whereby persistent bacteria cause increased inflammation that itself leads to increased microbial densities and in turn more inflammation [23, 45] (**Figure 2.2**).

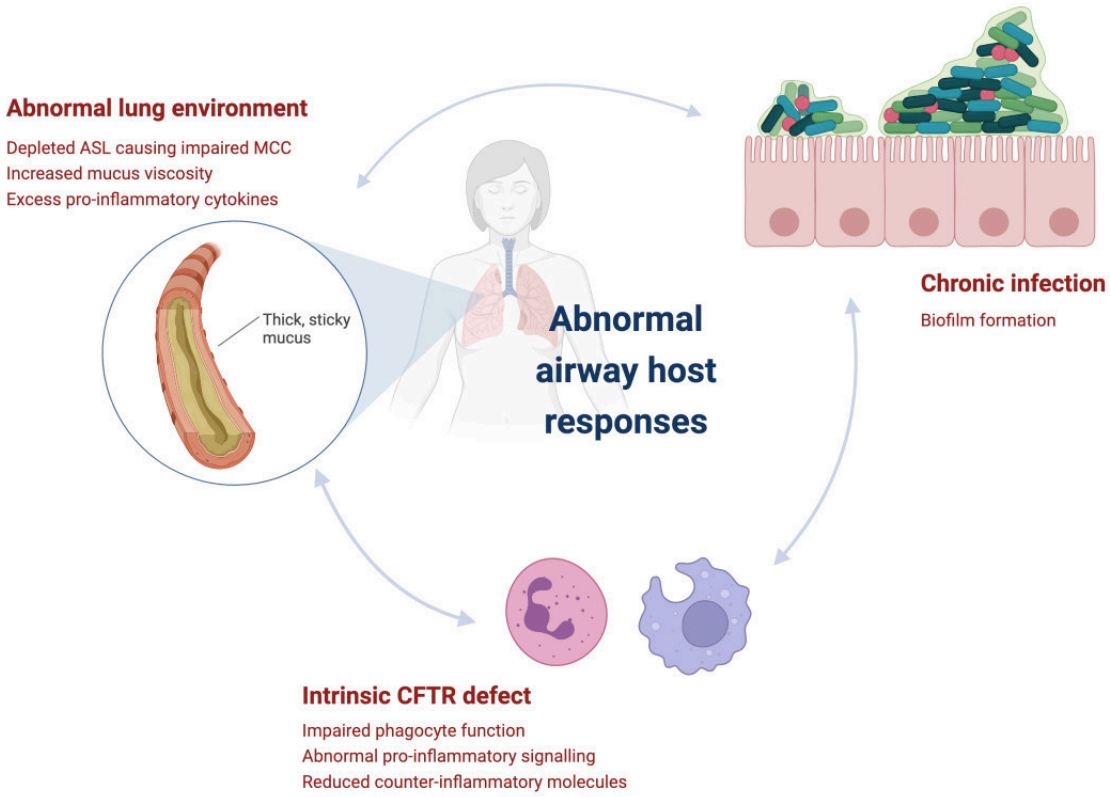


Figure 2.2. Factors affecting airway host immune responses. Defective CFTR results in dehydration of the airway surface liquid (ASL), which impairs mucociliary clearance (MCC) and host immune responses, thereby permitting a vicious cycle of airway infection, lung damage and a hyperinflammatory state in people with CF. Adapted from Turcios *et al.* [17]. Created with BioRender.com.

2.1.3 Microbiology

The CF airways offer a favourable environment for the colonization and proliferation of a large variety of organisms, including as bacteria, fungi and viruses, with bacterial species being the ones that are more frequently isolated [46–48]. Typically, *S. aureus, Haemophilus influenzae* and *Stenotrophomonas maltophilia* are considered the primary microorganisms because they are more prevalent in infections occurring during early childhood [49, 50]. Their increased prevalence at this so early stage it is associated to inflammation, damage of the airways and worse pulmonary function [51]. As patient gets older and the disease progresses, early colonizing species decrease their incidence and patients become more susceptible to a range of gram-negative bacteria, including *Burkholderia cepacia*, *Achromobacter xylosoxidans* and *P. aeruginosa* (**Figure 2.3**).

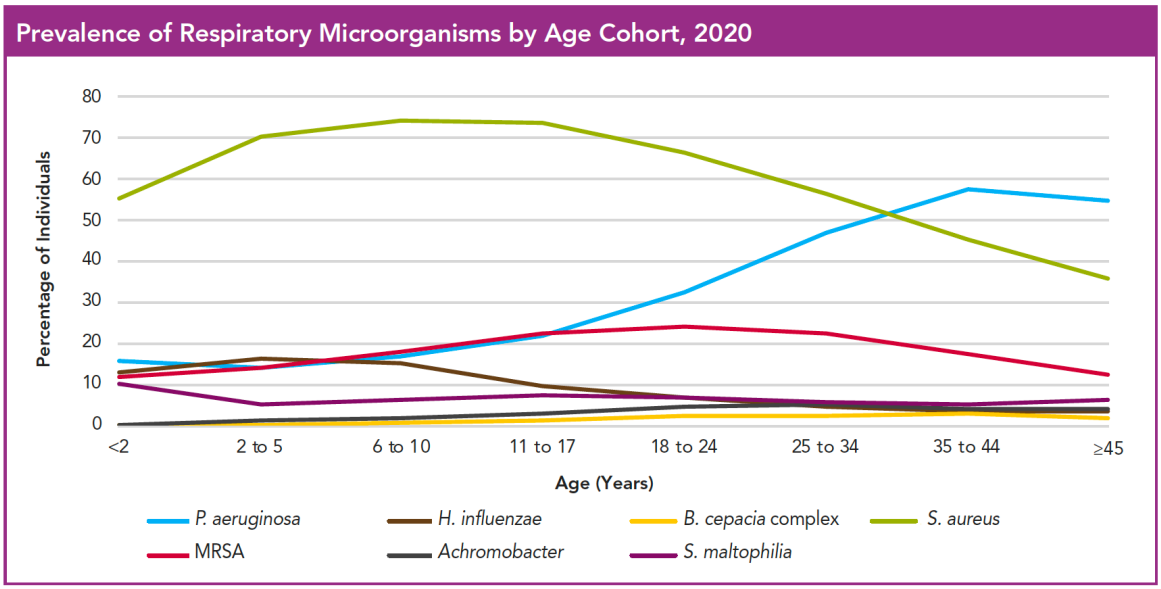


Figure 2.3. Prevalence of respiratory microorganism by age cohort during 2020. Adapted from Patient Registry 2020 Annual Data Report [52].

At this stage of disease, 2 to 8 % of CF 18-year-old patients are colonized by *Burkholderia cepacia complex* (Bcc), which encompass the 18 most threatening Burkholderia species, some of them highly transmissible [48, 53–55]. Bcc colonization is associated with severe respiratory symptoms such as lung function decline and fatal pneumonia through bacteraemia [56]. Nevertheless, the most problematic issue at this stage of CF disease is the detection of *P. aeruginosa* in CF sputum. After *P. aeruginosa* colonization CF patients suffer from frequent and consecutive episodes of re-colonization, resulting in the development of a chronic infection that can persist for years or even never be eradicated [57, 58]. Furthermore, patients with CF are more prone to develop severe biofilm-related infections that are thought to contribute greatly to the emergence and dissemination of antibiotic resistance [59]. Biofilm formation represents a protective mode of microorganisms growth that allows them to survive in hostile environments and disperse by seeding cells to colonize new niches [60]. Biofilm formation can be considered a continuous cycle, in which organized communities of microbial species are encased in a self-produced matrix of extracellular polymeric substances (EPS). For instance, in *P. aeruginosa* biofilm-associated infections, the EPS matrix often includes the polysaccharide alginate and polysaccharides like Psl/Pel, as well as proteins and nucleic acids, that hold bacterial cells together to a surface [61] (**Figure 2.4**). The limited penetration of antibiotics, immune defences cells and molecules through the EPS matrix is definitely a great contributor for their ineffective action and consequently successful microbial

persistence [61]. Nevertheless, biofilm resistance has been reported as complex and multifactorial resulting from the combination of several mechanisms, including the abovementioned restricted penetration of antimicrobials through the exopolysaccharide matrix [62], slow growth of bacteria within biofilms caused by nutrient and oxygen restriction [63], accumulated metabolic wastes and QS molecules [64].

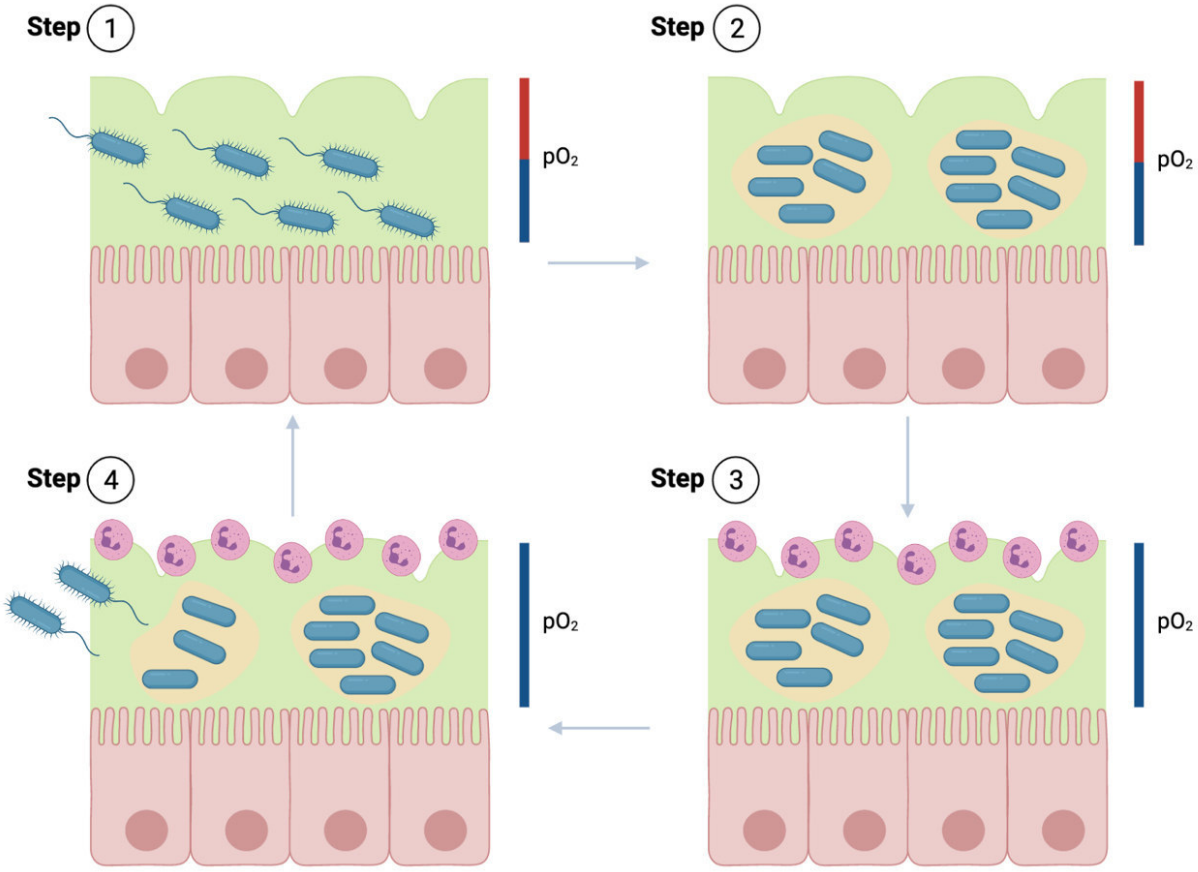


Figure 2.4. Schematic model of *P. aeruginosa* biofilm in the CF mucus: **(Step 1)** *P. aeruginosa* are deposited on the thickened mucus surfaces and can penetrate the mucus actively (e.g. by inhalation, flagellum- or pili-dependent motility) and/or passively (due to mucus turbulence) into the CF mucus. **(Step 2)** Afterwards, *P. aeruginosa* start to develop bacterial aggregates (the biofilms), which are protected by an alginate capsule. In this step, the consumption of O_2 (denoted by the red bar) is drastically increased by the bacterial cells and hypoxic and/or anaerobic pockets (blue colour in bar) are formed. **(Step 3)** In the final stages, where O_2 is almost depleted, the bacterial aggregates become highly resistant to the neutrophils and antibiotics, setting the stage for persistent chronic infection. **(Step 4)** In this phase of biofilm development, clusters of bacteria may detach from the biofilm in a process called dispersal. Adapted from Hassett *et al.* [15].

Clinical culture-based laboratory testing for specific pathogenic species has led to a reductionistic view of the microbiology within the CF niche. The list of new and/or emerging pathogens associated

with CF patients has been increasing with the advances in microbial diagnostic techniques, in particular the culture independent approaches, such as sequencing of bacterial 16S rRNA gene and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Indeed, the use of the cutting-edge methodologies revealed that CF lungs are an environment much more richer and complex than previously estimated [46, 65–69]. Inhabiting microorganisms of CF lungs range from beyond recognised pathogens, such as *P. aeruginosa, S. aureus* and *Burkholderia spp.*, to new and emerging bacteria such as *Prevotella spp., Veillonella spp., Propionibacterium spp., Inquilinus limosus* and *Dolosigranulum pigrum* [48]. Moreover, the advanced microbial diagnosis also allowed knowing that CF microbiome exists in a dynamic state changing its diversity and prevalence according to the patient age and disease progression [70, 71]. These findings represented a significant advance in CF management disease once antimicrobial treatments could be designed according to patient age and CF disease stage improving their effectiveness. However, the role of these emerging bacteria in the pathophysiology of infection and inflammation in chronic lung disease is still unclear, requiring thus further investigation.

2.1.4 Clinical treatment

The liberal and global use of antibiotics for CF has undoubtedly contributed to substantial improvements in respiratory health observed in current patient cohorts [72]. Antibiotic treatment is crucial to treat infection, for improving or maintaining lung function, improving quality of life and prolonging survival, as infection and inflammation still lead to progressive lung function decline [73]. Antibiotics are used in CF patients not only for treating pulmonary exacerbations but also as prevention for eradicating pathogens before evolving chronic stages [72, 74]. In other cases, antibiotics are used to reduce the bacterial burden in chronically infected patients when pathogens have already developed protective mechanisms, such as the formation of biofilm, and therefore cannot be eradicated once a chronic infection has been established [75]. Early *P. aeruginosa* infections usually have a low bacterial load, offering an opportunity for eradication [74]. Once chronic infection is established, *P. aeruginosa* is virtually impossible to eradicate with currently clinical strategies [76, 77].

People with CF receive huge numbers of antibiotics over the course of their lives, delivered via oral, inhaled, or intravenous routes [78]. The severity of the symptoms, condition of the patient and the susceptibility to antibiotics (depending on sensitivity of the microorganism) determine the

appropriate course of therapy [74]. In many countries, CF management starts from the time of diagnosis with anti-staphylococcal prophylaxis [79]. Although shown to be useful for reducing early *S. aureus* infections, some concern remains over whether such therapy increases the risk of acquiring other organisms, such as *P.* aeruginosa [80]. In patients infected with *S. aureus* who are experiencing an acute pulmonary exacerbation, vancomycin and linezolid are the first-line antimicrobial choices [81, 82]. For *P. aeruginosa* infections, the treatment usually starts with oral ciprofloxacin in mild to moderate exacerbations [83, 84]. In severe exacerbations, or when oral treatment has not been effective, different antibiotic therapies are available, including tobramycin or colistin inhalation or intravenous administration [83, 84]. A combination of antibiotics of different classes can be given at the same time, both to prevent the emergence of resistance and to achieve synergy, especially in the treatment of *P. aeruginosa*-associated infections [85–88]. Antimicrobial resistance is increasing worldwide and is a matter of particular concern in patients with CF, in whom bacteria and fungi are increasingly found to be resistant to multiple antimicrobial drugs [89].

Until recently, antimicrobial therapies such as antibiotic eradication treatment in early-stage infections and protocols for maintenance therapy of chronic infections could only control symptoms and restrict the complications of CF [78]. Advances in CFTR modulator therapies, to address the basic defect of CF, have been remarkable and the field is evolving rapidly [83]. In the last decades, CF patients survival has increased significantly due to the emergence of new drugs [83] (e.g., CFTR modulators, anti-inflammatory agents) and a better understanding of the vicious cycle of airway infection, inflammation, and progressive airway destruction. These new novel molecular targets and novel drugs approaches has launched different therapeutic strategies [90–92]. Recent studies, that have analysed the impact of CFTR modulators on airway microbiology in CF patients, reported that children receiving ivacaftor or ivacaftor/lumacaftor had a delay in the acquisition of *P. aeruginosa* infection [93, 94]. Ibuprofen, which at high doses reduces neutrophil influx into the lung, is currently the only chronic anti-inflammatory therapy in CF for which there is prospective evidence demonstrating a treatment-associated effect on the rate of pulmonary function decline [92].

Despite the recent breakthrough with CFTR modulators, the development of novel mucolytic, anti-inflammatory, and anti-infective therapies is still of upmost importance, especially for patients with more advanced stages of lung disease [78].

2.2 *P. aeruginosa* and *S. aureus*

P. aeruginosa is a well-known gram-negative opportunistic human pathogen capable of causing both acute and chronic infections [95]. Its great adaptability, phenotypic and genomic plasticity, ubiquity, and opportunistic sense [96–98] enable *P. aeruginosa* association with other types of infection, such as burns, wounds [99, 100], and those associated with biomaterials [101]. To aggravate this scenario, multidrug resistant (MDR) *P. aeruginosa* strains are emerging with increasing frequency, rendering ineffective many of the existing antibiotic treatments [102, 103]. In fact, the World Health Organization (WHO) has recently identified carbapenem-resistant *P. aeruginosa* as a critical threat for which there is an urgent need for new therapies [104]. *P. aeruginosa* displays intrinsic resistance to different types of antibiotics, e.g. aminoglycosides, quinolones, and β-lactams, due to resistance mechanisms such as low outer membrane permeability, multi-drug efflux systems, and inactivating enzymes (e.g. β-lactamase) [105]. *P. aeruginosa* can also acquire resistance genes from other microorganisms by horizontal gene transfer, while also being able to experience adaptive resistance, as is the case of biofilm formation [106].

The long persistence of *P. aeruginosa* infections seems to be related with complex mechanisms of adaptation in which virulence factors are expressed according to the infection stage. Several factors have been accounted for the pathogenic potential of this bacterium, with many playing a role in its biofilm formation and dispersal. Such factors include those related with motility (e.g. flagella, pili) [107], enzymes (e.g. proteases) [108], siderophores (pyoverdine and pyochelin) [109], surfactants (e.g. rhamnolipids) [110], toxins (e.g. exotoxin A, pyocyanin) [111, 112], and the type 3 secretion system (T3SS) [113].

S. aureus is a gram-positive human commensal bacteria frequently found in the mucosal surfaces of the nose and respiratory tract, and on the skin [114, 115]. Consequently, it is easily transmitted by direct contact, making most of the population prone to colonization. In the United States of America, a large part of healthy individuals is colonized with *S. aureus* (30-50%), within which 1% are colonized with methicillin-resistant *S. aureus* (MRSA) [116]. Because of this, *S. aureus* is very often associated with nosocomial infections, since it is commonly transmitted by colonized healthcare staff through direct contact or invasive medical procedures [116]. Its ability to evolve

and adapt to multiple settings has led MRSA to rapid disperse over the globe, being considered a high threat according to WHO [106, 117]. Additionally, MRSA has been developing resistance to virtually all antibiotics classes that are used to treat it, either by making use of its intrinsic resistance factors or by acquiring more through mutations or horizontal gene transfer from other microorganisms [118].

Biofilms of *S. aureus* are related to many serious acute and chronic infections whose treatment can be complicated since many of its clinical isolates are either MRSA or MDR [119, 120]. Furthermore, it has been reported that the presence of *S. aureus* in heterogeneous biofilms increases the rate of plasmid horizontal transfer, which increases the antibiotic resistance of the biofilm [121]. The ability of *S. aureus* to survive host immune defences and cause a diverse range of diseases has been attributed to the expression of a broad set of virulence factors [122]. For example, cell wall-anchored proteins (e.g. clumping factors, fibronectin proteins, protein A, and collagen adhesin) enable tissue attachment and evasion of the host immune system, allowing biofilm formation [123]. Extracellular toxins (e.g. haemolysin, leukotoxin, exfoliative toxin, enterotoxin, and toxic-shock syndrome toxin-1) and enzymes (e.g. coagulase, proteases, and staphylokinase) are secreted to help in tissue penetration and host invasion [122]. Surface-associated factors are down-regulated and surfactants are expressed in later stages leading to biofilm dispersion and infection spreading [114]. The phenotype of small colony variants (SCV) in *S. aureus* has also been linked to infection persistence, as they seem to be able to establish intracellular infection and have a reduced metabolic state, thus lowering antibiotic efficacy [124, 125]. SCV also stimulate a reduced immune response and express increased adhesins and reduced toxins [126].

2.2.1 Quorum-sensing regulation

QS is a communication mechanism that regulates gene expression in response to fluctuations in cell-population density [127]. In QS, bacteria produce signal molecules, termed auto-inducers (AI), whose concentration increases as a function of cell density [128]. Alterations in gene expression occur when the concentration of an AI reaches a minimal threshold [129]. Usually, AI regulate genes encoding virulence factors, such as those involved in biofilm formation and enhanced motility, but they can also coordinate interactions between microorganisms (intra- and inter-species) and between the microorganism and the host [130, 131].

Four main QS systems have been identified in *P. aeruginosa* (**Figure 2.5**), namely the LasI/LasR and the RhlI/RhlR systems [132], the PqsABCDE/PqsR system [133], and the AmbBCDE/IqsR system [134]. Each system has a corresponding AI: 3-oxododecanoyl-L-homoserine lactone (3-oxo-C12-HSL), N-butanoyl homoserine lactone (C4-HSL), 2-heptyl-3-hydroxy-4-quinolone (Pseudomonas Quinolone Signal - PQS), and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (Integrated Quorum Sensing Signal - IQS), respectively [108]. The QS systems regulate the expression of various genes related with motility, biofilm formation, immune evasion, iron scavenging, and antibiotic resistance [135], as well as each other, in a hierarchical manner (**Figure 2.5**).

The virulence factors expressed by *P. aeruginosa* are diversified and several of them play a role in the process of biofilm development [136]. For example, the PqsABCDE/PqsR system is responsible for extracellular DNA (eDNA) and lectin production, which are related to biofilm formation and structural stability [108, 137]. In addition, the RhlI/RhlR system controls the expression of rhamnolipids that influence the late stage of biofilm formation and its dispersal [138, 139]. The latter phenomenon is also controlled by the LasI/LasR system by downregulation the expression of *pel*, a major biofilm matrix component [140]. QS also influences swarming motility, which has been linked to early stages of biofilm formation [141], and is also related to the antibiotic tolerance found in *P. aeruginosa* biofilms but not in planktonic cells [9, 106].

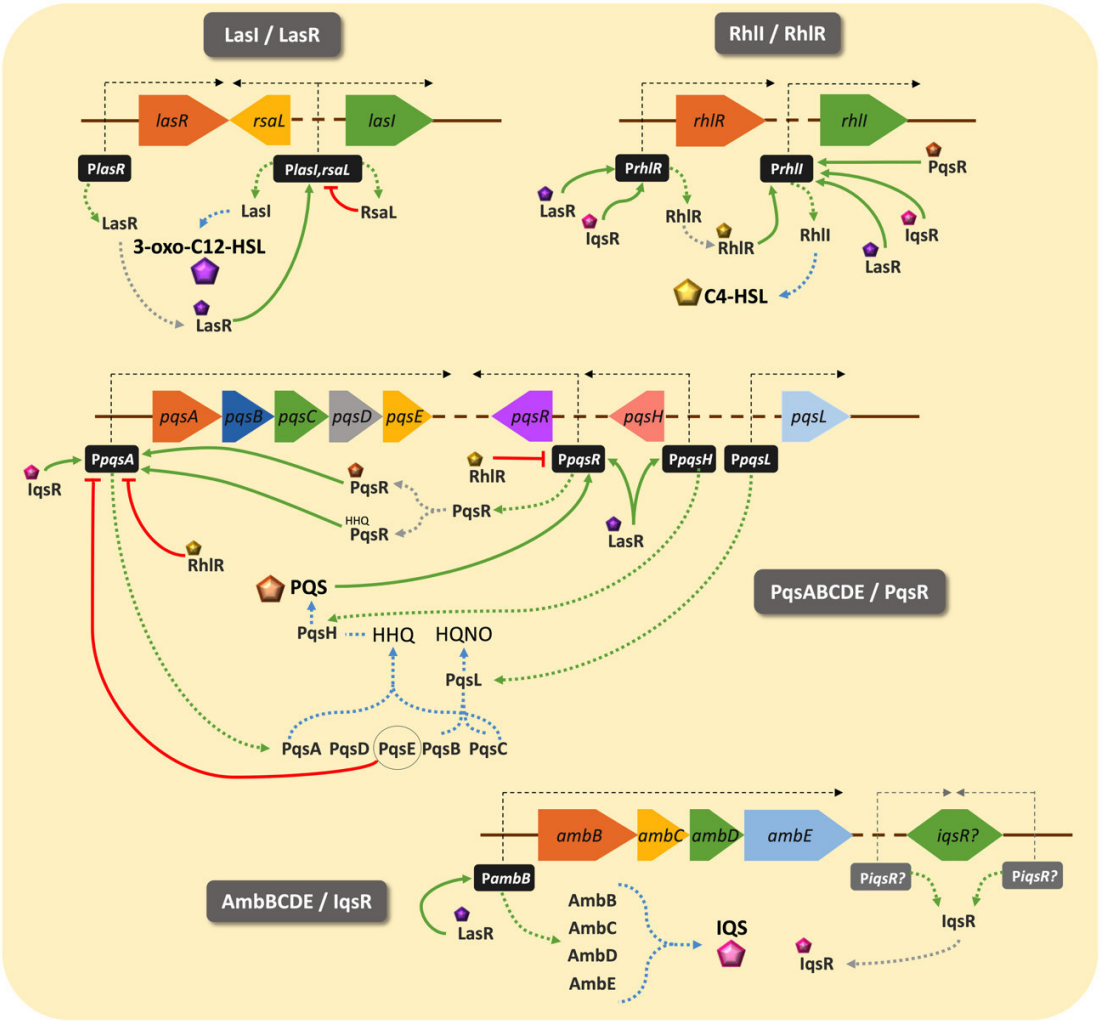


Figure 2.5. Hierarchical regulation of the four QS systems in *P. aeruginosa*. At the top of the hierarchy is the LasI/LasR system, which, when activated by 3-oxo-C12-HSL, upregulates itself (positive feedback loop) and the other three QS systems. The RhlrI/RhlR system also upregulates itself and downregulates the PqsABCDE/PqsR system. The latter also has a positive feedback loop and upregulates the RhlrI/RhlR system. It is noteworthy that this system is responsible for the synthesis of 2-alkyl-4-quinolones, namely PQS, HHQ and HQNO, which are known to influence *P. aeruginosa*/*S. aureus* interaction. Finally, the more recently described AmbBCDE/IqsR system upregulates the RhlrI/RhlR system. **Legend:** solid green arrows: upregulation of gene expression; dashed green arrows: gene expression; dashed blue arrows: synthesis process; red lines with flat ends: downregulation of gene expression; dashed grey arrows: receptor agonism; solid orange arrows: activation process.

As regards to QS in *S. aureus*, this bacterium deploys a wide collection of virulence factors in order to establish and sustain infection that are primarily coordinated by a complex global regulatory QS system known as accessory gene regulator (Agr). This QS system encodes a signaling circuit that produces and senses the extracellular AI autoinducing peptide (AIP) and the intracellular effector RNAIII (**Figure 2.6**) [142, 143], being responsible for inducing the expression of toxins, which

include hemolysins, phenol-soluble modulins (PSM), toxic shock syndrome toxin (TSST), enterotoxins, and Panton-Valentine leukocidin (PVL), among others [144–146]. Apart from toxins, the secretion of several *S. aureus* enzymes, such as proteases, staphylokinase (SAK), and lipases, has been related to the Agr system [143, 147]. Biofilm formation has also been strongly associated with Agr function, with down-regulation leading to excessive biofilm thickness and lack of structuring, and up-regulation inducing biofilm dispersal [148–150], indicating that the absence of Agr functionality may be advantageous for the success of persistent *S. aureus* infections [145, 151]. Also, the emergence of SCV, strongly associated with chronic and recurrent infections and lack of ability to produce cytolytic toxins, is due to reduced Agr activity and disrupted electron transport chain [152, 153].

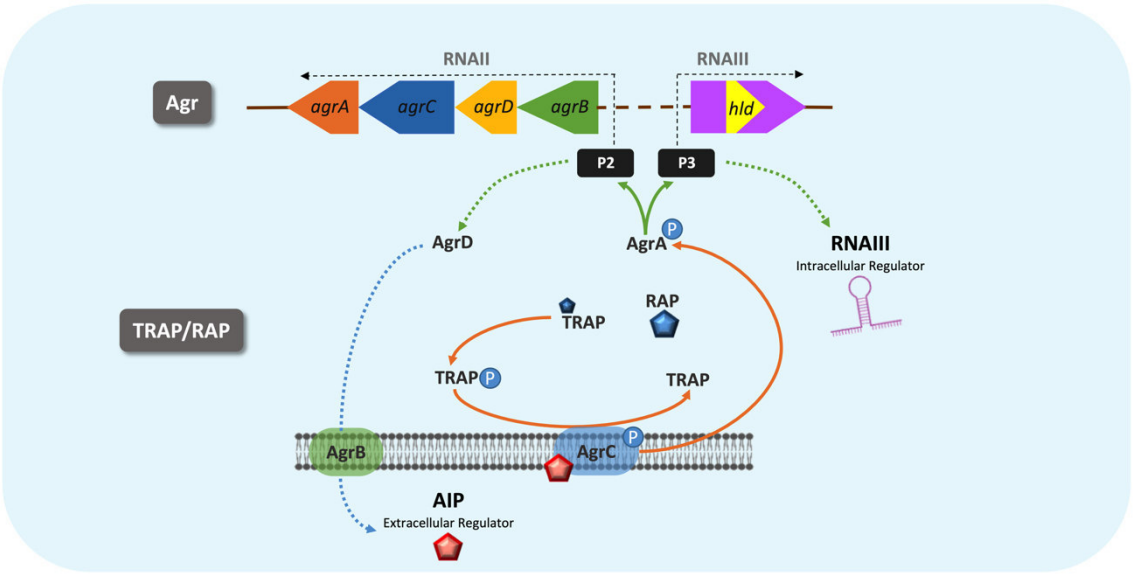


Figure 2.6. *S. aureus* Agr and TRAP/RAP regulatory systems. The *agr* operon consists of two transcriptional units, RNAII and RNAIII, driven by the promoters P2 and P3, respectively. RNAII is a four gene operon, *agr* BDCA, encoding AgrB responsible for processing and exporting AgrD, the AIP precursor. AIP is an exclusively extracellular AI produced in the mid exponential growth phase, whose threshold levels cause AgrC to autophosphorylate, leading to the phosphorylation of AgrA. AgrA activates RNAIII expression, an intracellular effector responsible for increasing the secretion of *S. aureus* toxins and enzymes. In turn, RAP (RNAIII-activating peptide) is another *S. aureus* AI that, in the initial exponential growth phase, induces the phosphorylation of TRAP, its target protein and master regulator of *S. aureus* pathogenesis, activating it. This causes the passage from the planktonic to the biofilm mode of growth and the activation of the Agr system. The two QS systems are therefore connected and are phase-dependent, with AIP indirectly downregulating TRAP phosphorylation. **Legend:** solid green arrows: upregulation of gene expression; dashed green arrows: gene expression; dashed blue arrows: synthesis process; red lines with flat ends: downregulation of gene expression; dashed grey arrows: receptor agonism; solid orange arrows: activation process.

A second QS system, closely related to Agr, is the TRAP/RAP system. The RNAIII-activating peptide (RAP) is an AI that causes phosphorylation of its target protein (TRAP). TRAP is responsible for inducing the production of adhesion proteins, stimulating biofilm formation, and also activates the Agr system. The TRAP/RAP system is mainly active during the early/mid exponential growth phase and it is followed by the Agr system, which is mainly active during the mid/late exponential phase [154]. In addition to these two QS systems, other regulatory systems, namely the staphylococcal accessory regulator (SarA), the *S. aureus* exoprotein (Sae) operon, and the staphylococcal alternative sigma factor B (SigB), regulate the production of virulence factors and intervene in *S. aureus* biofilm formation [143, 155, 156].

2.3 *P. aeruginosa* and *S. aureus*: friends or enemies?

Historically, microbial pathogenesis research has focused on the analysis of infections as monomicrobial occurrences. However, with the advent of culture-independent community analysis methodologies, several diseases had becoming increasingly recognized as having polymicrobial nature, including oral cavity infections [157], diabetic foot wounds [158], bacterial vaginosis [159] and chronic infection in the CF lung [160]. Polymicrobial communities may be defined as a varied collection of organisms, such as fungi, bacteria, and viruses, mostly found thriving in complex mixed biofilms embedded in a self-derived hydrated matrix [161]. Bacteria within polymicrobial biofilms can benefit from the presence of each other through the production and secretion of molecules that other bacteria may utilize (e.g., nutrients), or compete for resources and space (e.g., production of toxins) [162, 163]. These inter-species interactions can be mediated by a large number of mechanisms, which can encompass inter-species signalling, metabolite exchange and cell–cell contact, and are often implicated in the modulation of microbial behaviour, ultimately contributing to disease progression and clinical outcome [164].

2.3.1 Anti-staphylococcal behaviour of *P. aeruginosa*

There have been many studies investigating the inter-species interactions of *P. aeruginosa* and *S. aureus* when in co-culture, as discussed in the next sections. It has been widely accepted that when *P. aeruginosa* and *S. aureus* are grown together, *P. aeruginosa* becomes dominant, outcompeting *S. aureus* [165]. One of the main factors involved in their competitive interaction is the ability of *P. aeruginosa* to produce anti-staphylococcal compounds. Indeed, *P. aeruginosa*

produces several molecules that can affect *S. aureus* growth but without necessarily causing its death. These include HQNO (2-heptyl-4-hydroxyquinoline N-oxide), an exoproduct of the PQS system, that targets the electron transport chain (cytochrome system) and induces *S. aureus* SCV formation [165, 166] (**Figure 2.7A**). Likewise, the QS signal 3-oxo-C12-HSL was shown to inhibit *S. aureus* growth in a dose-dependent manner [167] (**Figure 2.7A**). *S. aureus* SCV, a frequent *S. aureus* phenotype isolated from 4 to 50% of CF patients [168], displayed increased antibiotic resistance and persistence and are associated with worse lung function [169]. Interestingly, it was demonstrated that *S. aureus* cells and their supernatant can also induce a SCV phenotype and antibiotic tolerance in CF strains of *P. aeruginosa* [170, 171].

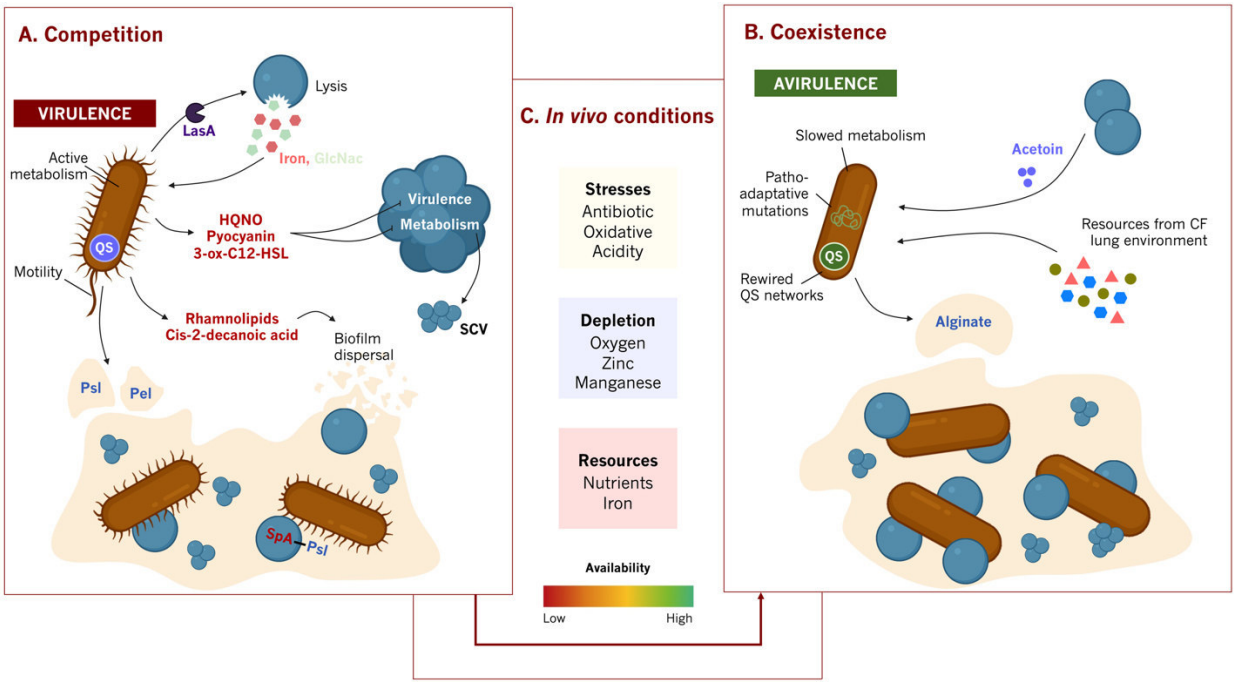


Figure 2.7. Bacterial interactions with *S. aureus* related to *P. aeruginosa* adaptation to CF environment. **(A)** Reference, environmental and acute infection *P. aeruginosa* isolates produce high amounts of virulence factors allowing *P. aeruginosa* to outcompete *S. aureus* by lysis mechanisms, growth, metabolism and virulence inhibition. **(B)** *P. aeruginosa* isolates evolved in CF context co-exist with *S. aureus* due to genetic and transcriptomic adaptations reducing the production of virulence and anti-staphylococcal factors. **(C)** Local conditions affecting interaction state between *P. aeruginosa* and *S. aureus.* QS, quorum-sensing; SCV, small-colony variants; HQNO, 2-heptyl-4-hydroxyquinoline N-oxide; 3-oxo-C12-HSL, N-3-oxo-dodecanoyl homoserine lactone; C4-HSL, butyryl homoserine lactone. Adapted from Camus *et al.* [172]. Created with BioRender.com.

Staphylococcus aureus is at an even further disadvantage by inducing the production of *P. aeruginosa* virulence factors through the release of the peptidoglycan component N-acetyl glucosamine (GlcNAc) upon *S. aureus* cell lysis [173, 174]. In addition, *P. aeruginosa* was shown to produce considerably higher amounts of exotoxin A during co-culture with S. aureus [175]. Exotoxin A is one of the most toxic *P. aeruginosa* virulence factor involved in tissue damage and bacterial invasion [111]. Pernet *et al.* [176] previously demonstrated that *P. aeruginosa* eradicates *S. aureus* through the manipulation of the host environment. Indeed, *P. aeruginosa* secreted the toxin ExoS into epithelial cells, which stimulated the production of the phospholipase sPLA2- IIA, an enzyme that kills *S. aureus*.

As a strategy to limit nutritional competition, *P. aeruginosa* is also able to inhibit *S. aureus* growth by altering its metabolic activity. Indeed, the secondary metabolites HQNO and pyocyanin induce a fermentative metabolism in *S. aureus* cells [124, 166, 167, 177]. Interestingly, *P. aeruginosa* also shifted *S. aureus* from aerobic respiration to fermentation and used the produced lactate as a carbon source [165, 178]. During iron depletion, it has been shown that *P. aeruginosa* uses *S. aureus* as an iron source by lysing its cells thanks to the LasA protease, whose production is regulated by the QS regulator LasR [167, 179, 180].

Another important feature of bacterial interaction is the formation of polymicrobial biofilms. Accordingly, it was demonstrated that *S. aureus* biofilm formation was enhanced by the secondary metabolites HQNO and PQS secreted by *P. aeruginosa* [170]. Conversely, *S. aureus* supernatant can either as well stimulate or inhibit *P. aeruginosa* biofilm formation in a strain-dependent manner [181]. By using *S. aureus* supernatant, it was discovered that Staphylococcal protein A (SpA), a protein known for its interaction with host targets, bound to both *P. aeruginosa* exopolysaccharide Psl and the PilA protein component of the type IV pili [181]. Conversely, *P. aeruginosa* strains that did not produce Psl exhibited inhibition of biofilm formation upon SpA binding to the type IV pili. *P. aeruginosa* strains that produced Psl did not exhibit inhibition of biofilm formation, instead SpA bound to Psl. The authors also revealed that the interaction between SpA and Psl protected *P. aeruginosa* from neutrophils phagocytosis. On the other hand, competitive *P. aeruginosa* strains were able to disperse *S. aureus* biofilm or limit its establishment through the secretion of rhamnolipids and cis-2-decenoic acid [167] (**Figure 2.7A**).

2.3.2 Co-existence interaction status characterization

While there are several *in vitro* experiments that showed that *P. aeruginosa* outcompetes *S. aureus* during co-culture, *P. aeruginosa* strains with more tolerant behaviour toward *S. aureus* have been isolated from chronic CF infections [182–187]. This would suggest that *P. aeruginosa* does not completely eradicate *S. aureus* during infection and they rather reach an equilibrium that balances the presence of both.

Several studies highlighted that the interaction pattern established with *S. aureus* is related with *P. aeruginosa* adaptation to the CF environment. In CF-adapted *P. aeruginosa* isolates , which were recovered several years after their clonal ancestor and presented common pathoadaptive traits, was indeed observed reduced anti-staphylococcal activity [171, 183, 188]. Using an *in vitro* evolution assay, the *P. aeruginosa* adaption to *S. aureus* was investigated the by studying *P. aeruginosa* in the presence and absence of *S. aureus* across 150 generations [189]. Intriguingly, mutations in the LPS biosynthesis pathway occurred only in the presence of *S. aureus* and increased *P. aeruginosa* fitness advantage and an increased resistance to β- lactam antibiotics. Of note, modifications of LPS production, increased antibiotic resistance and downregulation of QS are also frequently observed in CF-adapted *P. aeruginosa* isolates (**Figure 2.7B**) [76, 190, 191]. Baldan *et al.* [182] showed that during mixed-biofilm grown with *S. aureus* early and late CF *P. aeruginosa* isolates presented different behaviours. This latter exhibited better viability with the CF-adapted *P. aeruginosa* isolate and was also able to alter its biofilm formation ability. Other studies addressing co-existing mechanisms were performed using an *in vitro* co-evolution assay of *P. aeruginosa* and *S. aureus* over 21 days [192]. Initially, *P. aeruginosa* dominated over *S. aureus*, however, both species reached a dynamic equilibrium after its 14th passage. Interestingly, the authors also observed that the deletion of *lasR* in *P. aeruginosa* impaired its ability to inhibit *S. aureus* growth during co-culture, confirming that the downregulation of *P. aeruginosa* QS systems contributed to the co-existence of *S. aureus* over time. As *lasR* mutations and QS network rewiring occur during *P. aeruginosa* adaptation to CF lungs, protease activity and especially LasA production are frequently diminished in chronic isolates [193–197]. Indeed, numerous sequencing studies of longitudinal isolates of *P. aeruginosa* revealed that the bacterium accumulates a significant number of mutations during its evolution in CF lungs [191, 198–201]. In connection with this, adapted isolates of *P. aeruginosa* present reduced production of pyocyanin and HQNO [183, 188, 202,

203]. However, detection of pyocyanin and HQNO at active concentrations within CF sputum of chronically infected patients suggests that the isolates evolve differently according to their niches within the lungs, leading to different metabolic activities ([166, 204]. In fact, pyocyanin production among isolates has shown to display high variance within different patients, but also between lineages recovered from a single patient [197].

P. aeruginosa chronic isolates and the CF-adapted lineage DK2 were shown to produce less rhamnolipids than intermittent isolates and reference strains [183, 205]. Rhamnolipid promote *P. aeruginosa* motility [206] and stimulate a stronger inflammatory host response [207]. On the contrary, low rhamnolipid synthesis was linked to alginate overproduction and the common *P. aeruginosa* mucoid phenotype [188, 208]. Mucoid phenotypes of *P. aeruginosa* are associated with a decline of lung function and enhanced resistance to antibiotics [209]. Moreover, it was also discovered that the overproduction of alginate by mucoid strains inhibited *P. aeruginosa* anti-staphylococcal activity, therefore promoting its co-existence with *S. aureus* within the CF lung [188] (**Figure 2.7B**). In line with this findings, it was shown that exogenous alginate protected *S. aureus* biofilms from *P. aeruginosa* killing [208]. Additionally, exogenous alginate decreased the expression of siderophores, rhamnolipids, and PQS [188, 208]. Altogether, this suggests that increased alginate synthesis and reduced rhamnolipid production in adapted *P. aeruginosa* isolates contributes to improve *S. aureus* survival during mixed-biofilm formation (**Figure 2.7B**).

Although less studied, *S. aureus* may also impact the interaction with the co-colonizing *P. aeruginosa* strains and promote its long-term persistence within CF lungs. Repeated *in vitro* co-cultures with *S. aureus* induced a decrease of *P. aeruginosa* QS regulation and may provide a departure point for a co-existing interaction [192]. Additionally, the SCV phenotype appears to be crucial for *S. aureus* survival, as this colony variant presents increased resistance to *P. aeruginosa* mediated killing [167]. Once *P. aeruginosa* has evolved to a non-aggressive status, *S. aureus* can then switch to a non-defective growth mode and co-exist with its partner. Another feature promoting the persistence of *S. aureus* is the improvement of defence mechanisms against bacterial killing. By inhibiting the immune cell function *S. aureus* virulence factor alpha-toxin (AT) potentiates the expansion and rapid dissemination of *P. aeruginosa*, thereby reducing effective killing of both species [210]. The extracellular *S. aureus* protein SpA was also found to inhibit *P. aeruginosa* phagocytosis by neutrophils [181] and killing by tobramycin [211]. Moreover, the impact of *S.*

aureus on the physiology of *P. aeruginosa* was also investigated and the transcriptomic analyses revealed that *S. aureus* downregulated *P. aeruginosa* carbon and amino acid metabolism genes and upregulated genes involved in the acetoin catabolism (*aco*) pathway. In line with this, *P. aeruginosa* presented an enhanced ability to catabolize acetoin produced by *S. aureus* as an alternative carbon source, resulting in increased survival during co-culture (**Figure 2.7B**). Acetoin catabolism was also shown to benefit *S. aureus* in co-culture improving its survival, and therefore promoting persistence of both pathogens [187].

Overall, these studies highlight the complexity of *P. aeruginosa* - *S. aureus* interactions. The anti-staphylococcal behaviour of *P. aeruginosa* is centred on mechanisms of bacterial lysis and suppression of bacterial growth, as well as metabolic alterations, virulence and biofilm formation [167]. However, several studies demonstrated that such behaviour was not only conserved in *P. aeruginosa* CF-adapted strains and both species are able to co-exist [182, 187, 188, 208, 212].

2.3.3 Impact of environmental factors on *P. aeruginosa* - *S. aureus* interactions

As discussed above, bacterial features play an important role in driving *P. aeruginosa* - *S. aureus* inter-species interaction. However, environmental factors can also have a decisive impact on them. Indeed, *P. aeruginosa* adaptation and its interaction with *S. aureus* are critically influenced by environmental factors that characterize *in vivo* conditions. Environmental stressful conditions are known to drive the CF ecosystem by promoting a biofilm mode of growth in the colonizing microorganisms [213, 214]. This is highlighted by Trizna *et al.* [215] where polymicrobial biofilms of *P. aeruginosa* and *S. aureus* altered their spatial organization upon facing stressful conditions such as antibiotic treatment. Initially, *P. aeruginosa* dominated the lower layers within the biofilm whereas *S. aureus* was found within microcolonies in the upper layers. Upon vancomycin treatment the spatial organization of the biofilm changed, and *S. aureus* was found within the middle-lower layers. Recently, it was also observed a decreased production of *P. aeruginosa* virulence factors, such as proteases and pyocyanin, under reduced oxygen conditions [186]. In these conditions, different *P. aeruginosa* strains co-existed with *S. aureus* despite showing a competitive behaviour under anaerobic conditions. This finding is especially relevant as the availability of oxygen in the CF lung is limited due to excessive mucus production and oxygen consumption by host immune cells [186, 216]. Low oxygen conditions attenuate *P. aeruginosa* virulence, which would strongly support the co-existence of *P. aeruginosa* and *S. aureus* during infections [217].

In addition to oxygen, the interaction within biofilm members can also be shaped by nutrient availability [165, 167, 180, 218–220]. Under rich conditions, *S. aureus* is a better competitor of *P. aeruginosa*, the ability to replicate more quickly may explain its ability to outcompete *P. aeruginosa* in high-energy environments [219]. Apart from nutritive richness, limited alkalization was also shown to enhance *S. aureus* survival during interaction with *P. aeruginosa* [220]. Additionally, bacterial relationships are shaped by the presence of host proteins involved in the immune response. Calprotectin, a protein released from neutrophils or other phagocytes, sequesters essential nutrients including iron, zinc, and manganese away from bacteria in order to inhibit their growth [221]. Iron-, zinc-, and manganese-limited environments in turn repress the production of anti-staphylococcal factors in *P. aeruginosa*, promoting increased levels of *P. aeruginosa* and *S. aureus* co-colonization *in vitro* and *in vivo* [222] (**Figure 2.7C**).

Interestingly, the CF ecosystem seem to combine all the environmental factors promoting co-existence interaction between the two pathogens. Sputa from CF patients were shown to be relatively acidic [216], nutrient-rich, and containing limited oxygen concentrations [223]. Moreover, host-pathogen interfaces are known to be depleted in zinc and manganese, especially through sequestration by the host protein calprotectin [222, 224, 225]. Altogether, this evidence suggest that *in vivo* CF environmental conditions may promote co-existence interaction between *P. aeruginosa* and *S. aureus*. Different studies supported this hypothesis by demonstrating high levels of *S. aureus* in the presence of *P. aeruginosa* during *in vivo* mixed-species biofilm [226, 227]. But how co-existence relates to the general conception of worse clinical outcomes for CF patients infected with multiple pathogens? So far, few studies have investigated the impact of *P. aeruginosa* and *S. aureus* co-infection on clinical outcomes. Ahlgren *et al.* [228] did not find a significant clinical difference in adult patients co-colonized with *P. aeruginosa* and *S. aureus* compared to patients colonized solely by *P. aeruginosa*. Additionally, a higher respiratory decline and rate of hospital admission for patients infected by *P. aeruginosa* alone in comparison with patients co-infected by *P. aeruginosa* and *S. aureus* or by *S. aureus* alone was observed [229, 230]. In a recent study the co-existence status of *P. aeruginosa* and *S. aureus* in 52 CF chronically co-colonized patients was investigated [185]. While the authors identified that 65% of *P. aeruginosa* and *S. aureus* strains co-existed, there was no difference in clinical outcomes compared to *P. aeruginosa* mono-infection. In contrast, other studies reported that *P. aeruginosa* and *S. aureus* co-infection is associated with a worse clinical outcome [231–233]. Accordingly, it would be

interesting to study the long-term evolution of both *S. aureus* and *P. aeruginosa* in several co-infected CF patients to obtain information about the bacterial adaptations leading to the establishment of co-existence, together with the impact of that co-existence on clinical outcomes of CF patients.

2.4 *In vitro* models: A need to mimic the host environment

The polymicrobial nature of chronic airway infections can affect health outcomes of patients with CF, mainly due to biofilms formation and the interactions established between the bacterial members of these mixed consortia as discussed above [9], thus efforts have to be put in developing models resembling CF environment and able to study the polymicrobial biofilms infecting CF lungs.

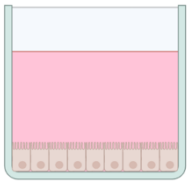
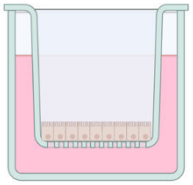
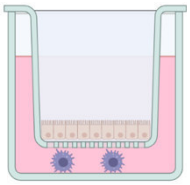
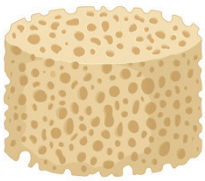
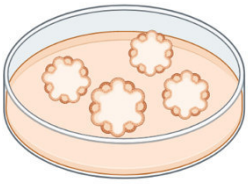
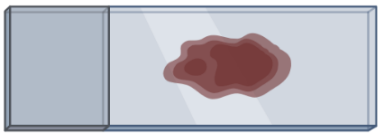
Animal models are thought to capture important aspects of human pathogenesis, such as host immunity and tissue structure, whilst many of the currently available *in vitro* models enable the high-throughput testing of an array of different conditions, antimicrobials and treatment strategies, they do not mimic biofilms found *in vivo* [234]. Notwithstanding, animal models present considerable ethical barriers, are complicated to run and cannot always accurately reflect human physiology and disease [235]. And as our understanding of the biofilms present at infection sites increases, more we uncover that the simplest *in vitro* models fail to consider the host environments. However, for now, no model system seems to capture the true complexity or spatial and temporal heterogeneity of the CF airway. Therefore, to step forward to more robust *in vitro* models, it is necessary to engineering CF models gradually integrating experimental conditions mimicking the *in vivo* scenario (e.g., airway microbiota, airway environment, and host) [236].

Human derived *in vitro* models (**Table 2.1**) offer an alternative approach for studying bacteria-bacteria or bacteria-host interactions and have been increasingly researched and developed in recent years [237–241]. The significant challenge in the development of *in vitro* lung models is finding a balance between a simple and robust system suited for high-throughput analysis and a system recapitulating the full complexity of the infection microenvironment [242]. Perhaps the most significant power of *in vitro* systems is that they can be tailored to address a specific biological question [236]. This configurability of an *in vitro* system, including choice of human cell types (e.g., epithelial, endothelial, immune cells), cell source (e.g., immortalized human cells or primary human cells), extracellular matrix components (e.g., collagen), and 2D- or 3D-dimensional

placement of these components relative to each other, grants exceptional control over the microenvironment and the ability to manipulate the *in vitro* system [237, 240, 243–245]. Further, *in vitro* models of higher level of complexity as the three-dimensional organoid model developed by Wu *et al.* can integrate host immune responses to biofilms upon antimicrobial treatments [246]. Nevertheless, complexity doesn't necessarily always translate to improve the similarity to the infection environment. Accordingly, Cornforth *et al.* [247] examined a variety of *P. aeruginosa* CF models for their accuracy via RNA sequencing data, and the infection transcriptome of *P. aeruginosa* in clinical samples had the highest genome-wide accuracy via an *in vitro* synthetic CF sputum medium model and a CF airway epithelial cell model over a common mouse CF lung infection model. As such, caution is needed when selecting the "right model for the job", and perhaps model selection does require a framework grounded in an evidence-based approach [236, 247].

In vitro models that recreate the host environment seem to be advantageous to explore the mechanisms of microbial interactions that drive clinically relevant phenotypes like persistence, antimicrobial recalcitrance, and virulence factors production, overcoming many of the limitations presented by simpler *in vitro* models and the ethical barriers posed by animal models. Therefore, developing or improving *in vitro* polymicrobial models is a clear and important challenge, and the benefits could be large in terms of understanding the inter-species interactions and host responses, as well as a screening platform for novel therapeutics that specifically target polymicrobial infections.

Table 2.1. Common human derived *in vitro* models to study host-pathogen interactions. Adapted from Barron *et al.* [237]. Cartoons created with BioRender.com.

Model type	Advantages	Disadvantages
Submerged cell line culture 	- Easy to culture - 2–5 days culture period - Less skill required - Readily available/cheap	- Representative of one cell type only - Usually a cancerous cell line; - 2D culture - Not representative of air liquid interface (ALI)
ALI monoculture 	- Representative of ALI condition found *in vivo* - Permits the study of viral entrance and metabolic pathways apically and basally	- More expensive - 3–4 weeks culture period with primary cells - 2D architecture
ALI co-culture 	- Most biomimetic static cell culture available - Representative of multiple cell types and systems found *in vivo* - 2.5D architecture.	- High level of skill needed to culture - 4–6 weeks culture period with primary cells
Polymer scaffolds 	- Ability to house multiple cell types - 3D architecture	- High level of skill and precision needed to slice and culture - Difficulty in monitoring cells within structure
Organoids 	- Derived from stem cells - Representative of the integrated tissue found *in vivo* - 3D structure	- High level of skill needed to culture. - 3–5 weeks culture period - Can't access/monitor apical and internal cell types without disrupting
Precision cut lung slices 	- Fully differentiated tissue - Representative of the heterogeneous phenotypes of population. - 3D architecture. - Culture times are less than that of ALI culture	- High level of skill and precision needed to slice and culture. - Expensive and limited supply.

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CHAPTER 3

P. aeruginosa and *S. aureus* communication in biofilm infections: insights through network and database construction

Summary

The polymicrobial nature of most infections is often characterized by complex biofilm communities, where pathogen interactions promote infection progression and severity. QS, the major regulator of virulence and inter-species communication, is a promising target for new anti-infective strategies. This study aimed at collecting and analysing experimental information on the molecular basis of *P. aeruginosa* and *S. aureus* interactions in biofilms. Data was systematically annotated from relevant full-text papers optimally retrieved from PubMed, reconstructed as networks, and integrated with specialized databases to identify promising antimicrobial targets. Network analysis revealed key entities regulating *P. aeruginosa*/*S. aureus* interactions, for instance the PqsABCDE/PqsR QS system, which affects *S. aureus* growth and biofilm formation. By identifying the most reported *P. aeruginosa* virulence factors affecting *S. aureus*, e.g., HQNO and siderophores, a list of experimentally validated agents affecting those factors, ranging from synthetic drugs to natural plant extracts, was constructed. The complex experimental data on *P. aeruginosa*/*S. aureus* interactions was for the first time systematically organized and made publically available in the new Inter-Species CrossTalk Database (www.ceb.uminho.pt/ISCTD).

Part of the work described in this chapter was published in 2019 in Critical Reviews in Microbiology 45:5-6, 712-728.

3.1 Brief introduction

Despite new insights into the complexity and impact of multi-species infections on human health, the impact of polymicrobial interactions on infectious diseases remains challenging to examine in laboratory context [1–4]. The gap is centred on the dearth of appropriate *in vitro* models that accurately reproduce the host environments, particularly the availability of required nutrients and the impact of host immune factors.

This study discusses various aspects of the social behaviour of *P. aeruginosa* and *S. aureus* interactions in infectious diseases, according to experimental findings reported in scientific literature. The main goals were to map the mechanisms underlying those interactions, through the collection and curation of scientific textual evidence, in order to gain insight into the implications that *P. aeruginosa* and *S. aureus* interactions have on the progression and outcome of polymicrobial infections, and analyse this information to pinpoint critical communication channels to be explored for antimicrobial therapy. Therefore, the main contributions of this work are the reconstruction of a knowledge network of up-to-date experimental results on this subject, the deposition of the gathered data in a newly constructed and publicly accessible database (www.ceb.uminho.pt/ISCTD), and the identification of promising therapeutic targets for *P. aeruginosa*/ *S. aureus* co-infections and their respective reported inhibitors.

3.2 Materials and Methods

This section describes the steps followed to obtain and curate the information on *P. aeruginosa* and *S. aureus* communication. Moreover, it presents the bioinformatics tools available to explore the gathered data.

3.2.1 Information Retrieval and Annotation

The information needed to reconstruct the *P. aeruginosa*/ *S. aureus* communication network was retrieved from PubMed [5], with emphasis put on the compilation of experimentally validated interactions between the two species. PubMed queries were optimized in terms of number of hits (too low: relevant papers were missed; too high: too many irrelevant papers and impracticable annotation effort) and scope, which was narrowed to experiments mentioning the names of the two species and common terminology denoting simultaneous culturing (e.g., co-cultivation, co-

infection, co-culture, polymicrobial, multispecies, mixed-biofilm, etc.). This ensured that PubMed results encompassed almost all the body of work in the field. Subsequently, the relevance of each retrieved document was assessed and relevant information (e.g., textual mentions to organisms, strains, mode of growth, interaction outcome, diseases, and experimental methods) was annotated from the full-text documents. The entity and interaction categories used in expert annotation are described in **Tables 3.1** and **3.2**, respectively.

Table 3.1. Annotated entity categories.

Entity Categories	**Examples**
Cell*	*P. aeruginosa*, *S. aureus*
Gene	*pvl*, *pvdE*, *pchA*
QS Molecule or AI	2-heptyl-4-hydroxy quinoline N-oxide (HQNO), Pseudomonas Quinolone Signal (PQS)
Virulence Mechanism	Biofilm, SCV
Virulence Factor	Siderophores, DesB
Other	Non-virulent proteins (e.g. TatC), metabolites (e.g. acetate), cell components (e.g. peptidoglycan), among others.

Note: * Entity and entity category were termed "cell" when the effector or target was unspecified.

Table 3.2. Annotated interaction categories.

Interaction Category	**Description**
Upregulation	Increase regarding the expression of a gene/protein.
Downregulation	Decrease regarding the expression of a gene/protein.
Stimulation	Increase in the appearance/amount of virulence mechanisms/factors (or other molecules) or bacterial growth/viability.
Inhibition	Decrease in the appearance/amount of virulence mechanisms/factors (or other molecules) or bacterial growth/viability.
Null Effect	No alteration in the expression of a gene/protein or in the appearance/amount of virulence mechanisms/factors (or other molecules) or bacterial growth/viability.
Protection	Protective effect of one bacterium onto another.
Synergism	Aggravation of the co-infection's severity/resilience in comparison with the single-species case.

3.2.2 Information Representation and Integration

The network analysis software Cytoscape [6] was used to represent the gathered data in network format. All the curated data was made publicly available in the newly constructed Inter Species CrossTalk Database (ISCTD), the first database solely focused on microorganism communication, at www.ceb.uminho.pt/ISCTD. All data was transformed into JSON format, and the online database constructed using Visual Studio software and HTML, CSS, JavaScript, and jQuery programming languages. Textual evidences were integrated with pertinent regulatory data in the literature for *P. aeruginosa*. Data on anti-QS agents experimentally validated for *P. aeruginosa* was retrieved from the PCQuorum database [7] and integrated with the new gathered data.

3.3 Results and discussion

3.3.1 Database and Knowledge Network Overview

The gathered data stemmed from an annotation effort that resulted in the reconstruction of a network comprehending a total of 670 interactions between *P. aeruginosa* and *S. aureus* described in 61 different scientific publications (dating from 1993 until October 2019). The annotated information on *P. aeruginosa/S. aureus* interactions was made publicly accessible at www.ceb.uminho.pt/ISCTD, in which users can perform searches and navigate through the annotated data. As illustrated in **Figure 3.1**, database users are allowed to do more or less refined searches depending on their specific interests. Specifically, users may search for interactions based on one or two specific entity categories (source and target) or considering a specific interaction direction (*P. aeruginosa* > *S. aureus* or *S. aureus* > *P. aeruginosa*) (**Figure 3.1A**). The respective data table is generated, showing an organized view of all interaction details, such as organism strains, mode of growth, experimental methods, observations made by the experts, and PubMed references. Users can then narrow down their search by querying for specific terms within the generated table (**Figure 3.1B**), for example "biofilm".

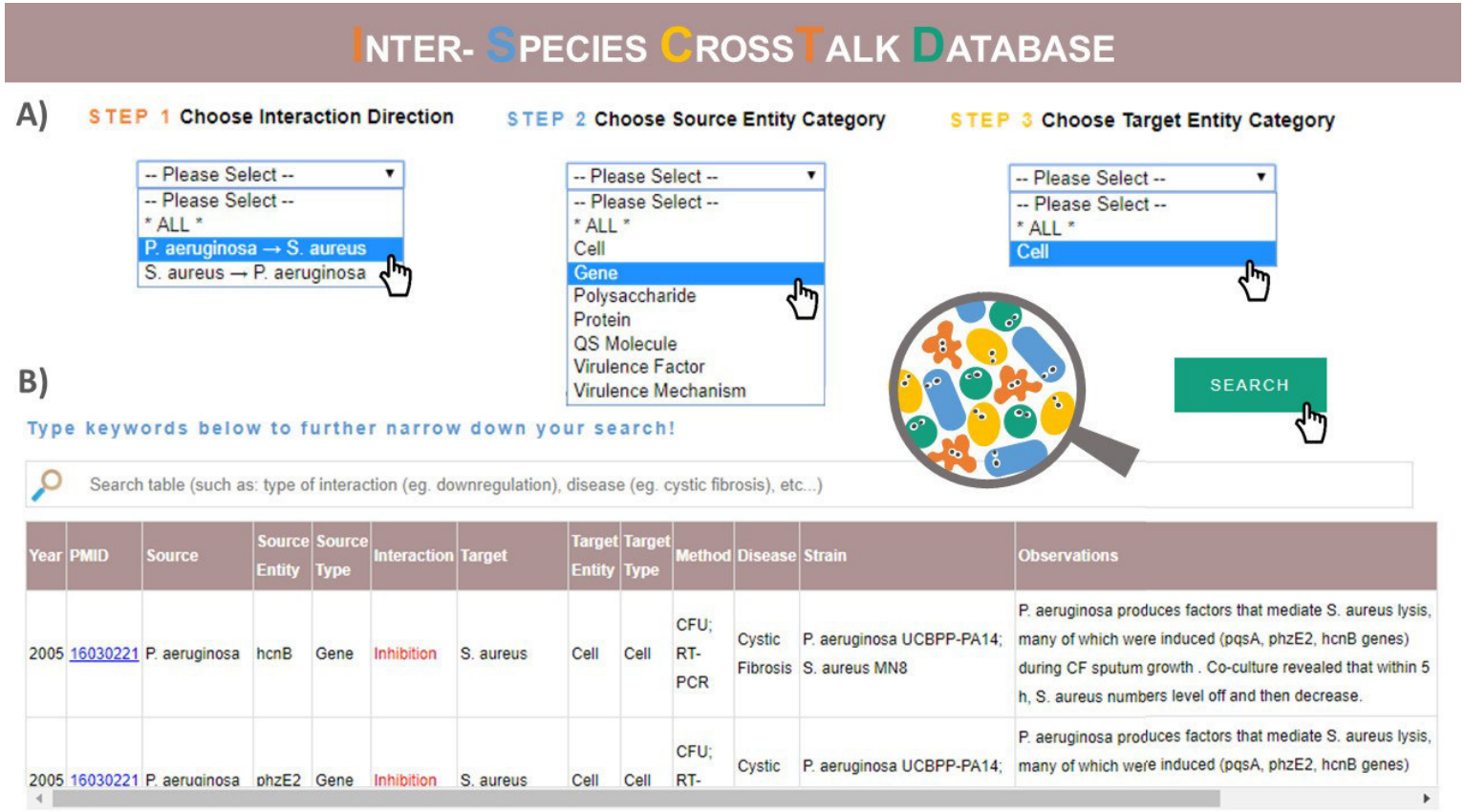

Figure 3.1. Schematization of the search flow available in the ISCTD. Users go through **(A)** a selection flow in which they can specify the interaction direction and the source and target entity categories. When hitting "Search", the database automatically outputs **(B)** a table containing all annotated data for that search. Users can then search keywords within the table in the search panel. In this figure, the exemplified table shows the effect of P. aeruginosa genes on S. aureus.

Figure 3.2 depicts a representation of this curated knowledge network, illustrating the complexity and the wide array of entities so far reported as involved in this inter-species interaction. This complexity is also demonstrated when taking into consideration the different types of interactions, both positive (e.g., stimulation, upregulation) and negative (e.g., inhibition, downregulation), that are established between both bacteria.

All the annotated data is summarized in **Table 3.3** according to interaction directionality, i.e., *P. aeruginosa* > *S. aureus* or *S. aureus* > *P. aeruginosa* interactions, and mode of growth. Interestingly, the majority of the annotated interactions have *P. aeruginosa* as the source microorganism (64%), which is corroborated by **Figure 3.2A** in which it is clear that the *S. aureus* node has a greater number of inward edges (interactions) than the *P. aeruginosa* node. In terms of the mode of growth used in all the analysed studies, no major differences were seen between biofilm and planktonic growth, with 46% of studies performed on biofilms and about 44% on planktonic bacteria. *In vivo* was the least studied setting, representing only 11% of the studies, and was mainly related to clinical studies of human samples rather than *in vivo* laboratorial testing. Looking into studies concerning a disease scenario, the majority were CF related (**Table 3.3**). Although *S. aureus* is typically one of the primary microorganisms isolated in CF lungs during early childhood, it is quickly followed by *P. aeruginosa*, which progressively increases its prevalence as patients grow older [1, 8]. The problematic of CF lung infection is a great example of the interplay between the two species and may explain why the majority of the studies focus on disclosing the mechanisms through which *P. aeruginosa* and *S. aureus* interact in the CF lung. Concerning the most reported effector (source) entities, most annotated interactions reported effects of bacteria as a whole (annotated as "cell") (**Table 3.3**), meaning that no molecular entity was identified/tested. Most annotated interactions, except those annotated in *in vivo* studies, have genes as the top target category (**Table 3.3**). However, this does not translate to a high number of studies analysing gene modulation, which comprise only 11% of the total analysed, but rather reflects the great amount of results that more in-depth molecular methodologies, such as microarrays and RNA sequencing, output, each translating into an annotated interaction. The concomitant analysis of both bacteria grown as a dual-species biofilm demands the adaptation and optimization of methodologies typically designed for single-species analysis. This setback can be what is currently impairing a greater use of in-depth methods for the analysis of *P. aeruginosa* and *S. aureus* interactions at the molecular level.

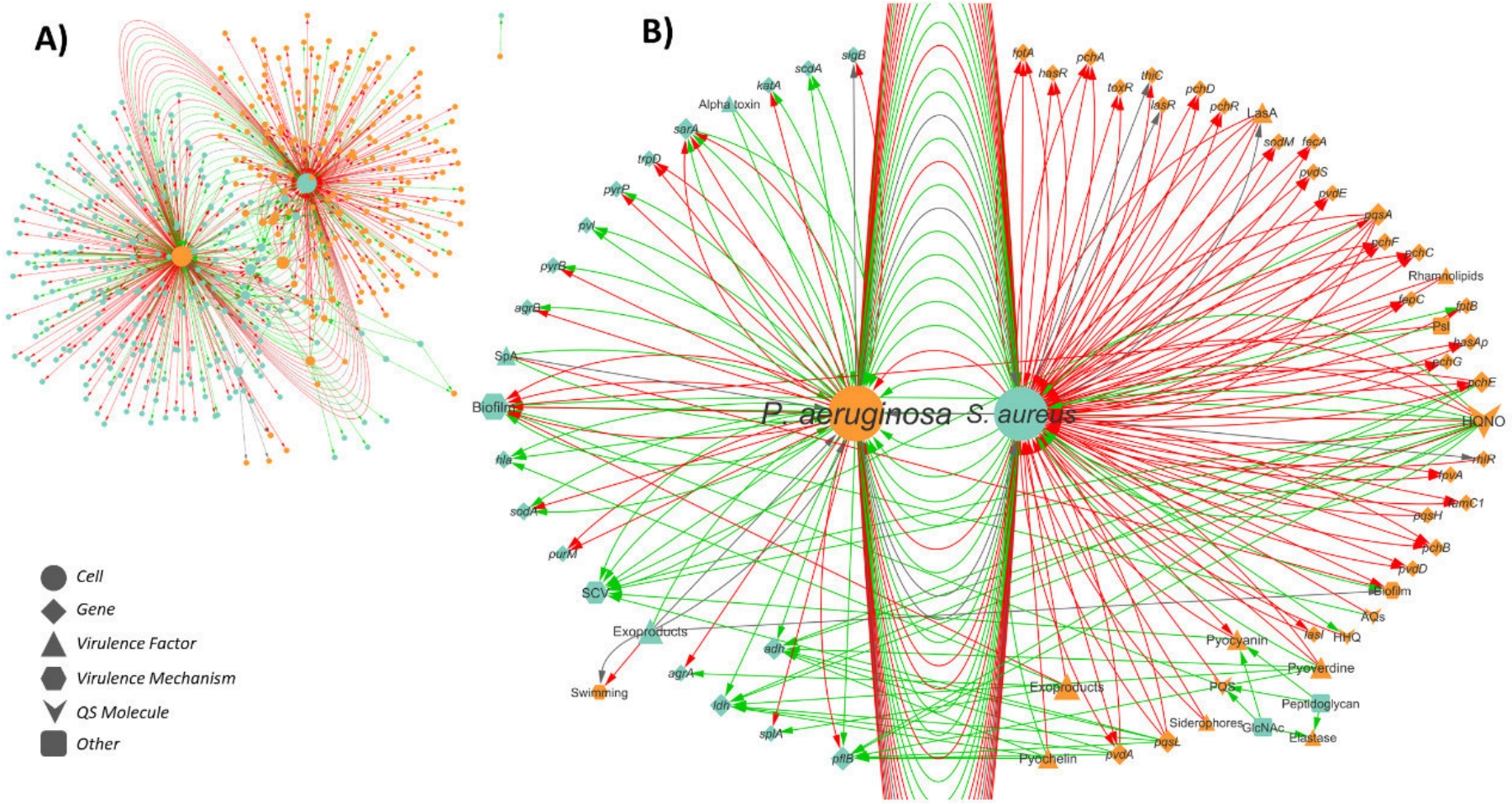

Figure 3.2. *P. aeruginosa* - *S. aureus* interaction network. **A)** Overview of all annotated interactions reported in the scientific literature; **B)** Most reported interactions (annotated more than once). **Legend:** green arrows: positive interactions (e.g.: upregulation, stimulation); red arrows: negative interactions (e.g.: downregulation, inhibition); grey arrows: null effect *P. aeruginosa* and *S. aureus* nodes are depicted in orange and blue, respectively; node and node label sizes are directly proportional to the number of related (outward and inward) edges (interactions).

Table 3.3. Top 3 curated information for *P. aeruginosa/S. aureus* interactions based on the source organisms and mode of growth.

Source organism	No. of interactions	Mode of growth	No. of interactions	Source entity[1]	Target entity category[1]	Interaction category[1]	Disease[2]	Experimental method[2]
P. aeruginosa	432 (64%)	Biofilm	308 (71%)	Cell (83%) HQNO (2.9%) Pyochelin, Pyoverdine (1.6%)	Gene (79%) Cell (25%) Virulence Mechanism (5.2%)	Downregulation (56%) Upregulation (23%) Inhibition (11%)	CF (54%) Wounds (21%) Chronic suppurative otitis media, Dental infections (3.6%)	CFU (37%) RT-PCR (12%) Crystal violet assay (7.7%)
		Planktonic	116 (27%)	Cell (60%) Exoproducts (7.8%) HQNO (6.9%)	Gene (55%) Cell (38%) Virulence Factor (4.3%)	Inhibition (34%) Upregulation (30%) Downregulation (25%)	CF (58%) Chronic lung infection, Wounds, Peritoneal infection (4.2%)	CFU (20%) RT-PCR (13%) Spectrophotometry (8.7%)
		In vivo[3]	8 (2%)	Cell (100%)	Cell (88%) Virulence Mechanism (13%)	Synergism (75%) Inhibition, Stimulation (13%)	CF (100%)	Lung function evaluation, Bacterial cultivability (38%) Bacterial lawn growth, Colony morphology analysis (13%)
S. aureus	238 (36%)	Biofilm	39 (16%)	Cell (82%) SpA (10%) Exoproducts (7.8%)	Gene (67%) Cell (26%) Virulence Mechanism (5.2%)	Upregulation (33%) Downregulation (28%) Null Effect (15%)	CF (45%) Wounds (36%)	CFU (23%) RT-PCR, Crystal violet assay (14%) RNAseq (9.1%)
		Planktonic	193 (81%)	Cell (91%) Exoproducts (3.1%) GlcNAc, Peptidoglycan (1.6%)	Gene (85%) Cell (5.2%) Virulence Factor (4.2%)	Downregulation (68%) Upregulation (15%) Stimulation (7.8%)	CF (57%) VAP, Wounds, Peritoneal infection (7.1%)	CFU, RT-PCR (13%) Motility assay in agar, Flow cytometry, Spectrophotometry (6.7%)
		In vivo[3]	6 (3%)	Cell (100%)	Cell (100%)	Synergism (100%)	CF (100%)	Lung function evaluation, Bacterial cultivability (50%)

Note: [1] % relative to the total number of annotated interactions for the respective mode of growth. [2] % relative to the sum of the number of different diseases or experimental methods per document for the respective source organism and mode of growth. [3] Clinical studies. HQNO – 2-heptyl-4-hydroxy quinoline N-oxide; GlcNAc – N-Acetylglucosamine; SpA - staphylococcal protein A; GlcNAc - N-Acetylglucosamine; VAP – ventilator associated pneumonia; CFU – colony forming units; RT-PCR – reverse transcriptase-polymerase chain reaction; RNAseq – RNA sequencing

Concerning the most reported effector (source) entities, most annotated interactions reported effects of bacteria as a whole (annotated as "cell") (**Table 3.3**), meaning that no molecular entity was identified/tested. Most annotated interactions, except those annotated in *in vivo* studies, have genes as the top target category (**Table 3.3**). However, this does not translate to a high number of studies analysing gene modulation, which comprise only 11% of the total analysed, but rather reflects the great amount of results that more in-depth molecular methodologies, such as microarrays and RNA sequencing, output, each translating into an annotated interaction. The concomitant analysis of both bacteria grown as a dual-species biofilm demands the adaptation and optimization of methodologies typically designed for single-species analysis. This setback can be what is currently impairing a greater use of in-depth methods for the analysis of *P. aeruginosa* and *S. aureus* interactions at the molecular level.

3.3.2 *P. aeruginosa* and *S. aureus* Biofilm Network

As stated, biofilms are known to be the mode of growth of up to 80% of human bacterial infections [9]. Thus, although planktonic testing is practical and informative, studies entailing biofilms do a better job at mimicking a real-life infection scenario. The biofilm, planktonic, and *in vivo P. aeruginosa/S. aureus* interaction networks are represented in **Figure 3.3**. Biofilms represent the mode of growth of 52% of the total annotated interactions, which is still far from ideal. Interestingly, when looking into *P. aeruginosa > S. aureus* interactions, most of them were reported in biofilm scenarios (71%), with only 27% using planktonic growth (**Table 3.3**). Regarding *S. aureus > P. aeruginosa* interactions, planktonic was the most used mode of growth, namely in over 80% of the annotated interactions. This is also observable in **Figure 3.3**, where there is a clear contrast between the number of edges, i.e., interactions, targeting each bacterium when comparing the biofilm (**Figure 3.3C**) and the planktonic (**Figure 3.3A**) networks. This observation illustrates a gap in studies concerned with the effect of *S. aureus* on *P. aeruginosa*, revealing an urgent need for more biofilm testing in order to originate a levelled understanding of the bidirectional interplay of both species within mixed consortia.

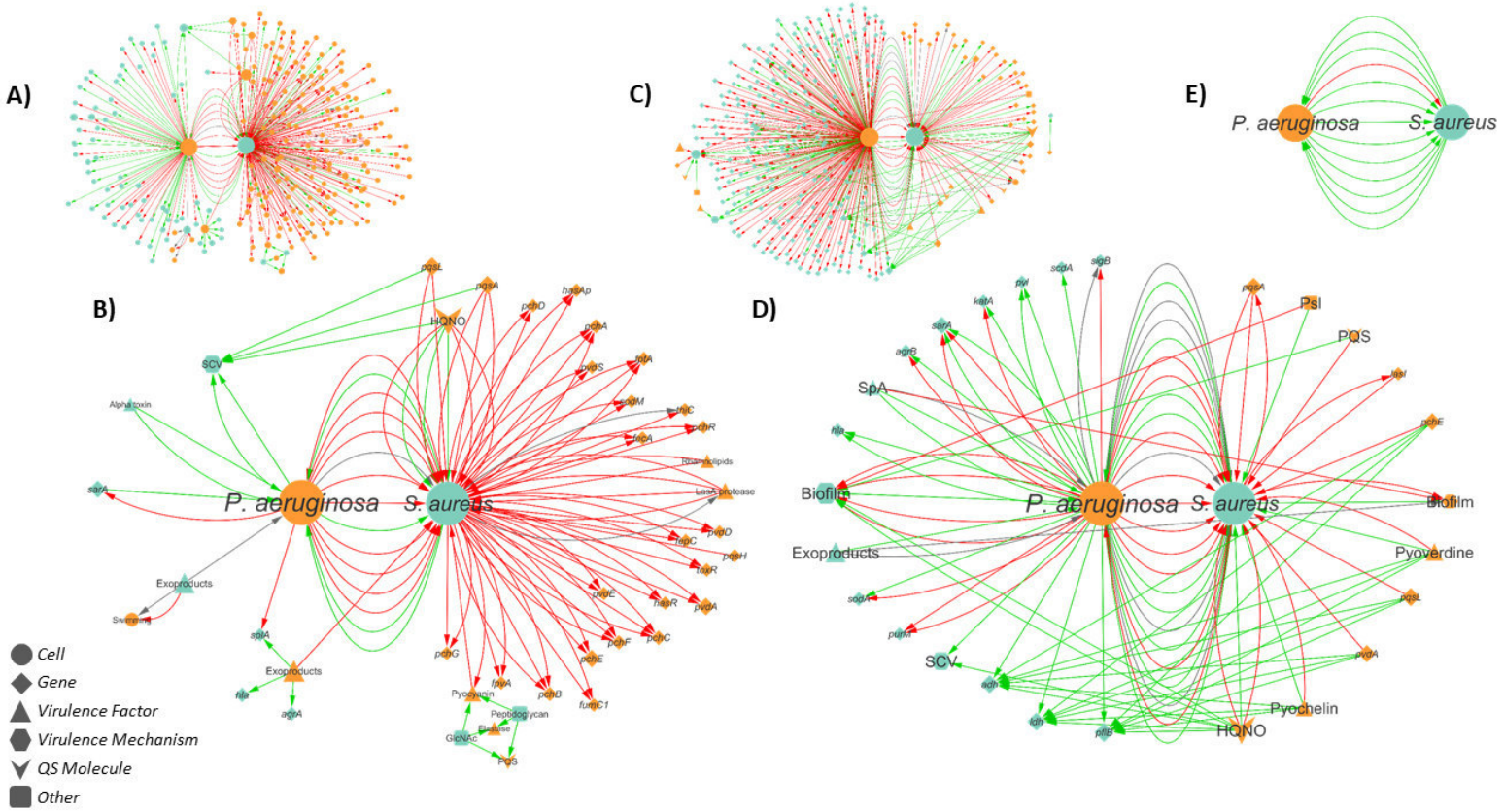

Figure 3.3. *P. aeruginosa*/ *S. aureus* interaction networks in planktonic, biofilm, and *in vivo* settings. A) All annotated interactions in the planktonic mode of growth; **B)** Most reported interactions (annotated more than once) in the planktonic mode of growth; **C)** All annotated interactions in the biofilm mode of growth; **D)** Most reported interactions (annotated more than once) in the biofilm mode of growth; **E)** All annotated interactions *in vivo*. **Legend:** green arrows: positive interactions (e.g.: upregulation, stimulation); red arrows: negative interactions (e.g.: downregulation, inhibition); grey arrows: null effect *P. aeruginosa* and *S. aureus* nodes are depicted in orange and blue, respectively; node and node label sizes are directly proportional to the number of related (outward and inward) edges (interactions).

The most annotated effector entities, apart from "cell", for *P. aeruginosa > S. aureus* biofilm interactions were HQNO, pyochelin, and pyoverdine (**Table 3.3**), as best represented in **Figure 3.3D**. HQNO is a major compound produced by the *pqsABCDE* operon of *P. aeruginosa* and is described as an anti-staphylococcal molecule acting through the inhibition of the oxidative respiration [10]. In turn, pyoverdine and pyochelin are the two major *P. aeruginosa* siderophores, i.e., iron-chelating molecules, produced in iron-limited conditions [11] that are able to inhibit *S. aureus* biofilm formation. In fact, it has been shown that HQNO and the two siderophores are both required for efficient killing of *S. aureus* by *P. aeruginosa* [4]. In turn, in S. *aureus > P. aeruginosa* interactions, the most annotated source entities, apart from "cell", were, exoproducts and the SpA (**Table 3.3**), as best shown in **Figure 3.3D**. SpA is a secreted factor that not only mediates interaction with host cells but has also been shown to bind cell surface structures of *P. aeruginosa* [12]. The testing of exoproducts was generally used in all scenarios and interaction directions, except for *in vivo* settings. Both HQNO and SpA are examples of exoproducts, but the general term here annotated was stemmed from studies where a specific molecule was not tested but rather the cell-free supernatant of the bacterial culture. This was an expected feature of these studies given that inter-species communication and QS are mainly based on the production and excretion of molecules to the extracellular media that are sensed by other cells.

As stated, gene was the most annotated target type in all scenarios except "*in vivo*". For *S. aureus > P. aeruginosa* interactions, most annotated target genes in planktonic settings related to the iron binding and transport. Iron is a vital nutrient for bacterial growth but relatively scarce in most infection sites, acting as a limiting factor [13]. *P. aeruginosa* secretes exoproducts that negatively impact *S. aureus* growth and survival causing cell death and subsequent iron release, which can be further utilized by *P. aeruginosa* for its own benefit [14]. The presence of *S. aureus* during co-culture seems to be related with a decreased transcription of *P. aeruginosa* iron regulated genes as 51% of annotated target genes pertained to this category, with 96% of them being downregulated, indicating that the presence of *S. aureus* increases usable iron for *P. aeruginosa*. In biofilm settings, QS related genes, such as *rhlR, pqsH*, and *lasI,* were one of the top annotated targets (23%). Looking at the types of established interactions, it is not surprising that *P. aeruginosa* QS genes were downregulated or unchanged in biofilms where *S. aureus* was present. *P. aeruginosa* produces multiple virulence factors that contribute to the removal of *S. aureus* from dual-species biofilms, with the LasI/LasR, PqsABCDE/PqsR, and RhlrI/RhlR systems being the

major regulators of these factors [15]. In fact, when *P. aeruginosa* mutants lacking one or both major QS genes (*lasI, rhlI*, and *lasI/rhlI*) were co-cultured in dual-species biofilms with *S. aureus*, a less effective removal of *S. aureus* was observed, suggesting that *S. aureus* thrives when *P. aeruginosa* QS is inactivated [16].

As to *P. aeruginosa > S. aureus* interactions in planktonic settings, most annotated target gene pertained to functions related to molecule biosynthesis (35%), mostly of purines and pyrimidines, key components of DNA/RNA synthesis and whose production was upregulated. This could point to an early state of nitrogen starvation in co-culture [17]. In biofilm settings, most annotated targets were related with ion and molecule transmembrane transport (17%) and biosynthesis (13%), mostly downregulated (77% and 93%, respectively), which may contribute to a state of metabolic quiescence, decrease in biofilm formation (Miller et al. 2017), and, possibly, SCV induction.

3.3.3 Discerning *P. aeruginosa > S. aureus* communication in biofilms

In clinical settings, biofilm infections caused conjointly by *P. aeruginosa* and *S. aureus* are more virulent than those caused by each independent species, and are frequently associated with increased disease severity and chronicity [18]. The effect of *P. aeruginosa* in *S. aureus* appears to be preferably studied and will be used here to showcase the complexity and diversity of established interactions. As demonstrated in **Figure 3.4**, a complex and intricate network of regulators dictates the expression of pathogenicity factors in *P. aeruginosa*, which play an important role in its social interplay with *S. aureus* within the dual-species biofilms. *P. aeruginosa* secretes several extracellular substances, such as hydroxy-2-alkylquinolines (PQS and HQNO) and hydrogen cyanide, which hinder the proliferation of *S. aureus* biofilms [19–21]. Both PQS and HQNO are products of the PqsABCDE/PqsR QS system, whose action accounted for 18 of the biofilm annotated interactions in over 5 different documents, including stimulation of *S. aureus* biofilm formation and *S. aureus* growth inhibition (**Figure 3.4**)

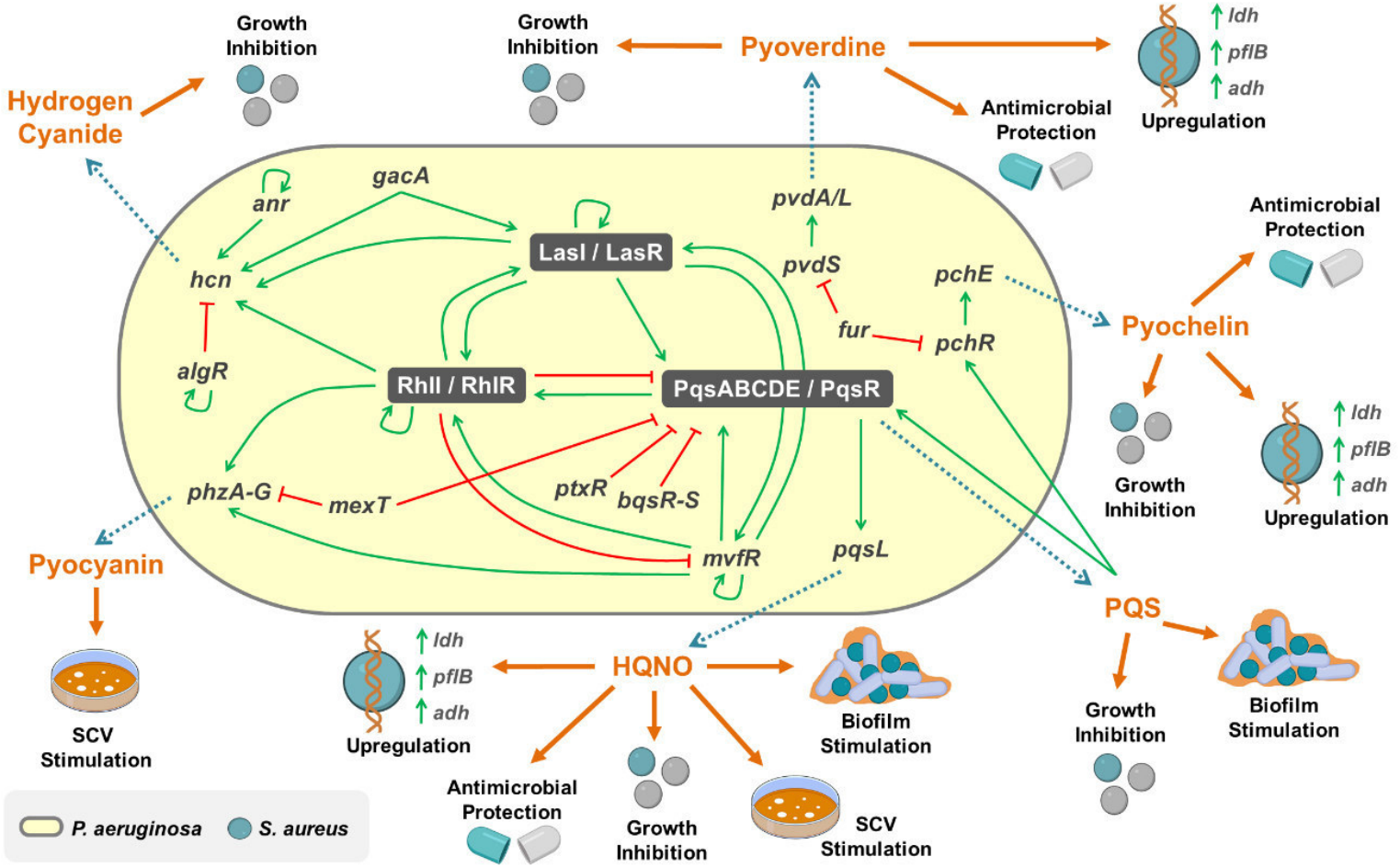

Figure 3.4. Schematic representation of the effect of *P. aeruginosa* on *S. aureus* in dual-species biofilms. Annotated interconnections between the molecules produced by *P. aeruginosa*, their effect on *S. aureus*, and the main regulatory network involved in *P. aeruginosa* > *S. aureus* interactions is depicted. **Legend:** solid green arrows: upregulation of gene expression; red lines with flat ends: downregulation of gene expression; dashed blue arrows: synthesis process; solid orange arrows: effect on *S. aureus.*

In fact, out of the existing four, this QS system was the most reported as acting, directly or indirectly, upon *S. aureus*, followed by the RhlI/RhlR system. PQS is a signalling molecule that positively regulates a subset of QS dependent genes involved in siderophore-mediated iron uptake and iron chelating activity in *P. aeruginosa* [22]. Specifically, PQS can prompt the expression of genes involved both in the regulation (*pvdS*) and biosynthesis of pyoverdine (e.g. *pvdA* and *pvdE*) and pyochelin (*pchE*) [23]. Despite the reported inhibitory effect of pyoverdine and pyochelin on *S. aureus* (Figure 3.4), both siderophores were found to be involved in its protection from antibiotic treatment in a biofilm model of CF infection [24].

Indeed, the growth of *S. aureus* is not completely inhibited by *P. aeruginosa. S. aureus* has defence mechanisms that help it outcompete *P. aeruginosa* in the same infection, thus coexisting as a persister [25]. For instance, *P. aeruginosa* excretes HQNO, which activates the alternative sigma factor B in *S. aureus*, and alters the expression of several virulence factors, including those that regulate adherence, invasiveness, and persistence within host cells, and facilitates the emergence of SCV [26]. The SCV phenotype can survive in proximity with *P. aeruginosa*, which allows *S. aureus* persistence. The stimulation of biofilm formation by PQS and HQNO also contributes to this persistence, as biofilm-enclosed cells become more protected from external stresses [27].

Although the exact regulatory mechanisms behind *P. aeruginosa/S. aureus* communication in biofilms are not fully clear, the reconstructed knowledge-networks highlight the complexity and multi-tiered regulatory processes by which *P. aeruginosa* controls expression of virulence factors that critically affect *S. aureus*, impacting the pathogenicity of the biofilm-related infection. Specifically, the networks revealed that *P. aeruginosa* QS signalling plays an important role in this dual-species interplay, since three QS systems, namely the systems LasI/LasR, RhlI/RhlR and PqsABCDE/PqsR, positively regulate the expression of specific *P. aeruginosa* virulence factors affecting *S. aureus*.

Besides QS, the integrated regulatory network of *P. aeruginosa* shows that other genes can also play key roles and control multiple pathways that trigger inter-species interactions. For example, PtxR is a transcriptional regulator that reduces the expression of the pqsABCDE operon (Figure 3.4), which negatively affects the production of pyocyanin [28]. Stressful environmental conditions may also be controlling the differential expression of the regulatory cascade. For instance, *mvfR* is involved in regulating critical physiological processes, being known to control the transcription of

iron-related genes, under low-iron concentrations [29], and upregulating the transcription of the three QS systems and pyocyanin synthesis (Figure 3.4). Once intracellular iron levels are high, uptake systems and their regulators, including *mvfR*, are repressed by the *fur* transcriptional regulator [30]. This negative feedback loop also turns down the production of all other systems under *mvfR* control (e.g., pyocyanin and HQNO synthesis) [31]. Under low oxygen or anoxia, *anr* controls hydrogen cyanide production and other cellular responses, including biofilm formation associated with chronic infections [32].

3.3.4 Targeting *P. aeruginosa/S. aureus* biofilm communication

Antivirulence agents are presented as alternative therapeutics that can circumvent antibiotic resistance by targeting virulence factors rather than bacterial growth pathways (i.e., the target of traditional antibiotics) [33]. This strategy is quite advantageous considering the large number of putative virulent targets [34]. Since QS is the main virulence regulator in bacteria, inhibition of QS mechanisms using quorum quenching (QQ) agents appears to be a promising strategy to modulate the virulence of bacterial pathogens [35]. Moreover, as the target may constitute an extracellular factor, the emergence and spread of resistance could be less likely [36]. Nevertheless, the selection of the target is of critical importance for the effectiveness of the antimicrobial strategy. In line with this, it is essential to understand the full dynamics of action of the targeted virulence factor as well as the dynamics of production [37]. The expression of certain virulence factors could be subjected to the control of several regulatory mechanisms other than the targeted QS system [38].

Figure 3.5 summarizes the antivirulence agents that have so far shown inhibitory effect against two major virulence factors produced by *P. aeruginosa* and annotated as affecting *S. aureus* in biofilm settings, HQNO and siderophores (pyoverdine and pyochelin). A search was made in http://pcquorum.org/ for inhibitors targeting not only the molecules of interest but also their coding genes. A total of 25 and 19 reported agents were encountered inhibiting HQNO and siderophores, respectively, with four of them being active against both pyoverdine and pyochelin (**Figure 3.5**).

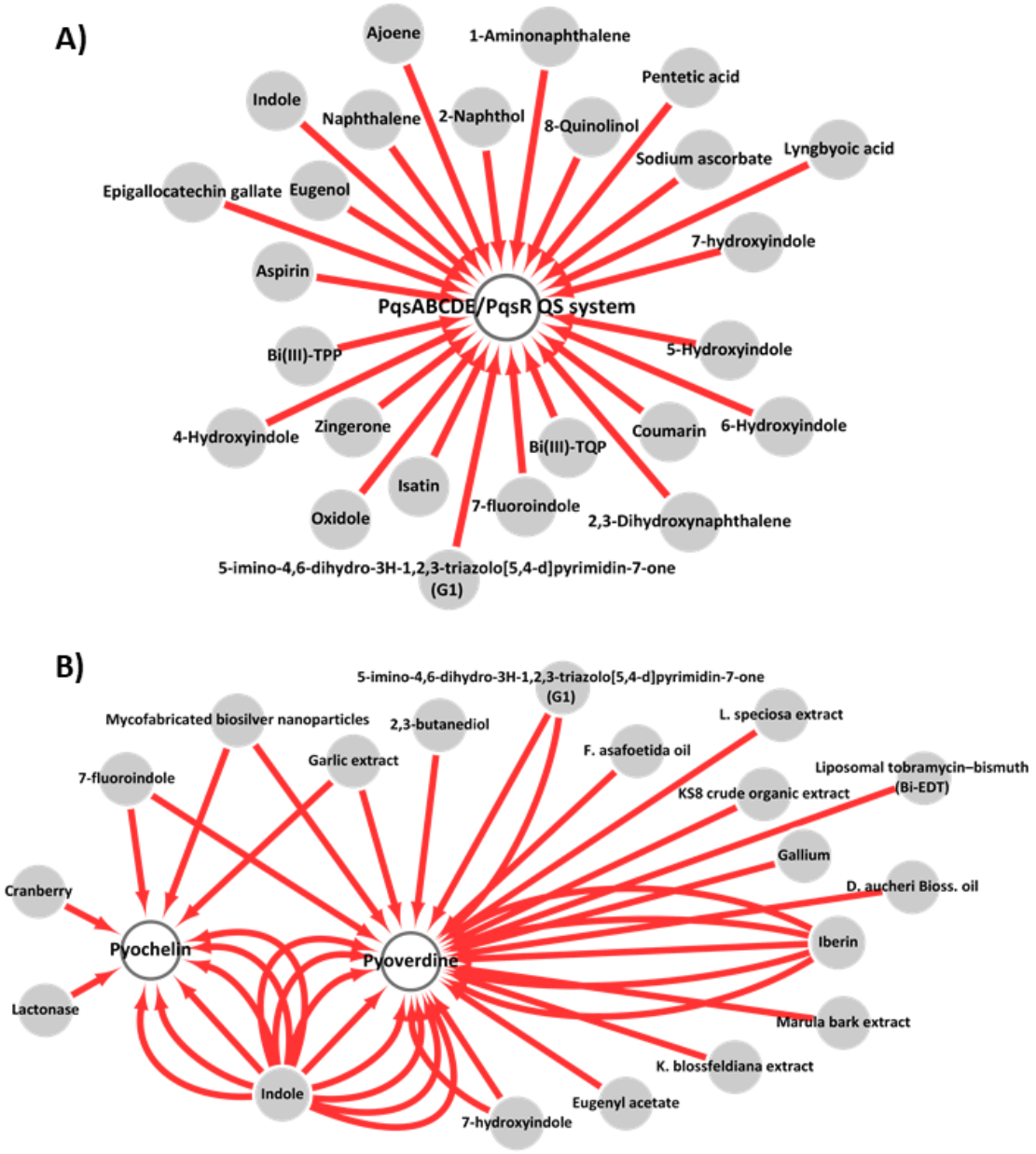

Figure 3.5. Networks of antivirulence agents targeting: A) the PqsABCDE/PqsR QS system and **B)** the siderophores pyoverdine and pyochelin.

Although these antivirulence agents may be promising for attenuating *S. aureus* and *P. aeruginosa* resilience in co-infection scenarios, this antivirulence strategy has to face several challenges to become a feasible therapeutic option. One of the challenges in disrupting QS networks is the fact that, in some cases, the interference with the target could promote virulence instead of attenuating it [39]. This point is even more relevant when considering polymicrobial infections. Because inter-species interactions could be mediate by QS-controlled factors, the interference with a QS system in a pathogen potentially could facilitate the pathogenicity of the co-infecting species [40]. For

example, the PqsABCDE/PqsR system influences the production of extracellular DNA [41] and lectins, which influences biofilm formation and enhances colonisation and infection establishment in *P. aeruginosa* [15]. In addition, this system positively regulates HQNO levels, involved in *P. aeruginosa/S. aureus* interactions (**Figure 3.4**). Depending on the environmental conditions found by these pathogens, different interactions were annotated, including stimulation of *S. aureus* SCV, antimicrobial protection, and inhibition of *S. aureus* growth. Therefore, the interference with the QS system could reduce the overall virulence, by inhibiting *P. aeruginosa* biofilm formation and possibly preventing the formation of *S. aureus* SCV. On the other hand, as HQNO inhibits *S. aureus* growth, the antivirulence therapies targeting this molecule could backfire and facilitate the spreading of *S. aureus.*

Consequently, the choice of antivirulence agents must pay attention to the multi-species community, i.e., the impact on the remaining co-occurring pathogens must be considered as it can have a positive, hence undesired, effect on them. Moreover, to increase antimicrobial effectiveness in polymicrobial communities, empiric therapy would probably require the combination with other antimicrobial agents (including other antivirulence drugs or even antibiotics). Very few studies have tackled this approach in double-species biofilms of *P. aeruginosa* and *S. aureus.* The only example found in the literature using an adapted optimized query is the use of the QS inhibitor hamamelitannin targeting the RAP/TRAP QS system in *S. aureus*, which was combined with vancomycin in a gauze and successfully reduced biofilm formation of both species in an *in vitro* mixed biofilm model of chronic wound [42]. Although antivirulence approaches have substantially progressed, it appears that the search for antivirulence agents is still a challenging and unexplored area of investigation, with most of these antivirulence drugs tested *in vitro* and against single-species populations [7, 43].

Overall, this work was able to decipher the current knowledge on the complex interactions between *P. aeruginosa* and *S. aureus* in the context of their competitiveness within the biofilm mode of growth, by implementing an innovative and systematic analysis of the scientific literature on this subject. The examination of the gathered information resulted in the clarification of the amount and types of studies being conducted, but more importantly, in the identification of the major and most studied molecular players in *P. aeruginosa/S. aureus* communication and/or interaction. More specifically, with *P. aeruginosa* revealed as key player, pseudomonal factors affecting *S.*

aureus were pointed out (e.g., HQNO and siderophores) and their respective antivirulence agents identified. Yet, we are still far away from fully understanding the dynamic and complex networks of interactions that occur in the natural infection environment. Although efforts are being made to deeply understand the interactions that those microorganisms experience during infection, as this work shows, the percentage of studies that have pointed out specific mechanisms of interaction remains relatively small. The improvement of more powerful and efficient genetic tools and the design of feasible in vitro models that reflect the *in vivo* environment are pivotal for exploring unanswered questions about the ecology of *P. aeruginosa/S. aureus* biofilm infections. Expectantly, this work and its resulting database (www.ceb.uminho.pt/ISCTD) will assist researchers aiming at diminishing the resilience of this bacterial consortium within biofilm related infections by downsizing time and work-related costs.

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CHAPTER 4

Viable but non-cultivable state: a strategy for *S. aureus* survivable in dual-species biofilms with *P. aeruginosa?*

Summary

P. aeruginosa and *S. aureus* are two of the most prevalent respiratory pathogens in CF patients. Both organisms often cause chronic and recalcitrant infections, in large part due to their ability to form biofilms, being these dual-species infections correlated with poor clinical outcomes. In this study, the hypothesis that *S. aureus* adopts phenotypes allowing its co-existence with *P. aeruginosa* during biofilm growth was put forward. It was noticed that *S. aureus* undergoes a viable but non-cultivable (VBNC) state in the dominated *P. aeruginosa* dual-species consortia, whatsoever the strains used to form the biofilms. Moreover, an increased expression of genes associated with *S. aureus* virulence was detected suggesting that the phenotypic switching to VBNC state might account for *S. aureus* pathogenicity and, in turn, influence the clinical outcome of the mixed-species infection. Thus, *P. aeruginosa* seems to induce both phenotypic and transcriptomic changes in *S. aureus*, helping its survival and co-existence in the dual-species biofilms. Overall, these findings illustrate how inter-species interactions can modulate bacterial virulence *in vitro*, contributing to a better understanding of the behaviour of *P. aeruginosa - S. aureus* dual-species biofilms.

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4.1 Brief introduction

Typically, the establishment of chronic infection begins with the acquisition of *S. aureus* during childhood [1, 2] and, as patients transition through adolescence to early adulthood, *P. aeruginosa* replaces *S. aureus* as the dominant pathogen [2, 3]. Although these bacteria seem to succeed one another, CF patients acquire coinciding *P. aeruginosa* and *S. aureus* pulmonary infections, being co-infection usually associated with decreased lung function and increased frequency of pulmonary exacerbations [4, 5].

In CF patients, the typical pattern of infection has led many researchers to speculate that *P. aeruginosa* eliminates *S. aureus* during infection, perhaps outcompeting *S. aureus* for limited nutrients [6] or producing anti-staphylococcal compounds [7–11], having *S. aureus* a minimal contribution to the overall course of the infection [6, 12, 13]. However, *P. aeruginosa* and *S. aureus* have been identified in the same lobe of CF lungs [14, 15], and are frequently diagnosed [4, 16] as co-infecting species in CF patients. Moreover, *P. aeruginosa* strains isolated from early infection outcompete *S. aureus*, while strains isolated from chronic infection are less aggressive and can be co-cultivated with *S. aureus* [17, 18], suggesting that these pathogens can in fact interact *in vivo*.

Although a significant progress has been made in studying *S. aureus* and *P. aeruginosa* [11, 19–24], the understanding provided by these studies is oversimplified and limited by inferring that *S. aureus* is no longer contributing to the overall course of the infection. Driven by this gap, the aim of this study was to investigate the survival strategy adopted by *S. aureus* during dual-species biofilm growth with *P. aeruginosa*.

4.2 Materials and Methods

4.2.1 Microorganisms and culture conditions

P. aeruginosa (strains UCBPP-PA14, anonymized clinical isolates 3626688 mucoid and 362668 non-mucoid) and *S. aureus* (strains ATCC 25923 and anonymized clinical isolate 352845) were used throughout this work. Strains 3626688 mucoid, 362668 non-mucoid and 352845 are clinical CF isolates kindly provided by the Hospital of Braga, Portugal [25, 26]. All bacteria were stored at -80 ± 2 °C in tryptic soy broth (TSB, Liofilchem, Italy) supplemented with 20% glycerol.

Prior to each assay, bacteria were sub-cultured from frozen stock preparations onto plates of TSB supplemented with 2% (W/V) agar and incubated aerobically at 37 °C for 24 h.

4.2.2 Formation of single- and dual-species biofilms

The formation of single- and dual-species biofilms formed by *P. aeruginosa* and *S. aureus* was performed as described previously, with minor modifications [27]. Briefly, overnight cultures of each strain, grown in TSB at 37 °C and 120 rpm in air conditions, were washed in sterile water and diluted in fresh TSB to obtain 1×10^7 CFU/mL as final concentration. Bacterial numbers were estimated using an ELISA microtiter plate reader (Sunrise-Basic Tecan, Männedorf, Switzerland) at an optical density (OD) of 620 nm. Calibrations were previously performed for each bacterial strain to relate the absorbance at 620 nm with the number of colony forming units (CFU). For dual-species cultures, a combination of 50% of the suspended inoculum of each species was used. Bacterial suspensions were further transferred to 24 well polystyrene plates (Orange Scientific, Braine-l`Alleud, Belgium) and incubated at 37 °C on a horizontal shaker (120 rpm) for 24 h, 48 h and 72 h. For the development of 48- and 72-h-old biofilms, the content of each well was replaced with fresh medium after each 24 h. To perform the biofilm biomass quantification assay, 96 well polystyrene plates (Orange Scientific, Braine L'Alleud, Belgium) were used. Following biofilm growth, the content of the polystyrene plates (planktonic fraction) was withdrawn, and the wells were washed twice with distilled sterile water. Biofilms cells were resuspended into 1 mL of 0.9% NaCl, detached by scraping and collected for further analysis.

4.2.3 Quantification of biomass and cultivability of biofilms

Biofilm biomass quantification was based on staining biofilms with 1% (vol/vol) of crystal violet (CV), as previously described [27]. Briefly, the wells were air dried for 10 min before fixing attached bacteria with pure methanol (Fisher Scientific, Leicestershire, UK) for 15 min. Then, biofilms were stained for 1 min with 1% (v/v) CV (Merck, Germany). The excess stain was removed by washing twice the wells with sterile distilled water. Lastly, the bound dye was dissolved in pure methanol (200 µL/well) and the OD was read at 570 nm in a microtiter plate reader.

After biofilm formation, the number of adhering bacteria was determined by plate counts. To undo any aggregates, biofilm harvested cells were first sonicated for 10 s at 30 % amplitude [28]

(Cole Parmer Ultrasonic Processor, IL, USA) and then the viable cells counted. Briefly, the disrupted biofilms were serially diluted (1:10) in 0.9 % NaCl, streaked onto different selective agar media and incubated at 37 °C for 18-24 h, for CFU counting. Pseudomonas isolation agar (PIA) and Mannitol Salt Agar (MSA) were the selective media used to discriminate *P. aeruginosa* and *S. aureus,* respectively. Values of cultivable biofilm cells were expressed as CFU/cm^2 or CFU/mL.

4.2.4 Determination of Competitive Index (CI) and the Relative Increase Ratio (RIR)

In order to determine whether the species could compete with each other in dual-species biofilms, the Competitive Index (CI) was defined as the *P. aeruginosa*/*S. aureus* ratio within the output sample (immediately after biofilm formation) divided by the corresponding ratio in the inoculum (input):

$$\mathbf{CI\ [}\textbf{\textit{P. aeruginosa}}\ \mathbf{vs}\ \textbf{\textit{S. aureus}}\mathbf{] = [N\ (}\textbf{\textit{P. aeruginosa}}\mathbf{/}\textbf{\textit{S. aureus}}\mathbf{)_{output}/N\ (}\textbf{\textit{P. aeruginosa}}\mathbf{/}\textbf{\textit{S. aureus}}\mathbf{)_{input}]}$$

where N is the value of CFU/cm^2 obtained for the species *P. aeruginosa* or *S. aureus* in the biofilm, in the output (at fixed times) and input (t = 0 or the inoculum) sample. For statistical analyses, CI values were first subjected to a Log transformation for normal distribution, then interpreted as follows: a CI value equal to 0 indicates equal competition of *P. aeruginosa* and *S. aureus* in the consortium; a positive CI value indicates a competitive advantage for the *P. aeruginosa*; a negative CI value indicates a competitive advantage for the *S. aureus*. Similarly, the Relative Increase Ratio (RIR) was calculated and based on the growth results obtained from the single-species biofilms formed by each strain. Calculations were adapted from [29]. Statistically significant differences between CI and RIR means for each case were suggestive of a meaningful competition between the species in the consortia.

4.2.5 Microscope observation

Scanning electron microscopy (SEM) was used to examine the biofilm structures of *P. aeruginosa* - *S. aureus* biofilms. For this, dual-species biofilms were formed on Thermanox® plastic coverslips (Thermo Scientific™; Rochester, NY, USA) placed in the bottom of the wells of 24 well polystyrene plates (Orange Scientific, Belgium). Thus, after 24 h, 48 h and 72 h of biofilm growth, the content of the polystyrene plates (planktonic fraction) was removed, and the wells were washed twice with distilled sterile water. The discs were then dehydrated with an ethanol series

(70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min), air dried for 20 min, and reserved in a desiccator until analysis.

Prior to observation, discs were added to aluminium pin stubs with electrically conductive carbon adhesive tape (PELCO Tabs™). Samples were coated with 2 nm of Au for improved conductivity. The aluminium pin stub was then placed inside a Phenom Standard Sample Holder or Phenom Charge Reduction Sample Holder. The analysis was conducted at 5 kV with intensity image. The samples were characterized using a desktop Scanning Electron Microscope (SEM) (Phenom ProX, Netherlands). All results were acquired using the ProSuite software.

Epifluorescence microscopy (EM) performed on 4',6'-diamidino-2-phenylindole DAPI (stains total cells), wheat germ agglutinin WGA (stains *S. aureus* cells) and propidium iodide PI (stains dead cells) stained harvested cells was used to evaluate biofilm cell viability. In brief, pre-formed biofilms were washed twice, scraped in 1 mL of 0.9% NaCl, collected by centrifugation (10000 rpm, 10 min, 4 °C) and suspended in 200 µL of 10 µg/mL of WGA conjugated with FITC (Molecular probes, ThermoFisher Scientific, MA, USA). This suspension was incubated at room temperature, in the dark, for 15 min, and then harvested cells were centrifuged to collect, and suspend in 1 mL of 0.9 % NaCl. *P. aeruginosa* biofilm harvested cells, stained with WGA-FITC, were included as negative control (**Figure 4.1**) and *S. aureus* biofilm harvested cells, stained with WGA-FITC, were included as positive control (**Figure 4.2**). Thereafter, 100 µg/mL of DAPI (Sigma) was added to the suspensions and let to incubate in the dark for 10 min. Lastly, the suspensions were incubated with 15 µM of PI (Invitrogen, Carlsbad, CA, USA) in the dark for 5 min.

Ethanol-fixation was used to kill and permeate biofilm samples used as controls. Briefly, 24-h-old biofilms were collected and submerged in 70 vol% ethanol in deionized water, incubated 1 h at room temperature. Ethanol-fixed biofilms were stained with PI and then visualized (**Figure 4.3**).

EM was carried out using Olympus BX51 microscope equipped with 100x oil immersion objective and filters sensitive to DAPI (BP 365–370, FT 400, LP 421), WGA-FITC (BP 470–490, FT 500, LP 516), and Propidium Iodide (PI) (BP 530–550, FT 570, LP 59). Images were captured with a CCD camera (DP72; Olympus) and CellB software.

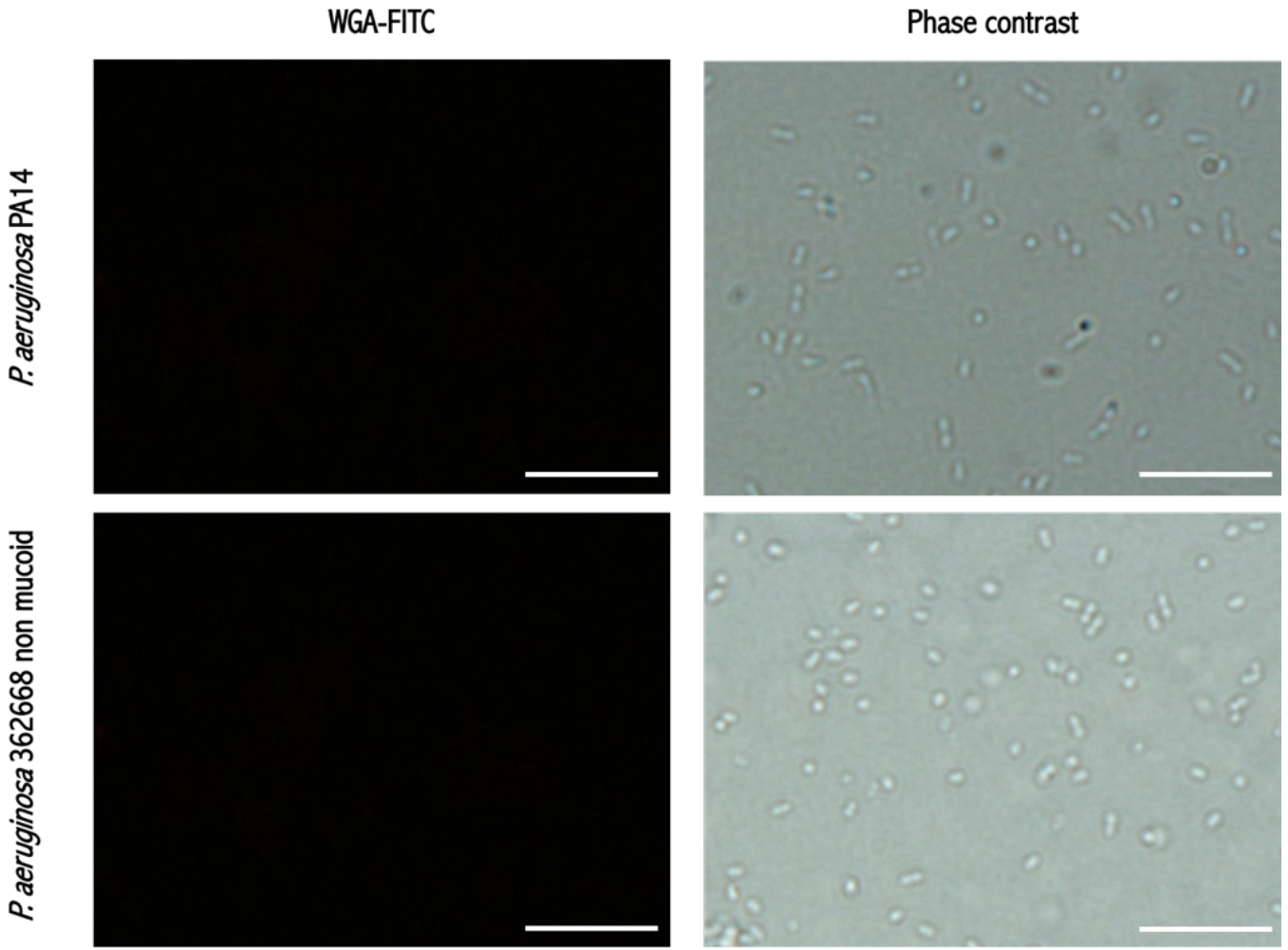

Figure 4.1. Epifluorescence and phase contrast images of *P. aeruginosa* PA14 and *P. aeruginosa* 362668 non mucoid biofilm cells stained with WGA-FITC. Fluorescence and phase contrast image are captured from the same view field. Scale bars correspond to 10 μm.

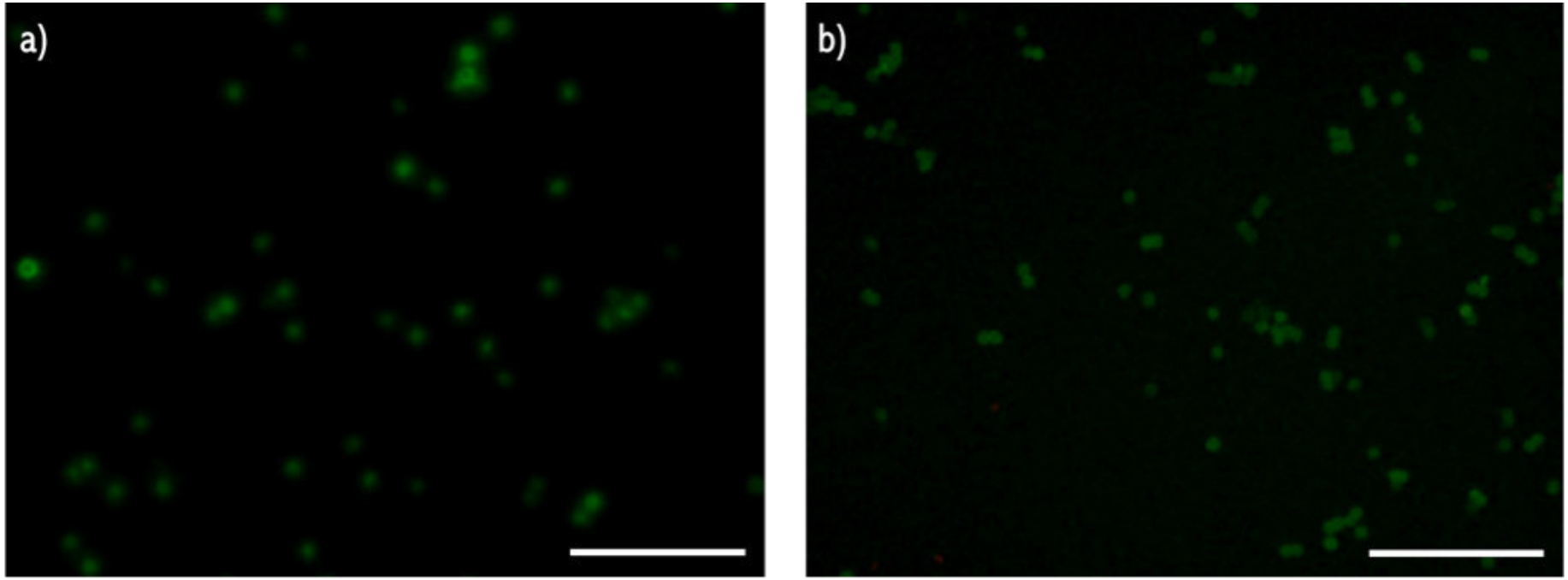

Figure 4.2. Epifluorescence images of **(a)** *S. aureus* ATCC 25923 and **(b)** *S. aureus* 352845 harvested biofilm cells with WGA-FITC. Scale bars correspond to 10 μm.

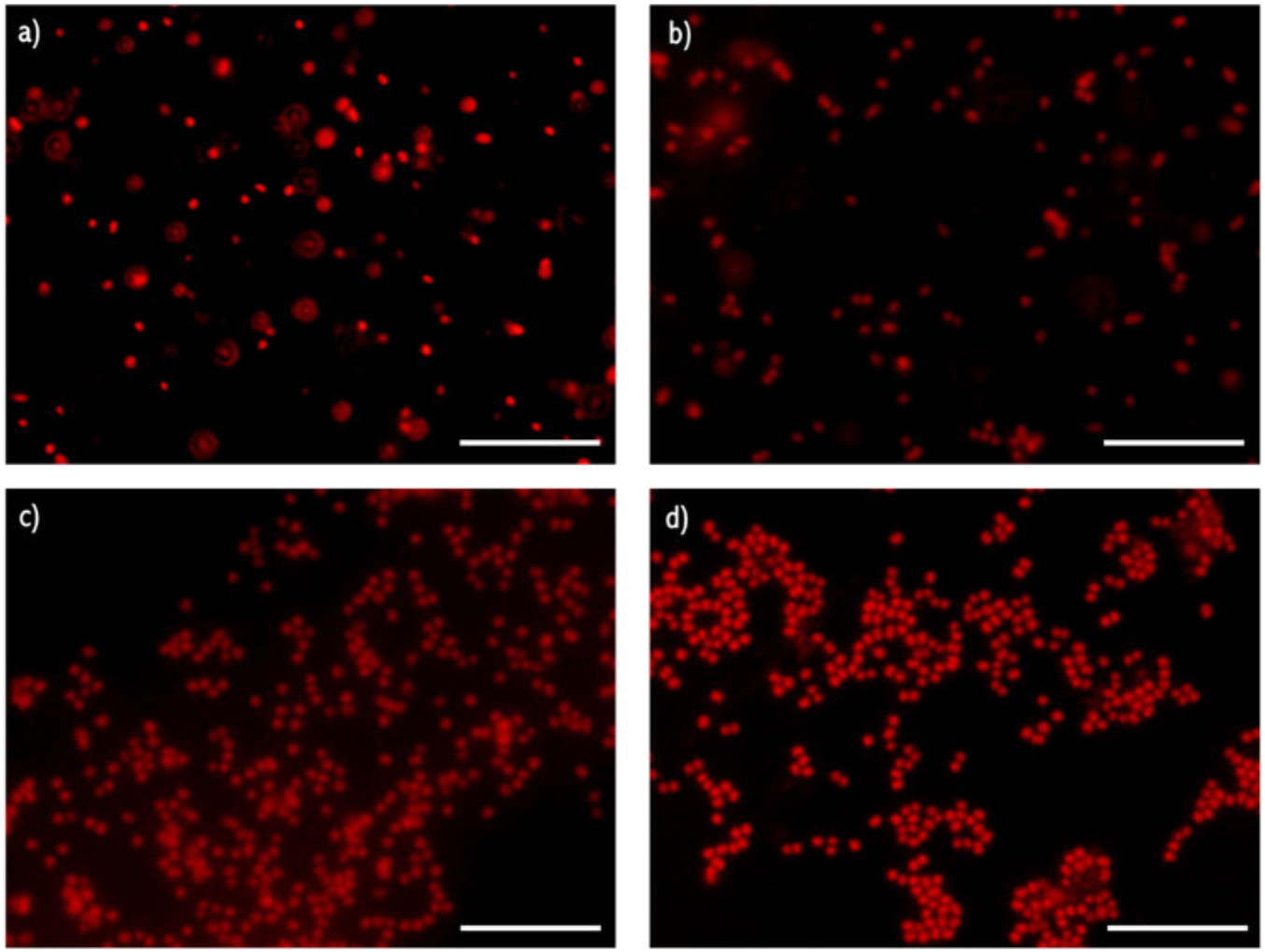

Figure 4.3. Epifluorescence fluorescence microscopy images of ethanol-fixed 24-h harvested biofilm cells stained with PI. **(a)** *P. aeruginosa* PA14, **(b)** *P. aeruginosa* 362668 non mucoid, **(c)** *S. aureus* ATCC 25923 and **(d)** *S. aureus* 352845. Scale bars correspond to 10 μm.

4.2.6 Effect of biofilm supernatants on *S. aureus* cell cultivability

The effect of *P. aeruginosa, S. aureus, P. aeruginosa + S. aureus* biofilm free-cell supernatants on *S. aureus* growth was tested in liquid. The planktonic fraction of 24-h-old biofilms was centrifuged, and the resulting supernatants were filtered (0.22 μm, Millipore). An overnight culture of *S. aureus* was adjusted in TSB to obtain 1×10^5 CFU/mL and 1×10^7 CFU/mL as final concentrations. 2 mL of this bacterial suspension was incubated with 2 mL of *P. aeruginosa*, *S. aureus, P. aeruginosa + S. aureus* biofilm free-cell supernatants. All mixtures were incubated at 37 °C, 120 rpm in air conditions and aliquots were recorded after 4 h and 8 h of incubation for CFU counting.

4.2.7 Flow cytometry

P. aeruginosa and *S. aureus* cells in single- and dual-species biofilms, as well as the BRC recovered from the bulk fluid of 48-h-old dual-species biofilms were analysed in terms of total counts and cell viability by flow cytometry. In brief, biofilm cells were collected by centrifugation (10000 rpm, 10 min, 4 °C) and suspended in 200 μL of a solution with 10 μg/mL of WGA-FITC (Molecular probes, ThermoFisher Scientific, MA, USA). This suspension was incubated at room temperature, in the dark, for 15 min. Thereafter, the harvested biofilm cells were collected and suspended in 1 mL of phosphate-buffered saline (PBS; 10 mM potassium phosphate, 150 mM NaCl; pH 7.0), and sonicated for 10 s at 33 % amplitude (Cole Parmer Ultrasonic Processor, IL, USA) to disrupt aggregates. Finally, the tested suspensions were incubated with 15 μM of PI (Invitrogen, Carlsbad, CA, USA) at room temperature, in the dark, for 5 min.

The bacterial fluorescence analysis was carried out using an EC800 Sony flow cytometer (Sony Biotechnologies Inc., CA, USA) equipped with a 488 nm laser. Multi-parametric analyses were performed on the scattering signals (forward scatter, FSC and side scatter, SSC), as well as on the FL1 (green fluorescence) and FL4 (red fluorescence) channels. WGA-FITC fluorescence was detected on the FL1 channel (PMT = 5) while PI fluorescence was detected on the FL4 channel (PMT = 3). Gating of dead and alive signal populations was executed on WGA-FITC (FL1)/PI (FL4) scatter plot as illustrated on **Figure 4.4**. All the detectors were set to logarithmic amplification. Samples were acquired with a flow rate of 10 μL/min and the analysis stopped when 80 000 events were detected. Each sample was analysed at least twice to ensure an

accurate counting. Data analysis was performed using FCS Express 6 analysis software (De Novo Software).

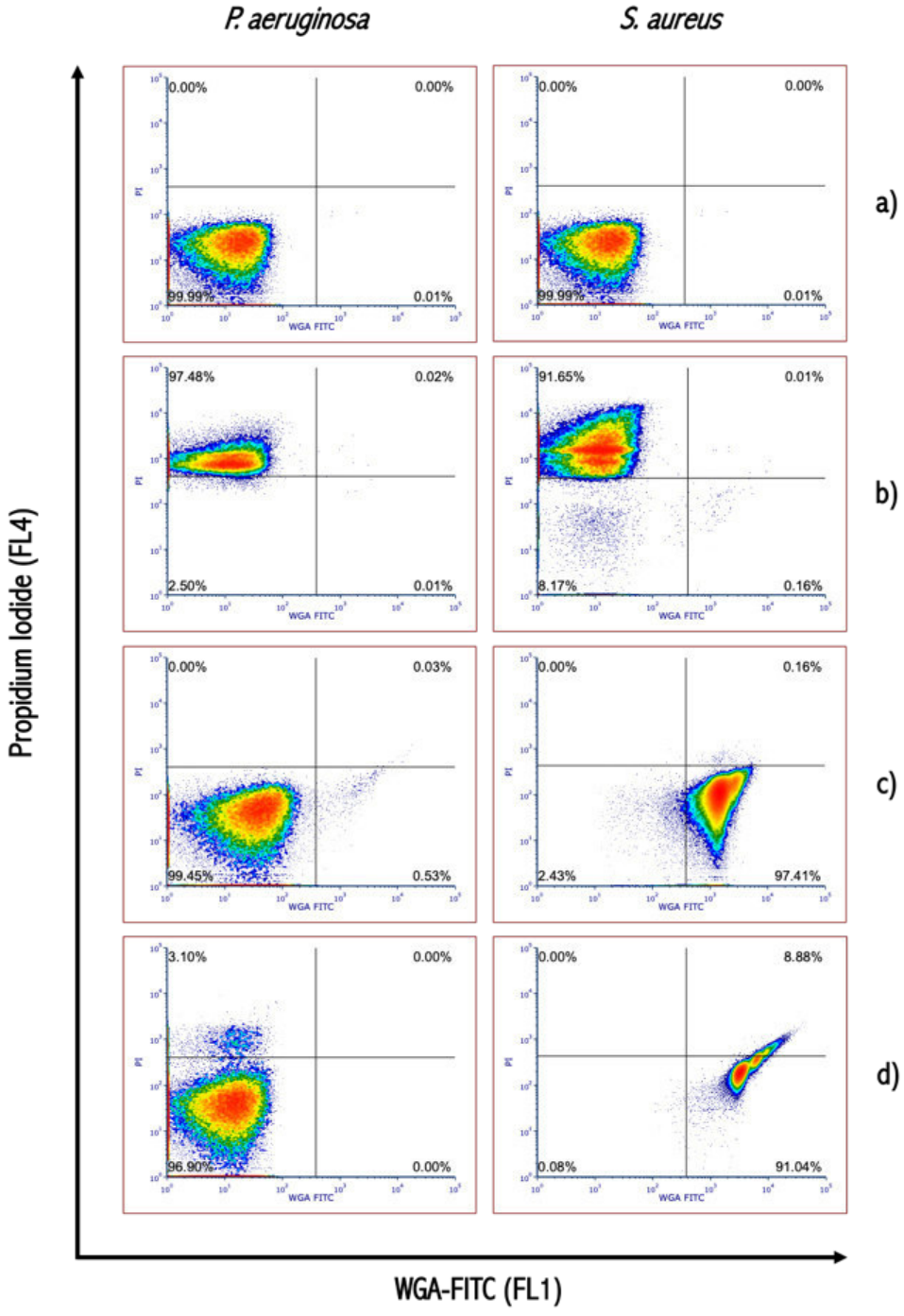

Figure 4.4. Representative Flow Cytometry (FC) density plots of *P. aeruginosa* and *S. aureus*: **(a)** PBS washed overnight planktonic cultures; **(b)** PBS washed and ethanol-killed overnight planktonic cultures stained with propidium iodide (PI); **(c)** PBS washed viable overnight planktonic cultures stained with WGA-FITC; **(d)** PBS washed harvested biofilm cells co-stained with WGA-FITC and PI.

4.2.8 Gene expression analysis

Single- and dual-species biofilms were grown as described above. For each condition, total RNA from a biofilm pooling (12 wells of a 24-well-plate) was extracted using RNeasy mini kit (Quiagen), with minor changes, as previously optimized [30]. Next, genomic DNA was degraded with one step of DNase treatment (Fermentas, Lithuania) following the manufacturer's instructions. RNA concentration, purity, and integrity were determined as described before [31]. The same amount of total RNA (500 ng) was reverse transcribed using the GRS cDNA Synthesis Kit (Grisp) in 10 µL of reaction volume by using random primers. The Quantitative PCR (qPCR) reaction was performed by mixing together 5 µL of master mix SYBR Xpert Fast SYBR (Grisp), 2 µL of 1:100 diluted cDNA, 0.5 µL of forward and reverse primes (5 µM), and water up to a total volume of 10 µL. The run was performed in a CFX96TM thermal cycler (Bio-Rad) with the following cycling parameters: 95 °C for 3 minutes, 40 cycles of 95 °C for 5 s, and 60 °C for 20 s. Reaction efficiency was determined by the dilution method [32]. At 60 °C all set of primers (**Table 4.1**) used had the highest and more similar efficiencies. Furthermore, the analysis of the obtained melting curves confirmed the presence of a single peak, demonstrating the specificity of the tested primers. Normalized gene expression was determined by using the delta Ct method ($E^{\Delta Ct}$), a variation of the Livak method, where ΔCt = Ct (reference gene) −Ct (target gene) and E stands for the reaction efficiency experimentally determined. A non-reverse transcriptase control was included in each reaction.

Table 4.1 - Oligonucleotide sequences of the primers used in this study.

Organisms	Primer	Forward	Reverse	Primer Efficiency	R^2	Melting Temperature (ºC)	Amplicon size (bp)
S. aureus	*16S RNA*	GGTCTTGCTGTCACTTATAGATGG	CGGAAGATTCCCTACTGCTG	90.4	0.996	59.2/59.8	164
	sodA	ATGGCGGTGGTCATTTTAAC	ACCAAGTCCAACCTGATCCA	110.0	0.993	59.7/60.4	171
	sarA	TGTTTGCTTCAGTGATTCGTTT	CATCAGCGAAAACAAAGAGAAA	94.0	0.997	59.8/59.5	233
	hld	TTTTTAGTGAATTTGTTCACTGTGTC	TAATTAAGGAAGGAGTGATTTCAATG	103.3	0.995	58.8/59.1	100
	icaA	CTCAAGGGGGACACGAAGT	CCAGCCATATTCTTTTTCTCGT	105.5	0.940	60.1/59.6	292
	clpP	AACAACAAATCGCGGTGAAC	TGCAGCCATACCGATACAAA	91.1	0.969	60.9/60.1	265
	uspA	ACTTACTGAAGAACGAAGCAAGC	AGGGATAGTCGCACGTTTTG	96.6	0.920	59.3/60.1	171
P. aeruginosa	*16S RNA*	GGAGAAAGTGGGGGATCTTC	CCGGTGCTTATTCTGTTGGT	90.6	0.997	59.9/59.9	316
	pqsE	GTCGTAGTGCTTGTGGGTGA	ATGATGACCTGTGCCTGTTG	92.4	0.995	59.8/59.7	188
	rhlR	GTTGCATGATCGAGTTGCTG	TGGATGTTCTTGTGGTGGAA	97.10	0.997	60.4/59.9	193
	lasI	CATGTAGGGGCCAGTGGTAT	CAAGTTGCGTGCTCAAGTGT	111.6	0.974	59.7/60.1	178

4.2.9 Statistical analysis

All numerical data were subjected to statistical analysis using the independent samples *t*-test, one-way analysis of variance (ANOVA), or non-parametric Mann–Whitney U, Kruskall-Wallis test for the data that did not follow a normal distribution according to Kolmogorov–Smirvon's test, with the statistical software Prism Version 7.0a for Macintosh. Results are presented as mean ± standard deviation (SD), unless stated otherwise.

4.3 Results

4.3.1 Interspecies competition in dual-species biofilms formed by *P. aeruginosa and S. aureus*

In order to inspect which bacterial species were prevailing in the biofilms and identify their social interaction, consortia of *P. aeruginosa* and *S. aureus* (ATCC type strains and clinical isolates) were initially selected to assess their growth as single- and dual-species biofilms over a course of 72 h. The consortia were first evaluated by determining the total biomass of single- and dual-species formed in 96 well plates. Dual-species biofilms encompassing *P. aeruginosa* PA14 and *S. aureus* ATCC 25923 strains produced similar values of biomass in comparison with their individual biofilms ($P>0.05$) (**Figure 4.5A**). The same trend was observed when *P. aeruginosa* 362668 non-mucoid was grown with *S. aureus* 352845 (**Figure 4.5B**). Overall, biofilms tend to significantly increase its biomass until reached 48 h of growth ($P<0.05$).

Biofilms were also characterized by enumerating the number of CFU for each *P. aeruginosa* and *S. aureus* strains on selective media (PIA and MSA, respectively). Plating experiments showed that single-species biofilms involving *P. aeruginosa* PA14 and *S. aureus* ATCC 25923 (**Figure 4.6A**) increased their cell numbers from 24 h up to 48 h of growth ($P<0.05$). Regarding *P. aeruginosa* 362668 non-mucoid and *S. aureus* 352845 strains (**Figure 4.6B**), both reached high CFU/cm^2 numbers as single-species biofilms, even with 362668 non-mucoid strain decreasing its cultivability over time. The analysis of the dual-species populations revealed that, overall, *S. aureus* cultivability was significantly decreased from 24 h up to 72 h in the presence of *P. aeruginosa* ($P<0.05$) (**Figure 4.6**), whilst *P. aeruginosa* was not affected by the presence of *S. aureus* in the consortia ($P<0.05$).

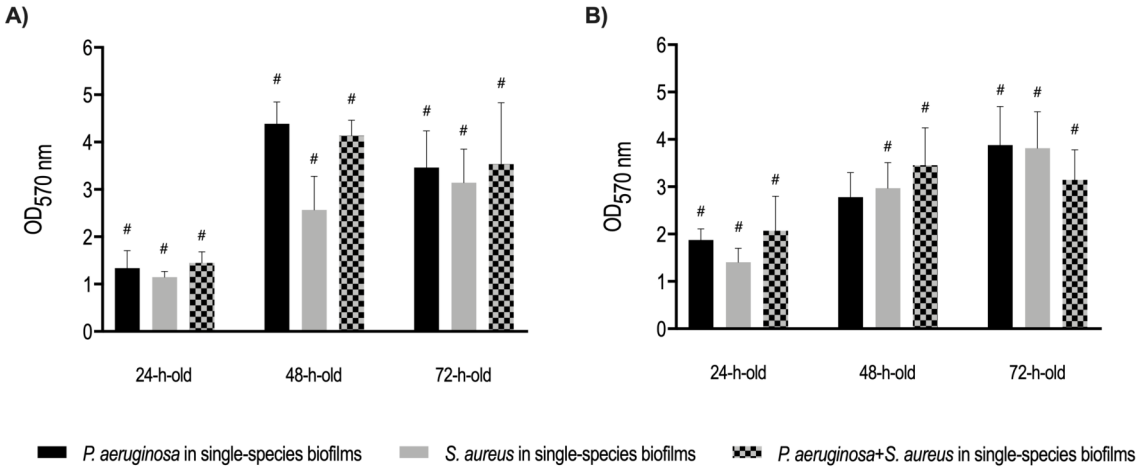

Figure 4.5. Biofilm biomass obtained for 24-, 48- and 72-h-old single- and dual-species consortia. **A)** *P. aeruginosa* PA14 and *S. aureus* ATCC 25923 and **B)** *P. aeruginosa* 362668 non-mucoid and *S. aureus* 352845. Mean ± SD for three independent assays is presented. Statistical significance was determined by performing an Kruskal-Wallis followed by a Dunnett's multiple comparison test to compare: (*) significantly differences between single- and dual-species biofilms (*, p<0.05); (#) significantly differences between 24- and 48- or 72-h (#, p<0.05), and (α) between 48 and 72-h of growth as single- or dual-species biofilms (α, p<0.05).

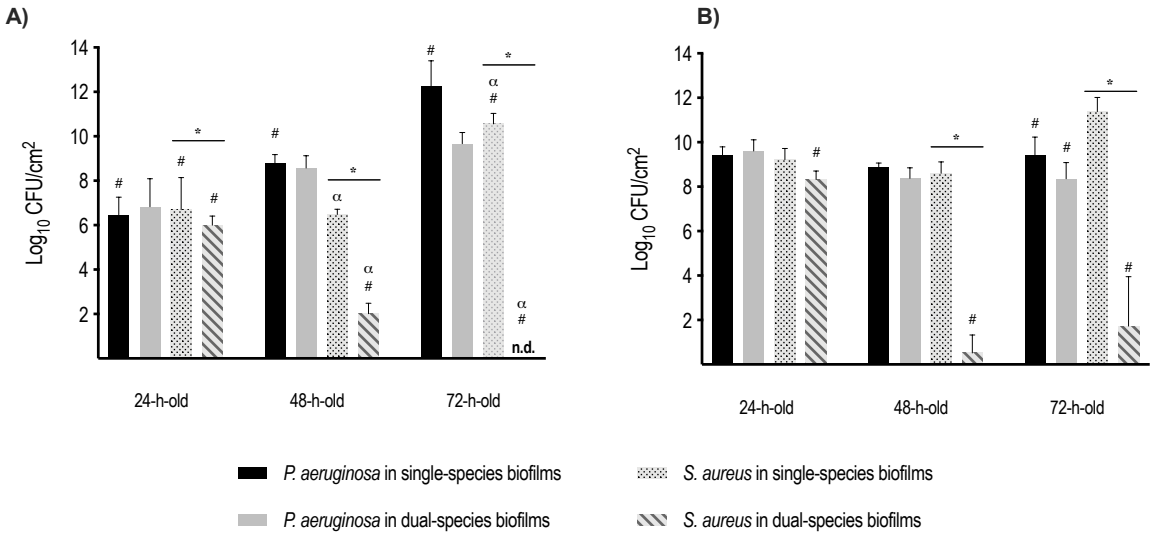

Figure 4.6. Biofilm quantification by plate count of single- and dual-species biofilms. **A)** *P. aeruginosa* PA14 and *S. aureus* ATCC 25923 and **B)** *P. aeruginosa* 362668 non-mucoid and *S. aureus* 352845 growing for 24-, 48- and 72-h-old. Mean ± SD for three independent assays is presented. Statistical significance was determined by performing an ANOVA followed by a Tukey's multiple comparison test to compare: (*) significantly differences between single- and dual-species biofilms of *P. aeruginosa* or *S. aureus* (*, p<0.05); (#) significantly differences between 24-h and 48- or 72-h (#, p<0.05), and (α) between 48- and 72-h of growth as single- or dual-species biofilms (α, p<0.05).

For a better comprehension of the differences in the growth between *P. aeruginosa* and *S. aureus* in single- *versus* dual-species biofilms, CI and RIR indexes were calculated (**Figure 4.7**). Whilst CI allows comparing the differences among the growth of each species in mixed cultures, the RIR index compares the growth of species within pure cultures [29]. As shown in **Figure 4.7A**, a positive CI index was observed for the *P. aeruginosa*/*S. aureus* consortia, meaning a clear competitive advantage of *P. aeruginosa* over *S. aureus* (CI >0, CI vs. RIR, P<0.01). Similar findings were obtained for the interaction between *P. aeruginosa* 362668 non-mucoid and *S. aureus* 352845 strains (**Figure 4.7B**), although their competition was only significant after 48 h of growth (CI vs. RIR, P< 0.01).

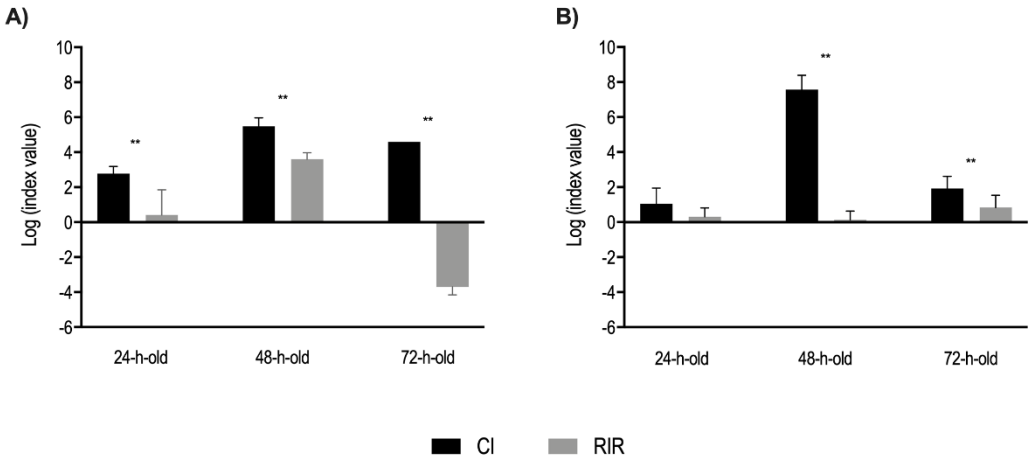

Figure 4.7. CI (black bars) and RIR (RIR; grey bars) calculated from 24-, 48- and 72-h-old biofilms. **(A)** *P. aeruginosa* PA14 and *S. aureus* ATCC 25923 and **(B)** *P. aeruginosa* 362668 non-mucoid and *S. aureus* 352845. Statistical significant differences between CI and RIR is suggestive of a meaningful competition between the species in the consortia. The results are shown as mean ± SD. ** p<0.01, CI vs. RIR, unpaired two-tailed *t*-test.

In order to inspect and compare biofilm structures, the dual-species consortia were directly examined using SEM (**Figure 4.8**). Images revealed that after 24 h of growth *P. aeruginosa* PA14 and *S. aureus* ATCC 25923 dual-species biofilms (**Figure 4.8A**) showed a non-contiguous layer of cells, while 48 h and 72 h of growth resulted in a remarkable increase in biofilm mass with considerable aggregation of bacterial cells. A same trend was observed for the mixed biofilms formed by *P. aeruginosa* 362668 non-mucoid and *S. aureus* 352845 (**Figure 4.8B**). In regard to biofilm populations, both rod-shape cells (*P. aeruginosa)* and coccus-shape cells (*S. aureus*) were detected in the 24-h-old consortia. Nevertheless, as 48- and 72-h-old biofilms presented dense structures, it was difficult to distinguish the species occupying the biofilm, and thus infer about the prevalence of the strains in the consortia.

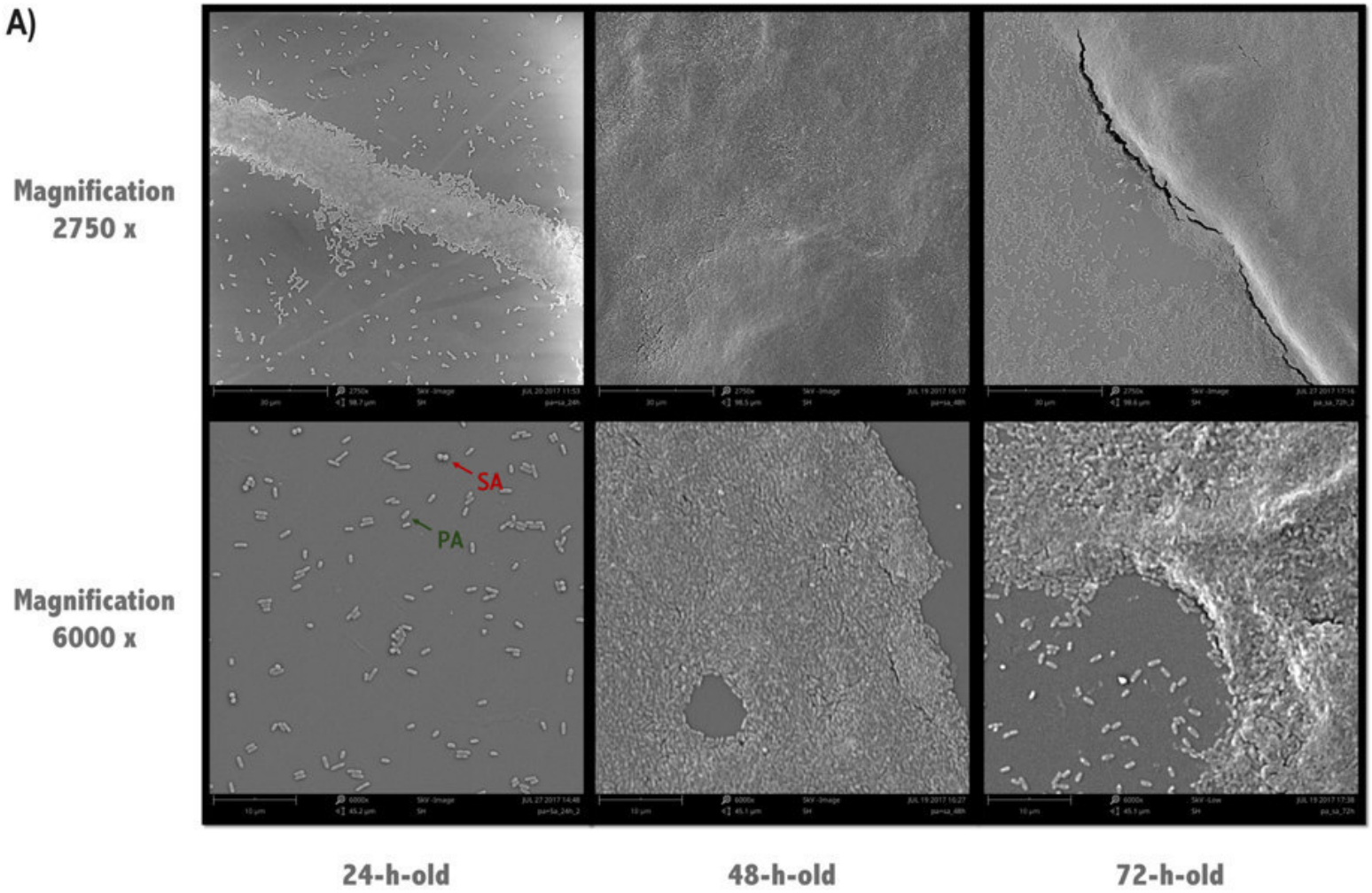

Figure 4.8. Representative scanning electron microscopy images of 24-, 48- and 72-h-old dual-species biofilms. **(A)** *P. aeruginosa* PA14 and *S. aureus* ATCC 25923 and **(B)** *P. aeruginosa* 362668 non-mucoid and *S. aureus* 352845. Magnifications are 2750× (scale bar; 30 µm) and 6000× (scale bar; 10 µm).

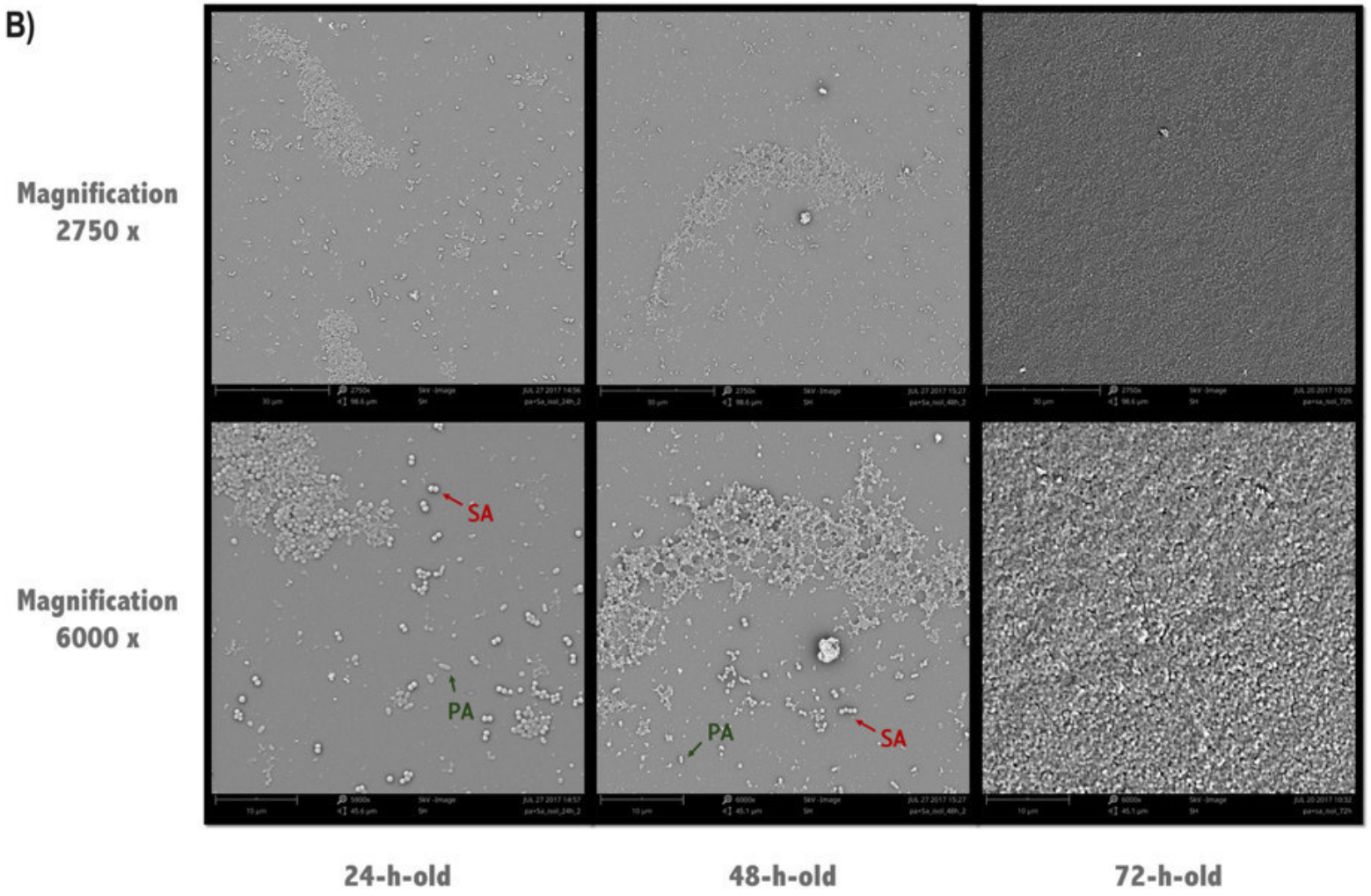

Figure 4.8. (continued)

To evaluate whether the observed bacterial competitiveness is influenced by the production of *P. aeruginosa* exoproducts, that potentially influence *S. aureus* growth, an overnight culture of *S. aureus* was exposed to different biofilm supernatants. As shown in **Figure 4.9**, the supernatant collected from 24 h biofilms of *P. aeruginosa* and *P. aeruginosa* + *S. aureus* significantly reduced cell viability of *S. aureus* ATCC 2523 and 352845 strains. A *S. aureus* biofilm supernatant sample was included as control (**Figure 4.9**), and no interference on cell viability was observed.

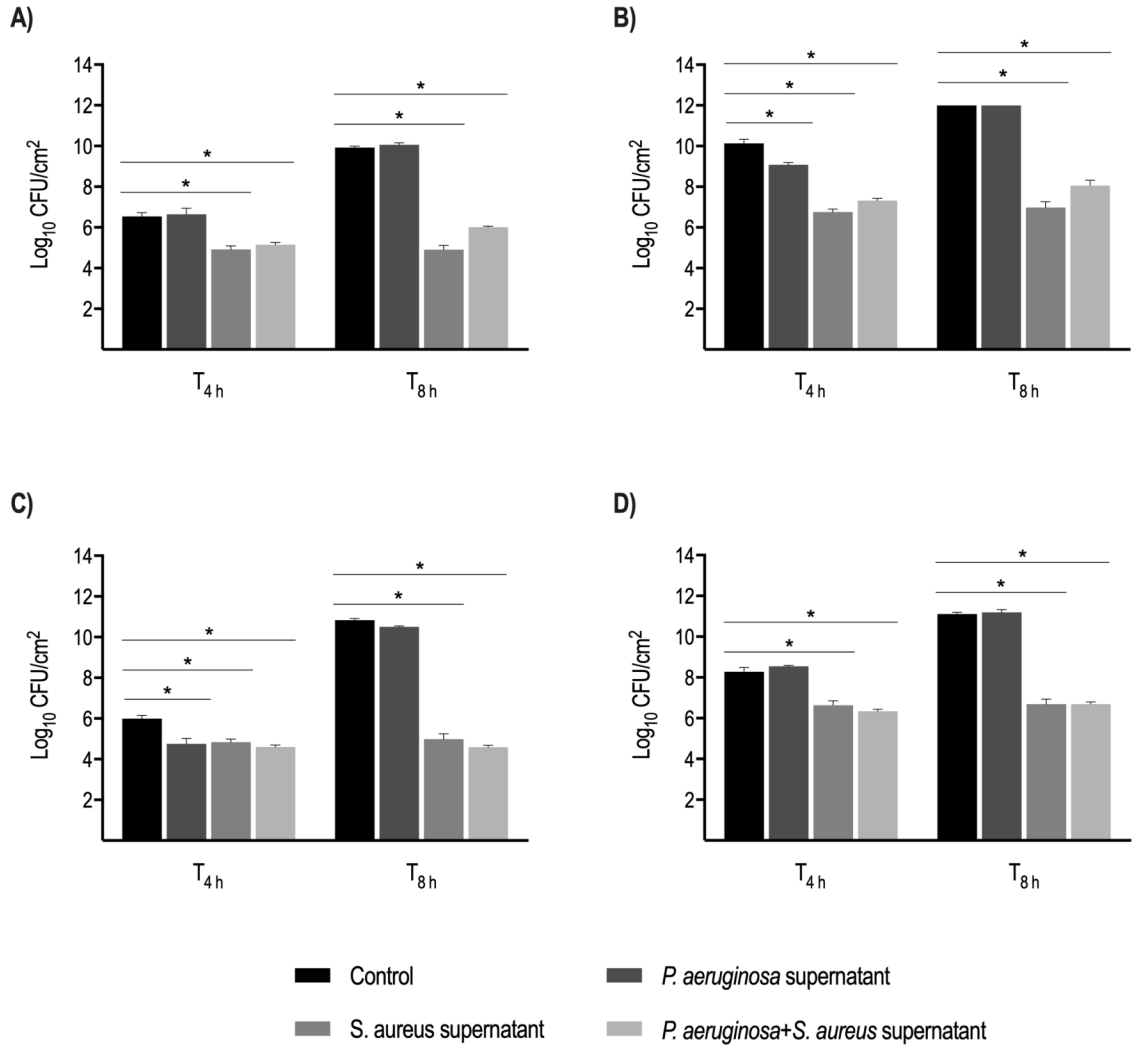

Figure 4.9. Effect of biofilm free-cell supernatants on *S. aureus* growth. Overnight cultures of *S. aureus* were adjusted in TSB to obtain **(A, C)** 1×10^{5} CFU/mL or **(B, D)** 1×10^{7} CFU/mL as final concentrations. **(A, B)** Biofilm free-cell supernatants of *S. aureus* ATCC 25923, *P. aeruginosa* PA14 and *P. aeruginosa* PA14 + *S. aureus* ATCC 25923 were incorporated on overnight cultures of *S. aureus* ATCC 25923; **(C, D)** biofilm free-cell supernatants of *S. aureus* 352845, *P. aeruginosa* 362668 non-mucoid and *P. aeruginosa* 362668 non-mucoid + *S. aureus* 352845 were incorporated on overnight cultures of *S. aureus* 352845 cells. The results for two independent assays are shown as mean +/- SDs. * $p<0.05$, unpaired two-tailed Mann-Whitney test with respect to control no supernatant.

4.3.2 Quantitative assessment of *P. aeruginosa* and *S. aureus* individual populations within dual-species biofilms

Inter-species competition in biofilms not only occurs by inhibiting or killing the other species but also by inducing its dispersal [20]. Thus, to deep understand the observed competitiveness of *P. aeruginosa* during dual-species biofilm growth, the cell numbers of the 48 h biofilm bulk fluid were also determined to inspect whether *S. aureus* cells were released from the dual-species biofilm after 24 h of growth (**Figure 4.10**). The biofilm released cells (BRC) obtained immediately after replacing the medium was also monitored along 24 h by CFU counting, in order to verify the influence of the medium replacement in the initial number of cells (**Figure 4.11**). Interestingly, despite the absence of cultivable *S. aureus* BRC, both species were microscopically visualized in the bulk fluid of 48-h-old dual-species biofilms (**Figure 4.12**).

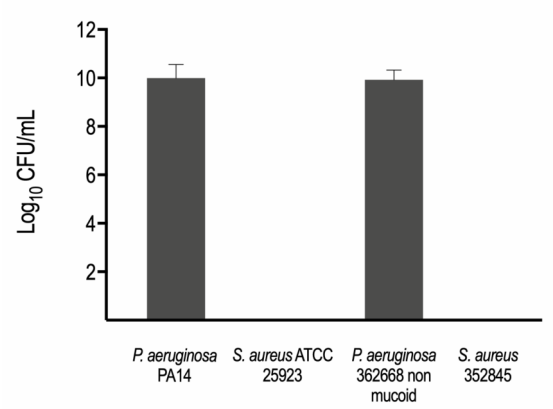

Figure 4.10. Assessment of *P. aeruginosa* and *S. aureus* BRC recovered from the bulk liquid of 48-h-old dual-species biofilms. The means ± SD for three independent assays are presented.

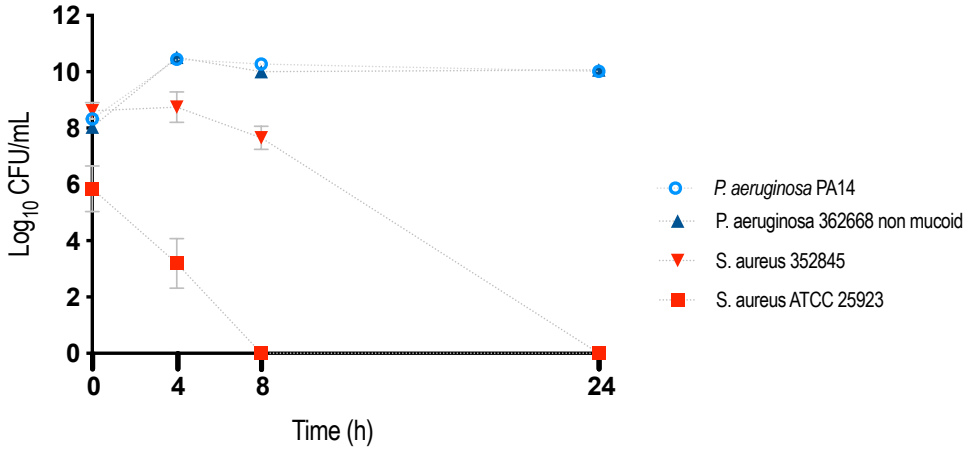

Figure 4.11. Growth of the BRC obtained immediately after medium replacement. Fresh TSB added to three wells of *P. aeruginosa* - *S. aureus* 24-h-old dual-species biofilms was then collected and incubated at 37◦C, 120 rpm in air conditions. Aliquots were recorded after 4-, 8- and 24-h of incubation for CFU counting in selective media. The results for two independent assays are shown as mean ± SD

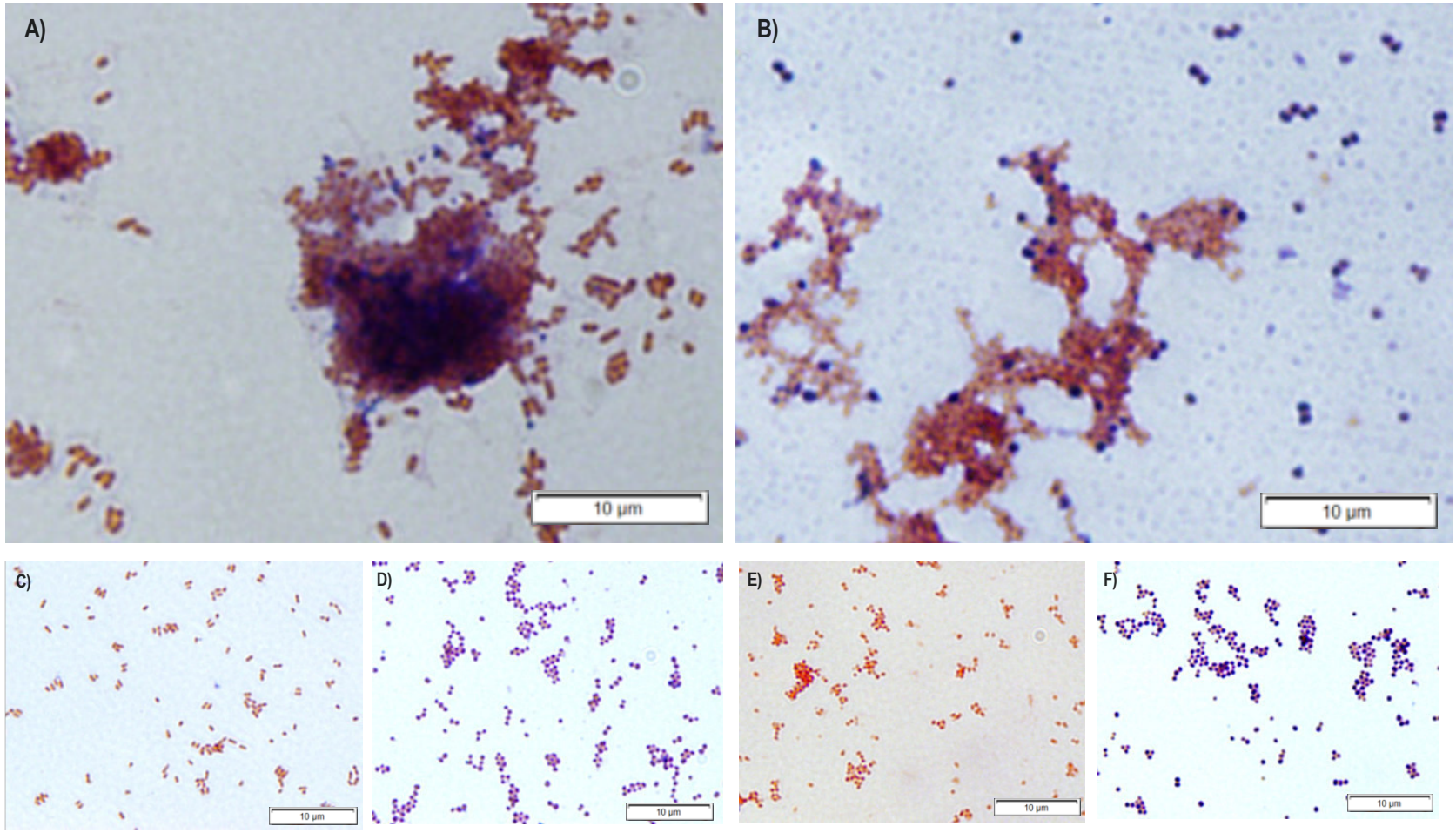

Figure 4.12. Visualization of BRC recovered from the bulk fluid of 48-h-old dual-species biofilms after gram staining under 100x light microscope. **A)** *P. aeruginosa* PA14 + *S. aureus* ATCC 25923, **B)** *P. aeruginosa* 362668 non mucoid + *S. aureus* 352845. Single-species gram staining as control: **C)** *P. aeruginosa* PA14, **D)** *P. aeruginosa* 362668 non mucoid, **E)** *S. aureus* ATCC 25923 and **F)** *S. aureus* 352845. Gram-positive bacteria stain violet (*S. aureus)* and Gram-negative stain pink (*P. aeruginosa).*

In its turn, this observation had led us to hypothesize that *S. aureus* cultivability is somehow affected by the prolonged interaction with *P. aeruginosa.* To address this hypothesis, a combination of flow cytometry (FC), epifluorescence microscopy (EM) and culture-based methods was used to reveal whether *S. aureus* cultivability was firstly underestimated. Cells were stained with PI, WGA conjugated with FITC and DAPI. Interestingly, the results from FC and culture-based method revealed that only slight differences (< 1 Log) were observed for both species as single-species biofilms, (**Figure 4.13**). While FC data revealed the presence of *S. aureus* in high numbers (~ 6-Log) in 48-h-old dual-species biofilms and in the biofilm bulk fluid, for both strains (**Figure 4.14 A and B**). It was also observed that *P. aeruginosa* viability was not affected by the presence of *S. aureus* as FC results are in agreement with cultivability data for all tested strains. Moreover, the VBNC switching phenomenon was further confirmed as not be strain dependent as four other *P. aeruginosa - S. aureus* consortia were tested with similar outcomes (**Figure 4.14 C-F**). Additionally, after WGA-FITC + DAPI + PI staining, *S. aureus* cells (stained in green with WGA-FITC) were also observed in both 24- and 48-h-old dual-species biofilms as can be seen on representative EM images (**Figure 4.15**). Also, the proportion of red-stained cells (dead cells) observed in both 24- and 48-h-old biofilms was low. These results suggest that most of the cells are viable, despite not being cultivable. Staining of single-species biofilms noted only a few dead cells among viable cells on both 24- and 48-h-old biofilms (**Figure 4.16 and 4.17**, respectively).

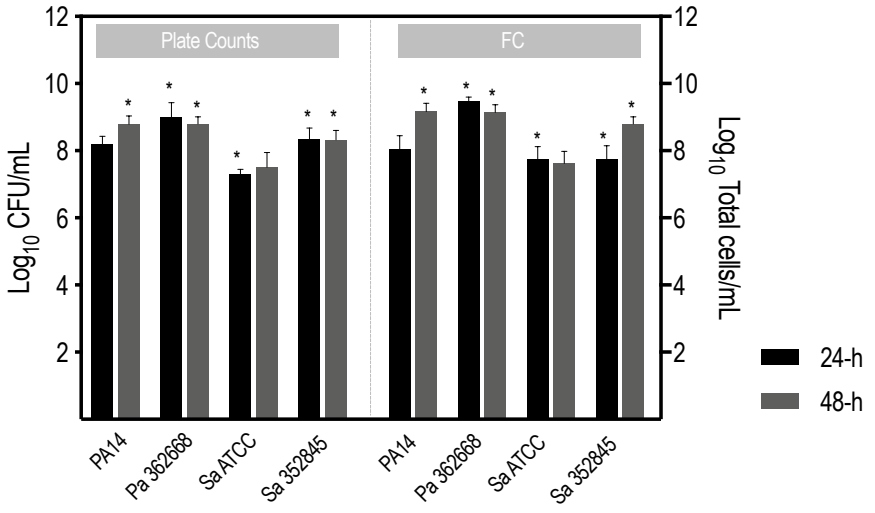

Figure 4.13. Populations of *P. aeruginosa* PA14 (PA), *P. aeruginosa* 362668 non mucoid (Pa), *S. aureus* ATCC 25923 (SA) and *S. aureus* 352845 (Sa) in 24- and 48-h-old single-species biofilms. The results for two independent assays are shown as mean +/- SD. *p<0.05, unpaired two-tailed Mann-Whitney test to compare significantly differences between CFU and FC counts.

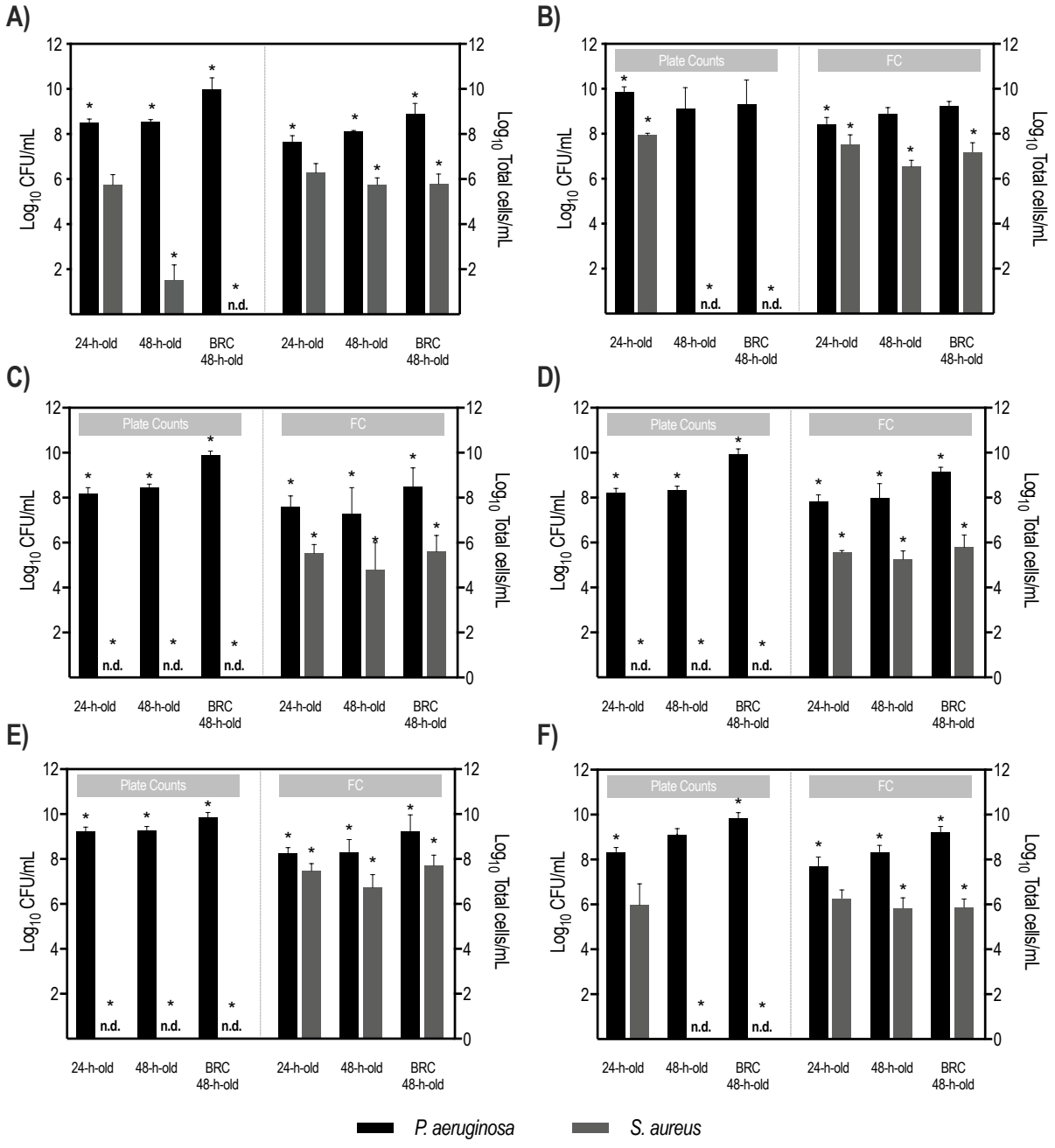

Figure 4.14. Populations of *P. aeruginosa* and *S. aureus* in dual-species biofilms, estimated by plate counts (CFU/mL) and flow cytometry (Total Cells/mL). Quantification was performed in 24- and 48-h-old dual-species biofilms and in the bulk liquid of 48-h-old dual-species biofilms. **A)** *P. aeruginosa* PA14 and *S. aureus* ATCC 25923 and **B)** *P. aeruginosa* 362668 non-mucoid and *S. aureus* 352845. **C)** *P. aeruginosa* 362668 mucoid and *S. aureus* ATCC 25923, **D)** *P. aeruginosa* 362668 mucoid and *S. aureus* 352845, **E)** *P. aeruginosa* 362668 non-mucoid and *S. aureus* ATCC 25923 and **F)** *P. aeruginosa* PA14 and *S. aureus* 352845. The results for two to three independent assays are shown as mean +/- SD. * $p<0.05$, unpaired two-tailed Mann-Whitney test to compare significantly differences between CFU and FC counts.

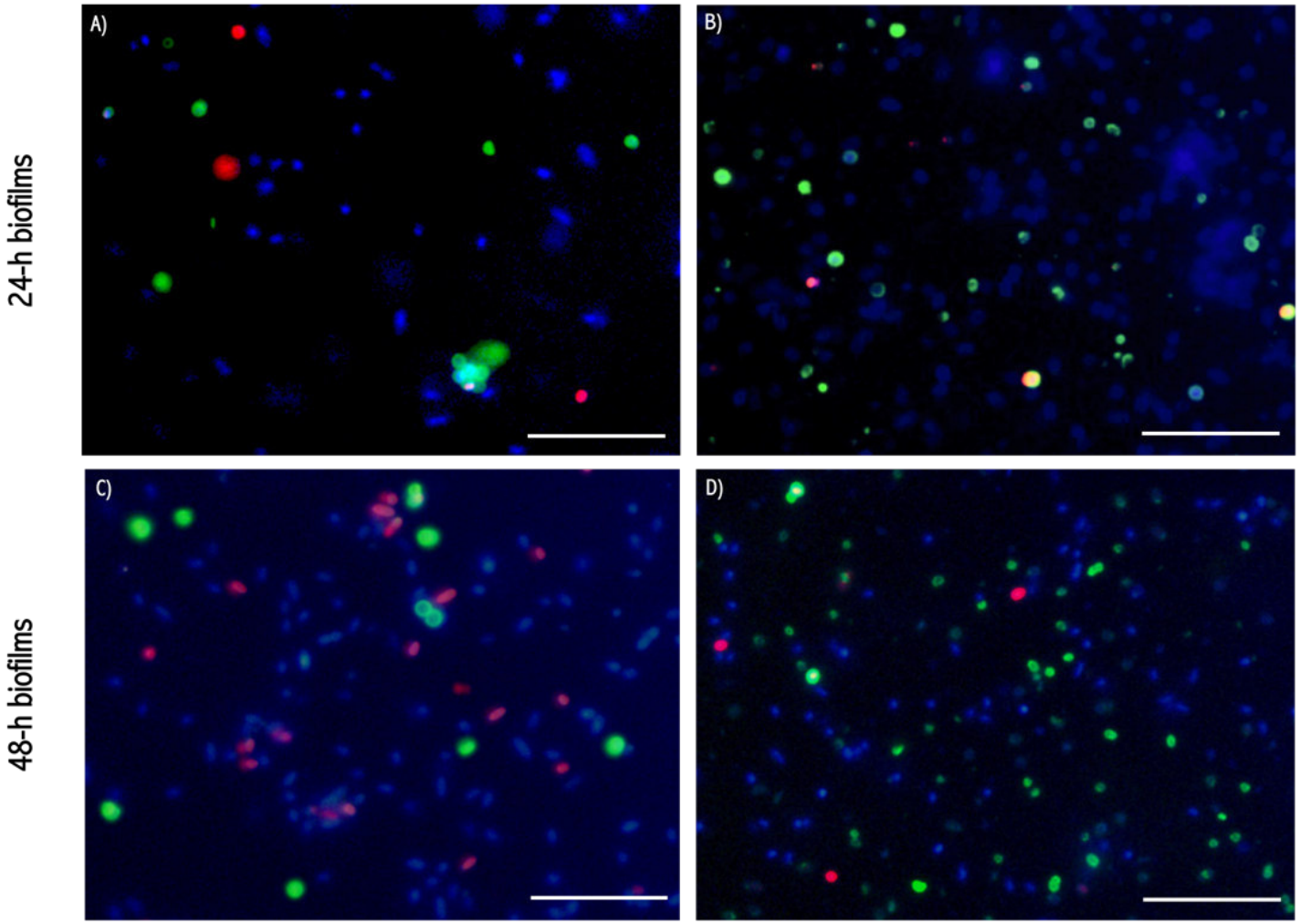

Figure 4.15. Representative epifluorescence microscopy images of 24 h and 48 h. **(A, C)** *P. aeruginosa* PA14 + *S. aureus* ATCC 25923 and (**B, D)** *P. aeruginosa* 362668 non mucoid + *S. aureus* 352845 dual-species harvested biofilms stained with DAPi + WGA-FITC + PI. Scale bars correspond to 10 μm.

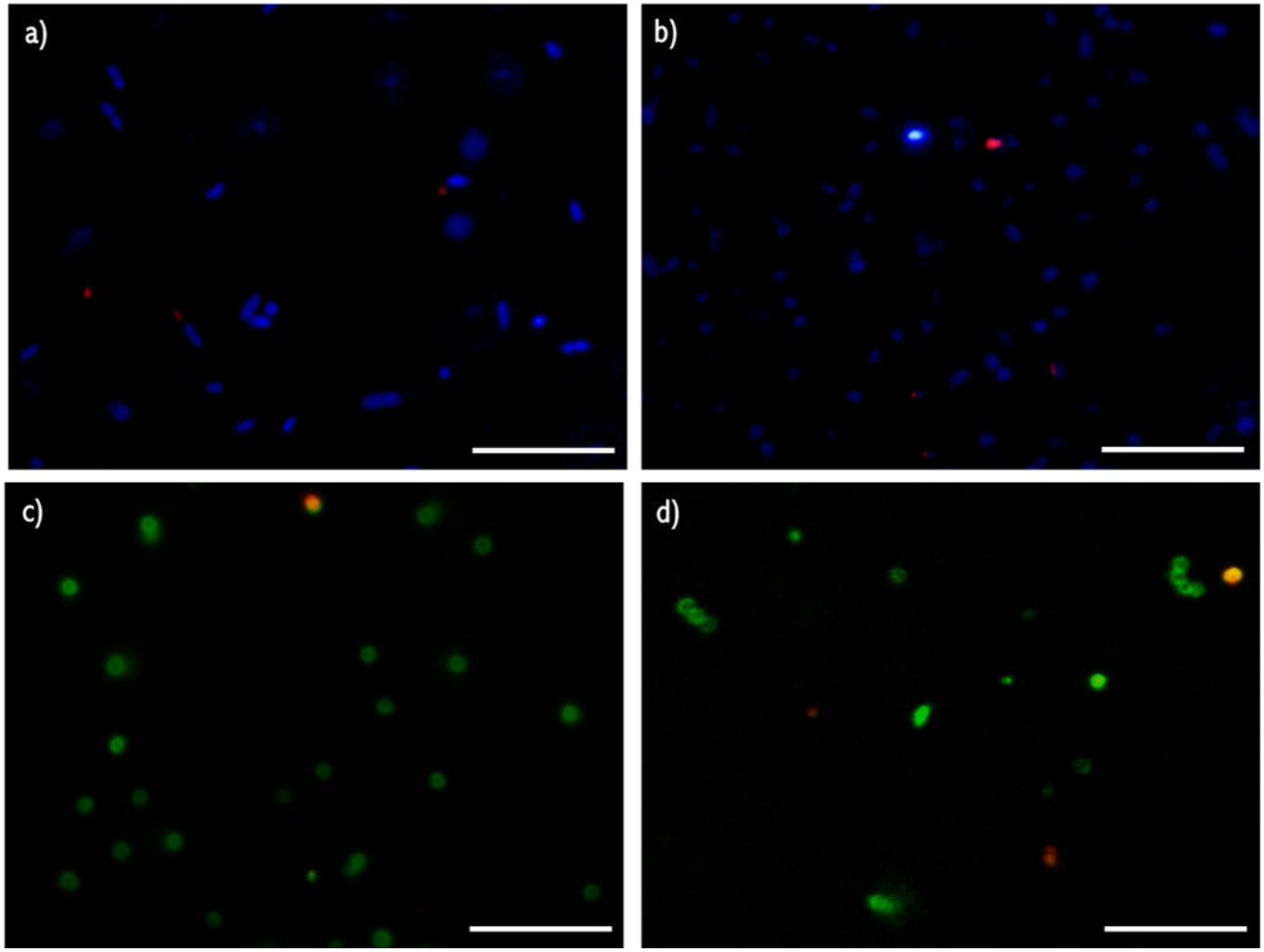

Figure 4.16. Representative epifluorescence microscopy images of 24-h single-species harvested biofilms of **(a)** *P. aeruginosa* PA14 **(b)** *P. aeruginosa* 362668 non mucoid stained with DAPI + PI, and 24-h single-species biofilms of **(c)** *S. aureus* ATCC 25923 and **(d)** *S. aureus* 352845 biofilms stained with WGA-FITC + PI. Scale bars correspond to 10 μm.

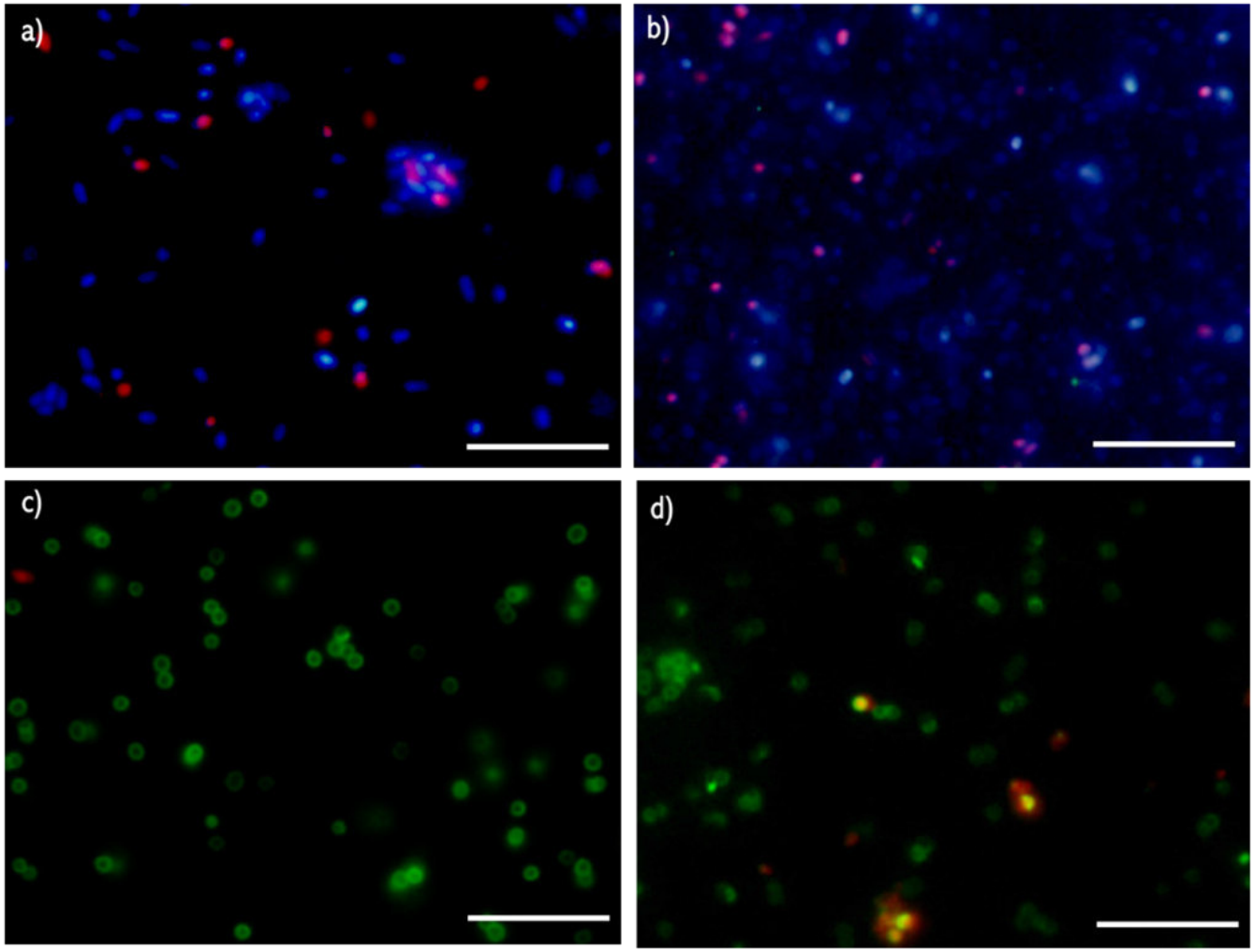

Figure 4.17. Representative epifluorescence microscopy images of 48-h single-species harvested biofilms of **(a)** *P. aeruginosa* PA14 **(b)** *P. aeruginosa* 362668 non mucoid stained with DAPI + PI, and 48-h single-species biofilms of **(c)** *S. aureus* ATCC 25923 and **(d)** *S. aureus* 352845 biofilms stained with WGA-FITC + PI. Scale bars correspond to 10 μm.

4.3.3 Expression profile of critical virulence-related genes in dual-species biofilms

Gene expression analysis was firstly used to test the hypothesis that the resulting phenotypic changes, observed after 48 h of interaction with *P. aeruginosa,* are directly correlated with *S. aureus* virulence potential. Thus, the expression of *S. aureus* genes related to QS (*sarA* and *hld*), biofilm formation (*icaA)*, cytotoxicity (*clpP)* and stress response (*sodA* and *uspA)* was evaluated by qPCR in 24- and 48-h-old dual-species biofilms (**Table 4.2**). Not surprisingly, results revealed that the two time points of interaction (24 h *versus* 48 h) generated distinct gene transcription profiles, with *S. aureus* virulence-related genes being significantly overexpressed (p<0.05) upon 48 h of growth as dual-species biofilms. When comparing the transcription of dual- *versus* single-species biofilms, significant downregulation (p<0.05) was observed at 24 h (except for *icaA* gene*)*, while all *S. aureus* virulence-related genes tended to be more expressed upon 48 h of co-culture (except for *hld* gene), comparing to single-species biofilm at identical culture time. Overall, the increased expression observed in 48-h-old dual-species biofilms suggests that *P. aeruginosa* plays a role in modulating the virulence potential of *S. aureus.*

Changes in the transcription of QS genes were only observed for strain PA14 with *pqsE* and *lasI* overexpressed (p<0.05) after 48 h of co-culture, comparing to 24-h-old dual-species biofilms. When *P. aeruginosa* biofilms were grown for 24 h*,* all QS genes (*pqsE, rhlR* and *lasI)* were significantly upregulated (p<0.05) in strain PA14, while no significant changes were noticed in strain 362668, comparing to individual biofilms. For *P. aeruginosa* 48 h biofilms no significant differences were observed in transcripts levels, although *P. aeruginosa* QS genes tended to be downregulated in co-culture.

Table 4.2. Gene expression profile of *P. aeruginosa* and *S. aureus* growing as single- and dual-species biofilm for 24 h and 48 h. Data indicate mRNA transcription normalized to 16S RNA. The mean ± SD for at least three independent assays is presented. Statistical significance was determined by performing an unpaired two-tailed Mann-Whitney test (p<0.05).

		Dual-species		Single-species	
Gene	**Strain**	**24-h-old**	**48-h-old**	**24-h-old**	**48-h-old**
sodA	*S. aureus* ATCC 25923	$8.14 \pm 5.68 \times 10^{-6}$	$3.75 \pm 5.76 \times 10^{-3}$ [a]	$4.04 \pm 2.35 \times 10^{-4}$ [b]	$5.86 \pm 0.48 \times 10^{-4}$
	S. aureus 352845	$6.91 \pm 4.57 \times 10^{-6}$	$1.02 \pm 1.24 \times 10^{-3}$ [a]	$6.12 \pm 2.13 \times 10^{-4}$ [b]	$3.94 \pm 0.06 \times 10^{-4}$
sarA	S. *aureus* ATCC 25923	$7.84 \pm 7.90 \times 10^{-6}$	$3.09 \pm 4.88 \times 10^{-3}$ [a]	$1.74 \pm 0.25 \times 10^{-4}$ [b]	$1.81 \pm 0.14 \times 10^{-4}$
	S. aureus 352845	$3.82 \pm 2.96 \times 10^{-5}$	$2.17 \pm 0.89 \times 10^{-3}$ [a]	$5.44 \pm 0.89 \times 10^{-4}$ [b]	$3.67 \pm 0.14 \times 10^{-4}$ [b]
hld	S. *aureus* ATCC 25923	$1.99 \pm 2.88 \times 10^{-5}$	$1.54 \pm 1.82 \times 10^{-3}$ [a]	$4.06 \pm 1.82 \times 10^{-3}$ [b]	$7.27 \pm 0.74 \times 10^{-3}$ [b]
	S. aureus 352845	$3.36 \pm 2.34 \times 10^{-5}$	$4.81 \pm 6.39 \times 10^{-4}$ [a]	$2.91 \pm 0.48 \times 10^{-2}$ [b]	$8.55 \pm 0.08 \times 10^{-3}$ [b]
icaA	S. *aureus* ATCC 25923	$1.14 \pm 1.29 \times 10^{-6}$	$4.09 \pm 5.53 \times 10^{-3}$ [a]	$1.74 \pm 0.99 \times 10^{-6}$	$1.43 \pm 0.40 \times 10^{-6}$ [b]
	S. aureus 352845	$3.36 \pm 5.18 \times 10^{-6}$	$4.09 \pm 6.11 \times 10^{-4}$ [a]	$2.79 \pm 0.30 \times 10^{-6}$	$2.09 \pm 0.59 \times 10^{-6}$ [b]
clpP	S. *aureus* ATCC 25923	$7.70 \pm 5.67 \times 10^{-6}$	$4.13 \pm 6.68 \times 10^{-3}$ [a]	$2.70 \pm 1.02 \times 10^{-4}$ [b]	$3.49 \pm 0.27 \times 10^{-4}$
	S. aureus 352845	$5.72 \pm 4.64 \times 10^{-5}$	$1.08 \pm 0.57 \times 10^{-3}$ [a]	$7.32 \pm 2.13 \times 10^{-4}$ [b]	$7.74 \pm 1.01 \times 10^{-4}$
uspA	S. *aureus* ATCC 25923	$1.33 \pm 0.55 \times 10^{-5}$	$3.46 \pm 5.51 \times 10^{-3}$ [a]	$5.91 \pm 3.05 \times 10^{-4}$ [b]	$5.13 \pm 0.81 \times 10^{-4}$
	S. aureus 352845	$5.35 \pm 2.07 \times 10^{-5}$	$2.39 \pm 0.16 \times 10^{-3}$ [a]	$7.90 \pm 3.70 \times 10^{-4}$ [b]	$5.81 \pm 0.50 \times 10^{-4}$ [b]
pqsE	*P. aeruginosa* PA14	$8.10 \pm 8.52 \times 10^{-6}$	$1.15 \pm 1.31 \times 10^{-4}$ [a]	$2.12 \pm 0.84 \times 10^{-6}$ [b]	$1.40 \pm 1.88 \times 10^{-3}$
	P. aeruginosa 362668 non mucoid	$2.08 \pm 3.49 \times 10^{-5}$	$2.38 \pm 4.03 \times 10^{-4}$	$0.84 \pm 1.36 \times 10^{-4}$	$3.76 \pm 5.96 \times 10^{-4}$
rhlR	*P. aeruginosa* PA14	$0.98 \pm 1.13 \times 10^{-4}$	$0.67 \pm 1.13 \times 10^{-4}$	$2.72 \pm 0.37 \times 10^{-5}$ [b]	$2.57 \pm 3.50 \times 10^{-3}$ [b]
	P. aeruginosa 362668 non mucoid	$3.83 \pm 5.88 \times 10^{-5}$	$3.61 \pm 6.03 \times 10^{-4}$	$1.30 \pm 2.10 \times 10^{-4}$	$6.26 \pm 9.78 \times 10^{-4}$
lal	*P. aeruginosa* PA14	$7.15 \pm 8.41 \times 10^{-5}$	$3.07 \pm 2.00 \times 10^{-4}$ [a]	$1.01 \pm 0.06 \times 10^{-5}$ [b]	$3.21 \pm 3.33 \times 10^{-3}$ [b]
	P. aeruginosa 362668 non mucoid	$4.02 \pm 4.80 \times 10^{-5}$	$0.79 \pm 1.04 \times 10^{-3}$	$1.75 \pm 2.46 \times 10^{-4}$	$0.96 \pm 1.11 \times 10^{-3}$

[a] Significant differences between 48 h and 24 h in dual-species biofilms.

[b] Significant differences between dual- and single-species biofilms in 24 h or 48 h.

4.4 Discussion

Pseudomonas aeruginosa and *S. aureus* are the most prevalent pathogens in CF infections and, although displaying an inverse correlation with one another, they are not mutually exclusive since patients are frequently diagnosed as being co-infected by both species [2, 4]. Despite co-colonization of *P. aeruginosa* and *S. aureus* is frequent and documented, the consequences of their interaction and the role of each species within multi-species biofilms is mostly unknown.

Previously, it was reported that *S. aureus* alters its cultivability growing as dual-species biofilms with *P. aeruginosa* [33]. These findings driven us to design a following study to gain insights into the possible mechanisms governing their co-existence.

In the competition assay, *P. aeruginosa* is confirmed to be the dominant species during *in vitro* evolution, as both tested strains were able to outcompete *S. aureus* after 24 h of growth as dual-species biofilms, without their own growth being adversely affected. Previous studies have confirmed that *P. aeruginosa* QS-controlled exoproducts can inhibit *S. aureus* growth in dual-species cultures *in vitro* and *in vivo* [6, 8, 20]. The exposure of *S. aureus* to the supernatant resulting from both *P. aeruginosa* and *P. aeruginosa + S. aureus* biofilm phase significantly reduced the growth of all *S. aureus* strains after just 4 h of interaction. However, the data gathered here also revealed that both staphylococcal species are present in the bulk fluid of 48-h-old dual-species biofilms while no cultivable *S. aureus* cells were detected. Indeed, compared to FC data, plate cell counts seem to underestimate the presence of *S. aureus* in 48-h-old dual-species biofilms. Moreover, it was also evident that *S. aureus* viability was not compromised since biofilm cells were co-stained with PI + WGA-FITC. Fluorescence microscopy confirmed FC results, indicating that the majority of the harvested biofilms cells are truly viable since PI-staining was considerably low. The fact that *S. aureus* viability, estimated based on culture-based method, was significantly lower than that pointed out by FC and EM suggests that *P. aeruginosa* induces a VBNC state in *S. aureus* cells embedded within the 48-h-old dual-species biofilms. This hypothesis was further strengthened by inspecting four additional *P. aeruginosa - S. aureus* consortia through FC and culture based-methodologies with similar outcomes.

The VBNC state is a unique survival strategy adopted by many bacteria in response to adverse environmental conditions such as antimicrobial pressure [34], high/low temperature [35] and starvation [36]. Additionally, the ability of microorganisms to enter the VBNC state may be

advantageous for cells [37] but the underestimation or non-detection of viable cells in clinical samples induces a serious risk to human health [38].

Recently, it was reported that *P. aeruginosa* co-infected with *S. aureus* induces the expressions of staphylococcal virulence factors, contributing to the severity of polymicrobial wound infections [39]. Therefore, understanding how *S. aureus* regulates gene expression is critical to provide insights into the impact of the observed phenotypic switching on the *S. aureus* pathogenesis. The transcription of several virulence-related genes that are directly involved in QS (*sarA* and *hld*), biofilm formation (*icaA)*, cytotoxicity (*clpP)* and stress response (*sodA* and *uspA)* were examined and compared in 24 h and 48 h dual-species biofilms. The transcriptome analysis showed that all *S. aureus* genes were significantly upregulated in 48-h-old dual-species biofilms compared to 24 h of co-culture. Also, *S. aureus* genes tended to be more expressed upon 48 h of co-culture compared to individual biofilms. Overall, our results suggest that the presence of *P. aeruginosa* in dual-species biofilms strengthens the switching of *S. aureus* to a VBNC state, with increased virulence potential, demonstrating the ability of *S. aureus* to persist within dual-species biofilms encompassing both *S. aureus* and *P. aeruginosa.* Unluckily, the mechanisms underlying this adaptation are unknown and its unveiling will require further investigation.

Considering that *S. aureus* is not truly outcompeted when embedded in dual-species biofilm, the expression level of *P. aeruginosa* QS genes was also assessed since it has been proposed that deficient QS regulation in *P. aeruginosa* strains contributes to the reduced removal of *S. aureus* from dual cultures [18, 40, 41]. Overall, it was found that when in dual-species biofilms the *P. aeruginosa* QS genes were slightly up-regulated from 24 h to 48 h of co-culture. Even so, their expression is lower than those observed for the 48 h old single-species. Some studies reported that *P. aeruginosa* strains with deficient QS regulation could form a symbiotic relationship with *S. aureus* [18, 40, 41]. Therefore, the decreased QS-related competitiveness of *P. aeruginosa* (dual- *versus* single-species biofilms) could promote the co-existence of the competing species in the *P. aeruginosa* dominated community. It is plausible to assume that during the course of infection, *P. aeruginosa* might find helpful to maintain *S. aureus* population. This possibility is supported by evidences that *P. aeruginosa* populations benefit from the presence of toxin-producing *S. aureus* strains in both the context of wound and lung infection [39, 42]. The idea that *P. aeruginosa* benefits from polymicrobial infection provides an additional explanation for the

occurrence of numerous *P. aeruginosa* strains with reduced anti-staphylococcal capacity isolated from chronically-infected CF patients [10, 17, 18, 43].

Overall, our findings revealed the ability of *S. aureus* to persist within a dual-species biofilm containing *P. aeruginosa*. Nevertheless, this biofilm model embraces a few limitations (e.g. it is *an in vitro* model, it includes only four different strains; it comprises biofilms grown in nutrient-rich environment and no other conditions were used; it embodies a mixture of two species rather than the full diversity and abundance of microbes in CF infections). Thus, further studies are needed to completely explore interspecies interactions and the mechanisms that occur overtime in both *S. aureus* and *P. aeruginosa,* and whether the modulated bacterial virulence is clinically significant. The role of polymicrobial biofilms in infectious diseases, such as CF, is of utmost importance and will probably assist novel therapies targeting the multiplicity of species within the consortia by avoiding the enhanced pathogenesis that results from interactions among the causative microbes of such infections.

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CHAPTER 5

Bidirectional protection of *P. aeruginosa* and *S. aureus* dual-species biofilms upon antibiotic treatment

Summary

P. aeruginosa and *S. aureus* often adopt a biofilm mode of growth, which is being increasingly acknowledged as contributing to high tolerance to antibiotic treatment and the recalcitrant nature of CF chronic co-infections, leading to significant patient morbidity and mortality. While *P. aeruginosa* infections undoubtedly cause deterioration in patient health, the role of *S. aureus* in infection progression remains controversial. Therefore, to better understand the co-existence state allowing *S. aureus* to survive in the presence of *P. aeruginosa,* and to gain insight into the role that interactions between both species might have on antimicrobial tolerance, single- and dual-species biofilms were characterized upon ciprofloxacin and vancomycin treatment. The role of VBNC and morphological diversification on antibiotic effectiveness was also ascertained. Data showed that *P. aeruginosa* decreased the sensitivity of *S. aureus* biofilm populations toward vancomycin and ciprofloxacin. Likewise, *S. aureus* also protected *P. aeruginosa* from the antimicrobial activity of ciprofloxacin. Following antibiotic therapy, *S. aureus* persisted in the dual-species biofilm in a VBNC state. It was also observed that *P. aeruginosa* triggered *S. aureus* SCV selection, however this phenotypic diversification did not disturb antibiotic efficacy toward *S. aureus* within the dual-species biofilms. These results reinforce the role of *S.* aureus VBNC throughout *P. aeruginosa – S.* aureus interactions and support the premise that *S. aureus* increased tolerance to antibiotic treatments is related with the VBNC state.

Taken together, these findings pointed out that, beyond *P. aeruginosa – S. aureus* co-existence, both bacteria can even interact synergistically, leading to increased tolerance of the consortia to antimicrobial therapy, which helps to explain the clinical failure of current antibiotic treatment regiments in CF infections.

5.1 Brief introduction

Interactions between *P. aeruginosa* and *S. aureus* have been widely studied and it is commonly admitted that *P. aeruginosa* outcompetes *S. aureus*. As discussed in chapter 2, *P. aeruginosa* negatively impacts *S. aureus* by producing several compounds, eventually leading to reduced *S. aureus* viability [1–4] and transition into SCV [5]. The switch to the SCV phenotype improves the survival of *S. aureus* under unfavourable conditions [6, 7]. Alongside with *S. aureus*, long-term persistence of *P. aeruginosa* in CF lungs has also been associated with its clonal diversification into specialized phenotypes, such as mucoid variants and SCV [8]. *P. aeruginosa* SCV are frequently isolated from chronic CF lung infections mainly after long antibiotic courses [9, 10]. These colony variants are known to exhibit hyper biofilm formation [11] and increased resistance to several classes of antibiotics, contributing to *P. aeruginosa* persistence and decline of lung function [9, 12]. Indeed, *in vivo* models indicate that co-infection increases disease severity and antimicrobial resistance [1, 13–16]. This cooperative co-existence is expected to influence pathogenic behaviour such as biofilm formation and antibiotic tolerance [15, 17].

Therefore, it was considered of utmost importance to investigate whether *P. aeruginosa* and/or *S. aureus* alters its antibiotic response depending on the inter-species interactions occurring between them. Moreover, taking into account the results described in chapter 4, it was also intended to inspect the presence of VBNC cells within the dual-species biofilms and, also, determine whether this state has a role on antibiotic effectiveness. The occurrence of clonal diversification driven by the inter-species interactions or trigged by antimicrobial treatments was also inspected to gain insights into the driving forces that can elicit that diversification and, thus, more comprehensively understand their impact on infection progression.

5.2 Materials and Methods

5.2.1 Microorganisms and culture conditions

P. aeruginosa (UCBPP-PA14 and an anonymized clinical isolate 362668 non-mucoid) and *S. aureus* (ATCC 25923 and an anonymized clinical isolate 352845) strains were used throughout this work. The strains were preserved and cultured as described in section **4.2.1**.

5.2.2 Determination of Minimum Inhibitory Concentration (MIC)

The antibiotic susceptibility of *P. aeruginosa* and *S. aureus* strains was ascertained by determining the MIC using the microdilution assay following the recommendations of the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards) [18]. Overnight cultures of *P. aeruginosa* and *S. aureus* strains were washed and diluted with Mueller Hinton Broth (MHB) to 5×10^5 CFU/mL and transferred to 96 well plates. Further, bacterial strains were exposed to different concentrations of ciprofloxacin (CIP) and vancomycin (VAC), ranging from 0.0625 to 16 mg/L, at 37 °C, 120 rpm, for 18–21 h in air conditions. MIC was established as the minimum concentration of antibiotic required to inhibit 90% of growth, measuring the OD at 620 nm using an ELISA microtiter plate reader (Sunrise-Basic Tecan, Männedorf, Switzerland). MHB alone and bacterial cultures free of CIP and VANC were used as negative and positive controls, respectively. VAC, is a frontline antibiotic for the treatment of infections caused by *S. aureus* but typically inefficient against *P. aeruginosa*, and CIP, is a broad-spectrum antibiotic active against both *S. aureus* and *P. aeruginosa* infections

5.2.3 Application of ciprofloxacin and vancomycin treatments to single- and dual-species biofilms

Single- and dual-species biofilms of *P. aeruginosa* and *S. aureus* were formed as described in section **4.2.2**, with some minor modifications. In brief, after 24 h of growth the content of each well was replaced with fresh TSB medium and then, single- and dual-species biofilms, were exposed for an additional 24 h period to peak serum concentrations (PSC) of CIP (4.5 mg/L) and VAC (40 mg/L) by adding 10 μL of antibiotic solution to each well. For comparison purposes, 24-h-old biofilms were not exposed to antibiotics, being the content of the respective wells replaced solely with fresh TSB medium, achieving 48-h-old non-treated biofilms. Following biofilm growth, the planktonic liquid content of the polystyrene plates was withdrawn, and the wells were washed twice with distilled sterile water. Biofilms cells were resuspended into 1 mL of 0.9% NaCl, detached by scraping and collected for further analysis.

5.2.4 Quantification of single- and dual-species biofilms

P. aeruginosa and *S. aureus* cultivable cells within single- and dual-species biofilms were determined as described in section **4.2.3**. Further, total counts and cell viability by flow cytometry (FC) were also analysed following the procedure described in section **4.2.7**.

5.2.5 Phenotypic diversity and pathogenic potential of *P. aeruginosa* and *S. aureus* populations

Phenotypic diversity of *P. aeruginosa* and *S. aureus* bacterial populations was assessed through colony morphology observation. Briefly, single- and dual-species biofilms were grown as mentioned in section **4.2.2** and species population was inspected by serially dilute and plate bacteria on TSA plates and let them grow at 37 °C for 48 h, as previously optimized [19–21]. Colonies were evaluated according to their form, margin, type of surface, texture, size, sheath, elevation, opacity, consistency and colour as described by Sousa *et al.* [22]. The relative abundance of each colony morphotype was estimated based on the number of each morphotype comparatively to the number of total colonies in the TSA plates.

The different morphotype-forming bacteria were inspected in terms of pathogenic potential by determining their biofilm formation ability and antibiotic susceptibility.

The ability of each colony morphotype-associated bacteria to form biofilms was assessed by the CV method, as previously described in section **4.2.3,** with minor modifications. Briefly, 1×10^7 CFU/mL of cell suspension of each colony morphotype was incubated in the 96 well microtiter plates.

The antimicrobial susceptibility of of each morphotype was determined using the disk diffusion method, performed in accordance with the recommendations of CLSI [18]. The inhibition zones were measured (mm) after 18-21 h of bacterial growth at 37 °C in air conditions. Bacteria were assigned as resistant, intermediate resistant or sensitive according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [23].

5.2.6 Statistical analysis

All numerical data were subjected to statistical analysis using the non-parametric Mann–Whitney U test since the data that did not follow a normal distribution according to Kolmogorov–Smirvon's

test, with the statistical software Prism Version 7.0a for Macintosh. Results are presented as mean ± standard deviation (SD), unless stated otherwise.

5.3 Results

5.3.1 Effect of antibiotic treatments on microbial composition of *P. aeruginosa* and *S. aureus* dual-species biofilms

First, the antimicrobial susceptibility profile of all tested strains to both VAC and CIP was determined. As shown in **Table 5. 1** *P. aeruginosa* PA14 and *S. aureus* ATCC 25923 strains were susceptible to CIP action, while *P. aeruginosa* 382668 non mucoid and *S. aureus* 352845 CF isolated strains were resistant. Regarding VAC, both *S. aureus* strains were susceptible.

Table 5.1. MIC values (in mg/L) of CIP and VANC against *P. aeruginosa* and *S. aureus* planktonic cultures.

	CIP	**Susceptibility category** [a)]	**VANC**	**Susceptibility category** [a)]
***P. aeruginosa* PA14**	0.125	susceptible	N/A	N/A
***P. aeruginosa* 362668 non mucoid**	1	**resistant**	N/A	N/A
***S. aureus* ATCC 25923**	0.5	susceptible	2	susceptible
***S. aureus* 352845**	2	**resistant**	2	susceptible

N/A - Not applicable

Since biofilms are known to be more tolerant than planktonic cultures [24], pre-formed biofilms were challenged to PSC (supra-MIC concentrations) of each antibiotic [25, 26]. Then, the effect of CIP and VAC on cell viability and cultivability of the single-species biofilms was determined using FC and culture-based methods, respectively. As already noticed in Chapter 4, FC data did not entirely corroborate with CFU counts when *P. aeruginosa* and *S. aureus* biofilms were challenged with both antibiotics. Looking at FC data **(Figure 5.1)**, *P. aeruginosa* viability was significantly affected (~ 2.5 Log reduction) when exposed to CIP regardless the antimicrobial susceptibility profile of the strains provided in **Table 5.1** ($p < 0.05$). Conversely, *S. aureus* ATCC 25923 viability was not significantly disturbed by either of the antibiotics, while *S. aureus* 352845 biofilm cell viability was slightly affected by CIP (< 1 Log) and significantly reduced for VAC ($p<0.05$).

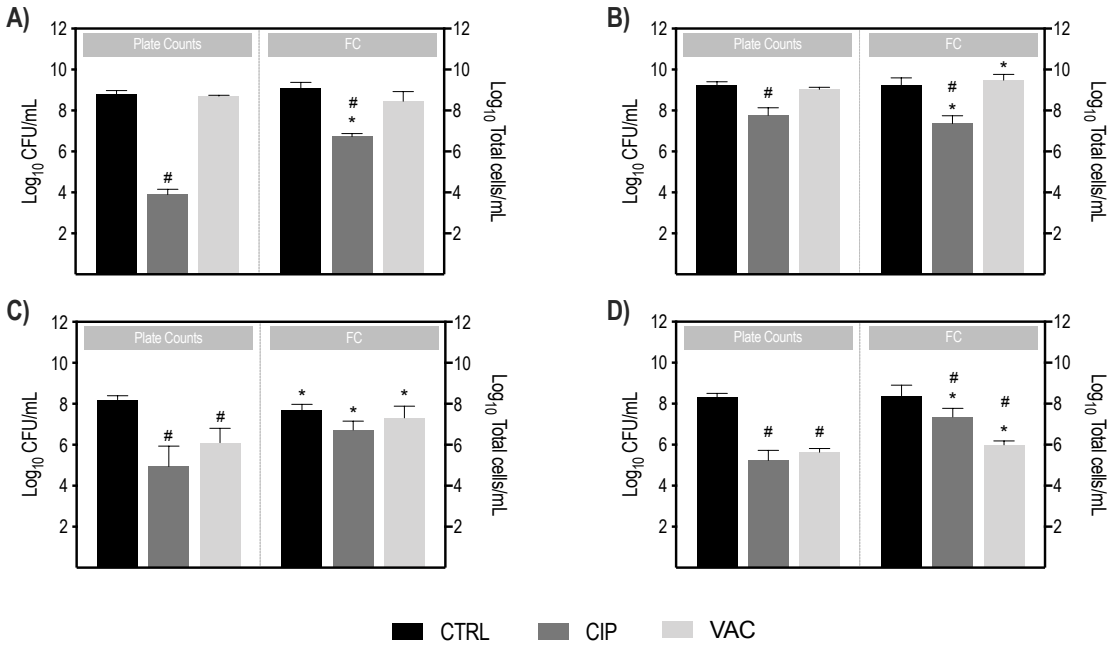

Figure 5.1. Quantitative assessment of *P. aeruginosa* and *S. aureus* 48-h-old single-species biofilms unexposed (CTRL) and exposed to CIP and VAC treatments, estimated by plate counts (CFU/mL) and FC (Total Cells/mL). **(A)** *P. aeruginosa* PA14, **(B)** *P. aeruginosa* 362668 non mucoid, **(C)** *S. aureus* ATCC 25923 and **(D)** *S. aureus* 352845. The results for three independent assays are shown as mean +/- SD. *p<0.05, unpaired two-tailed Mann-Whitney test to compare significantly differences between CFU and FC counts; #p<0.05, to compare significantly differences between CTRL and antibiotic treatments.

To ascertain whether *P. aeruginosa* - *S. aureus* interactions could have a role on antibiotic effectiveness and gain knowledge to step forward the understanding gathered in Chapter 4, the microbial composition of the 48-h-old dual-species biofilms, unexposed and exposed to antibiotic treatments, was also inspected by FC and CFU counts. As can be seen in **Figure 5.2**, without antibiotic exposure, *P. aeruginosa* clearly predominated in the dual-species biofilms. This predominance was however less marked after CIP and VAC treatment, once a greater number of viable cells was detected for *S. aureus* strains, which were unaffected by both antibiotics at the concentration tested. Conversely, *S. aureus* cultivability was significantly reduced with both antibiotics ($p<0.05$), suggesting that *S. aureus* undergoes the VBNC state when challenged by antibiotics. Once again, differences in cell counts were less pronounced for single-species biofilms (**Figure 5.1**), strengthening that the loss of cultivability is related with *P. aeruginosa* – *S. aureus* interactions, as pointed out in Chapter 4.

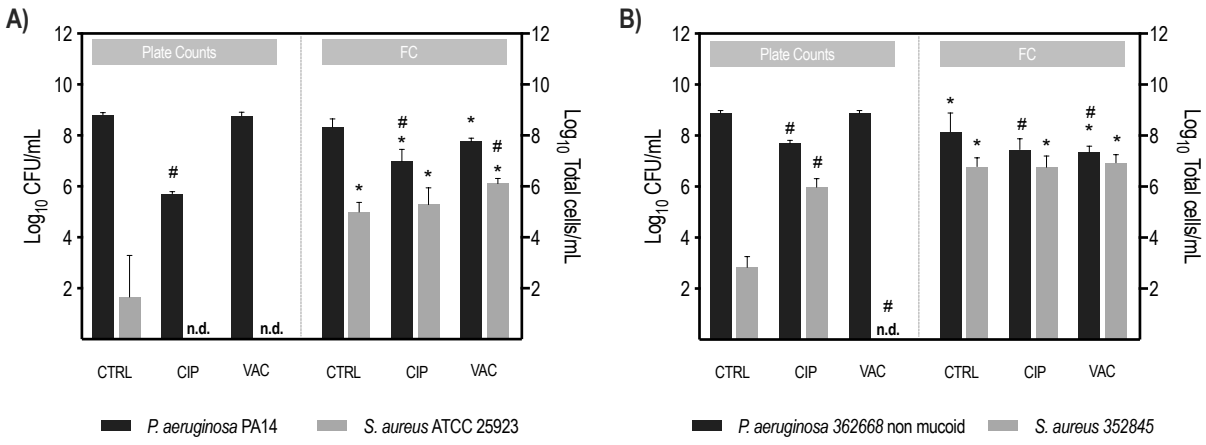

Figure 5.2. Quantitative assessment of *P. aeruginosa* and *S. aureus* 48-h-old dual-species biofilms unexposed (CTRL) and exposed to CIP and VAC treatments, estimated by plate counts (CFU/mL) and FC (Total Cells/mL). **(A)** *P. aeruginosa* PA14 + *S. aureus* ATCC 25923 and **(B)** *P. aeruginosa* 362668 non mucoid + *S. aureus* 352845. The results for three independent assays are shown as mean +/- SD. *p<0.05, unpaired two-tailed Mann-Whitney test to compare significantly differences between CFU and FC counts; #p<0.05, to compare significantly differences between CTRL and antibiotic treatments.

For a more comprehensive assessment of how the single- and dual-species biofilms were affected by the antibiotics, bacterial reductions were determined based on the FC counts after both antibiotic treatments. This data strengthened that *S. aureus* strains were unaffected by CIP and VAN treatments while growing as dual-species biofilms (**Figure 5.3**). Likewise, *P. aeruginosa* strains were also significantly less affected by CIP action when embedded in the dual-species biofilms. (**Figure 5.3A**). About VAC, no differences were noticed it terms of bacterial reduction for *P. aeruginosa* single- and dual-species biofilms as this antibiotic, due to its mode of action, is not a therapeutic choice for gram-negative bacteria [27] (**Figure 5.3B**). Even though some *P. aeruginosa* and *S. aureus* strains were susceptible to CIP and VAC (**Table 5.1)**, the dual-species biofilms presented a high level of tolerance to both tested antibiotics. Overall, these outcomes suggest bidirectional benefits of both bacteria toward antimicrobial treatment once encased in dual-species biofilms.

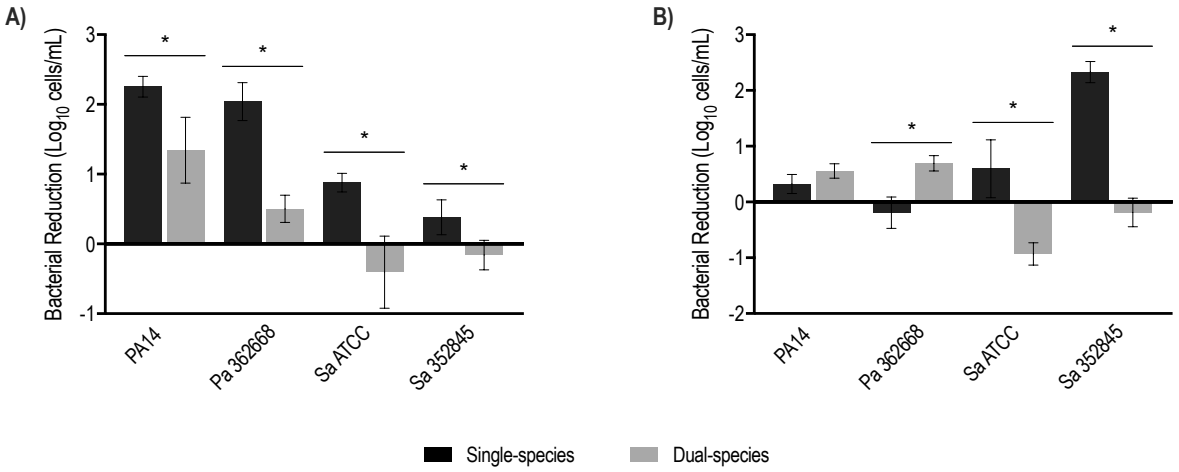

Figure 5.3. Effectiveness of **(A)** CIP and **(B)** VAC treatments against *P. aeruginosa* and *S. aureus* populations encased in 48-h-old single- and dual-species biofilms determined by FC. The results for three independent assays are shown as mean +/- SD. *p<0.05, unpaired two-tailed Mann-Whitney test to compare significantly differences between single- and dual-species biofilms.

5.3.2 Phenotypic diversity of *P. aeruginosa* and *S. aureus* populations in dual-species biofilms

The so-called SCV appear to be crucial for *S. aureus* survival, as these morphotypes present increased resistance to *P. aeruginosa* mediated killing [4]. Indeed, it has been shown that, compared to *S. aureus* WMT, SCV phenotype cells can better withstand external stresses [28]. So, it was hypothesized whether the observed antibiotic treatment failure toward *S. aureus*, when in dual-species biofilms, could be potentially attributed to the presence of SCV. To test this hypothesis, any SCV and specialized phenotype that may arise under biofilms unexposed and exposed to CIP and VAC treatments were inspected.

Based on the standardised morphological characterisation system described by Sousa *et al.* [22], distinct morphotypes of *P. aeruginosa* and *S. aureus* (**Figure 5.4**) were classified as described in **Table 5.2.** With exception of *P. aeruginosa* 362668 non mucoid, that did not exhibit colony diversification although its WMT was already a small colony, all other strains generated one new morphotype, catalogued as SCV.

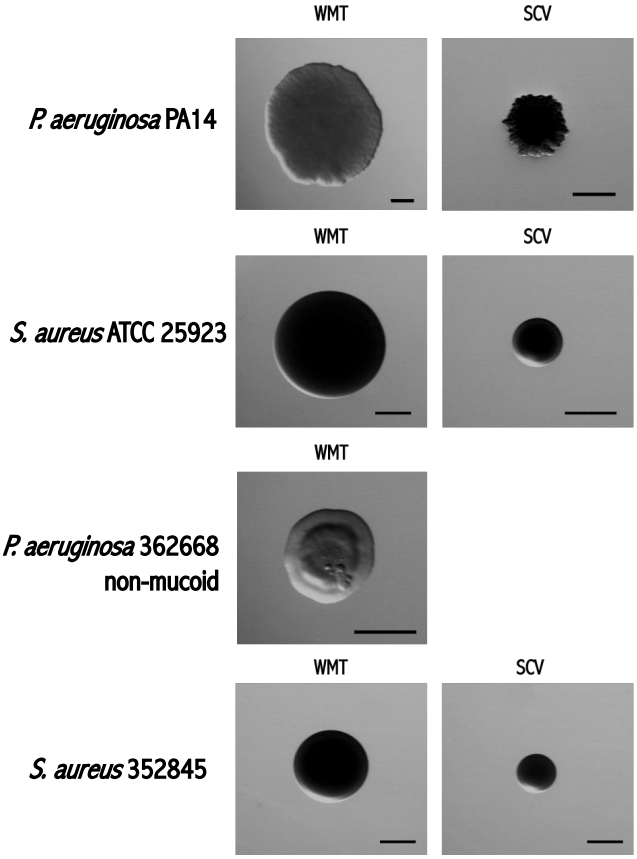

Figure 5.4. Colony morphotypes isolated from *P. aeruginosa* and *S. aureus* biofilm cells unexposed and exposed to CIP and VAC treatments. Black bar = 1 mm.

Table 5.2 - Detailed morphological description of the colonies generated by *P. aeruginosa* and *S. aureus* strains after 48 h of growth on TSA.

Morphotype		Form	Margin	Sheath	Type of Surface	Texture	Consistency	Elevation	Opacity	Size	Colour
***P. aeruginosa* PA14**	WMT	Circular	Entire	Absent	Homogenous	Smooth	Dry	Flat	Opaque	Large	Green
	SCV	Irregular	Undulate	Absent	Homogeneous	Wrinkled	Dry	Flat	Opaque	Small	Green
***P. aeruginosa* 362668 non mucoid**	WMT	Circular	Entire	Absent	Homogeneous	Smooth	Dry	Flat	Opaque	Small	Green
***S. aureus* ATCC 25923**	WMT	Circular	Entire	Absent	Homogeneous	Smooth	Moist	Flat	Opaque	Large	Beige
	SCV	Circular	Entire	Absent	Homogenous	Smooth	Moist	Flat	Opaque	Small	Beige
***S. aureus* 352845**	WMT	Circular	Entire	Absent	Homogeneous	Smooth	Moist	Flat	Opaque	Large	Beige
	SCV	Circular	Entire	Absent	Homogenous	Smooth	Moist	Flat	Opaque	Small	Beige

The analysis of the population diversity of dual-species biofilms showed that, for both consortia, a *S. aureus* SCV emerged after 48 h of growth with *P. aeruginosa* (**Figure 5.5**). These findings seem to suggest that *P. aeruginosa* triggered the selection of *S. aureus* SCV, even if these new morphotypes had only reached around 1% of estimated prevalence. The *P. aeruginosa* PA14 SCV variant detected when this strain grown together with *S. aureus* ATCC 25934 within a dual biofilm had already been noticed in the single-species biofilms.

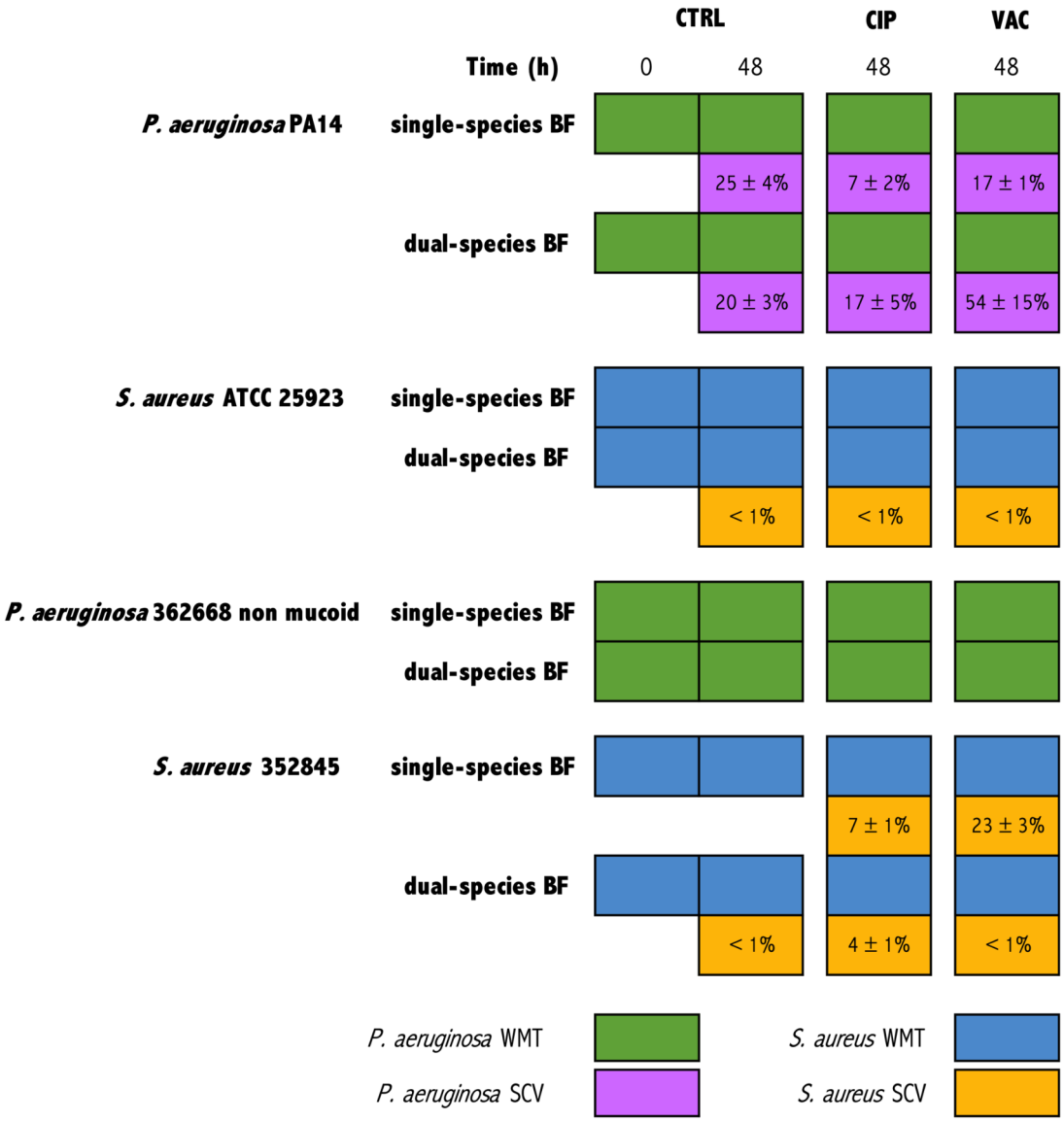

Figure 5.5. Population diversity of *P. aeruginosa* and *S. aureus* during their growth as single- and dual-species biofilms (BF) unexposed and exposed to CIP and VAC treatments. The percentages of the squares indicated the estimated frequency of SCV in the respective single- or dual-species biofilm populations.

Regarding to antimicrobial stress, CIP and VAC did not trigger the emergence of new colony variants. Indeed, the SCV morphotype previously observed in *S. aureus* ATCC 25923 dual-species biofilms was also detected when this consortium was exposed to antibiotic treatments. Moreover, the *S. aureus* SCV, previously detected in the *S. aureus* 352845-*P. aeruginosa* 362668 non-mucoid consortia, was also noticed in single- and dual-species biofilms after CIP and VAC exposure (**Figure 5.5**).

Overall, due to the low value of the estimated frequency of SCV in single- or dual-species biofilm populations, it can be speculated that the ineffective action of CIP and VAC against *S. aureus* within the dual-species biofilms is unrelated to SCV formation and rather governed by interspecies interactions stablished between both bacteria.

SCV have also been referred to as having enhanced ability to form biofilms, as a result of EPS overproduction [29], and decreased antibiotic susceptibility [9, 12]. The biofilm formation ability of the SCV evolved in this work **(Figure 5.6**) showed that only *P. aeruginosa* PA14 SCV has a significant increase in biofilm biomass when compared with the WMT strain. Regarding the antibiotic susceptibility profiles, *P. aeruginosa* 362668 SCV and *S. aureus* 352845 SCV exhibited resistance to CIP (**Table 5.3**) as did the corresponding planktonic cultures **(Table 5.1),** while *S. aureus* 352845 WMT was susceptible to CIP. Moreover, the estimated prevalence of the *S. aureus* 352845 SCV increased when the pre-formed single- and dual-species biofilms were exposed to CIP treatment (**Figure 5.5)**, highlighting the potential role of SCV in the increased antibiotic tolerance of the whole biofilm community. Even though, most of the colony variants exhibited antibiotic susceptibility against the other clinically relevant antibiotics. In overall, the new colony variants still preserve most of the WMT features and pathogenic potential.

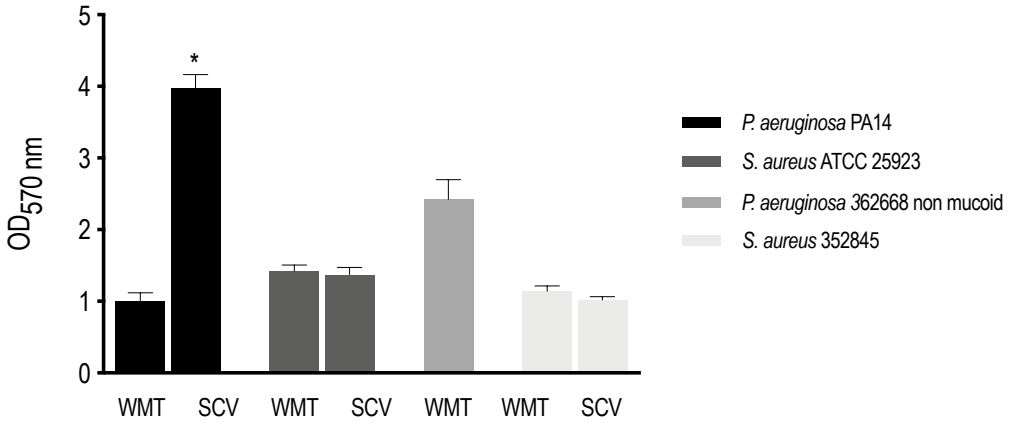

Figure 5.6. Biofilm formation ability of the *P. aeruginosa* and *S. aureus* colony morphotypes-associated bacteria detected in single- and dual-species biofilm populations. *$p<0.05$, unpaired two-tailed Mann-Whitney test to compare significantly differences between WMT and SCV.

Table 5.3. Antibiotic susceptibility of *P. aeruginosa* and *S. aureus* colony morphotypes-associated bacteria detected in single- and dual-species biofilm populations.

	P. aeruginosa **PA14**		***P. aeruginosa*** **362668 non mucoid**		***S. aureus*** **ATCC 25923**		***S. aureus*** **352845**	
Antibiotic	**WMT**	**SCV**	**WMT/SC**	**Antibiotic**	**WMT**	**SCV**	**WMT**	**SCV**
Aztreonam 30 μg	S	S	S	Gentamicn 10 μg	S	S	S	S
Imipenem 10 μg	S	S	R	Ciprofloxacin 5 μg	S	S	S	R
Cefepime 30 μg	S	S	R	Linezolid 30 μg	S	S	S	S
Ceftazimide 30 μg	S	S	R	Rifampicin 5 μg	S	S	S	S
Piperacilin 100 μg	S	S	S	Clindamycin 2 μg	S	S	S	S
Tobramycin 10 μg	S	S	R	Tetracyclin 30 μg	S	S	S	S
Ciprofloxacin 5 μg	S	S	R	Quinupristin + Dalfopristin 15 μg	S	S	S	S
Ticarcilin + Clavulanic acid 85 μg	S	S	S	Vancomycin 30 μg	S	S	S	S

5.4 Discussion

In **chapter 4**, it was demonstrated that *P. aeruginosa* and *S. aureus* strains do not necessarily display antagonistic interaction when grown as dual-species biofilms [30]. Moreover, increasing evidence has been showing that cooperative behaviours between *P. aeruginosa* and *S. aureus* can occur during co-existence, impacting biofilm production [31], antibiotic tolerance [32], and *in vivo* virulence [33, 34]. Thus, exploring the effects of *P. aeruginosa – S. aureus* interaction on the antibiotic tolerance of both microbial species was the research step taken to better understand how *P. aeruginosa* and *S. aureus* co-existence could influence the antimicrobial outcome. Specifically, it was tested whether *P. aeruginosa* influences the antimicrobial tolerance of *S. aureus* to VAC and CIP in pre-formed dual-species biofilms, and vice versa.

Our results demonstrated that the bacterial composition of *P. aeruginosa* and *S. aureus* dual-species biofilms changes after antibiotic treatment. While *S. aureus* cultivability was significantly affected after exposure to both antibiotics, FC data revealed that the prevalence of *S. aureus* increased in the treated dual-species biofilms. Interestingly, when comparing the antimicrobial activity of both antibiotics between dual- and single-species biofilms, a lower efficiency in the dual-species biofilms was observed, especially denoted for *S. aureus* strains, which suggests that the interplay between *P. aeruginosa* and *S. aureus* could promote increased tolerance to CIP and VAC. Coherently with data displayed in chapter 4, it was observed that *S. aureus* strains also undergo a VBNC state when the dual-species biofilms were treated with both antibiotics. It was been reported that VBNC cells are more resistant to antimicrobial therapy [35, 36] and can reinitiate infection when appropriate conditions are established [37, 38]. Thus, it is conceivable that the observed enhanced antibiotic tolerance of *S. aureus* to VAC and CIP may be, in part, mediated by its switching to the VBNC state when in the presence of *P. aeruginosa,* once the loss of cultivability was less evident for treated single-species biofilms. Another reasonable possibility is related to the bioavailability of the antibiotics to the target species. The biofilm matrix is been considered as a shield against the antimicrobial action by delaying its penetration and/or reducing its bioavailability [39, 40]. Recently, it was demonstrated that *S. aureus* completely avoided vancomycin, ampicillin, and ceftriaxone by 'hiding' in *P. aeruginosa* biofilm [41]. Therefore, the protection conferred by the biofilm matrix of *P. aeruginosa* to *S. aureus* cannot also be excluded.

In vitro evidence of bacterial cooperation leading to increased antimicrobial tolerance has been previously pointed out [41- 44]. Efflux of antibiotics from cells via transport pumps is a well-known mechanism of antimicrobial resistance, and several antibiotic pumps belonging to the Nor family were upregulated in *S. aureus* during co-culture, leading to an increase in antibiotic resistance of *S. aureus* to tetracycline and ciprofloxacin [42]. Interestingly, other study showed that *S. aureus* increases the susceptibility of *P. aeruginosa* to ciprofloxacin and aminoglycosides in mixed biofilms [41]. The HQNO molecule produced by *P. aeruginosa* has been shown to protect *S. aureus* from the antimicrobial activity of vancomycin [43]. Moreover, HQNO has been reported to increase *S. aureus* biofilm formation, and long-term exposure induced *S. aureus* SCV formation [44]. Remarkably, the survival of *S. aureus* under unfavourable conditions is significantly improved after its switch to the SCV phenotype, as it exhibits increased resistance to antibiotics [45–49], as well as intracellular survival [50, 51]. Based on the data obtained herein, no direct role for SCV in *P. aeruginosa*-mediated protection of *S. aureus* from VAC or CIP action was found. Nevertheless, it should be taken into account that *S. aureus* 352845 SCV exhibited resistance to CIP, while the WMT did not, and the estimated prevalence of the SCV increased after CIP exposure, highlighting the potential role of SCV in the antibiotic tolerance of the biofilms. Moreover, SCV are frequently isolated from chronic CF lung infections mainly after long antibiotic courses [9, 10], and our model included only 48-h-old treated biofilms. Additionally, our results should be also interpreted in light of some other limitations. First, the biofilms included only *P. aeruginosa* and *S. aureus* species, and it was not explored how other CF-related species would affect the antimicrobial tolerance of the whole consortia. Furthermore, the dual-species biofilms were performed in polystyrene plates rather than on host epithelial cells and, therefore, did not taken into account the presence of host-derived factors, which could impact the antimicrobial treatment and the microbial composition of the dual-species biofilms. Despite these constraints, this work is the first (to the best of our knowledge) demonstrating that *S. aureus* species exhibit enhanced tolerance to antibiotics under dual-species biofilms, as compared with single-species biofilms, clearly related with *S. aureus* VBNC. Furthermore, bidirectional protection of both species was also observed during biofilm growth. Evidences from this study point toward the assumption that *P. aeruginosa* and *S. aureus* co-existence can impact the treatment outcome, ultimately modifying the course of the disease. Consequently, these data is particularly meaningful in clinical practices, once CF infections involve polymicrobial communities, and culture-based techniques (CFU counts) are the primary methods used to identify the microbes in

the infected samples [52] and to assess the efficacy of antimicrobial agents *in vitro* [43, 53–55]. As displayed in chapter 4, these outcomes confirm that at least two quantification techniques are required to obtain reliable measures of the microbial composition of polymicrobial biofilms, mainly when exposed to antimicrobial treatments, otherwise misleading outcomes regarding antimicrobial efficiency could be assumed, due to the presence of VBNC.

Although this study provides new findings regarding the impact of *P. aeruginosa – S. aureus* co-existence in the biofilm-related tolerance to antibiotics, a mechanistic explanation was not provided. Therefore, it could be very informative to assess the biofilm structure (biomass and bacterial distribution), the antimicrobial susceptibility and the gene expression profile of dual-species biofilms unexposed and exposed to antimicrobial treatments [56–59].

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CHAPTER 6

Uncovering inter-species interactions upon *in vivo*-like conditions: *P. aeruginosa* promotes *S. aureus* adhesion and persistence in epithelial cells

Summary

P. aeruginosa and *S. aureus* frequently colonize the same niche influencing CF pathogenesis. While Chapter 4 and 5 showed that *P. aeruginosa* and *S. aureus* can co-exist and even interact synergistically, data from infection models resembling traits of the CF disease are needed to deep investigate the interactions between these microorganisms and, thus, better understand the clinical course associated with their co-infection. We sought to evaluate whether key factors driving CF infections, such as bacteria-bacteria-host interactions, can shape *P. aeruginosa* - *S. aureus* dual-species biofilms and have a role in inter-species interactions. Interestingly, after 24 h of epithelial cells infection, an ecological shift from a Pseudomonas-dominated to a Staphylococcus-dominated community was observed. A significant higher adhesion and successful invasion of *S. aureus* to epithelial cells while growing together with *P. aeruginosa* was also detected. The intracellular survival of *P. aeruginosa* - *S. aureus* consortia into epithelial cells was also significantly higher than the observed in the mono-infections. Interestingly, the cytotoxic effects on host cells seems to be attenuated during co-infection, particularly for *P. aeruginosa* PA14 strain.

Overall, these results suggest that the inter-species interactions occurring between *P. aeruginosa* and *S. aureus* are ruled by their interplay with the host cells. Beyond disclosing some novel insights that may help to comprehend the progression of CF-associated infections, this study also stressed the importance of developing *in vitro* models integrating factors resembling the *in vivo* environment.

6.1 Brief introduction

The respiratory epithelium is a common point of initial host contact for many pathogens and provokes highly varied and multifaceted host responses [1]. In addition to being a structural barrier, airway epithelia cells (AEC) are also critical for innate host defence. Indeed, AEC express several pattern recognition receptors (PRR) and secrete antimicrobial molecules and mucins that aid in the defence against invading pathogens [2]. However, pathogens have developed multiple mechanisms to avoid epithelial resistance mechanisms, as intracellular invasion [3]. These mechanisms have been recognised as an advantageous process used by *P. aeruginosa* and *S. aureus* to access a nutrient-rich environment suitable to its replication and, at the same time, to avoid immune detection or antimicrobial therapy, which consequently promotes infection persistence and recurrence [4]. *In vitro* uptake of *P. aeruginosa* has been demonstrated using several types of AEC, including A549 cells [5–8]. Within AEC, intracellular *P. aeruginosa* viability is not reduced and *P. aeruginosa* replicates in plasma membrane blebs [9]. Following uptake of *P. aeruginosa*, epithelial cells isolated from CF patients undergo significantly delayed apoptosis allowing higher rates of intracellular replication and sustained host damage [10]. Similarly, *S. aureus* is also internalised and survives within AEC leading to bacterial persistence and establishment of chronic infections [11–14]. Despite significant efforts to characterize *P. aeruginosa* and *S. aureus* co-existence occurring in CF infections, the role of the host in driving the dynamics between both species is not yet well understood [8, 15, 16]. *P. aeruginosa* and *S. aureus* co-infection is related with increased morbidity and mortality [17–19] and *in vivo* studies indicate that co-infection increases disease severity and antimicrobial resistance [16, 20–23]. So, as the polymicrobial nature of chronic airway infections affects health outcomes of CF patients, efforts have to be put in developing models resembling the inherent polymicrobial characteristic of biofilms infecting CF lungs. Several CF models have been developed, but most of the time, biofilms are formed in basic *in vitro* systems that do not entirely mimic biofilms found *in vivo* [24]. To go deep in the investigation of the interactions between *P. aeruginosa* and *S. aureus* and, thus, more comprehensively understand the clinical observations associated with their co-infection, the aim of this work was to evaluate the factors driving *S. aureus* survival in the presence of *P. aeruginosa*, under *in vivo*-like conditions. Thus, a *vivo*-like conditions model was engineered to promote and follow bacterial infection on lung epithelial cells, evaluate the adhesion, internalization, and intracellular survival of single- and dual-species consortia of *P. aeruginosa* and *S. aureus*.

6.2 Materials and Methods

6.2.1 Microorganisms and culture conditions

P. aeruginosa (UCBPP-PA14 and an anonymized clinical isolate 362668 non-mucoid) and *S. aureus* (ATCC 25923 and an anonymized clinical isolate 352845) strains were used throughout this work. The strains were preserved and cultured as described in section **4.2.1**.

6.2.2 *In-vitro* and *in vivo-like* experimental setup:

(i) Single-species and dual-species biofilm model.

For this assay, biofilm formation was performed as described in section **4.2.2** with minor modifications. Briefly, *P. aeruginosa* or *S. aureus* (single-species biofilm model) and *P. aeruginosa* - *S. aureus* (dual-species biofilm model) were grown in 24 well culture plates (**Figure 6.1A**) during 4 h. Afterwards, non-adherent bacteria were removed by washing twice with warm PBS, and fresh TSB medium was added to each well to an additional 20 h of growth. After the 24 h period of biofilm formation, the number of *P. aeruginosa* and *S. aureus* cultivable cells was determined as described in section **4.2.3**.

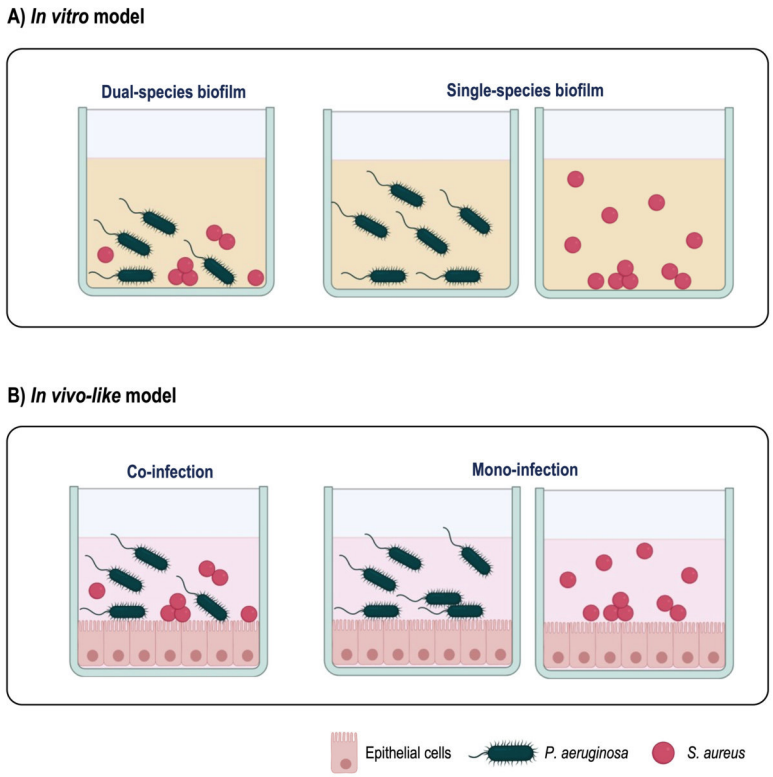

Figure 6.1. Schematic representation of the experimental setups used to study *P. aeruginosa* and *S. aureus* under **(A)** *in vitro* and **(B)** *in vivo*-like models.

(ii) *In vivo*-like model: Bacterial adhesion, internalization and intracellular persistence

In this assay, a comparison is made between mono-infection (*P. aeruginosa* or *S. aureus* plus epithelial cells) and co-infection (*P. aeruginosa* and *S. aureus* plus epithelial cells) infection setups (**Figure 6.1B**). Briefly, A549 cells were seeded into 24 well culture plates (Orange Scientific, Braine-l`Alleud, Belgium) at $1x10^5$ cells/mL in Dulbecco modified eagle medium (DMEM, Sigma) supplemented with 10 % of fetal bovine serum (FBS, Gibco) and 1% antibiotics (ZellShieldTM, Biochrom) at 37 °, and 5 % CO2. Epithelial cells were grown during 72 h to achieve confluence and then washed twice with 1 mL of PBS and infected at a multiplicity of infection (MOI) of 10 using single-species (mono-infection) and dual-species (co-infection) cultures of *P. aeruginosa* and *S. aureus* in antibiotic-free DMEM for 4 h, as previously described [8].

To determine bacterial adhesion (total cells: adhered and internalized cells), cell monolayers were gently washed twice with warm PBS and lysed with 1 mL of deionized water for 30 min. The number of adhered cells was determined based on the difference between the total cells and the number of internalized cells. To quantify bacterial internalization, the gentamicin survival assay was used with some modifications. Briefly, after 4 h of infection, monolayers were washed twice with warm PBS and then incubated with serum- and antibiotic-free DMEM supplemented with 400 µg/mL of gentamicin (Sigma) and 100 µg/mL of polymyxin B (Sigma) to kill extracellular bacteria. After 1 h of incubation, cells were washed twice with warm PBS and subsequently lysed with 1 mL of deionized water for 30 min, as used for adhesion evaluation. Cell lysates were enumerated as described in section **4.2.3.**

For biofilm formation on A549 cells, after 4 h of infection, non-adherent bacteria were removed by washing twice with warm PBS, and fresh medium was added to each well to an additional 20 h period of growth. After incubation, cells were washed twice with warm PBS and subsequently lysed with 1mL of deionized water for 30 min. Again, cell lysates were enumerated accordingly to section **4.2.3**.

For intracellular persistence experiments, after 4 h of infection, monolayers were washed twice with warm PBS and then incubated with serum- and antibiotic-free DMEM supplemented with 400 µg/mL gentamicin (Sigma), 100 µg/mL polymyxin B (Sigma) to kill extracellular bacteria. After 1 h of incubation, cells were washed twice with warm PBS and then incubated in antibiotic-free DMEM for additional 20 h of growth. After incubation, epithelial cells were lysed in 1 mL of

deionized water for 30 min and the bacterial numbers enumerated as CFU/mL by plating out serial dilutions on selective media as described in section **4.2.3**.

6.2.3 Light microscopy

Overall morphology and integrity of epithelial cell monolayers were assessed with an inverted phase contrast microscope (LEICA DMIL). Imaging was performed with a 10x, 20x or 40x objectives.

6.2.4 Fluorescent staining and microscopy

To visualize biofilm formation on human A549 cells, infected monolayers were stained with DAPI, a membrane permeable fluorescent dye (ThermoFisher Scientific). Briefly, A549 cells were placed in a 24 well microtiter plate containing the microscopic cover glass at the bottom (Sarstedt) and incubated under the above-described conditions (section **6.2.2**). Bacterial suspensions were then added to each well of the 24 well microtiter plate. After 4 h of infection, monolayers were washed twice with PBS and air dried at 37 °C for 1 h.

Infected monolayers were then fixed with methanol (100% v/v) for 20 min, followed by 4% (w/v) paraformaldehyde and 50% (v/v) ethanol (10 min each), at room temperature. After the fixation step, the surfaces of the microscopic cover glass were covered with 5 μM DAPI and cells were stained at room temperature for 10 min. Cells were then observed under a fluorescence microscope (Olympus BX51) equipped with 100x oil immersion objective and filters sensitive to DAPI (BP 365–370, FT 400, LP 421). Images were captured with a CCD camera (DP72; Olympus) and CellB software.

6.2.5 Metabolic activity

The metabolic activity of cells was determined using the MTS [(3-(4,5-dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] inner salt (Promega) assay to evaluate the viability of the cell epithelial monolayers after 20 h of bacterial internalization. Briefly, A549 cells were placed in a 96 well microtiter plate (Orange Scientific, Braine-l`Alleud, Belgium) and incubated under the above-described conditions (section **6.2.2**).

After 20 h of bacterial internalization, in the dark, 20 μL of MTS was added to each well and the plate was further incubated for 1 h at 37 °C, 5% CO2. The optical density of the resulting solution

was measured at 490 nm. Results were presented as percentage of viable cells compared to the positive control (non-infected monolayers).

6.2.6 Lactase dehydrogenase (LDH) activity

The extracellular activity of LDH released from A549 cells after 20 h of internalization was monitored as an indicator of tissue damage. LDH released in the maintenance medium of the control A549 cells which was devoid of bacteria, as well as infected monolayers was measured using the CytoTox-ONE Homogeneous Membrane Integrity Assay kit (Promega) following the manufacturer's instructions. Briefly, cell medium was collected and centrifuged at 300 *g*, 4 °C during 5 min and then free-cell supernatant was collected to LDH measurement.

The LDH released during mono-infection or co-infection with *P. aeruginosa* and *S. aureus* was then expressed as relative LDH activity to the non-infected monolayers. LDH activity was analysed spectrophotometrically (FLUOstar OPTIMA; BMG Labtech, Ortenberg/Germany) by measuring the NADH disappearance rate at 544 nm excitation and 590 nm emission during the LDH-catalysed conversion of pyruvate to lactate.

6.2.7 Statistical analysis

All numerical data were subjected to statistical analysis using the non-parametric Mann–Whitney U test, Kruskal-Wallis test or two WAY-ANOVA since the data that did not follow a normal distribution according to Kolmogorov–Smirvon's test, with the statistical software Prism Version 7.0a for Macintosh. Results are presented as mean ± standard deviation (SD), unless stated otherwise.

6.3 Results

6.3.1 *P. aeruginosa* and *S. aureus* interaction under *in vivo*-like conditions

To ascertain whether host may have a role on *P. aeruginosa - S. aureus* interplay and gain insights helping to explain CF-associated infections progression more in depth, the dual-species biofilm assay was adapted and optimized to integrate human lung epithelial cells in a *in vivo*-like model (**Figure 6.1**). Throughout the entire infection, it was observed that epithelial cells formed confluent monolayers after mono- and co-infection for almost all *P. aeruginosa* and *S. aureus* strains (**Figure 6.2**). The exception was for *P. aeruginosa* PA14, wherein many of the epithelial cells had lifted off the adhesion surface (**Figure 6.2A**).

In order to inspect eventual changes in biofilm formation ability on the different attachment surfaces, *P. aeruginosa* and *S. aureus* strains were individually grown during 24 h in an epithelial cell monolayer (*in vivo*-like model) and the number of cultivable cells was determined and compared with the growth of each species in polystyrene plates (*in vitro* model) (**Figure 6.3A**). Significant differences were observed in the number of biofilm cells for *P. aeruginosa* and *S. aureus* strains in both experimental models ($p<0.05$). Interestingly, under *in vivo*-like conditions both species exhibited a more close ability to form biofilms, although *S. aureus* strains reached a higher number of CFU. Regarding *P. aeruginosa - S. aureus* consortia, a different trend in their composition can be observed depending on the experimental model used to mimic the interactions within the consortia (**Figure 6.3B**). Interestingly, the bacterial communities retrieved from the *in vivo*-like model were dominated by *S. aureus* strains, whereas the *in vitro* model was characterized by high abundance of *P. aeruginosa* strains in both consortia. These findings underscore that *P. aeruginosa - S. aureus* biofilm formation ability is highly dependent on the attachment surface and pointed out the role of the host cells on the interplay occurring between both microorganisms.

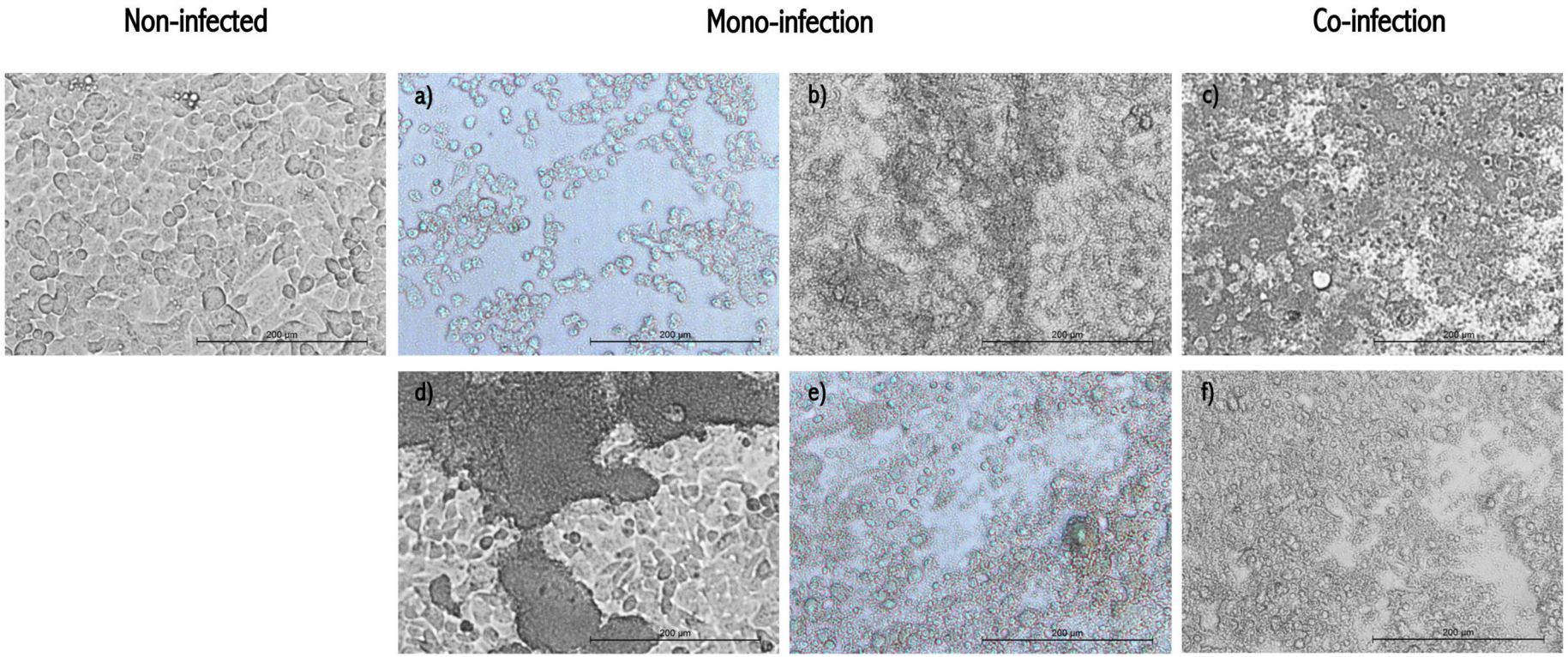

Figure 6.2. Representative light microscopy images of 24 h non-infected and infected epithelial cells. **(a)** *P. aeruginosa* PA14, **(b)** *S. aureus* ATCC 25923, **(c)** *P. aeruginosa* PA14 + *S. aureus* ATCC 25923, **(d)** *P. aeruginosa* 362668 non mucoid, **(e)** *S. aureus* 352845, **(f)** *P. aeruginosa* 362668 non mucoid + *S. aureus* 352845. After 4 h of initial infection, extracellular and non-adherent bacteria were removed, and cells were grown for additional 20 h. Scale bars correspond to 200 µm.

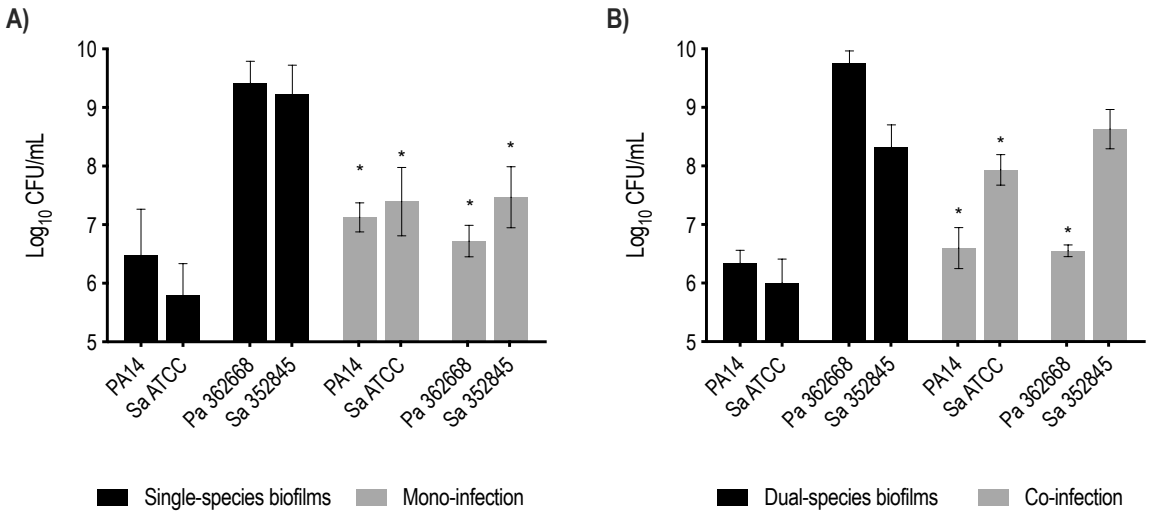

Figure 6.3. Quantitative assessment of *P. aeruginosa* and *S. aureus* individual populations estimated by plate counts (CFU/mL) under *in vitro* and *in vivo*-like models. The number of cultivable cells was determined for **(A)** *P. aeruginosa* PA14, *P. aeruginosa* 362668 non mucoid, *S. aureus* ATCC 25923 and *S. aureus* 352845; and for **(B)** *P. aeruginosa* PA14 + *S. aureus* ATCC 25923 and *P. aeruginosa* 362668 non mucoid + *S. aureus* 352845. The results for three independent assays are shown as mean +/- SD. *$p<0.05$, unpaired two-tailed Mann-Whitney test to compare significantly differences between *in vitro versus in vivo*-like model (single-species biofilms *versus* mono-infection; dual-species biofilm *versus* co-infection).

6.3.2 *P. aeruginosa - S. aureus* - host responses during *in vivo*-like conditions

To increase our understanding regarding the role of the host cells in shaping the behaviour of *P. aeruginosa - S. aureus* communities, further experiments were conducted by evaluating bacteria-bacteria-host interactions in the *in vivo*-like model. First, as bacterial adhesion to epithelial cells followed by internalization is crucial for bacteria-host interactions and can be shaped by inter-species interactions [43, 44], both parameters were evaluated after *P. aeruginosa* and *S. aureus* mono- and co-infection. To ensure that the epithelial cell monolayers remained intact throughout the experiment, the bacterial infections were performed during 4 h (**Figure 6.4**). While the adhesion of *P. aeruginosa* strains to epithelial cells was not affected in mono- *versus* co-infection, as confirmed by fluorescence microscopy analysis of infected epithelial cell monolayers (**Figure 6.5)**, a significant higher number of adhered cells was observed for *S. aureus* strains in the presence of *P. aeruginosa* ($p < 0.05$) (**Figure 6.6A**). Likewise, *S. aureus* strains exhibited a higher successful invasion into epithelial cells than did *P. aeruginosa* during co-infection, while no differences were observed compared to the corresponding mono-infections (**Figure 6.6B**).

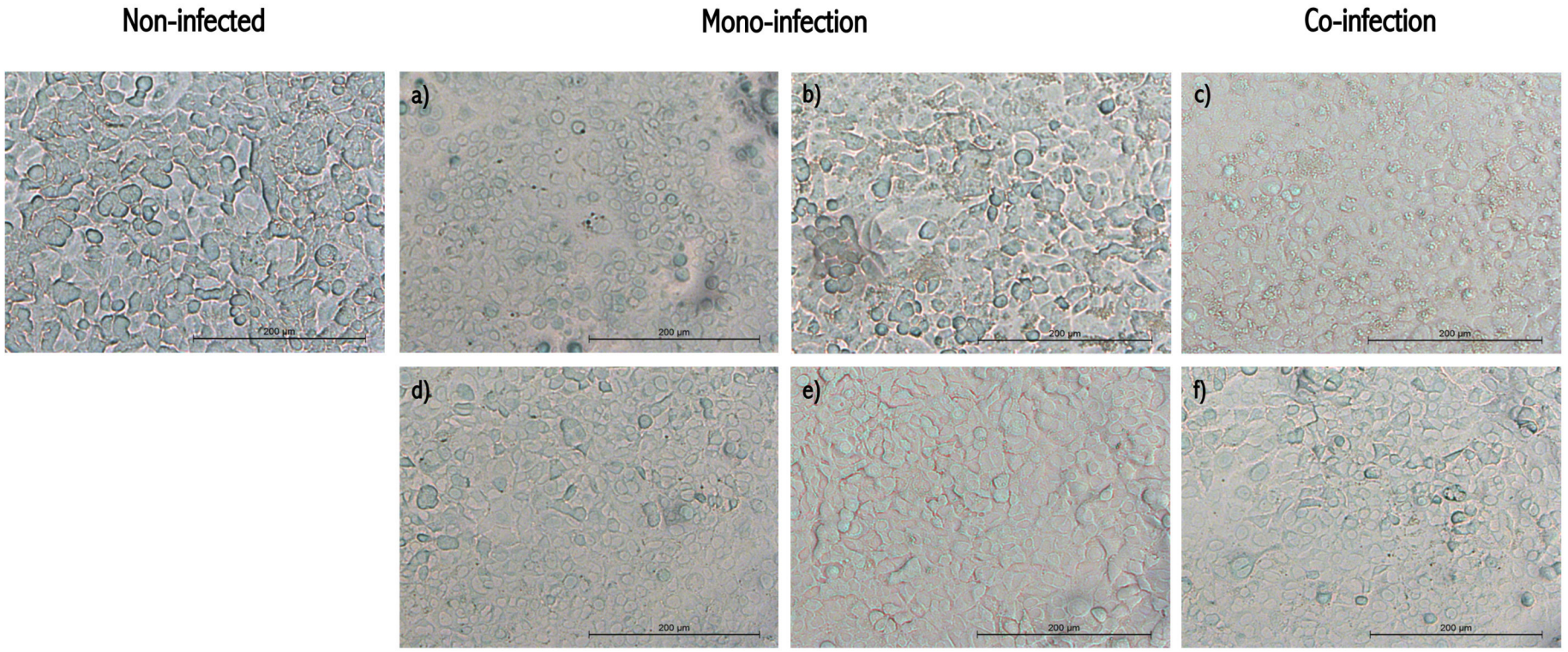

Figure 6.4. Representative light microscopy images of 4 h non-infected and infected epithelial cells. **(a)** *P. aeruginosa PA14*, **(b)** *S. aureus ATCC 25923*, **(c)** *P. aeruginosa PA14 + S. aureus ATCC 25923*, (**d)** P*. aeruginosa 362668 non mucoid*, **(e)** S*. aureus 352845*, **(f)** *P. aeruginosa 362668 non mucoid + S. aureus 352845*. Scale bars correspond to 200 µm.

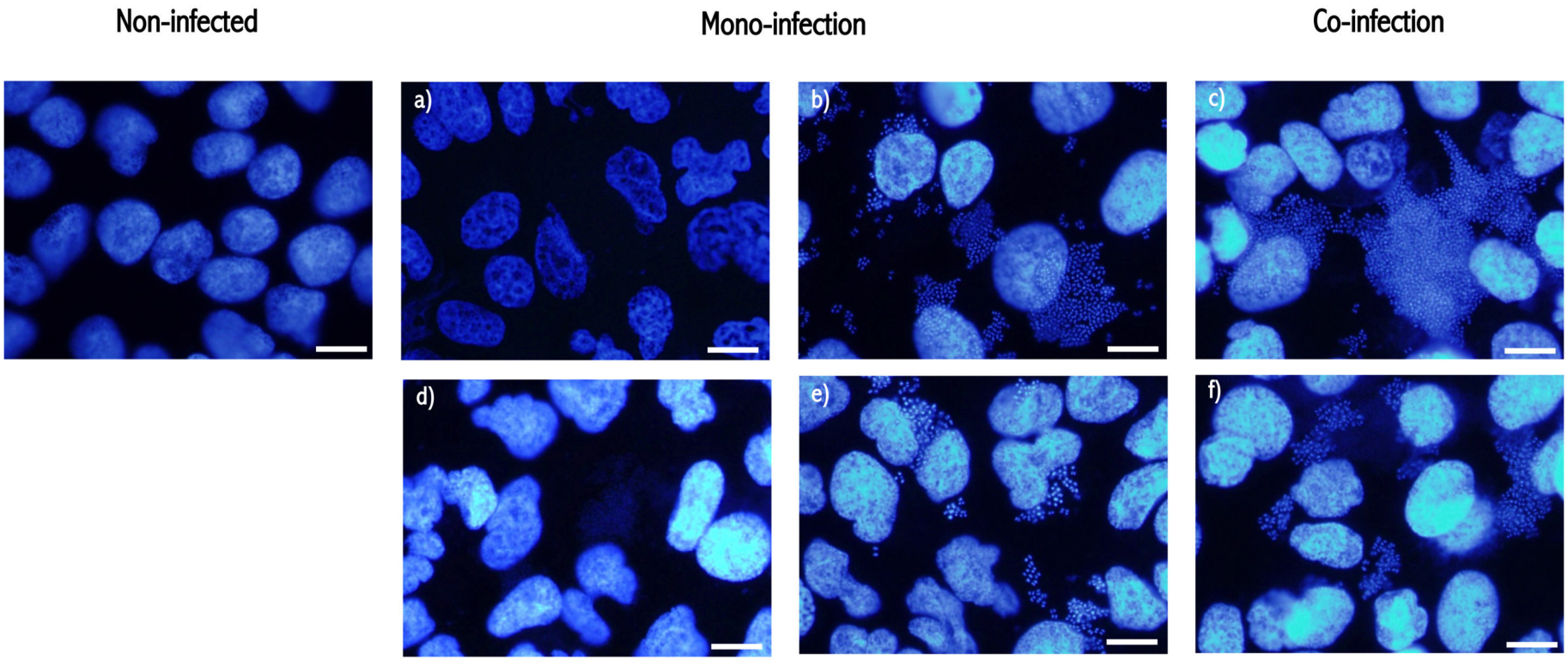

Figure 6.5. Representative epifluorescence microscopy images of 4 h non-infected and infected epithelial cells stained with 5 µM of DAPI. **(a)** *P. aeruginosa* PA14, **(b)** *S. aureus* ATCC 25923, **(c)** *P. aeruginosa* PA14 + *S. aureus* ATCC 25923, **(d)** *P. aeruginosa* 362668 non mucoid, **(e)** *S. aureus* 352845, **(f)** *P. aeruginosa* 362668 non mucoid + *S. aureus* 352845. Scale bars correspond to 10 µm.

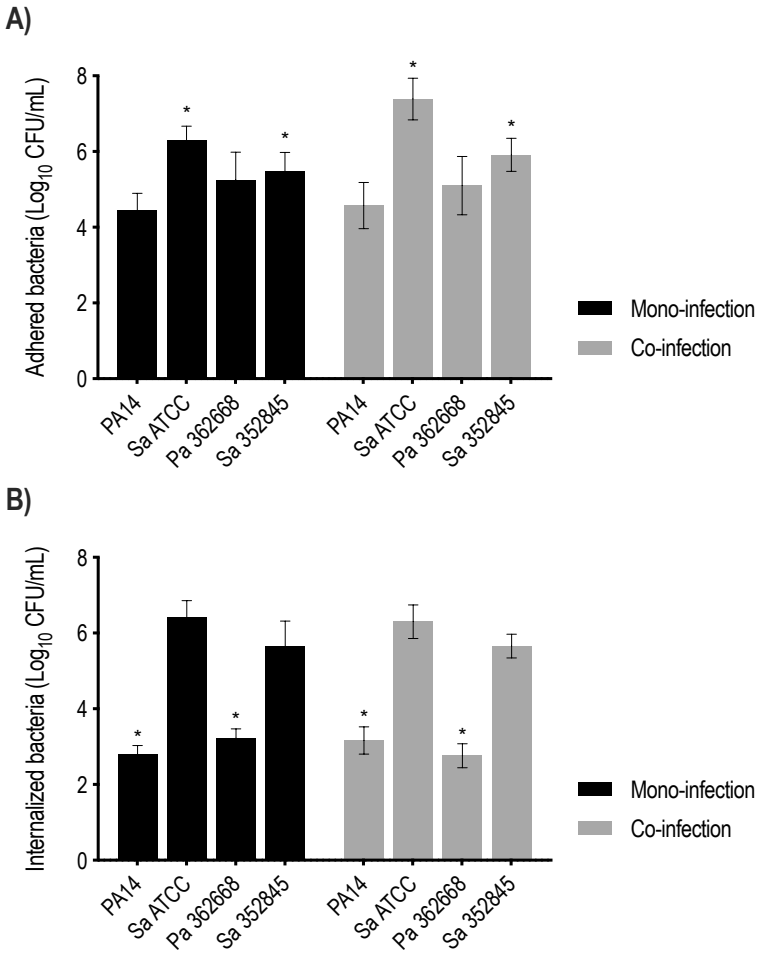

Figure 6.6. **(A)** Adhesion and **(B)** internalization of *P. aeruginosa* and *S. aureus* strains to epithelial cells after 4 h of mono- and co-infection. Data represent the mean ± standard deviation of triplicates from at least three independent experiments. *p<0.05, unpaired two-tailed Mann-Whitney test to compare significantly differences between mono- *versus* co-infection.

To better explore the observed cellular invasion, additional experiments were carried out to evaluate the intracellular survival of *P. aeruginosa* and *S. aureus* during mono- and co-infection. In these experiments, the number of bacteria were recovered after an additional period of 20 h of intracellular growth (**Figure 6.7**). Results demonstrated that *S. aureus* was able to survive intracellularly in higher CFU numbers than *P. aeruginosa* during both mono- and co-infection. Moreover, a significant increase in the number of intracellular bacterial was observed for both species during co-infection, suggesting that the inter-species interactions occurring between *P. aeruginosa* and *S. aureus* also exert influence on host cells. Finally, as can been seen in **Figure 6.8,** both species were able to proliferate intracellularly throughout the entire course of mono- and co-infection ($p<0.05$).

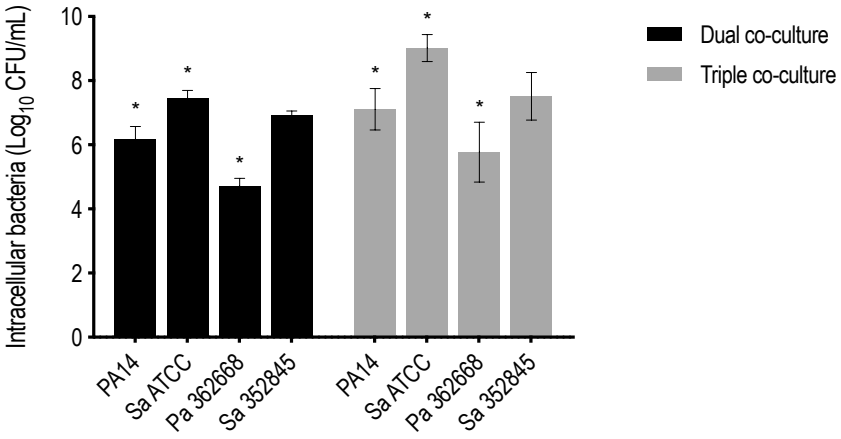

Figure 6.7. Intracellular survival of *P. aeruginosa* and *S. aureus* strains during mono- and co-infection. Data represent the mean ± standard deviation of triplicates from at least three independent experiments. *p<0.05, unpaired two-tailed Mann-Whitney test to compare significantly differences between mono- *versus* co-infection.

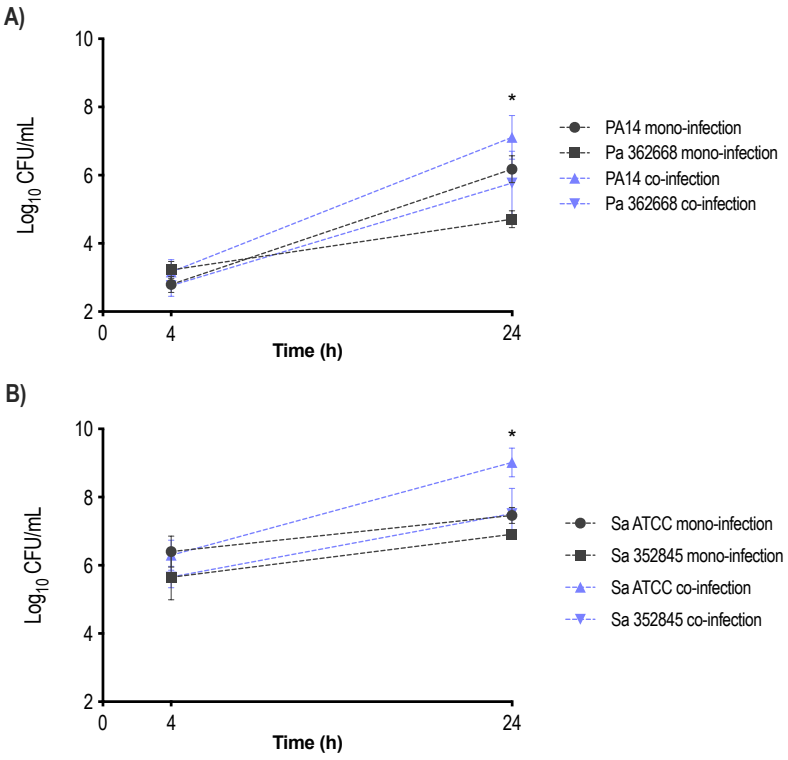

Figure 6.8. Quantification of intracellular **(A)** *P. aeruginosa* and **(B)** *S. aureus* strains after 4 h and 24 h of mono- and co-infection. Data represent the mean ± standard deviation of triplicates from at least three independent experiments. *p<0.05, two-way ANOVA and Sidak's multiple comparison test o compare significantly differences between 4 h *versus* 24 h.

Finally, the cytotoxicity of the various strains under mono- and co-infection toward epithelial cells was tested. Interestingly, the viability of the infected epithelial cells was only slightly affected in all

conditions (p<0.05), except for *P. aeruginosa* PA14 mono-infection (**Figure 6.9A**). PA14 is reported as a highly cytotoxic strain due to the T3SS effector ExoU [25], so the cytotoxicity level observed for this strain was not surprising. Curiously, our data showed that, when evolved in co-infection, *P. aeruginosa* and *S. aureus* seem to reduce its cytotoxic activity, specially noticed for *P. aeruginosa* PA14 - *S. aureus* ATCC 25923 consortium.

To better understand the observed phenomena, the release of a cytosolic enzyme, LDH, into cell culture supernatants was also measured (**Figure 6.9B**). As expected, LDH release after *P. aeruginosa* PA14 infection was significantly increased, whereas reduced LDH release was detected for all other conditions, suggesting that host cell permeabilization/death is in an initial stage. The microscopic observation of the infected cells also demonstrated that epithelial cell monolayers were greatly damaged when infected by the individual *P. aeruginosa* PA14, while this effect is clearly not observed during co-infection with *S. aureus* ATCC 25923 strain (**Figure 6.10**). No lysis of uninfected cells present in the same field was observed. Overall, these data demonstrate that *P. aeruginosa* PA14 was somehow able to delay host cell death during co-infection with *S. aureus* ATCC 25923.

It was also examined whether intracellular *P. aeruginosa* and *S. aureus* cells were released from host cells in a viable state. Interestingly, both species were detected into the extracellular medium in high CFU (**Figure 6.11**).

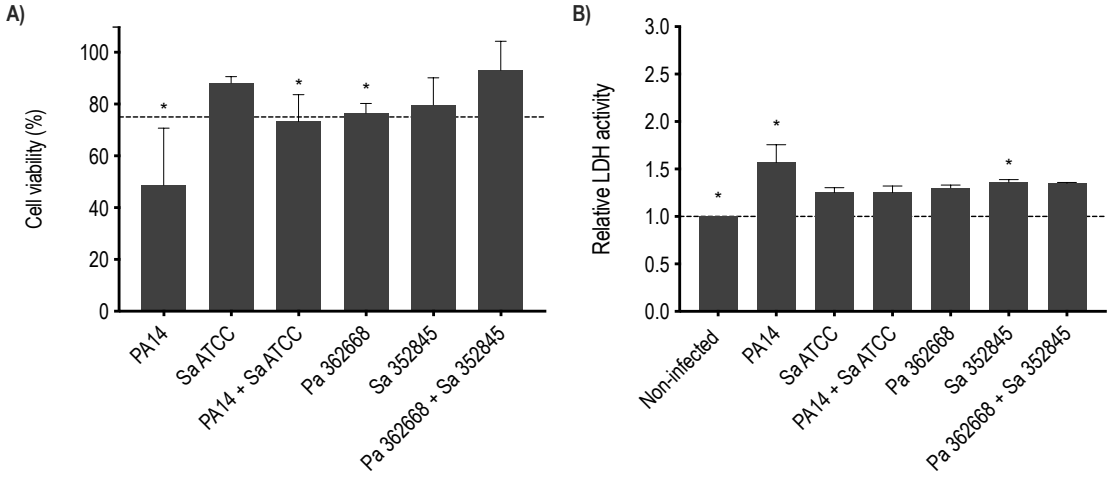

Figure 6.9. Viability of the epithelial cell monolayers after 20 h of intracellular survival of *P. aeruginosa* and *S. aureus* during mono- and co-infection determined by **(A)** MTS assay and **(B)** LDH activity. Data represent the mean ± standard deviation of duplicates from at two independent experiments. *p<0.05, Kruskal-Wallis followed by a Dunnett's multiple test to compare significant differences between the viability of infected *versus* non-infected cells.

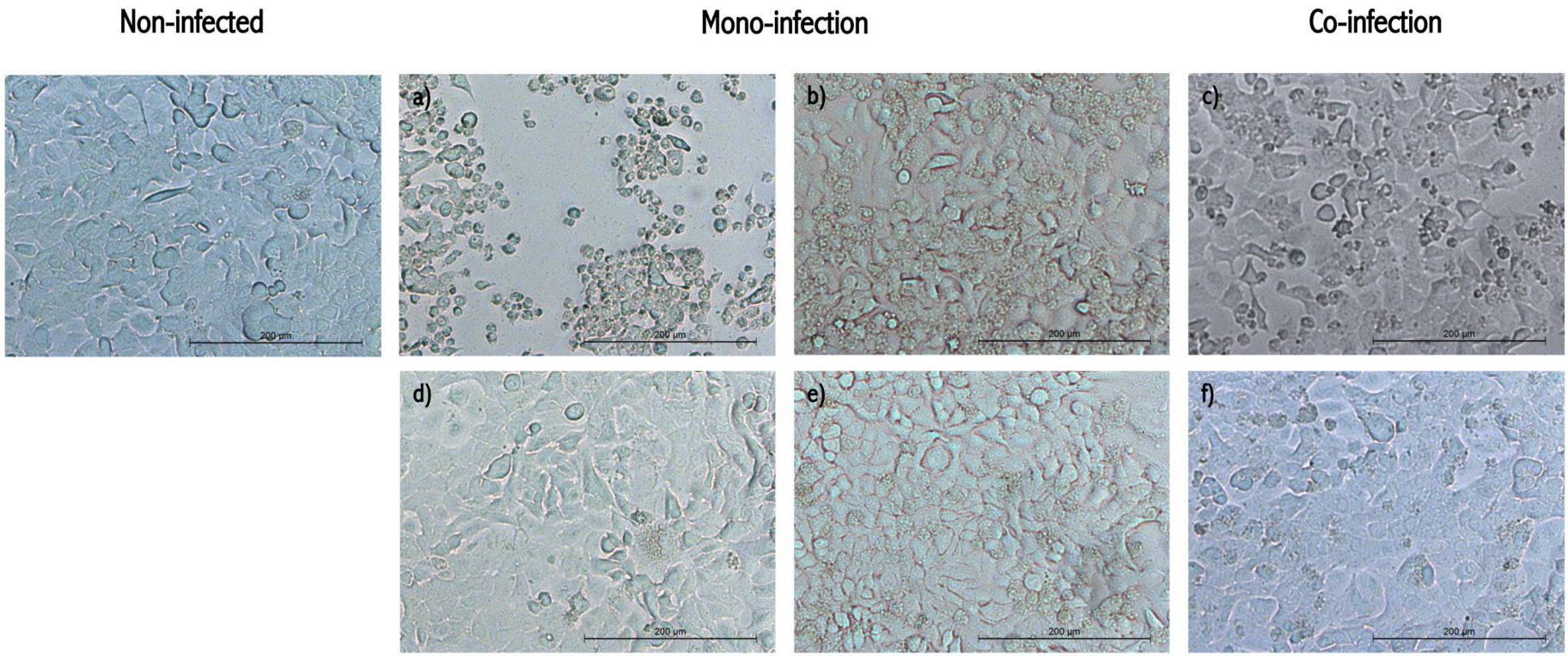

Figure 6.10. Representative light microscopy images of 24 h non-infected and infected epithelial cells growing intracellularly during 20 h. **(a)** *P. aeruginosa* PA14, **(b)** *S. aureus* ATCC 25923, **(c)** *P. aeruginosa* PA14 + *S. aureus* ATCC 25923, **(d)** *P. aeruginosa* 362668 non mucoid, **(e)** *S. aureus* 352845, **(f)** *P. aeruginosa* 362668 non mucoid + *S. aureus* 352845. Scale bars correspond to 200 µm.

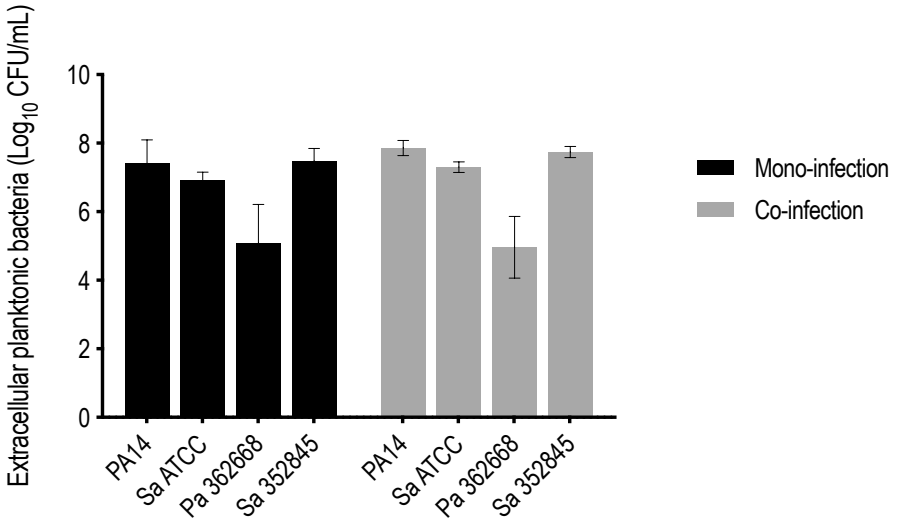

Figure 6.11. Release of viable intracellular bacteria into the medium after 20 h of intracellular survival of *P. aeruginosa* and *S. aureus* during mono- and co-infection. The number of bacteria present were enumerated using the drop plate technique. Data represent the mean ± standard deviation of triplicates from at least three independent experiments. *p<0.05, unpaired two-tailed Mann-Whitney test to compare significantly differences between mono- *versus* co-infection

6.4 Discussion

Even though the knowledge on CF inter-species interactions is increasing, studies involving key factors driving lung infection are scarce, hampering the full understanding of infection pathogenesis, progression, and recurrence. Taking this into account, efforts have to be put in engineering CF models gradually integrating experimental conditions resembling the CF *in vivo* infections. Thus, studying *P. aeruginosa – S. aureus* under *in vivo*-like conditions is a step forward to obtain new insights into the inter-species interactions governing co-infection. In this work, an *in vivo-like* model was employed to obtain further insights helping to understand *P. aeruginosa - S. aureus* interactions when challenged by the presence of epithelial host cells. Based on these results, it can be pointed out that, following epithelial cells infection, an ecological shift occurred regarding the bacteria who dominates. In fact, data showed that the predominance of *P. aeruginosa* observed in the dual-species biofilms (*in vitro* model) ceased in co-infection (*in vivo-like* model), passing *S. aureus* to dominate the consortia. This outcome highlighted the key role of the experimental models used to evaluate the bacterial composition of polymicrobial biofilm-associated infections. Importantly, these findings underline two main issues: (i) the role of the host cells in driving the interplay occurring between both bacterial species and (ii) the need of implementing *in*

vitro models capturing as much as the complexity of the *in vivo* lung environment (airway microbiota, airway environment, and host) to better understand the interactions occurring between *P. aeruginosa* and *S. aureus*.

By exploiting the *P. aeruginosa* - *S. aureus* - host interactions in the *in vivo*-like model, it was possible to further evaluate the ability of both bacteria, *per se* or in co-infection, to colonize and survive in the host cells. A significant higher adhesion and successful invasion of *S. aureus* into epithelial cells were observed while growing together with *P. aeruginosa,* that led us to speculate that *P. aeruginosa* has a role on *S. aureus* colonization. It has been shown that the activation of specific transcription factors involved in innate immune response increases *S. aureus* invasion of pulmonary cells cultured *in vitro* [14]. The same authors also concluded that by delaying the induction of apoptosis in infected host cells, *S. aureus* reach a higher density per cell [14]. Furthermore, Millette *et. al* [16] reported that *P. aeruginosa* increases the expression of specific host cell components that might benefit *S. aureus* to adhere to host cells and increase its colonization of the lung tissue during co-infection. Briaud *et. al* [8] also demonstrated that *S. aureus* internalization into epithelial cells were increased in the presence of co-existing *P. aeruginosa* strains.

Pathogenic bacteria are commonly classified as intracellular or extracellular pathogens [26]. Intracellular survival may provide substantial advantages for bacterial persistence within the host. Apart from protecting the pathogens from the host immunity defences, the intracellular localization might provide protection against antibacterial treatments or local inflammation during infection, which consequently promotes infection persistence and recurrence [12, 27, 28]. In contrast, extracellular pathogens avoid phagocytosis or exhibit cytotoxicity towards phagocytic cells, to promote their extracellular multiplication [3]. However, recent data have emphasized that several extracellular pathogens can enter host cells, resulting in a phase of intracellular residence which can be of importance in addition to the typical extracellular infection. Once considered extracellular pathogen, it is now established that *S. aureus* can survive within many mammalian cell types [29–32]. *P. aeruginosa,* while often regarded as an extracellular pathogen, can also invade multiple types of epithelial cell *in vivo* [33, 34] and *in vitro* [35–38].

By investigating the intracellular survival of *P. aeruginosa* and *S. aureus* during co-infection, it was detected a significant increase in bacterial numbers compared to the mono-infections, suggesting that the interplay occurring between both species play a role in its intracellular survival within

epithelial cells. Surprisingly, the cytotoxic effect of *P. aeruginosa* PA14 strain observed during mono-infection was impaired in co-infection with *S. aureus*. Paradoxically, some *P. aeruginosa* T3SS effectors can have both antiphagocytic and cytotoxic activities. T3SS, through its effectors, is the major determinant of *P. aeruginosa* cytotoxicity [39].The T3SS, more specifically its effector ExoS, is required for invasion and intracellular survival in both epithelial cells and neutrophils [40, 41], and can even confer this capacity on some cytotoxic strains that do not naturally encode ExoS [37]. Moreover, the expression of T3SS and its ExoS effector has been recently reported as intracellularly regulated [37], and the induction of T3SS only after cell invasion delays host cell death, enabling time for intracellular replication [38]. These works provided further understanding to the conundrum of how invasive strains of *P. aeruginosa* invade host cells while encoding potentially antiphagocytic effectors. In addition, induction of cell death has been linked with the ability to escape from host cells [42, 43]. Herein, all *P. aeruginosa* and *S. aureus* strains were detected as free-living cells in the extracellular environment after mono- and co-infection. In agreement with these observations, several studies have reported intracellular bacterial survival by both species, with subsequent lysis of host cells [12, 27, 28, 38, 44]. Thus, survival of *P. aeruginosa* and *S. aureus* within epithelial cells and subsequent bacterial release may play a role in the establishment and dissemination of co-infection. The results of our study provide evidence that both invasion and intracellular replication occur during *P. aeruginosa – S. aureus* co-infection, which certainly contribute to the ability of both species in persisting during CF co-infection. Nevertheless, more research would be need to explore the mechanistic details of how co-infection seems to delay host cell death following bacterial internalization, and the contribution of this phenomenon to the pathogenesis of *P. aeruginosa – S. aureus* co-infection.

Overall, by employing an *in vivo*-like model, it was unveiled that the presence of host cells drives the interactions occurring between *P. aeruginosa* and *S. aureus*. During co-infection *P. aeruginosa* promoted increased adhesion and intracellular survival of *S. aureus*, which certainly contribute to its ability to persist during CF infection. Of note, the *in vivo*-like model used in this study is limited by the fact that cell monolayers are not polarized, as are lung epithelial cells *in vivo*. Still, cell-based *in vitro* model can be very informative, and be key to further study the virulence potential of *P. aeruginosa - S. aureus* co-infections.

To better understand the impact of the inter-species interactions between *P. aeruginosa* and *S. aureus* it is necessary to recreate the *in-situ* environment through the design of more robust *in vitro*

CF-models integrating more factors driving the CF airway milieu. Even so, the implemented model could benefit from further characterization. The presence of the host cells generated complex changes in the microbial composition of *P. aeruginosa - S. aureus* consortia, eventually having potential implication in the course of the infection. Thus, evaluating the impact of the host in the antimicrobial effectiveness against the co-infecting species, can be also valuable for understanding the mechanisms beyond *P. aeruginosa - S. aureus* persistence in CF infections.

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CHAPTER 7

Concluding remarks and future work

Summary

This chapter presents a summary of the major findings, limitations, and suggestions for future directions.

7.1 Concluding remarks

This work aimed at providing novel insights into *P. aeruginosa* and *S. aureus* inter-species interactions in a CF environment airway and gaining knowledge helping to understand how this ecological interaction can contribute to the recognized enhanced virulence and persistence of these bacteria in CF-related infections. CF patients who are co-infected with *P. aeruginosa* and *S. aureus* have worse outcomes than those who are infected with either organism alone [1–3]. However, it remains unclear whether this is due to increased virulence of each bacterial species or because of the whole multi-species community benefits. Thus, understanding the circumstances allowing *P. aeruginosa* and *S. aureus* co-existence, and how the inter-species interactions influence the behaviour of the overall biofilm community, may enable the development of strategies to improve the clinical success of treatments.

7.1.1 Major findings

Despite extensive knowledge of the complex nature of CF lung infections, there is still a lack of understanding regarding how microbial diversity and dynamics is maintained, particularly, the interactions established between *P. aeruginosa* and *S. aureus*. Therefore, the work developed under the scope of this PhD project gave rise to new findings important to unveil the ecological interactions established between these pathogens in a CF environment and inferred their role in CF polymicrobial biofilm infections recurrence and persistence. Indeed, the evidences that, within a dual-species biofilm, both bacteria co-exist seemingly in a non-competitive manner, and *S. aureus* converts to a VBNC phenotype, disclose a new facet of microbial interaction between these two species traditionally thought of as competitors. Their co-existence also led to an increased tolerance of the consortia towards antibiotic treatment, in which *S. aureus* preserved its VBNC phenotype, indicating that both bacteria benefit within the consortia. The use of an *in-vivo* like conditions model mimicking co-infection on host cells revealed that *P. aeruginosa - S. aureus* continue co-existing. Interestingly, even keeping their co-existing state, the predominance of *P. aeruginosa,* observed in the dual-species biofilms, was no longer noticed during co-infection, becoming instead *S. aureus* the dominant bacteria. Moreover, while the intracellular survival of both bacteria was increased, their cytotoxic effects on host cells seem to be delayed during co-infection.

Overall, this PhD project provided novel insights regarding *P. aeruginosa - S. aureus* interactions that may help to deep comprehend their role in the progression of CF-associated infections.

7.1.2 Answering the research questions

The research findings reported throughout this PhD generated important information allowing to answer the four main research questions formulated in chapter 1 of this PhD project:

1. **What is the current knowledge regarding inter-species interactions occurring between *P. aeruginosa* and *S. aureus* and how it can help to identify promising antimicrobial targets?**

In chapter 3, experimental data on the molecular basis of *P. aeruginosa* and *S. aureus* interactions was for the first time systematically organized and made publically available in the new Inter-Species CrossTalk Database (www.ceb.uminho.pt/ISCTD). The retrieved information was systematically annotated from relevant scientific literature, reconstructed as networks, and integrated with specialized databases to identify promising antimicrobial targets. Network analysis revealed key entities regulating *P. aeruginosa* - *S. aureus* interactions, namely the PqsABCDE/PqsR QS system, which affects *S. aureus* growth and biofilm formation. Additionally, by identifying the most reported *P. aeruginosa* virulence factors affecting *S. aureus*, it was created a set of experimentally validated agents affecting those factors, ranging from synthetic drugs to natural plant extracts.

2. **Can *P. aeruginosa* and *S. aureus* increase its virulence features when they are growing as dual-species biofilms?**

The findings obtained in chapter 4 clearly demonstrated phenotypic and transcriptomic differences between single- and dual-species biofilms of *P. aeruginosa* and *S. aureus* that could impact their persistence during CF infections. Growing as dual-species biofilms, *P. aeruginosa* was confirmed to be the dominant species during *in vitro* evolution, while *S. aureus* undergone a VBNC state. Moreover, an increased expression of genes associated with *S. aureus* virulence was detected suggesting that the phenotypic switching to VBNC state might account for *S. aureus* pathogenicity and, in turn, rule the clinical outcome of the infection. Interestingly, *P. aeruginosa* decreased the expression level of key QS genes in the presence of *S. aureus.* All these changes are likely important for bacterial persistence and, consequently, for the virulence of the entire dual-species community, suggesting that inter-species interactions indeed play a key role in *P. aeruginosa* - *S. aureus* pathogenesis.

3. What happens to *P. aeruginosa* - *S. aureus* dual-species biofilms upon exposure to antibiotic therapy?

Data from chapter 5 showed that *P. aeruginosa* and *S. aureus* can interact synergistically, leading to increased tolerance to antibiotics. Under dual-species biofilm growth, *P. aeruginosa* decreased the sensitivity of *S. aureus* to CIP and VAC. Similarly, *S. aureus* also protected *P. aeruginosa* from the antimicrobial activity of CIP. Interestingly, following antibiotic therapy, *S. aureus* persisted in the dual-species biofilm in a VBNC state, which certainly contributes to its increased tolerance to antibiotic treatments. Despite *P. aeruginosa* triggers the selection of *S. aureus* SCV, this phenotypic diversification seems not determine *S. aureus* susceptibility in the dual-species biofilms.

4. What happens to *P. aeruginosa* and *S. aureus* interactions when growing under *in vivo*-like conditions?

In chapter 6 was shown that, after co-infection, an ecological shift occurred in terms of microbial composition in the *P. aeruginosa* - *S. aureus* consortia. Interestingly, the predominance of *P. aeruginosa* observed in the dual-species biofilm (*in vitro* model) ceased under *in vivo*-like conditions, passing *S. aureus* to dominate the consortia. This data underlines the role of the host in driving the interactions occurring between both species, clearly evidencing the importance of gradually integrate the *in vivo*-like conditions found in CF infections in the *in vitro* models used to study the dynamics of these communities.

By studying further bacteria-bacteria-host interaction it was shown that *S. aureus* has a higher ability to colonize and survive into host cells while growing individually. During co-infection with *P. aeruginosa*, *S. aureus* significantly increased adhesion to host cells. Additionally, *S. aureus* and *P. aeruginosa* were able to survive intracellularly in higher cell numbers during co-infection and seems to reduce their cytotoxicity effect on host cells. These observations suggest that the interplay of the consortia with the host cells shapes *P. aeruginosa* and *S. aureus* interactions, which may subsequently have a role in infection progression and outcome.

7.2 Study Limitations

Herein, the role of *P. aeruginosa* and *S. aureus* co-existence to the enhanced virulence and persistence during CF co-infection was appraised. Even if the use of two bacteria to mimic the polymicrobial nature of CF-associated infections is a step forward, we have also to acknowledge, on the other hand, that this can also be looked as a constraint, as this study only embodies *P. aeruginosa* - *S. aureus* dual-species biofilm communities rather than the full diversity and abundance of microbes found associated with CF infections, which would certainly impact the behaviour of the both species used [4]. Secondly, it comprises biofilms grown in nutrient-rich environment and no other medium resembling CF sputum (as, for instance, artificial sputum media) was used to ascertain *P. aeruginosa* and *S. aureus* co-existence. Lastly, in the *in vivo*-like model, *P. aeruginosa* and *S. aureus* did not deal with the full complexity found in CF infections, namely the airway environment and immune cells. Together, these limitations highlight the challenges in assessing the impact of *P. aeruginosa* - *S. aureus* interactions on CF outcomes using *in vitro* models.

7.3 Future perspectives

The described work highlighted several aspects regarding the pathogenic potential of *P. aeruginosa* and *S. aureus* co-infection. However, several questions remain open and may be taken into consideration in the near future.

Firstly, it should be interestingly to confirm the phenotypic observations using an *in vitro* model that includes host immune cells, particularly neutrophils and macrophages, which would allow mimicking more closely the *in vivo* conditions. Moreover, evaluating the antimicrobial effectiveness against *P. aeruginosa* - *S. aureus* under the *in vivo*-like conditions above mentioned would be useful for the optimal use of current CF antibiotic therapy. Additionally, assess which role could have the occurrence of VBNC and SCV, driven by the presence of the host cells, would be certainly valuable for understanding the mechanisms beyond *P. aeruginosa* - *S. aureus* persistence in CF infections.

Secondly, in order to expand the knowledge regarding the pathogenesis of *P. aeruginosa* and *S. aureus* dual-species biofilms, it would be interesting to select bacterial strains of distinguishable phenotypes, such as the case of non-mucoid and mucoid *P. aeruginosa*, or early and late CF isolates. In fact, *P. aeruginosa* acquiring a mucoid physiology are associated with a decline of lung

function and enhanced resistance to antibiotics [5]. Since early and late CF *P. aeruginosa* isolates have been shown different behaviours during biofilm grown with *S. aureus* [6]. Furthermore, other experimental conditions, namely oxygen concentration, artificial sputum media and exposure to a wide range of in-use antibiotics [7], should be taken into account as changes in the surrounded environment alter how bacteria may interact with one another [8–10].

Thirdly, more research is needed to understand the molecular basis and biological effect of the complex interplay between *P. aeruginosa* and *S. aureus.* In this thesis an initial transcriptome analysis was performed but ideally, an RNA sequencing analysis of *P. aeruginosa* and *S. aureus*, consisting of single- and dual-species biofilms might shed a new light on virulence-related genes and provide novel information necessary to define new targets and strategies for the development of new tailored therapies.

Lastly, despite remarkable research efforts, our current understanding into the relevance and pathogenesis of other microbes for CF lung disease is still lacking, especially as it is based mostly on studies of mono- or dual-species biofilms. Therefore, there is a pressing need for more research directed at delineating ecological interactions within multi-species biofilms in CF models resembling the CF infections.

This is just a small fraction of all the work that can be done...

7.4 References

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