Universidade do Minho
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Functionalization of PVC using a mussel-inspired coating strategy to target the polymicrobial nature of ventilator-associated pneumonia

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Tese de Doutoramento

Engenharia Química e Biológica

Trabalho efetuado sob a orientação da

Professora Doutora Maria Olívia Pereira

E da

Doutora Diana Filipa Barros Alves

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"O mundo está nas mãos daqueles que têm a coragem de sonhar e de correr o risco de viver os seus sonhos"

Paulo Coelho

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STATEMENT OF INTEGRITY

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Abstract

Ventilator-associated pneumonia (VAP) is a common nosocomial infection with high mortality and morbidity rates. The endotracheal tube (ETT) is a risk factor for developing VAP as they are prone to microbial adhesion and biofilm formation; hence, strategies to impart these devices with antimicrobial properties are in great need. As such, this PhD project aimed to engineer an antimicrobial coating for ETTs to prevent VAP occurrence. Since the polymicrobial (intra- and interkingdom) nature of VAP is of particular concern, this work also aimed to understand the interactions within these consortia as well as to inspect their impact on the efficacy of the engineered surfaces.

Through an *in silico* approach, experimental data on the molecular basis of *P. aeruginosa – C. albicans* interactions, two VAP-relevant pathogens, were systematically curated and deposited in the new Inter-Species CrossTalk Database (www.ceb.uminho.pt/ISCTD). Data reconstructed as networks revealed key entities regulating these interactions, potential therapeutic targets, and possible inhibitors, which helped in antimicrobial compound selection for the *in vitro* tasks.

In parallel, polyvinyl chloride (PVC) was functionalized using an adhesive dopamine-based strategy, applying safe-by-design criteria, to prevent single, dual, and triple adhesion and biofilm formation of VAP-relevant pathogens: *P. aeruginosa, Staphylococcus aureus*, and *C. albicans*. The coating strategy was successful in immobilizing nine compounds (natural and synthetic) on PVC. Coatings containing ciprofloxacin (CIP) inhibited *P. aeruginosa* and *S. aureus* while those containing amphotericin B (AmB) prevented *C. albicans* single-species biofilms. Co-immobilization of these agents imparted PVC with broad-spectrum activity, impairing the formation of single, dual, and triple-species biofilms up to 48 h, while also displaying biocompatibility. Longer application times showed reduced efficacy after 72 h against *S. aureus* and after 5 days against *P. aeruginosa* and *S. aureus,* but still demonstrated activity against *C. albicans*. Bacteria recovered from the surfaces after 5 days revealed enhanced CIP tolerance, probably due to CIP exposure and, in the case of *S. aureus*, such traits could be attributed to its interaction with *P. aeruginosa*. Still, the application of a single CIP dose at 48 h boosted triple consortia inhibition for up to 5 days. As such, this coating strategy holds great potential to be further explored in ETT design to fight VAP.

Keywords: Antimicrobial coatings; endotracheal tube; polymicrobial biofilms; ventilator-associated pneumonia.

Resumo

A pneumonia associada à ventilação mecânica (PAV) é uma infeção nosocomial comum e apresenta elevadas taxas de mortalidade e morbilidade associadas. A presença do tubo endotraqueal (TET) é um fator de risco para o desenvolvimento da PAV pois é propenso à adesão microbiana e formação de biofilme; por isso, são necessárias estratégias para conferir propriedades antimicrobianas aos TETs. Como tal, este projeto de doutoramento teve como objetivo desenvolver um revestimento antimicrobiano para aplicar no TET e prevenir a ocorrência da PAV. Uma vez que a natureza polimicrobiana (intra- e inter-reino) da PAV é de particular interesse, este trabalho também teve como objetivo contribuir para uma melhor compreensão das interações dentro destes consórcios bem como avaliar o seu impacto nos resultados antimicrobianos das superfícies desenvolvidas.

Usando uma abordagem *in silico*, os dados experimentais sobre a base molecular das interações *P. aeruginosa* - *C. albicans,* dois agentes patogénicos importantes relacionados com a PAV, foram sistematicamente curados e depositados *online*. Os dados curados reconstruídos como redes revelaram entidades-chave que regulam estas interações, potenciais alvos terapêuticos e possíveis inibidores, o que ajudou na seleção dos compostos antimicrobianos para os estudos *in vitro*.

Paralelamente, foi feita a funcionalização de policloreto de vinilo (PVC) usando uma estratégia de adesão baseada na dopamina, utilizando critérios de segurança, para prevenir a adesão única, dupla e tripla e a formação de biofilmes de agentes patogénicos importantes relacionados com a VAP: *P. aeruginosa*, *Staphylococcus aureus* e *C. albicans*. A estratégia foi aplicada com sucesso na imobilização de 9 compostos (naturais e sintéticos) em PVC. Os revestimentos que continham CIP apresentaram boa atividade inibitória contra biofilmes de espécies únicas de *P. aeruginosa* e *S. aureus*, enquanto que aqueles que continham AmB foram capazes de prevenir biofilmes de *C. albicans*. A co-imobilização destes agentes conferiu ao PVC atividade antimicrobiana de amplo espectro capaz de prevenir a formação de biofilmes de espécies únicas, duplas e triplas até 48 h, apresentando também características biocompatíveis. Para tempos mais longos, os revestimentos mostraram eficácia inferior após 72 h contra *S. aureus* e após 5 dias contra *P. aeruginosa* e *S. aureus*, mas ainda demonstraram atividade inibitória contra *C. albicans*. Por outro lado, a aplicação de uma única dose de CIP às 48 h aumentou o efeito antimicrobiano do PVC funcionalizado, garantindo a sua atividade de inibição tripla até 5 dias. Desta forma, esta estratégia de revestimento possui grande potencial para ser explorada no desenvolvimento de TET para combater a PAV.

Palavras-chave: Pneumonia associada à ventilação mecânica; tubo endotraqueal; biofilmes polimicrobianos; revestimentos antimicrobianos.

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CHAPTER 3

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CHAPTER 4

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List of abbreviations

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

°C: Celsius degrees

%: Percent

µL: Microliter

AgNPs: Silver nanoparticles

AI: Autoinducers

ANOVA: Analysis of variance

AmB: Amphotericin B

BAI: Biomaterial-associated infections

BIP: "Bactiguard®" Infection Protection

C4-HSL: N-butanoyl homoserine lactone

CIP: Ciprofloxacin

CF: Cystic fibrosis

CFU: Colony-forming units

COPD: Chronic obstructive pulmonary disease

COVID-19: Coronavirus disease 2019

DNA: Deoxyribonucleic acid

eDNA: extracelular DNA

EPS: Extracellular polymeric substances

***et al.*:** (*et alli*) and others

ETT: Endotracheal tube

FDA: US Food and Drug Administration

***g*:** Relative centrifugal force

GM: Gentamicin

h: Hour

HAI: Hospital-acquired infection

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

ICU: Intensive care units

IQS: Integrated Quorum Sensing Signal

L: Liter

$\log_{10}$: Logarithm with base 10

LPS: Lipopolysaccharides

MBC: Minimum bactericidal concentration

MDR: Multidrug resistant

mg: Milligram

MIC: Minimum Inhibitory Concentration

min: Minute

mL: Milliliter

MRSA: Methicillin resistant *Staphylococcus aureus*

MSSA: Methicillin sensitive *Staphylococcus aureus*

MV: Mechanical ventilation

NaCl: Sodium chloride

NMA: Noble metal alloy

P: Probability

PBS: Phosphate-buffered saline

pDA: polydopamine

PDMS: polydimethylsiloxane

pH: potential hydrogen

PKA: Protein kinase A

PQS: Pseudomonas quinolone signal

PVC: Polyvinyl chloride

QQ: quorum quenching

QS: Quorum-sensing

RNA: Ribosomal Ribonucleic Acid

RNA-seq: Ribosomal Ribonucleic Acid sequencing

rpm: Revolutions per minute

RPMI: Roswell Park Memorial Institute

s: Second

SarA: Staphylococcal accessory regulator

SCV: Small colony variants

SD: Standard deviation

SDA: Sabouraud dextrose agar

SDB: Sabouraud dextrose broth

spp.: Species

TSA: Tryptic soy agar

TSB: Tryptic soy broth

US: United States

V: Volume

VAP: Ventilator-associated pneumonia

VF: Virulence factors

VM: Virulence mechanisms

WHO: World Health Organization

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List of publications

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

Papers in peer-reviewed journals

Diana Alves; **Tânia Grainha**; Maria Olívia Pereira; Susana Patrícia Lopes. Antimicrobial materials for endotracheal tubes: A review on the last two decades of technological progress. Acta Biomaterialia, 158, 32-55, 2023.

Grainha, Tânia; Jorge, Paula; Alves, Diana; Lopes, Susana Patrícia; Pereira, Maria Olívia. Unraveling *Pseudomonas aeruginosa* and *Candida albicans* Communication in Coinfection Scenarios: Insights Through Network Analysis. Frontiers in Cellular and Infection Microbiology 10(550505), 2020.

Papers in preparation

Grainha, Tânia; Alves, Diana; Neiva, Joana; Pereira, Maria O. Multi-functional PVC coating addressing the polymicrobial nature of Ventilator-associated Pneumonia.

Grainha, Tânia; Alves, Diana; Pereira, Maria O. Polydopamine-based strategies to design anti-infective surface coatings in the fight against biomaterial-associated infections.

Poster communication at international scientific conferences

Grainha, Tânia; Alves, Diana F.; Pereira, Maria Olívia. A broad-spectrum antimicrobial coating targeting polymicrobial biofilms in ventilator-associated pneumonia. The 6th Stevens Conference on Bacteria-Material Interactions. Hoboken, USA, May 31 – June 1, 2023.

Grainha, Tânia; Alves, Diana F.; Nogueira, Eugénia; Pereira, Maria Olívia. Design of multi-functional PVC coating addressing the polymicrobial nature of Ventilator-Associated Pneumonia. ESB 2021 - 31st Annual Conference of the European Society for Biomaterials together with 43rd Annual Congress of the Iberian Society for Biomechanics and Biomaterials (SIBB). September 5-9, 2021, Online.

CHAPTER 1

Introduction

This chapter introduces the context and the motivation as well as the goals behind this work. The significance and the outline of this PhD project are also provided.

1.1 Context and Motivation

The use of medical devices is undoubtedly crucial in current healthcare practices. In particular, the endotracheal tube (ETT) is essential to provide airway patency for patients under mechanical ventilation (MV). MV involves the use of a machine to help a person breathe when they are unable to do so on their own, in cases like surgeries using general anaesthesia, critical care situations, or a traumatically compromised airway [1]. During the coronavirus disease 2019 (COVID-19) pandemic, the usage of these devices was particularly high in order to mitigate the virus-induced lung injuries [2]. Although this technology can be lifesaving, it can also put patients at risk for developing infections, including ventilator-associated pneumonia (VAP) [3]. VAP is a lung infection that develops after patients have been intubated and received MV. This infection occurs when bacteria, or other pathogens, enter the lungs through the ETT, usually by colonization and subsequent biofilm formation on the biomaterial itself [4]. Its rapid surface colonization and biofilm formation are critical events for VAP pathogenesis and relapses. Studies in this field have demonstrated a broad look at the diverse microbial communities in VAP, reporting distinct polymicrobial profiles and varied microbial causes [5–8]. Polymicrobial communities, encompassing inter-kingdom species, are frequently related to biofilm-associated infections, playing extensive ecological roles and substantially increasing the resistance and the burden of VAP infections [9,10].

VAP can be serious and even life-threatening, especially in patients who are already critically ill. There are several factors that can increase the risk of developing VAP, including prolonged MV, previous antibiotic use, and certain medical conditions, such as immunodeficiency [11]. Preventing VAP involves a combination of measures, such as minimizing ventilator exposure, oral care, aspiration of subglottic secretions, maintaining optimal positioning, and encouraging mobility [12]. Early diagnosis and treatment are also important in improving outcomes for patients with VAP. Despite the efforts made in clinical practices, up until now, there is no universal method able to prevent the occurrence of VAP. Therefore, it is urgent to find effective alternative therapeutic strategies targeting such infections that take into consideration their particularities, such as the polymicrobial aetiology of VAP, which is a critical issue in this infection but still often neglected. Indeed, the coexistence of a set of different microorganisms, including bacterial and fungal species, is frequent in nosocomial infections such as VAP [13–16]; however, the mechanisms of microbial interactions remain unclear or even contradictory. A better understanding of the social behaviour within the consortium and how microbes change their virulence as

a result of established interactions is also clinically relevant to better tackle this kind of infection. It is urgent to tailor effective therapeutic strategies aligned with the challenges faced *in vivo*.

The development of materials that can resist or prevent microbial adhesion constitutes an emerging approach to deal with biomaterial-associated infections (BAI), such as VAP, and it has gained great attention over the years [17]. Modern biomaterial science has provided several modification strategies to impart biomaterials, such as ETTs, with anti-infective properties. However, most current strategies have raised some concerns, particularly their deficient antimicrobial efficacy, as most studies only address single-species adhesion, and their short-duration effectiveness [18]. Indeed, even though VAP is often mediated by biofilms, only a few studies address the effectiveness of ETT coatings towards biofilms and even less bear in mind its polymicrobial nature. Likewise, though significant work has been placed in combating bacterial pathogens, substantially less has been focused on combating ETT-related fungal infections. Additionally, some of the current studies involving the design of anti-infective materials do not evaluate the period of time that the antimicrobial activity lasts (the long-term stability). These abovementioned features should be taken into consideration in the search for a new strategy in order to boost the worth of the surface's modification approaches. Thus, the key goal of this PhD project was to tailor a coating strategy with lasting and broad-spectrum antimicrobial activity to be applied to ETTs, addressing the biofilm-mediated and polymicrobial nature of VAP, features often overlooked in current research.

1.2 Research Aims

The main goal of the present thesis was to engineer a functional broad-spectrum antimicrobial coating on polyvinyl chloride (PVC), the polymer constituting ETTs, based on the immobilization of antimicrobial compounds to impair the formation of polymicrobial inter-kingdom biofilms. This work was designed applying safe-by-design criteria appraising aspects such as the cytotoxicity of the coating, the development of antimicrobial tolerance, in addition to long-term antimicrobial activity.

It was hypothesized that the combination of different antimicrobial agents with different microbial targets immobilized onto PVC would assist the development of a wide-spectrum antimicrobial surface. To establish such coating, it was therefore required to screen suitable antimicrobial combinations and respective concentrations to generate surfaces able to simultaneously prevent single, dual, and triple microbial adhesion and biofilm formation by relevant VAP-related pathogens (*Pseudomonas aeruginosa, Staphylococcus aureus*, and *Candida albicans*) and be non-cytotoxic to mammalian cells (Figure 1).

As the interactions underlying polymicrobial biofilms can alter the behaviour of the microbial players enrolled in it and account for the (in)success of the antimicrobial strategies, this work also intended to contribute to a better comprehension of the interactions and regulatory targets of the abovementioned VAP pathogens. As in-group efforts had already disclosed this information for the bacterial pair *P. aeruginosa* and *S. aureus*, this work focused on the still unexplored inter-kingdom duet of *P. aeruginosa* and *C. albicans*.

To accomplish these purposes, the following specific aims were delineated:

- *In silico* reconstruction of the inter-species communication network between *P. aeruginosa* and *C. albicans* in co-infection scenarios.

- Immobilization of antimicrobial candidates, single and in combination, onto PVC using a polydopamine (pDA)-based approach for the intermediate coupling of the compounds.

- Screening of the antimicrobial-modified PVC by assessing its contact-killing and leaching properties.

- Examination of the antimicrobial performance of the functionalized PVC surfaces against single-, dual, and triple-species biofilms.

- Evaluation of the antimicrobial stability overtime of the functionalized PVC.

- Physicochemical characterization of the functionalized PVC surfaces.

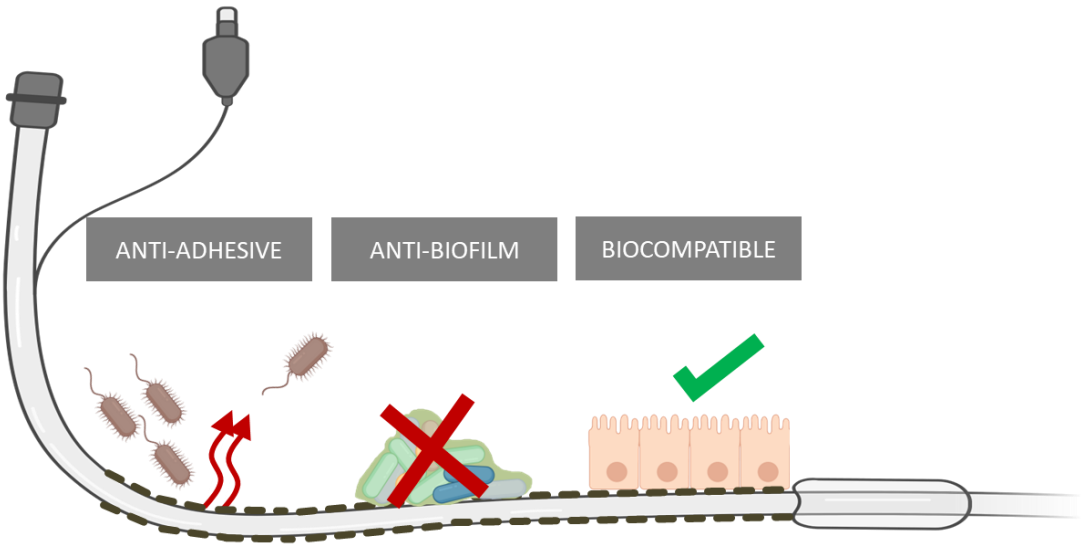

Figure 1. Schematic representation of the main goal of the present thesis: to tailor a wide-spectrum antimicrobial coating with both anti-adhesive and anti-biofilm properties, while ensuring no toxicity towards mammalian cells. The ultimate goal of this project is that the present technology could be applied in ETT design.

1.3 Thesis Outline

This thesis is organized into six chapters that cover the work performed for the research aims previously mentioned. Following this introductory chapter, Chapter 2 presents a literature review providing a general outline of the major aspects of the specific microbiological and clinical features of VAP, carefully emphasizing the presence of the ETT and biofilm development as key factors for VAP. Relevant information on *C. albicans*, *S. aureus*, *P. aeruginosa*, and their interactions is also given. A general outline of the different antimicrobial modifications that have already been applied to ETT is provided. Finally, the relevance of dopamine chemistry for the functionalization of biomaterials is enlightened. Chapter 3 presents the *in silico* approach used for the retrieval and analysis of experimental information from the scientific literature on the molecular basis of *P. aeruginosa* and *C. albicans* interactions its deposition on a public database, and its integration with other online resources to extrapolate candidate antimicrobials. Chapter 4 is dedicated to the optimization of a PVC coating to impair the development of a polymicrobial inter-kingdom biofilm model. After a preliminary screening of the compounds based on their antimicrobial release and contact-killing features, an optimization related to compound immobilization concerning their concentration was performed against single-species biofilms. The most promising antimicrobial compounds were further co-immobilized onto PVC at different concentrations to inspect their activity to combat single-, dual, and triple-species biofilms. In this chapter, a full physicochemical characterization of un- and modified PVC surfaces was also performed. Chapter 5 presents an in-deep study regarding the antimicrobial performance of PVC-coated surfaces to be used for long-term applications. Finally, the main conclusions of the present work are presented in Chapter 6 and clues for future work are also suggested.

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CHAPTER 2

Literature Review

This chapter provides a general outline of the major aspects of the specific microbiological and clinical features of VAP, carefully emphasizing the presence of the ETT and biofilm development as key factors in infection establishment. Relevant information on *P. aeruginosa*, *S. aureus,* and *C. albicans* pathogenicity and the interactions that can occur between these microorganisms are also addressed. A general outline of the different antimicrobial approaches that can be used for surface modification is provided, with a special focus on the strategies that have been proposed in the last years for ETT. Current challenges that need to be directed and potential avenues that may assist upcoming advances in this field to tackle VAP are also elucidated. Finally, the relevance of dopamine chemistry for the functionalization of biomaterials with antimicrobial properties is mentioned.

2.1 Ventilator-associated pneumonia

Ventilator-associated pneumonia is a common nosocomial infection that arises in patients under MV, remaining the second most common hospital-acquired infection (HAI) and the most common in mechanically ventilated patients [1]. VAP can be described as a lung infection that develops at least 48 h or more after intubation and MV, presenting, in some cases, non-specific symptoms or clinical signs. Its primary symptoms overlap with those of other medical conditions, which difficult and makes diagnosis delayed or even missed [2]. Despite the improvements in microbiological technologies, the epidemiology and diagnostic criteria for VAP are still controversial topics. Usually, after clinical suspicion of VAP, the diagnosis is often based on a combination of criteria, including, radiographic indication (the presence of new or progressive lung infiltrate), laboratory evidence (leukocytosis or leukopenia), and clinical evidence (temperature <36 °C or >38 °C, new onset or increase of purulent aspirates, wheezing, rales, rhonchi, or worsening gas exchange) [3]. Depending on the time it occurs after the start of MV, VAP is usually classified as early- or late-onset VAP. It is categorized as early-onset VAP if occurs within four days of MV and late-onset pneumonia when takes place after 5 or more days after MV onset [4]. The main attributes of each type of VAP often found across the studies are summarized in Table 1.

Table 1. Comparative overview of the main attributes of early- vs. late-onset VAP.

	Early-onset VAP	Late-onset VAP	Ref(s).
Time of occurrence	2-4 days post intubation/MV	≥ 5 days post intubation/MV	[5–7]
MV days	14	23	[8–10]
ICU/Hospital length of stay	13-20/26.5 days	20-26.5/35.5 days	[8–10]
Incidence	11-16%	81-84%	[9,11]
Hospital mortality rate	16-23%	31-44%	[8–10]
Microbial aetiology	Antibiotic-sensitive bacteria: ● MSSA ● *Streptococcus pneumoniae* ● *Haemophilus influenzae* ● *Klebsiella pneumoniae* ● *Escherichia coli* ● *Proteus* spp. ● *Serratia* spp.	Antibiotic-resistant or MDR bacteria: ● *P. aeruginosa* ● MRSA ● *Acinetobacter* spp. ● *Enterobacter* spp. ● VRE	[8,12,13]

Legend: MDR - multidrug-resistant; MRSA - methicillin-resistant *S. aureus*; MSSA - methicillin-sensitive *S. aureus*; VRE - Vancomycin-resistant *Enterococcus*

In general, early-onset VAP is a result of antibiotic-sensitive pathogens whereas late-onset VAP has been commonly associated with the worst prognosis being essentially caused by antibiotic- or multidrug-resistant (MDR) pathogens. It has also a significantly longer duration of MV, length of stay in intensive care units (ICU), and higher mortality rates than the early-onset VAP.

Regarding VAP incidence, discrepant rates have been reported among the studies, which can probably be associated with the different interpretations of VAP, limitations regarding the diagnostic methods, and the use of nonstandardized microbiological sampling methodologies [14]. Nevertheless, incidence rates founded associated with late-onset VAP are greatly higher than those related to early-onset VAP.

Generally, VAP incidence greatly varies from 5% to 40%, based on the diagnostic criteria, type of healthcare unit, and studied patient population [15,16]. In 2009, a report from the International Nosocomial Infection Control Consortium which includes data from Latin America, Asia, Africa, and Europe gives a pooled density of 13.6 per 1000 ventilator-days [17]. In Northern United States (US) hospitals, VAP rates are referred to as low as only 1 to 2.5 episodes per 1000 ventilator days are noticed [18]. Much higher VAP rates are reported by European centres. Indeed, in a study, which enrolled patients from 27 ICU in 9 European countries, the VAP incidence was 18.3 episodes per 1000 ventilator-days [19]. On average, the incidence rate of VAP varies from 2.8 in the US [18], to 14.5 in Europe [20], and 22 episodes per 1000 ventilator-days in developing countries [21]. A systematic review covering 22 countries in Asia, reported that the pooled incidence density of VAP was high in lower- and upper-middle-income countries and lower in high-income countries (18.5, 15.2, and 9.0 per 1000 ventilator-days, respectively) [22]. Another systematic review and meta-analysis of VAP cases focused on China reported an incidence of 24.14 episodes per 1000 ventilator-days [23].

Some clinical conditions (e.g. comorbidities; severity of illness) can account for a high risk to contract VAP in intubated patients. For instance, high incidence rates are reported in cancer patients (24.5 cases per 1000 ventilator days) [24] or in patients with traumatic injuries (at a rate of 17.8%) [25]. Specific risk factors, such as prolonged invasive MV, high incidence of microaspiration and airway bacterial colonization, defective mucociliary clearance, and altered local and general host immune defences, account all for the increased VAP incidences in intubated chronic obstructive pulmonary disease (COPD) patients. VAP incidence in COPD patients ranges widely from 6.2% to 56% [26–30]. Also, a recent study has suggested a higher risk of VAP occurring among coronavirus disease 2019 (COVID-19) patients compared with the general ICU population (25.5 vs 15.4 cases per 1000 ventilator-days) [31]. VAP all-cause mortality has been reported to be as high as 50%, and patients admitted to ICU are generally at increased risk for VAP, even though there is still considerable controversy regarding the attributable

mortality of VAP in ICU patients [32]. However, VAP has an undeniable clinical relevance and remains an unresolved problem, associated with a longer duration of MV, prolonged stays in ICU and hospital, and increased risk of disability and mortality [33,34]. In addition, VAP accounts for approximately 50% of antibiotic prescriptions within ICU [34,35].

VAP has also a significant worldwide economic impact, accounting for increased healthcare costs that range from $28-$33 billion per year in the US [36], which represents approximately 32% of the total HAI annual costs [37]. A study conducted by Kollef and colleagues reported average costs of $99 598 for patients with VAP, compared to $59 770 for patients without VAP [38]. In the European healthcare system, the economic impact of VAP is estimated between €13 and 24 billion per year [39–41].

2.1.1 Endotracheal tube: the gateway for VAP

The use of medical devices is undoubtedly crucial in current healthcare practices. Specifically, the ETT is essential to provide airway patency for patients under MV in cases like surgeries using general anaesthesia, critical care situations, or a traumatically compromised airway [42]. MV can be defined as the maintenance of the oxygenation and/or ventilation of patients in an artificial manner [43,44]. Although MV can be noninvasive, involving various types of face masks, there are cases where it is necessary to resort to invasive therapy, namely endotracheal intubation. In the latter case, an ETT is usually inserted through the nose or mouth into the trachea. MV is a life-saving procedure but it is also associated with some complications, presenting risks for patients, including the development of infection [45]. Although the duration of MV, bed positioning, enteral feeding, witnessed aspiration, prior antibiotic therapy, and multiple comorbidities, may all contribute to VAP [46], the ETT alone has been recognized as a major risk factor contributing for up to 25–56% of the intubated patients to contract VAP [33,47]. In other words, the risk of acquiring VAP increases by 6 to 20 folds every time an ETT is placed in a patient [48,49]. The intubation process modifies the anatomy of the larynx, causing damage in the mucociliary system, and acting as an entry point for microorganisms to the lower airways [49]. Infectious microorganisms can obtain access to the lower respiratory tract around the ETT (microaspiration) and through the ETT surface (microbial colonization following biofilm formation) [50]. Microaspiration comprises the distal migration of microorganisms from secretions which can occur during intubation itself or by pooling and trickling of secretions around the cuff [47,51]. A cuff is an inflatable balloon at the distal end of the ETT to seal the trachea to carry out positive pressure ventilation and prevent the aspiration of fluid and pharyngeal contents into the lower airways. However, it has been reported that an inflated ETT cuff may be a reservoir of infecting microorganisms being a source of microaspiration due to the variations in the cuff pressure

alongside the formation of folds in the inflated cuff [52] leading to tracheobronchial colonization and VAP. Overall, the ETT surface provides conditions for microbial attachment and proliferation, ultimately resulting in microbial colonization over the whole external and internal ETT surface [45,53,54].

The pathogens involved in this process can arise from different sources including endogenous or exogenous origin. The exogenous sources of microbial pathogens responsible for VAP include aerosols of the contaminated air and medical devices (ventilatory circuit, catheter, bronchoscope, and humidifier) while the endogenous sources are mostly from the oral, pharyngeal and gastric flora of the patient [55,56] (Figure 2).

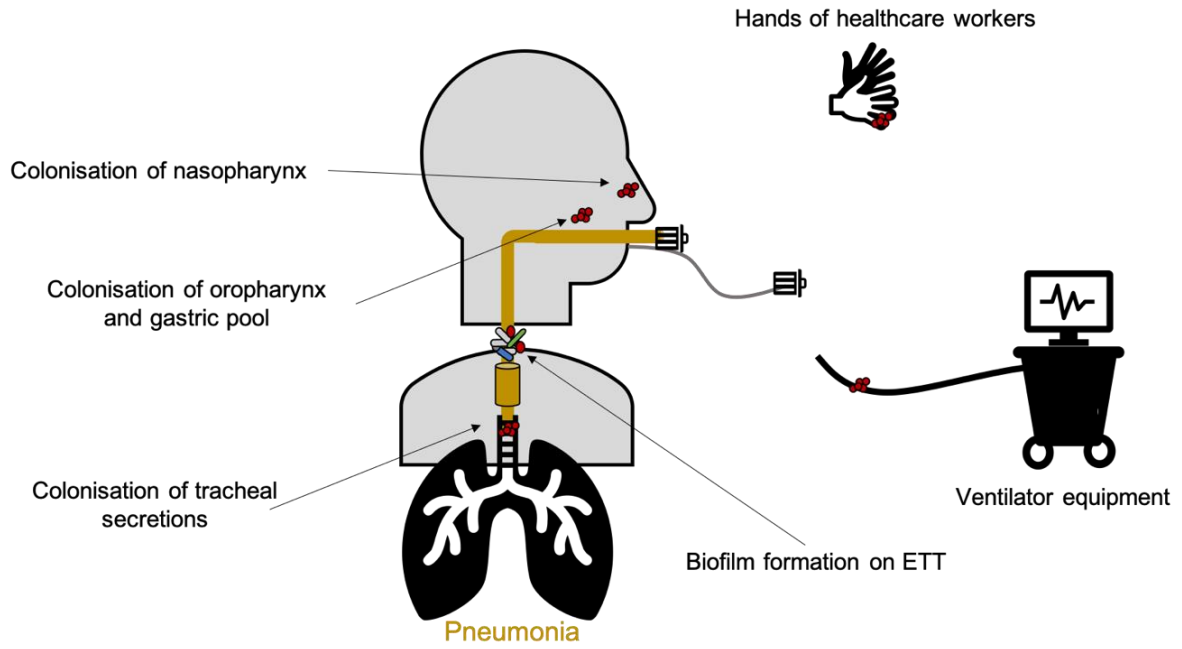

Figure 2. Pathogenesis of ventilator-associated pneumonia. The colonization of the upper airway can be caused by the contaminated hands of healthcare workers, contamination of the ventilator equipment, a biofilm within the ETT, and from contaminated secretions from the nasopharynx, oropharynx, and trachea. The aspiration of colonized fluids from any of these sources into the lungs can result in pneumonia (adapted from [57]).

2.1.2 ETT biofilms

An important feature of VAP pathogenesis is its association with biofilms. Microbial adhesion to medical devices including ETT and consequent biofilm formation remains one of the greatest current challenges in healthcare, becoming the focus of persistent infections. Biofilms are well-developed communities that represent the most dominant and active mode of microbial life in nature. They are matrix-enclosed communities harder to eradicate due to their inherent tolerance to antimicrobial therapies and the host immune system, which leads to the persistence and recurrence of infections [58]. It was previously reported that 80% of nosocomial infections are biofilm-mediated [59].

Biofilm development is a dynamic process that involves the following main stages: initial attachment; aggregation; maturation and dispersion (Figure 3). Following the initial attachment of planktonic cells on the surface, the microbial cells divide and proliferate to establish colonization. Cells within the consortia produce an extracellular matrix of polymeric substances (EPS) leading to a mature biofilm architecture. Microbial cells inside this mature biofilm can subsequently disperse, leading to the colonization and infection of other sites. During biofilm formation, attachment of different species can occur, leading to a multi-species biofilm [60].

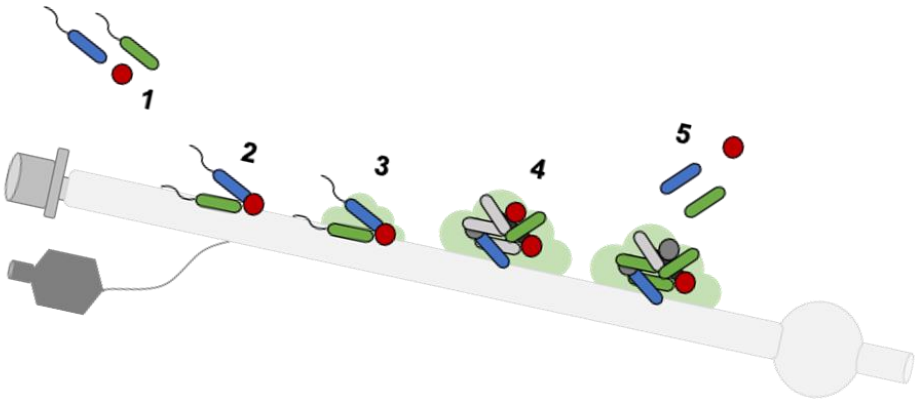

Figure 3. Schematic representation of biofilm formation on ETT surface. (1) Free cells initiate attachment to the ETT surface; (2) reversible attachment of the planktonic cells is followed by the adhesion on the surface; (3) cells then form a monolayer and irreversibly attach by producing EPS; (4) microcolonies are formed and multilayers appear leading to a mature biofilm architecture; (5) the cells within a mature biofilm can subsequently detach and disperse, leading to the colonization of other sites.

Generally made of flexible materials such as PVC or polydimethylsiloxane (PDMS), the ETT acts as a reservoir for infecting microorganisms [61], thereby increasing the patient's opportunity of getting pneumonia. The ETT provides an optimal surface for microbial colonization and biofilm proliferation which may occur in both its inner luminal and outer surface [62]. Microbial colonization of the ETT combined with the injured host-defense mechanisms of the patients significantly contributes to the development of the ETT biofilm and the consequent spread of the infection [63].

It was previously proposed by Vandecandelaere and Coenye (2015) a model elucidating how bacteria propagate and develop a multi-species biofilm at the distal end of the ETT. In this model, the first stage of biofilm formation results from the leak of nasopharyngeal secretions with non-pathogenic oral bacteria (mostly *Streptococcus* spp.) around the cuff of the ETT. After bacterial attachment and accumulation, the next step comprises streptococci co-aggregation with other commensal members of the subgingival flora such as *Veilonella* spp. and *Actinomyces* spp., followed by the recruitment of a variety of opportunistic oral species (e.g. *Fusobacterium nucleatum* and *Prevotella* spp.). This may allow further nosocomial pathogens such as *P. aeruginosa* or *S. aureus*, to consequently join and interact with oral bacterial species within the pre-formed biofilm, with harmful consequences leading to VAP development [63]. Other factors such as the supine patient positioning, alongside the shape, thickness, and permeability of the ETT cuff wall, together with variations in the cuff pressure, are sufficient to allow deposited contaminated secretions to migrate to the lower airways and easily lead to pneumonia [64–66]. Portions of the biofilm can also be easily dislodged during suctioning, bronchoscopy or simply by gravity, spreading into the lower airways, and invading the lungs [67]. The ETT poses, therefore, a crucial role in VAP pathogenesis, allowing microorganisms to colonize and form resilient biofilms on its surface, and likely shaping lung ecology during invasive MV at a microbiome scale [68]. The ETT biofilm colonization was early evidenced, first by culturing methods [69] and later, through advanced assessment techniques (e.g. scanning electron microscopy; quantitative polymerase chain) [70,71] . Increasing research has shown that microbial colonization and biofilm formation on ETT is an early and frequent event in intubated patients, contributing to the pathogenesis, lack of treatment and relapses of VAP [3,63,72–76]. When settled on the ETT surface, the biofilm becomes hard to treat due to its inherent recalcitrance nature [76]. Overall, bacteria in biofilms can require 500 to 5000 times more antibiotic doses than that necessary to kill planktonic counterparts [77].

2.1.3 The polymicrobial nature of VAP

Microbial pathogenesis research was originally focused on the analysis of infections as monomicrobial events. However, this idea has been refuted by mounting evidence which was supported by the presence of a distinct microbiome in several diseases, including VAP. Several studies have demonstrated that ETT biofilms generally harbour multiple microbial pathogens, which makes VAP a polymicrobial infection, with considerable inter-patient and intra-patient diversity [61]. Data have demonstrated a broad look at the diverse microbial communities in VAP, reporting distinct polymicrobial profiles and varied microbial causes [13,63,76,78]. A wide range of microorganisms is often involved in VAP, with bacteria being the most predominant. Bacteria from the ESKAPE (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* spp.) group are frequently found in nosocomial environments. Particularly *P. aeruginosa*, A*. baumannii*, *K. pneumoniae*, and *S. aureus* play a dominant role in VAP aetiology. Gram-negative bacterial species generally account for 80% of the total isolates [79], and Gram-positive bacteria represent 20-30% of VAP cases [80]. *P. aeruginosa* is the single-most common pathogen cause of VAP followed by Gram-positive organisms like *S. aureus* [81–84]. Alongside bacterial pathogens, there is an increasing recognition that a considerable part of nosocomial pneumonia may be also associated with viruses (e.g. Influenza and Cytomegalovirus) and fungi (e.g. *Candida* spp. and *Aspergillus* spp.) playing a more important role in immunocompromised patients [85,86]. For instance, *Candida* spp, especially *C. albicans*, has been found as part of the array of microorganisms isolated from the respiratory tract of patients diagnosed with VAP [81,83,84,87]. Polymicrobial communities, composed of inter-kingdom species, are often related to biofilm-associated infections playing extensive ecological roles, and substantially increasing the resistance and the burden of VAP infections [88,89]. For instance, it has already been demonstrated that *C. albicans* instillation in rats increased the probability to develop experimental *P. aeruginosa* and *S. aureus* pneumonia, fostering the production of lung inflammatory cytokines [90,91]. Understanding the impact of microorganisms in VAP and their interactions is essential to clearly unveil the infection pathogenesis. Co-infection studies involving bacterial and fungal species, such as *P. aeruginosa* and *S. aureus* with *C. albicans*, have emerged in the last decade [92–97]. The co-existence of these pathogens is devastating in a variety of human-associated infections, including lung infections [98,99], because of their propensity to develop biofilms highly resilient to conventional antimicrobial therapy [96,100–102]. In general, mixed bacterial and bacterial-fungal biofilms are more resistant to antibiotic treatment than the corresponding single-species biofilms [103]. Therefore, it is urgent to develop effective therapeutic strategies targeting this particular trait of infections.

2.1.3.1 *P. aeruginosa*

P. aeruginosa is a motile Gram-negative bacterium ubiquitously found in human environments. It is an opportunistic pathogen with the ability to cause life-threatening acute and chronic infections, especially in hospitalized and immunocompromised individuals. *P. aeruginosa* is frequently associated with different types of infections, such as cystic fibrosis (CF) lung infection, burn wounds, otitis media, urinary tract infections, and those associated with biomaterials [104,105] such as VAP. There are essentially three key factors for *P. aeruginosa*'s pathogenic abilities: production of virulence factors (VF), resistance to antimicrobial agents, and biofilm formation [106]. *P. aeruginosa* is widely recognized for producing an arsenal of VF associated with diverse functions, such as motility (e.g. flagella, pilli), tissue invasion and damage to the host cells (e.g. proteases such as elastase, alkaline phosphatase, haemolysins, pyocyanin, siderophores, endotoxin A, lipopolysaccharides (LPS), exotoxin A), as well as surface adhesion and biofilm formation (e.g. alginate, Pel, Psl, lectins) [105,107,108]. In addition to these factors, *P. aeruginosa* is known for its remarkable ability to resist antibiotics. Actually, the World Health Organization (WHO) has listed carbapenem-resistant *P. aeruginosa* in the top three species for which the development of new treatments is in critical need [109]. The major resistance mechanisms of *P. aeruginosa* that allow it to withstand the action of antibiotics can be intrinsic or innate (e.g. restricted outer membrane permeability, efflux pumps, antibiotic-degrading enzymes), acquired by either horizontal transfer of resistance genes or by mutations, or adaptive (e.g. biofilm formation, persister cells) [110]. The ability of *P. aeruginosa* to form biofilms comprises an advantage in many infections [111]. In effect, it is considered the hallmark of chronic infections and the revealing of disease progression. The biofilm environment triggers the development of small colony variants (SCV) and persister cells that can resist higher doses of antibiotics [112,113].

2.1.3.2 *C. albicans*

C. albicans is a polymorphic fungus that has the ability to grow in several different morphological forms, namely yeast, hyphae, and pseudohyphae, depending on the environmental conditions [114]. This microorganism is the most common fungal species in the human microbiota colonizing the skin, oral cavity, gastrointestinal and reproductive tract, being harmless in the majority of cases [115]. However, *C. albicans* can take advantage of immunocompromised patients and cause several opportunistic infections [116]. It is the most frequent fungal species isolated from medical devices being one of the most common fungi found in the ETT microbiome [87]. Similarly to *P. aeruginosa*, there are three important features for

the pathogenesis of *C. albicans*: its ability to switch from yeast-to-filamentous growth and vice versa, secretion of VF, and biofilm formation [117]. Each morphology assumed by *C. albicans* provides different advantages in the development of infection. The filamentous forms (hyphae and pseudohyphae) are one of its most important virulence mechanisms (VM), being responsible for host cell invasion and macrophage destruction, thus having an important role in the establishment of an infection process [118,119]. In turn, the yeast form is believed to be important for dissemination through the bloodstream and adhesion to endothelial surfaces [120]. This phenotypic switch can be induced by temperature, pH, nutrient concentration, cell density, and human serum [121]. Besides this morphological plasticity, there are other VF contributing to *C. albicans* pathogenesis, such as the production of adhesins (biomolecules that enable binding to the host cells) and hydrolytic enzymes (e.g. aspartyl proteinases and phospholipases) [122,123]. It is now well acknowledged that biofilm formation is one of the main virulence features involved in the pathogenesis of *C. albicans*, as these organized communities confer protection against antimicrobial therapy and host defenses [124].

2.1.3.3 *S. aureus*

S. aureus is a Gram-positive commensal bacterium frequently found on mucous surfaces of the nose and respiratory tract, and on the skin [125,126]. This microorganism is also recognized as a human pathogen often associated with community-acquired and nosocomial infections. Indeed, *S. aureus* is responsible for a wide range of infections, from mild skin and soft-tissue infections to bacteremia, endocarditis, and osteomyelitis [127] displaying also the ability to adhere to medical devices as ETTs, resulting in biofilm formation and infection [128,129]. Its ability to evolve and adapt to multiple settings has led to the emergence of MRSA, a drug-resistant phenotype, being considered a high threat according to WHO [109]. Additionally, MRSA has been developing resistance to virtually all antibiotics classes that are used to tackle it, either by making use of its intrinsic resistance factors or by acquiring more through mutations or horizontal gene transfer [130]. The presence of MRSA seriously hinders the treatment of infections caused by this pathogen [131]. *S. aureus* biofilms have been implicated in serious acute chronic infections due to the presence of either MRSA or MDR [132,133]. Furthermore, the heterogeneous nature of biofilms comprising this bacterium increases the chances of plasmid horizontal transfer, leading to antibiotic resistance which can consequently culminate in ineffective treatment regimens and consequent recalcitrant infections [134]. *S. aureus* also possesses a set of VF which contributes to the success of this pathogen in a wide range of infections. These staphylococcal VF enable attachment to host cells, breaking down the host immune shield, tissue invasion, causing sepsis, and eliciting toxin-mediated

syndromes. These factors include the production of surface components such as cell wall proteins (e.g. collagen, fibronectin, protein A, and clumping factors) that are involved in attachment and evasion to the host-tissue. Extracellular toxins (e.g. haemolysin, leukotoxin, exfoliative toxin, enterotoxin, and toxic-shock syndrome toxin-1) and enzymes (e.g. proteases, lipases, and staphylokinase) are also secreted by *S. aureus* to help in tissue penetration and host invasion [135]. The phenotype SCV has also been strongly linked to pathogenic traits of *S. aureus* contributing to infection persistence [136].

2.1.3.4 Quorum-sensing

Microorganisms interact by means of a major process that allows them to coordinately sense and respond to the fluctuating conditions of the surrounding environment. This cell-to-cell communication is called quorum-sensing (QS) and is mediated by small diffusible signalling molecules, termed autoinducers (AI). AI regulate the expression of target genes, namely those related to virulence, pathogenicity, resistance, and competition when a threshold concentration is reached regarding population density [137–140]. AI-mediated signalling in QS allows microbial communication and the control of pivotal processes, namely VF production, biofilm formation, motility, sporulation, production of secondary metabolites, and stress adaptation through, for example, secretion systems [141].

The structure and functioning of the QS machinery deployed by *P. aeruginosa* have been well elucidated [142–144]. *P. aeruginosa* possesses four well-known QS systems: LasI/LasR, RhlI/RhlR, PqsABCDE/PqsR, and AmbBCDE/IqsR. Each of these systems produces an AI, namely 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 [143]. The hierarchical regulation of *P. aeruginosa* QS systems allows the triggering of massive changes in genetic expression, namely in genes involved in motility, biofilm formation, iron sequestration, antibiotic resistance, and immune system evasion [145,146]. For example, the LasI/LasR system controls the production of multiple VF involved in acute infection and host cell damage, such as LasA and LasB elastases, exotoxin A, and alkaline protease [147–149]. It also controls the expression of Pel, a major biofilm matrix component [150]. In turn, the excretion of bacterial biosurfactants, such as rhamnolipids, is regulated by the Rhl system [151], while the PqsABCDE/PqsR system controls biofilm formation and its structural stability through extracellular DNA (eDNA) and lectin production [144,152].

In the case of *S. aureus*, among the regulatory mechanisms described for this pathogen, its main system is called the accessory gene regulator (Agr) and ensures its adaptation to the environment and its

coordinated pathogenesis. This complex coordinated QS system encodes a signalling circuit that produces and senses the extracellular autoinducing peptide and the intracellular regulator RNA III [153]. Agr system is responsible for the increased expression of many toxins (e.g. haemolysins and enterotoxins) and degradative exoenzymes (e.g. proteases, lipases) [154] being also related to the decreased expression of several colonization factors. Accordingly, biofilm formation is strongly associated with Agr function, with down-regulation leading to excessive biofilm thickness and lack of structuring, while up-regulation induces biofilm dispersal [155,156]. The emergence of SCV in *S. aureus* is also associated with reduced Agr activity [157]. A second regulatory QS system in *S. aureus*, closely related to Agr, is the TRAP/RAP system. The RNAIII-activating peptide (RAP) is an AI that induces the phosphorylation of its target protein (TRAP). TRAP induces the production of adhesion proteins, stimulating biofilm formation, and 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 [158]. Another QS regulator system, the staphylococcal accessory regulator (SarA) of *S. aureus,* regulates the expression of many VF, including biofilm formation, to mediate pathogenesis and evasion of the host immune system in the late phases of growth [154,159]

Contrary to the enormous information available about QS in prokaryotes, QS in the fungal kingdom was somewhat concealed until the discovery of farnesol, a common sesquiterpene produced by *C. albicans* and similar in structure to bacterial 3-oxo-C12-HSL, in 2001 [160]. Although other molecules mediating QS in *C. albicans* have been identified since then, including farnesoic acid [161] and aromatic amino acid-derived alcohols like tyrosol [162], tryptophol, and phenylethanol [163,164], farnesol is undoubtedly the most explored AI. Regulation of fungal virulence by farnesol is thought to be particularly confined to inhibiting yeast-to-hypha transition [165], promoting reverse morphogenesis [166], and inhibiting biofilm development [167]. Farnesol is known to directly inhibit the fungal Ras1-cyr1-cAMP-protein kinase A (PKA) signaling pathway, ultimately blocking cAMP synthesis, and consequently suppressing hyphal development [168–170]. Following the same event cascade, the catalytic subunits of PKA, Tpk1 and Tpk2, are also stimulated. Tpk1 and Tpk2 share redundant functions in hyphal growth, adhesion, and biofilm formation, but also have distinct roles in stress responses and pathogenesis, respectively [171,172]. *RAS1, CYR1*, and *EFG1* (other important hypha-associated genes) have also been documented as important effector genes targeted by farnesol [173]. Apart from farnesol, the AI farnesoic acid, tyrosol, tryptophol, and phenylethanol may also affect important processes such as morphogenesis, biofilm development, limitation of cell population density, control of nutrient competition, and control of infection dissemination [174]. Tyrosol, for instance, stimulates *C. albicans* filamentation, biofilm

formation, and germ tube development [162,175], in contrast to farnesol. However, understanding their underlying regulatory mechanisms is yet to be fully elucidated.

2.1.3.5 *P. aeruginosa* and *C. albicans* interplay

Bacteria and fungi often coexist in competitive ecological niches in a myriad of ways, communicating through the excretion of metabolic by-products, physical interactions, chemical signaling exchanges, and alterations in the environment [176]. *P. aeruginosa* and *C. albicans* are likely the best-studied models in the investigation of these inter-kingdom interactions since reports on their impressive interactions and communication have greatly evolved in the last decade [91,95,177–179]. Although *P. aeruginosa-C. albicans* interplay is fundamentally antagonistic, its interaction is rather complex, as synergistic, and antagonistic effects can occur simultaneously, mostly dictated by physical associations and by secreted factors *via* QS [97,180,181].

P. aeruginosa and *C. albicans* are frequently co-isolated in polymicrobial infections, such as those related to skin, lung, and medical devices [182]. Their genetic and phenotypic plasticity, along with their propensity to assemble as recalcitrant polymicrobial biofilms, places a considerable burden on infections in which they are involved, explaining the increasing interest and the bulk of research on this topic. For example, it has been well-documented that *P. aeruginosa* and *C. albicans* colonize the lungs of CF patients and readily form biofilms on ETT surfaces [116]. Interestingly, *C. albicans* is only implicated in airway colonization in critically ill (elderly, immunocompromised, and/or hospitalized) individuals undergoing invasive MV. However, despite their antagonistic relationship, those who display *C. albicans* tracheobronchial colonization are at increased risk of acquiring severe VAP infections due to *P. aeruginosa* [89].

The inhibitory effect of *P. aeruginosa* on *C. albicans* growth was first reported in the 1970s [183,184]. In 2002, Hogan and Kolter reported the killing of *C. albicans* hyphal cells by *P. aeruginosa*, showing, however, no such effect on fungal yeasts [185]. The deadly effect demonstrated by *P. aeruginosa* was further confirmed to be largely dependent on the distinct morphotypes exhibited by *C. albicans* [185–187]. Often, a reversion of germ tube formation may occur in the presence of *P. aeruginosa*. The most common event elucidating the physical interaction between *P. aeruginosa* and *C. albicans* is likely the extensive bacterial attachment to fungal hyphae [97,188]. Physical association among *P. aeruginosa* and *C. albicans* occurs via cell wall-associated compounds in bacteria, such as type IV pili, lectin-carbohydrate interactions, and mannans [189]. The bacterial attachment to fungal hyphae is hypothesized to be caused by nutrient competition at the first hours of co-isolation, after which the bacterial-fungal interaction tends

to be of parasitism [178]. Secreted LPS, another bacterial cell-wall component, also functions on polymicrobial intertwining, by interfering with fungal filamentation, metabolism, and growth [190,191]. The *P. aeruginosa* QS signal 3-oxo-C12-HSL plays major roles in interaction, namely the bacterial binding to *C. albicans* filaments and in the inhibition of yeast to hyphae switch [187,188]. Because of the inhibition of yeast-to-hypha transformation, *C. albicans* ability to adhere or invade tissues is compromised [192]. The 2-heptyl-4-quinolone (HHQ), the immediate precursor of the PQS signal, has been shown to repress *C. albicans* biofilm formation [193]. PQS induces the expression of the VF phenazines, such as pyocyanin, which are toxic products that produce a deleterious effect in eukaryotic cells [194,195]. This effect may be associated with the generation of highly toxic reactive oxygen species [196,197]. Pyocyanin was shown to reduce cAMP [186], which is required for yeast-to-hyphae transition [94,198]. The effect of phenazines, including pyocyanin, in disturbing *C. albicans* biofilm formation and hyphal growth, has also been evidenced [199,200]. Certain phenazines have even shown the potential to act synergistically in concert with several azole agents against fungal infection [201]. Along with secreting inhibitory molecules, *P. aeruginosa* also produces substances such as the proteolytic enzyme elastase LasB, which increases the virulence of *C. albicans* [176].

C. albicans secreted factors, such as farnesol, tyrosol, ethanol, oxylipins, and eicosanoids, have been shown to affect *P. aeruginosa* growth and biofilm. *C. albicans* generally uses farnesol to resist oxidative stress and to induce the generation of ROS, thus providing a competitive advantage over bacteria [202]. Farnesol has a deleterious effect on *P. aeruginosa*, modulating PQS-controlled virulence in a dose-dependent manner [203]. Farnesol is able to inhibit pyocyanin [203] and rhamnolipid-mediated swarming motility [204] through such modulation, while also inhibiting other virulence-related proteins. Tyrosol, another *C. albicans* AI, has been shown to inhibit the secretion of haemolysin and protease (toxins usually involved in tissue damage) by *P. aeruginosa* at high concentrations [205]. Ethanol is a common fermentation product produced by many bacteria and fungi known to influence *P. aeruginosa* in diverse polymicrobial settings. In the context of infections where *P. aeruginosa* coexists with *C. albicans*, exogenous fungal-produced ethanol may alter phenazine production and promote biofilm development on biotic and abiotic surfaces. In addition, ethanol enhances bacterial Pel matrix production and represses surface motility [206]. Its production is continuously stimulated by the enhanced *P. aeruginosa* biofilm formation and production of antifungal phenazines, in a positive feedback loop [207]. Eicosanoids (e.g. prostaglandin E2, PGE2) are often secreted by *Candida* spp., including *C. albicans.* Although the role of such fatty acid metabolites is still to be determined in the bacterial-fungal cross-talk, it has been suggested that they act as immunomodulatory mediators that affect the dynamics of mixed bacterial-fungal infections

and their outcomes [97,208]. Competition for iron is also one of the main antagonistic interactions occurring in *P. aeruginosa-C. albicans* infections. Enhanced secretion of *P. aeruginosa* siderophore pyoverdine in mixed-species biofilms leads to a decrease in iron availability in *C. albicans* and consequently the inhibition of yeast growth [209].

2.1.3.6 *P. aeruginosa* and *S. aureus* interplay

Many studies have investigated the inter-species interactions established between *P. aeruginosa* and *S. aureus* when co-cultured. The interplays between this consortium are particularly studied using CF models due to the high impact of these pathogens in this disease. Regarding *P. aeruginosa-S.aureus* interaction, it has been widely accepted that *P. aeruginosa* becomes dominant, outcompeting *S. aureus* when co-cultured [210]. However, despite many contradictory studies, there is growing evidence that both bacteria can co-exist [211–213]. In fact, it has been reported a range of interactions between *P. aeruginosa* and *S. aureus* from competitive to coexistence. It was also shown that the type of interaction between the two bacteria is dependent on the genetic background and the environmental adaptation they undergo during infection [214]. The interactions between *P. aeruginosa* and *S. aureus* are mainly mediated by the production of several bacterial compounds. Indeed, *P. aeruginosa* produces different virulence products that can affect the growth and/or induces *S. aureus* lysis. These include the QS signal 3-oxo-C12-HSL that can affect *S. aureus* growth in a dose-dependent manner [215]. Furthermore, it was also demonstrated that 2-heptyl-4-hydroxyquinoline N-oxide (HQNO), an exoproduct of the PQS system, interfere with the electron transport chain (cytochrome system) on *S. aureus* and induces SCV formation [210,216]. This frequent *S. aureus* phenotype displayed increased antibiotic resistance leading to chronic and persistent infections [217]. Interestingly, it was demonstrated that *S. aureus* cells and their supernatant can also induce a SCV phenotype and antibiotic tolerance in *P. aeruginosa* [218,219]. Additionally, it was also shown that *S. aureus* biofilm formation is mediated by the secondary metabolites HQNO and PQS produced by *P. aeruginosa* [218]. Conversely, *S. aureus* supernatant can either stimulate or inhibit *P. aeruginosa* biofilm formation in a strain-dependent manner [220]. Competitive *P. aeruginosa* also secreted HQNO and pyocyanin to inhibit *S. aureus* oxidative respiration, a strategy to limit nutritional competition. Moreover, *P. aeruginosa* produces the LasA protease that lyses *S. aureus* cells and cis-2-decenoic acid and rhamnolipids which promote biofilm dispersal of *S. aureus* [216]. *S. aureus* is at an even further disadvantage by inducing the production of *P. aeruginosa* virulence through the release of the peptidoglycan component N-acetyl glucosamine (GlcNAc) upon cell lysis [221,222]. While several experiments confirmed that *P. aeruginosa* outcompetes *S. aureus* during early co-culture, chronic

infections may promote co-existence. This suggests that chronicity modulates the virulence of *P. aeruginosa*, and consequently promotes the co-existence with *S. aureus*. Likewise, this indicates that *P. aeruginosa* does not completely eradicate *S. aureus* during co-infection and they somewhat reach a dynamic equilibrium that balances the presence of both. In fact, some investigations into coexisting mechanisms have been performed. In CF-adapted *P. aeruginosa* isolates, it was observed that *S. aureus* biofilm formation was not as affected as by an early clonal isolate [223]. 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* [213]. Another study into coexisting mechanisms revealed a downregulation of the QS system of *P. aeruginosa* during several passages in co-culture contributing to the coexistence of *S. aureus* over time. Interestingly, in the same study, it was found that *S. aureus* did not have any effect on *P. aeruginosa*, suggesting that *P. aeruginosa* is the main pathogen that controls the infection process [224].

2.1.3.7 *C. albicans* and *S. aureus* interplay

In different niches in the host, *C. albicans* coexists with numerous bacterial species, including *S. aureus.* The incidence of infections comprising these pathogens has steadily increased due to the growing use of implanted medical devices. Indeed, besides their co-existence in a variety of diseases, these pathogens are frequently found together in biofilms formed on medical implants [225,226]. It is well established that *C. albicans* and *S. aureus* display mostly a synergistic interaction with important clinical and therapeutic implications such as higher mortality rates [227–229]. Previous studies demonstrated that *S. aureus* exhibits high affinity to the *C. albicans* hyphal form as these species co-adhere and interact synergistically, forming a dense and complex biofilm, where the fungus creates a scaffold for the bacteria [230,231]. Recently, in a study that characterized *S. aureus* and *C. albicans* polymicrobial biofilms on different surfaces, including a catheter model, it was observed that the fungus was the most contributor to the whole density of the biofilm. Additionally, it was observed that *C. albicans* adhered preferentially to all the surfaces tested while *S. aureus* mostly adhered to *C. albicans* surface [231]. The attachment of bacteria to fungal hyphae may provide staphylococci with an invasion strategy because candidal hyphae can penetrate through epithelial layers. In a recent work, it was identified *C. albicans* Als1 and Als3 adhesins as the molecular players involved in the interaction with *S. aureus* and consequent bacterial dissemination [232]. Besides, it was demonstrated that the enhanced pathogenesis of *S. aureus* may not only be attributed to physical interactions with *C. albicans* but can also be due to the differential regulation of VF during polymicrobial growth [233]. Recently, it was shown that in co-infected catheters, *C. albicans*

induced the activation of the *S. aureus* biofilm formation network, increasing eDNA production and extracellular polysaccharide matrix which enhanced *S. aureus* tolerance to vancomycin [234]. On the other hand, it was also demonstrated that *C. albicans* also secreted matrix polysaccharides that similarly confer *S. aureus* with enhanced tolerance to vancomycin by impeding drug penetration through the biofilm [96]. Additionally, it was previously reported that the QS molecule farnesol, induces antimicrobial tolerance in *S. aureus* by inducing oxidative stress, triggering the activation of staphylococcal drug efflux pumps [235].

2.1.3.8 *C. albicans*, *S. aureus* and *P. aeruginosa* interplay

Studies of polymicrobial biofilms have gained special attention in the last years since it has been well established the polymicrobial nature of most biofilm-mediated infections. Likewise, efforts have been made to better mimic the real scenarios and respond to the *in vivo* faced challenges. However, regarding the available scientific literature, the study of polymicrobial biofilms encompassing triple species is still limited. Indeed, most of the current research addresses polymicrobial studies as dual-species consortia [92–97]. When going further with the increased complexity of the consortia, a further level of complexity will also be added to accurately characterize these communities, however, it is necessary this step forward. As previously discussed, several interactions have been reported regarding dual-species biofilms, especially those encompassing *P. aeruginosa* (i.e. *P. aeruginosa*-*C. albicans* and *P. aeruginosa*-*S. aureus*). Furthermore, it is known that *C. albicans*, *S. aureus,* and *P. aeruginosa* have been considered aetiological agents of VAP [76,87,236], and even, they have been co-isolated from ETT samples [61]. However, the interactions between these three species in the consortium are poorly reported. It is known that microbial interactions and adaptations behind mixed consortia are undoubtedly implicated in clinical and therapeutic outcomes. In the case of ETT, a wide range of strains has been observed and it has been shown that the clinical outcome generally became worse if *Pseudomonas* strains are present, being the key predictor of patient survival [87]. In this niche, these strains also appeared to outcompete strains of *Candida* and *Staphylococcus*. Conversely, *Staphylococci* and *Candida* are thriving well together. Furthermore, it was observed that biofilm quantity on the device or a general presence of bacteria on the tubes was not directly correlated with disease. Instead, it was observed that the composition of the consortium has a more important role in the clinical outcome [87]. These findings reinforce the necessity of *in vitro* testing taking into account these complexities during research and development. Additionally, having in mind the complexity of interactions that can occur during dual-species biofilms, as previously

discussed, this emphasizes the importance of studies using these more complex communities since more interactions may occur leading to altered and multifaceted outcomes.

2.1.4 Treatment of VAP

When there is a suspicion of VAP, but microbiologic results are not available, empirical antibiotics should be administered. However, inappropriate antibiotic therapies have been found to be associated with an increased mortality rate [237,238]. The selection of initial empiric antimicrobials may differ among different clinical scenarios [3,239]. The most relevant information to start VAP treatment, until there is no specific clinical information on the causative infection agent, is whether it comes from early- or late-onset VAP. The duration of MV before the onset of pneumonia is, therefore, an important determinant of the likely pathogen [4]. The accurate identification of aetiologic pathogens might improve therapy procedures and the control of the infection. It is also very important to know the whole clinical history of the patient with infection to choose the appropriate treatment. A critical question when initiating empirical antibiotic treatment is the possibility of the chosen(s) antibiotic(s) failing to target the microorganism causing the infection. Additionally, the characteristics intrinsic to biofilms and their polymicrobial nature can significantly alter the optimal treatment options leading to an unsuccessful treatment [240]. Thus, aggressive and extended antibiotic therapy regimens may be needed to control the infection. The major concern related to this approach is the development of resistance to antibiotics, one of the most important concerns in modern healthcare [241]. Studies have reported that inadequate and excessive use of antibiotics (essentially of broad-spectrum) therapy in ICU are associated with an increase in antibiotic resistance, biofilm formation and patient mortality [16,242,243]. The hurdles with diagnosing VAP coupled with the urgent need of getting an effective treatment to combat VAP make prevention of ETT microbial colonization the most reliable approach for staving off biofilm proliferation and preventing VAP occurrence. Some preventive measures to prevent VAP have included subglottic secretions aspiration [244,245], control of ETT cuff pressure [246], to prevent microaspiration, intensive oral care, maintenance of optimal positioning and mobility encouragement; and administration of prophylactic probiotics [3]. Despite the efforts made in clinical practices, until now there is no universal method able to prevent the occurrence of VAP. Likewise, their benefit in terms of diminishing the outcomes associated with VAP (e.g. MV duration, mortality, cost-benefit ratio) remains unclear, limiting its widespread use [247]. Therefore, it is urgent to find effective therapeutic strategies targeting this infection and having into consideration its particularities.

2.2 Antimicrobial surface modifications for ETTs

Given the recognized role of ETT in VAP development, a promising approach to tackle VAP establishment is to prevent its colonization through the modification of the surface materials used to design these medical devices [248]. ETT is usually designed using medical-grade plasticized PVC or PDMS whose intrinsic properties are prompt to the establishment of infection. Current research aims, therefore, to endow these materials with the ability to prevent microbial colonization [249]. In general, biomaterial surface modifications can incorporate passive or active approaches if the aim is to prevent microbial establishment and growth (anti-adhesive) or to possess a biocidal effect (antimicrobial releasing and contact-killing), respectively. Anti-adhesive or repelling strategies aim to prevent the initial attachment of the cells to the biomaterial surface. This strategy includes physical surface modifications or immobilization of molecules that can resist protein adsorption, being recognized as a non-cytotoxic approach. While these surfaces may mitigate microbial adhesion, they will not kill microorganisms that manage to adhere to them [250]. Moreover, their antimicrobial properties can be quickly masked by the adsorption of a conditioning film which may be different depending on the kind of environment the surface is exposed [251]. Antimicrobial-releasing is another type of functionality that can be added to biomaterials such as ETT and it involves the release of loaded antimicrobial compounds over time, which allows the killing of cells adhered to the surfaces as well in the bulk phase. This strategy presents some disadvantages as it becomes inactive over time and may induce the development of microbial resistance. Contact-killing surfaces aim to kill microorganisms when they are able to reach and adhere to the surface. In this last strategy, antimicrobial compounds are anchored to the material surface without release into the surroundings, therefore only killing microorganisms upon contact. This type of coating was developed in order to prevent the reservoir exhaustion problem of release-based materials. Furthermore, this strategy minimizes the risk of resistance development since the antimicrobial agents are permanently linked to the surfaces [252,253]. However, contact-killing surfaces present a disadvantage since they can be masked by biomolecules or residues of dead cells blocking their antimicrobial effect [254].

Several surface modifications intended to be applied to ETT have been explored, including passive, active and even combinatorial approaches (Table 2). Reports covering active approaches include metal-based coatings, impregnation with antiseptics/antibiotics, and coatings based on bio-inspired antimicrobials. Passive approaches that have been proposed for ETT modifications include hydrophobic surfaces, micropatterned surfaces, and even nano-rough surfaces. Combinatorial strategies also have been investigated to render ETT with both active and passive properties. Among the wide variety of antimicrobial approaches that have been reported, active materials are the ones that have attracted more attention,

especially metal-based coatings, in which silver has endured as the most well-documented metal. Active antimicrobial materials are particularly useful for ETT applications since they often promptly led to the elimination of microorganisms. Although less representative than active strategies, passive approaches encompassing nanomodified surfaces are the most reported ones. Furthermore, there is a notable growing interest by researchers in merging active antimicrobial and passive anti-biofouling strategies for ETT modification surfaces [67,255].

Of all the strategies reported, there is one that has gained greater emphasis due to the high number of studies in which it has been implicated since it was reported. Furthermore, it has given important clues regarding its potential to be used in the clinic. This technology, reported in 2015, called Bactiguard®' Infection Protection (BIP), is based on the coating of ETT with a sub-micron layer of a noble metal alloy composed of silver-gold-palladium. After their use on urinary tract and central venous catheters, their application on ETT showed potential due to its clinical tolerability, safety and performance during short-term intubation (3-8 h) in a small sample size (29 patients) [256]. Very recently, it was reported the results of a pilot study enrolling 300 ventilated patients from different ICU and hospital centres (n=9, Belgium), with the aim to test the impact of BIP ETT in reducing VAP incidence, antibiotic consumption days and tracheal colonization by pathogenic bacteria [257]. Results showed that the length of ICU stays, MV duration, tracheostomy rate and hospital mortality were not significantly different between BIP-coated ETT and non-coated ETT groups. However, this study still provided evidence to support the benefit of applying the mixed noble metal coating to prevent VAP, by reducing its incidence by 5.1%, the number of antibiotic days by 6.6% and tracheal pathogen colonization by 3.7% in the BIP-coated ETT compared with non-coated subglottic suctioning ETTs in the ICU patients. Additionally, another study that involved a shorter number of ICU patients showed a reduction of the ventilation days from 5.03 ± 1.88 in the control group to 3.2 ± 0.78 in the intervention (BIP-coated) group, in addition to a decreased incidence of VAP and ICU stays for ventilated patients [258]. The BIP ETT is currently seeking FDA approval.

Table 2. Antimicrobial surface modifications reported for ETT in last twenty years.

Type of designed material	Approach(es)	Source material	Antimicrobial compound/feature	Microorganism(s) tested	Mode of growth/ Testing model	Main Results/Remarks	Ref.
Active Materials	Metal-based coatings (Silver)	Commercially available ETT	Polyamide/AgNPs composite	· *A. bambini* (including carbapenem-resistant) · *C. albicans* · *Enterococcus faecalis* · *P. aeruginosa* (including carbapenem-resistant) · *K. pneumoniae* (including extended-spectrum b-lactamase producing strain) · *MRSA* · *S. aureus*	*In vitro*	· Coated ETT resulted in a significant difference in reducing both planktonic growth and microbial adhesion of single and mixed-species cultures, compared with uncoated ETT. · A time-kill assay demonstrated rapid bactericidal effects of the coating on bacterial growth and cell adhesion to ETT surface. · Biofilm formation by *P. aeruginosa* and *S. aureus* was inhibited (4-6 log) after 72 h. · Broad-spectrum activity against Gram-positive, and Gram-negative bacteria as well as *C. albicans*. · Prolonged antimicrobial activity (4 weeks).	[54]
		Commercially available ETT	Silver ions	· *P. aeruginosa*	*In vivo* (dog model)	· Delay (1.4 days) in bacterial colonization of ETT and reduced number of attached bacteria. · Reduced lung inflammation.	[259]
		PVC	Silver ions	· *P. aeruginosa*	*In vitro*	· Complete reduction of initial bacterial adhesion. · Reduced biofilm formation over a prolonged period of time (72 h).	[260]
		Commercially available ETT	Silver ions	· *P. aeruginosa*	*In vitro*	· 2-log inhibition of bacterial attachment to the ETT, after overnight challenge. · Zone of inhibition, indicative of silver release from the ETT.	[261]
		PVA hydrogel	AgNPs	· *P. aeruginosa* · *S. aureus*	*In vitro*	· Reduction of biofilm formation of both species, especially *P. aeruginosa* (about 1 log after 18 h). · No toxicity against lung epithelial cells.	[262]

Table 2. (continued)

Type of designed material	Approach(es)	Source material	Antimicrobial compound/feature	Microorganism(s) tested	Mode of growth/ Testing model	Main Results/Remarks	Ref.
		Commercially available ETT	AgNPs	· *P. aeruginosa* · *S. aureus*	*In vitro*	· Inhibition of adhesion of both species to ETT (99,9%). · No toxicity against human lung epithelial cells. · Better efficacy than a commercially available silver-coated ETT.	[263]
		Polyethylene ETT	Silver-silicon dioxide	· *E. coli*	*In vitro* *In vivo* (rabbit and golden hamster model)	· Antimicrobial activity reduced after contact (93,7%). · In rabbits, no pyrogenic effect was observed and haemolysis test showed biocompatibility with red blood cells. · In gold hamsters, no irritation of oral mucosa was found.	[264]
		Commercially available ETT	NMA of gold/silver/palladium (BIP ETT)	· *Enterococci spp.* · *Haemophilus parainfluenzae* · *Neisseria spp.* · *Staphylococci* · *Streptococcus*	*In vivo* (ICU patients)	· The BIP ETT was well tolerated and presented good clinical short-term performance (5 h), while presenting low level of bacterial colonization.	[256]
		Commercially available ETT	NMA of gold/silver/palladium (BIP ETT)	· Unknown	*In vivo* (ICU patients)	· No significant reduction in ICU length of stay, MV duration, tracheostomy rate and hospital mortality. · VAP incidence reduced by 5.1%. · Number of antibiotic days reduced by 6.6%. · Tracheal pathogen colonization reduced by 3.7% in the NMA-coated compared with non-coated subglottic suctioning ETTs.	[257]
		Commercially available ETT	Silver sulfadiazine	· *P. aeruginosa*	*In vitro* *In vivo* (sheep model)	· No bacterial adhesion up to 72 h on ETT. · In sheep, after 24 h of ventilation, no bacterial growth was detected on the ETT, ventilator tubing or lower respiratory tract.	[265]

Table 2. (continued)

Type of designed material	Approach(es)	Source material	Antimicrobial compound/feature	Microorganism(s) tested	Mode of growth/ Testing model	Main Results/Remarks	Ref.
		Commercially available ETT	NMA of gold/silver/palladium (BIP ETT)	· Unknown	*In vivo* (ICU patients)	· No significant reduction in ICU length of stay, MV duration, tracheostomy rate and hospital mortality. · VAP incidence reduced by 15.3%. · Mean MV duration reduced by 1.8 days.	[258]
		Commercially available ETT	NMA of gold/silver/palladium (BIP ETT)	· Unknown	*In vivo* (ICU patients)	· Reduction in the volume of secretions, incidence of purulent secretions, fever, leukocytosis, culture positive, and the onset of VAP symptoms. · Incidence of VAP reduced from 26% to 18% (intervention vs control groups).	[266]
		Commercially available ETT	Nanosilver (AgNPs)-polyurethane polymer	· *E. coli* · *S. aureus*	*In vivo* (rat model)	· Potent antimicrobial and antibiofilm proliferation properties. · Thickness and cell numbers of biofilm formed by catheterization 48 and 72 h after rat operation was significantly lower than that in the control group.	[267]
		Commercially available ETT	Silver ions	· *A. baumannii* · *Enterobacteriaceae* · *H. influenzae* · *P. aeruginosa* · *S. aureus* · *S. pneumoniae* · *Streptococcus viridans* group · Other Non-fermentative Gram-negative bacilli	*In vivo* (ICU patients)	· Reduced colonization rates by patient (29%). · Delayed colonization on the inner tube surface (1.8 vs. 3.2 days in control device) and on the tube · Reduced bacterial burden in tracheal aspirates.	[268]

Table 2. (continued)

Type of designed material	Approach(es)	Source material	Antimicrobial compound/feature	Microorganism(s) tested	Mode of growth/ Testing model	Main Results/Remarks	Ref.
	Metal-based coatings (ZnO)	Commercially available ETT	ZnO-NPs	· *S. aureus*	*In vitro*	· Reduction of biofilm formation (55%) after 72 h. · After 24 h challenge, membrane's compromise was observed after live/dead staining.	[269]
		PVC taken from a commercially available ETT	ZnO-NPs	· *S. aureus*	*In vitro*	· Reduction (87%) of biofilm formation after 24 h.	[270]
	Metal-based coatings (TiO_2)	Commercially available ETT	TiO_2-NPs (commercial and N-doped)	· *P. aeruginosa* · *S. aureus*	*In vitro*	· Inhibition of bacterial growth under visible fluorescent light, after 24 h. · N-doped NPs more efficient against *S. aureus*.	[271]
		PVC medical grade	Iodine-modified TiO2	· *E. coli*	*In vitro* *In vivo* (pig model)	· Photocatalytic activity able to kill bacteria under visible light irradiation after 30 min. · Decrease of bacterial attachment and biofilm formation after 72 h. · Reduced inflammation of tracheal and lung tissues in pigs, after 72 h ventilation.	[272]
	Metal-based coatings (Se)	PVC medical grade	SeNPs	· *S. aureus*	*In vitro*	· Bacterial colonization was reduced by 80%. · Better antimicrobial efficacy than a silver-coated ETT.	[273]
	Biocides impregnation	Commercially available ETT	Gardine and Gendine	· *A. baumannii* · *C. albicans* · *E. cloacae* · *K. pneumoniae* · MRSA · *P. aeruginosa*	*In vitro*	· Complete inhibition of the adhesion of all species after 24h. · Antimicrobial activity was prolonged for 2 weeks against MRSA. · Better antimicrobial efficacy than a silver-coated ETT.	[274]
		PVC	Hexetidine	· *P. aeruginosa* · *S. aureus*	*In vitro*	· Reduction of *P. aeruginosa* adhesion by 52%, after 7 days. · Reduction of S. aureus adhesion by 75%, after 7 days.	[275]

Table 2. (continued)

Type of designed material	Approach(es)	Source material	Antimicrobial compound/feature	Microorganism(s) tested	Mode of growth/ Testing model	Main Results/Remarks	Ref.
		Commercially available ETT	Gendine	· MRSA · *P. aeruginosa* · *E. coli* · *C. parapsilosis*	*In vitro*	· Antimicrobial activity evidenced by an inhibition zone up to 3 weeks against all species. · Complete inhibition of MRSA, *C. parapsilosis* and *E. coli* after 24 h. · No toxicity against mouse fibroblast cells.	[276]
		Commercially available ETT	Poly(lauryl acrylate)-based nanocapsules encapsulating clove oil or eugenol	· *K. pneumoniae* · MRSA	*In vitro*	· Both species were affected by the antibacterial loaded nanocapsules in a dose dependent manner, with >50% inhibition at a concentration of 0.625 mg mL−1 of eugenol or clove oil. · The observed surface-binding reduction of bacteria, biocompatibility and slow release of eugenol demonstrate the potential of this strategy for clinical applications.	[277]
		PVC	NO (SNAP as NO donor)	· *P. aeruginosa*	*In vitro*	· NO release over a 7-day period without altering the mechanical properties of the ETT. · Reduction of *P. aeruginosa* adhesion by 92.72 ± 0.97% (1.5 log) compared with the control ETT, after 24 h.	[278]
		Commercially available ETT	Styrylbenzene-based (BCP3)	· MSSA and MRSA · *P. aeruginosa*	*In vitro*	· A concentration-dependent release of BCP3 was observed for at least 31 days. · After 24 h, functionalized surfaces inhibited mainly the growth of MSSA (a maximum of 95%) and MRSA (a maximum of 80%) and to a smaller extent *P. aeruginosa* (maximum of 63%). · No cytotoxicity against L929 fibroblasts.	[279]

Table 2. (continued)

Type of designed material	Approach(es)	Source material	Antimicrobial compound/feature	Microorganism(s) tested	Mode of growth/ Testing model	Main Results/Remarks	Ref.
	Bio-inspired antimicrobials (AMPs)	Commercially available ETT	Lasioglossin-III	· *S. epidermidis* · *S. pneumoniae* · Pooled human microbiome samples	*In vitro*	· Prolonged, linear peptide release over 1 week. · Inhibition of planktonic bacterial growth and adhesion to tubes after 24 h. · No toxicity against laryngotracheal fibroblasts or lung epithelial cells.	[280]
	Bio-inspired antimicrobials (Ceragenins)	Commercially available ETT	Ceragenin CSA-131	· *C. albicans* · *C. auris* · *K. pneumoniae* · MRSA · *P. aeruginosa*	*In vitro* *In vivo* (porcine model)	· Prevention of the colonization of ETT and biofilm formation for several days (from 4 to 16 days), depending on the species tested. · Prevention of mixed biofilms formation of MRSA/ *P. aeruginosa* and *P. aeruginosa*/ *C. auris* for up to 2 and 3 days. · In pigs ventilated for 24 h, no abnormalities were found in the oropharyngeal area, trachea or lungs. · Histological analyses showed no inflammation and CSA-131 could not be detected in blood system.	[281]
	Bio-inspired antimicrobials (Surfactants)	Commercially available ETT	Cholesterol and lecithin (different ratios)	· *P. aeruginosa* · *S. aureus*	*In vitro*	· Reduction of bacterial adhesion by more than 90%, after 8h.	[282]
		Commercially available ETT	Sphingosine	· *A. baumannii* · *P. aeruginosa* · *S. aureus*	*In vitro* *In vivo* (mice)	· Prevention of biofilm formation of all bacterial species after 24 h *in vitro*. · Prevention of bacterial colonization in vivo. · Coatings stable with no side effects on tracheal epithelial cells or inflammation.	[283]
	Bio-inspired antimicrobials (Phages)	Commercially available ETT	Phage cocktail	· *P. aeruginosa*	*In vitro*	· Antimicrobial action was strain dependent. · Phages-coated tubes did not promote substantial changes in metabolic activity. · Limited anti-biofilm effect after testing the tubes in artificial sputum medium up to 168 h.	[284]

Table 2. (continued)

Type of designed material	Approach(es)	Source material	Antimicrobial compound/feature	Microorganism(s) tested	Mode of growth/ Testing model	Main Results/Remarks	Ref.
		Commercially available ETT	Phages ΦJHS-PA1139 and ΦSMK-PA1139	· MDR *P. aeruginosa*	*In vitro*	· Phage-coated ETT segments of 12 -mm in length inhibited bacterial colonization by 1.2 log up to 3.2 log comparing with non-coated segments following 6 h coating. · Phage treatment of ETT segments yielded 1.0 log up to 1.6 log reductions in MDR biofilms for phage ΦJHS and 1.6 log up to 2.4 log reductions for phage ΦSMK.	[285]
Passive Materials	Hydrophobic/ Hydrophilic surfaces	PVC	Oxygen plasma treatment	· *P. aeruginosa*	*In vitro*	· Reduction of the number of adhered bacteria by 70%.	[286]
		PVC	Superhydrophobic surfaces	· *P. aeruginosa*	*In vitro*	· No bacterial attachment in the first 6 h. · Reduction of biofilm formation after 24 h.	[287]
	Nanomodified surfaces	Commercially available ETT	Nanoscale surface features created by a fungal lipase	· *S. aureus*	*In vitro*	· Reduction of 1.5 log of the total number of adhered bacterial cells, after 24 h.	[288]
		Commercially available ETT	Nanoscale surface features created by a fungal lipase	· *P. aeruginosa*	*In vitro*	· Reduction of 2.7 log of the total number of adhered bacterial cells, after 24 h.	[289]
		Commercially available ETT	Nanoscale surface features created by a fungal lipase	· *P. aeruginosa*	*In vitro*	· Reduction of the number of bacterial cells adhered by 40%, after 24h.	[290]
	Micropatterned surfaces	Silicon wafers	Engineered micro-pattern of surfaces using the Sharklet pattern design	· *A. baumannii* · *E. coli* · *K. pneumoniae* · MRSA · *P. aeruginosa*	*In vitro*	· Reduction on the attachment of all species (from 1 to 3 log). · Reductions of MRSA and *P. aeruginosa* biofilms (67% and 58%).	[291]
		Thermoplastic polyurethane ETT	Engineered micro-pattern of surfaces using the Sharklet pattern design	· *P. aeruginosa*	*In vitro*	· Reduction of biofilm accumulation by 71%. · Reduction of artificial mucus accumulation.	[292]

Table 2. (continued)

Type of designed material	Approach(es)	Source material	Antimicrobial compound/feature	Microorganism(s) tested	Mode of growth/ Testing model	Main Results/Remarks	Ref.
Combinatorial Materials		Commercially available ETT	CS-AgNPs@PAAm-Gelatin composite (CS=chitosan; PAAm=polyacrylamide)	· *P. aeruginosa* · *S. aureus*	*In vitro* (broncho-lung model) *In vivo* (porcine MV model)	· Excellent antibacterial properties in both *in vitro* and *in vivo* models. · Antibiofouling property by decreasing lumen occlusion · Good biocompatibility (tested after 1, 4 and 7 days against fibroblasts). · Good stability, by keeping antimicrobial performance to up to 21 days. · Coated ETTs could decrease in vivo lumen occlusion by artificial mucus for up to 97%.	[67]
		Commercially available ETT	Chlorhexidine and silver carbonate	· *A. baumannii* · *Enterobacter aerogenes* · MRSA · *P. aeruginosa* · *S. aureus*	*In vitro*	· Reduction of 4-6 log of bacterial colonization of ETT, after 5 days, using an airway model.	[293]
		Commercially available ETT	Nanoscale surface features created by a fungal lipase and Fructose	· *S. aureus*	*In vitro*	· Fructose coating alone caused a reduction of 38% of bacterial adhesion. · Nanocoated surfaces alone caused a reduction of 45% of bacterial adhesion.	[294]
		Commercially available ETT	Copper and zeolite	· MDR *A. baumannii*	*In vitro*	· A maximum reduction of immobilized cells of 14%, after 24 h. · Antimicrobial efficacy was lower than the silver coating.	[295]
		Commercially available ETT	Antimicrobial lipid (octadecylamine), mucolytic (N-acetylcysteine), and antibiotics (doxycycline and levofloxacin)	No inoculation with exogenous bacteria	*In vivo* (pig model)	· Pigs ventilated with coated tubes were less hypoxic, had less bacterial colonization of the lungs and survived longer than pigs ventilated with uncoated tubes for 72 h.	[255]

Table 2. (continued)

Type of designed material	Approach(es)	Source material	Antimicrobial compound/feature	Microorganism(s) tested	Mode of growth/ Testing model	Main Results/Remarks	Ref.
		Commercially available ETT	Silver, zeolite and tyrosine	· MDR *A. baumannii*	*In vitro*	· Combination of tyrosine to natural zeolite with silver increased the antimicrobial activity against immobilized cells (from 2.5 to 2.8 log achieving almost 100% reduction) after a contact of 24 h. · Combination of both strategies caused a higher reduction of 60% of bacterial adhesion, evidence of a synergistic effect.	[296]
		Commercially available ETT	Hydrogels of hydroxyethyl methacrylate (HEMA):methacrylic acid (MAA) entrapped with nebulized gentamicin	· *P. aeruginosa* · *S. aureus*	*In vitro*	· No bacterial adherence occurred to gentamicin-containing HEMA:MAA copolymers. · 70:30 HEMA:MAA hydrogel exhibited >20 days persistence against *S. aureus* and *P. aeruginosa.* · Viable bacteria were not observed on the gentamicin-treated p(HEMA: MAA) copolymers, whereas growth was observed on gentamicin-treated p(HEMA).	[297]
		Commercially available ETT	Curcumin and photodynamic action	· *E. coli* · *P. aeruginosa* · *S. aureus*	*In vitro*	· After 24 h, the combined effect of curcumin-functionalized ETT and light application caused a reduction of about 95%, 72% and 73% on the adhesion and biofilm formation of *S. aureus*, *E. coli* and *P. aeruginosa*, respectively. · Curcumin-ETT remains active after six photodynamic sessions every 24h up to 6 days with 23.76% of microbial reduction.	[298]

Table 2. (continued)

Type of designed material	Approach(es)	Source material	Antimicrobial compound/feature	Microorganism(s) tested	Mode of growth/ Testing model	Main Results/Remarks	Ref.
		Commercially available ETT	Combinations of silver, TiO_2 and Degussa (metallic alloy)	· *P. aeruginosa* · *S. aureus*	*In vitro*	· Combination of TiO_2 and silver reduced *P. aeruginosa* growth after 24 h. · Combination of Degussa with TiO_2 reduced *P. aeruginosa* growth up to 48 h. · No reduction was found against *S. aureus* up to 5 days.	[299]

Legend:
AgNPs - Silver nanoparticles;
BIP - Bactiguard®'Infection Protection;
ETT - Endotracheal tube;
HEMA:MAA - hydroxyethyl methacrylate: methacrylic acid;
MDR - Multidrug resistant;
MRSA - methicilin-resistant *S. aureus*;
MSSA - methicilin-sensitive *S. aureus*;
NMA - Noble metal alloy;
NO - Nitric oxide;
NPs -nanoparticles;
PVA - polyvinyl alcohol;
PVC - polyvinyl chloride;
Se - Selenium;
SeNPs - Selenium nanoparticles;
SNAP - S -Nitroso-N - acetylpenicillamine;
TiO2-NPs - Titanium dioxide nanoparticles;
ZnO-NPs - zinc oxide nanoparticles.

2.2.1 Current challenges in the field of antimicrobial approaches for ETTs

Having in mind the key role of ETT in VAP establishment, the development of innovative antimicrobial materials to be used in ETT design could be a promising way to deal with this matter. Indeed, high developments in this field have been achieved with a significant increase in the proposed antimicrobial modifications for such devices during the last years (Table 2).

Although promising results have been accomplished in the current investigations, most of them are not being translated into clinical practices. In fact, silver-based materials for ETTs are the best-studied approaches with their progress reaching the market in the US (BIP ETT). However, its widespread use has been limited by the relatively high costs [300] in addition to some concerns that have been raised regarding their safety, antimicrobial activity and long-term stability [301].

Designing antimicrobial materials to be implemented in the design of medical devices requires safe-by-design criteria, including (but not limited to) antimicrobial performance, biocompatibility evaluation, the potential to induce antimicrobial resistance, and long-term stability [302,303]. The best way to achieve such outcomes is by integrating multidisciplinary expertise into the full developmental processes, thus ensuring that reliable and robust testing is efficiently employed [304]. A comprehensive analysis of the current proposed antimicrobial materials for ETTs has allowed to discern some gaps in this field. While encouraging results have been reported in the literature regarding ETT improvements, there are still important opportunities to be further explored, namely the development of coating strategies to target polymicrobial communities, especially composed of inter-kingdom biofilms (bacterial and fungal species). Indeed, the approaches reporting ETT antimicrobial coatings often neglected VAP polymicrobial aetiology despite it has been increasingly recognized. Even though some proposed strategies have increased the number of microorganisms tested, including more bacterial species and even fungal species [54], most of the studies only use bacteria and consider one single species to demonstrate its efficacy [272,278,284,285,289,305]. While it is widely recognized that VAP is frequently caused by biofilms, there is a limited number of studies examining the efficacy of ETT coatings against biofilms, and even fewer studies investigating their effectiveness against polymicrobial biofilms. The only study attending this issue was found in 2018, evaluating the performance of an immobilized ceragenin into a polyurethane-based hydrogel against polymicrobial, including bacterial-fungal biofilms [281]. However, the antimicrobial efficacy of the coated tubes was reduced in preventing mixed-species consortia for multiple days compared to their single-species biofilms. Also challenging is the shortage of *in vitro* models that can effectively predict long-term *in vivo* antibacterial and anti-biofilm performance. Indeed, some of the

current studies involving the design of anti-infective materials demonstrate antimicrobial activity for short-term applications but do not test their ability to prevent infections for longer applications [256,261,262,270,271]. In effect, it is not possible to predict its long-term performance based on the results obtained for their application in a short time. Actually, short-lived anti-biofilm coatings may serve as microbial reservoirs over the longer-term [306,307].

Overall, there are still several challenges that need to be addressed in the field of antimicrobial materials for ETTs. One major challenge is the development of materials effective against a broad range of microorganisms and mainly grown in biofilms. Another challenge is the durability of antimicrobial coatings, as they can wear off over time or become less effective. Additionally, the safety and biocompatibility of these materials must be carefully evaluated to ensure that they do not cause adverse reactions or compromise patient health. Finally, the cost-effectiveness of antimicrobial materials must be considered, as they can be more expensive than conventional materials. Addressing these challenges will be critical to the successful development and implementation of effective antimicrobial materials for ETTs.

2.3 Polydopamine: a bioinspired adhesive and surface modification approach

Lessons drawn from the observation of Nature can serve as an inspiration to engineer solutions to the challenges in many real situations. For instance, several examples of adhesive mechanisms used by living organisms have been a source of inspiration to develop new strategies for modifying surfaces. Marine mussels, in particular, have a remarkable ability to attach to wet surfaces in the sea. Their adhesion is fast, strong and tough. These adhesive mechanisms have been well characterized in the blue mussel *Mytilus edulis,* which anchors itself to substrates through acellular byssal threads composed of collagen and silk-like proteins as well as unique adhesive proteins (Figure 4A). These proteins, called mussel foot proteins (Mfps), attracted great interest for having high contents of catechol (3,4-dihydroxyphenylalanine, DOPA, a hydroxylated version of the natural amino acid tyrosine) in combination with primary and secondary amines (lysine, Lys, and histidine), (Figure 4D) motifs that contribute to the robust interfacial adhesion [308]. Assuming that the coexistence of DOPA and Lys groups may be essential for successful adhesion to a wide range of materials, in 2007, Messersmith and co-workers, identified a small molecule that combines both functionalities, dopamine (Figure 4C and Figure 4E) [309]. They reported that dopamine, as a simple structural mimic of Mefp-5, under oxidative and alkaline conditions, polymerizes and leads to the deposition of a thin adhesive polymer film called pDA on different material surfaces. In spite of the widespread use of this approach, there is currently no consensus on the pDA formation mechanism and therefore it remains under investigation. It is well accepted that the first steps of pDA

formation include auto-oxidation of dopamine, originating dopamine-quinone, which cyclizes to form dihydroxy-indole (DHI), a crucial precursor to pDA (Figure 4F). Several hypotheses have been proposed for the structure of pDA, all falling into two main categories. The most common is that pDA is a supramolecular aggregate of monomeric and/or oligomeric species, for example consisting of dopamine-quinone, DHI, dopamine eumelanin-like derivatives that are held together through relatively weak interactions such as hydrogen bonding, charge transfer, π-π stacking and π-cation assembly [310,311]. It has also been proposed that pDA has a polymeric nature formed by covalent coupling of the oxidized and cyclized dopamine monomers via aryl-aryl linkages [312]. Another study showed that pDA films contain high molecular weight polymer chains with covalently connected subunits [313]. These findings represent the first direct evidence for the polymeric nature of pDA and provide a foundation upon which to understand and tailor its physicochemical properties.

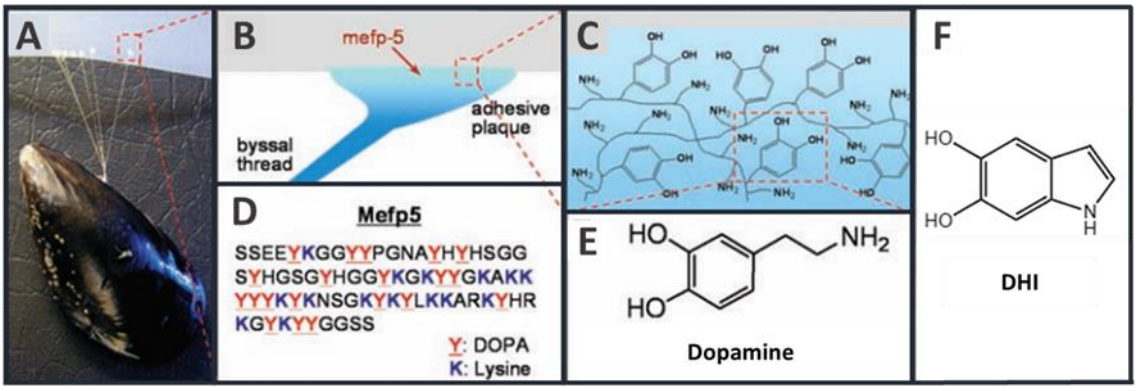

Figure 4. (A and B) Photograph of a mussel and schematic illustration of the interfacial location of Mefp-5. (C and D) Schematic representations of the molecular structure of Mefp-5. (E) Schematic representation of the molecular structure of dopamine. (F) Schematic representation of the molecular structure of DHI. [Adapted from [309]].

Polydopamine is composed of dense hyperbranched oligomers that coalesce into colloidal particles in the bulk solution and conformably coat interfaces including substrates immersed in a buffer. This approach is compatible with a wide number of substrate materials including metals, polymers and inorganic materials. The resulting pDA coating contains functional groups that can afterwards act as a versatile platform for post-modification, by immobilization of compounds via Schiff-base reactions (amine-containing molecules) or Michael-type reactions (amine- and thiol-containing molecules) [314]. The properties of the secondary coating can be tailored to the desired application.

Besides this strategy, recognized as a 2-step strategy, that was first reported, pDA chemistry has also been explored in a single step (1-step) (Figure 5), in which self-polymerization of dopamine occurs in the presence of compounds to be immobilized, leading to a homogeneous mixing of the compounds

throughout the layer of pDA [315]. The main advantage of this approach is the fact that it is not limited to molecules presenting either amine or thiol groups and the fact that it confers a more homogeneous coating with less roughness [316]. Both strategies have been investigated with different materials since their report. It has been successfully applied for the immobilization of antifungals, antimicrobial peptides (AMPs) and biocides to render the surfaces with antimicrobial properties [316–318].

The widespread adoption of pDA surface chemistry is mainly due to its process simplicity, low cost, material independence, strong reactivity for secondary functionalization and positive interactions with mammalian cells [319].

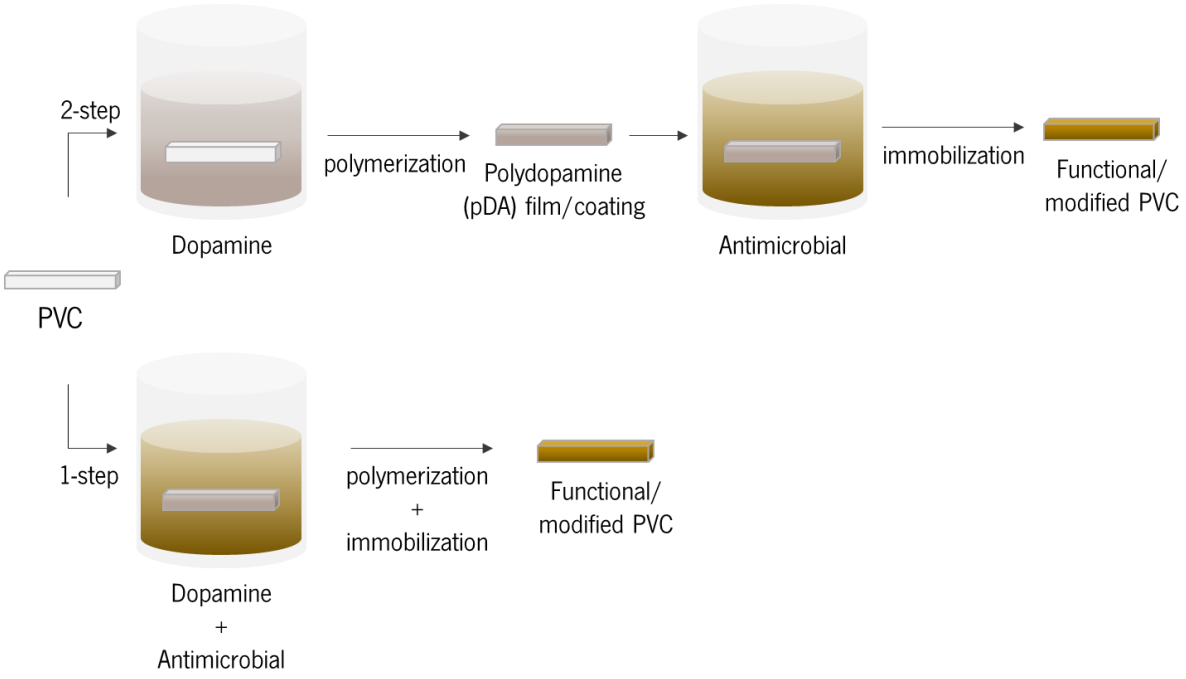

Figure 5. Schematic illustration of the main steps to obtain a functional surface using a pDA-based strategy. In 2-step approach, PVC is firstly immersed in an alkaline solution (pH 8.5) of dopamine resulting in its polymerization and deposition of an adhesive film called pDA. Afterwards, antimicrobial immobilization occurs by immersion of pdA-coated PVC in a solution containing the antimicrobial resulting in a functional PVC. In 1-step approach, modified PVC is obtained by immersion of PVC in a solution containing dopamine combined with the antimicrobial(s).

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CHAPTER 3

Unravelling *Pseudomonas aeruginosa* and *Candida albicans* communication in coinfection scenarios: insights through network analysis

Modern medicine is currently facing huge setbacks concerning infection therapeutics as microorganisms are consistently knocking down every antimicrobial wall set before them. The situation becomes more worrying when taking into account that, in both environmental and disease scenarios, microorganisms present themselves as biofilm communities that are often polymicrobial. This comprises a competitive advantage, with interactions between different species altering host responses, antimicrobial effectiveness, microbial pathogenesis and virulence, usually augmenting the severity of the infection and contributing to the recalcitrance towards conventional therapy. *P. aeruginosa* and *C. albicans* are two opportunistic pathogens often co-isolated from infections, such as VAP. Despite the billions of years of co-existence, this pair of microorganisms is a great example of how little is known about cross-kingdom interactions, particularly within the context of coinfections. Given the described scenario, this study aimed to collect, curate, and analyse all published experimental information on the molecular basis of *P. aeruginosa* and *C. albicans* interactions in biofilms, in order to shed light into key mechanisms that may affect infection prognosis, increasing this area of knowledge. Publications were optimally retrieved from PubMed and Web of Science and classified as to their relevance. Data was then systematically and manually curated, analysed, and further reconstructed as networks. All annotated data was made publicly available at www.ceb.uminho.pt/ISCTD, a database already containing similar data for *P. aeruginosa* and *S. aureus* communication.

3.1 Brief Introduction

In actual scenarios, including natural, clinical, and industrial settings, microorganisms team up to survive the hostile environmental conditions they are continuously exposed to by building complex communities called biofilms. Their organization in these surface-attached microbial communities, embedded in a self-produced matrix, facilitates the intercellular exchange of metabolites, genetic material, and signalling molecules [1]. Once established, biofilms are less susceptible to predatory action and antimicrobial agents compared to their planktonic counterparts [2]. In addition to this advantageous form of microbial life, one type of microorganism often exists in close association with other microbial species. Interactions between different microorganisms may provide an advantage to one party (antagonism) or both parties (mutualism) [3]. Although most natural biofilms are polymicrobial, most of our current knowledge about these communities comes from the study of single-species biofilms, using model bacteria [4,5]. Recognition of the real impact of polymicrobial biofilms has triggered a recent interest towards the study of multi-species communities to learn more about their behaviour [6].

This is particularly true for the clinical field, since the human body is well known to be colonized by trillions of microorganisms pertaining to more than 10000 different species [7–9]. Examples of human polymicrobial diseases include VAP, cystic fibrosis (CF) lung infection, otitis media, biomaterial-associated infections, urinary tract infections, periodontitis, wound infections, and diabetic ulcers [6,10]. The interactions between the microorganisms comprising polymicrobial communities can exacerbate the severity of infections, compromising their antimicrobial treatments [11,12]. Among the great panoply of interactions found within the context of human infections, communication between fungi and bacteria has been the focus of great interest in the last years [13,14]. *C. albicans* and *P. aeruginosa* comprise an example of a clinically relevant fungal-bacterial consortium commonly found in the respiratory tract and skin [3].

Given the abovementioned information, the purpose of this study was to gather, curate and analyse all published experimental data regarding the molecular basis of *P. aeruginosa* and *C. albicans* interactions, with emphasis on biofilms. The objective is to provide insights into the key communication mechanisms and molecular players affecting *P. aeruginosa* and *C. albicans* coexistence, hopefully facilitating the current understanding of inter-species and inter-kingdom interplays, and ultimately guiding the design for novel and effective tailored therapies.

3.2 Material and Methods

3.2.1 Information Retrieval

Medline (PubMed) database [15] and Web of Science [16] were searched in order to extract information from the scientific literature related to *P. aeruginosa* and *C. albicans* interactions. The scope of the search was optimized so that relevant papers were not overlooked but not so broad that irrelevant papers made the curation effort infeasible. Documents were narrowed down to those whose title, abstract or MeSH (Medical Subject Headings) terms mentioned "*Pseudomonas aeruginosa*", "*Candida albicans*" and, at least, one term related to interaction (e.g. "co-cultivation", "mixed biofilms", "double species biofilms", "multispecies", "polymicrobial", "consortia", "communication", "quorum-sensing"). The query was not specific for VAP, or other infections, to ensure that sufficient data was extracted for network reconstruction and analysis, not excluding a priori any information gathered from other diseases and non-disease scenarios that could possibly be extrapolated or give hints for VAP research.

3.2.2 Selection of Studies and Data Extraction

Articles searched on PubMed and Web of Science were classified as relevant or irrelevant by analysing each title and abstract for its suitability for the subject of *P. aeruginosa*-*C. albicans* interactions. When necessary, a full-text article analysis was performed to confirm its relevance. Irrelevant articles were deemed as those out of scope, but also reviews, as they would output duplicated data, and non-English written articles, as they would not be possible to annotate. Out of scope articles were those that did not include testing of the two species in combination or of the effect of exoproducts of one species on the other. Many articles were deemed irrelevant as specific terms pertaining to polymicrobial biofilms included in the query (see section 2.1. Information Retrieval) were only mentioned as part of the introduction or contextualization.

As for relevant publications, the full-texts were curated and important information was annotated, namely the interactions between the two microorganisms and, where possible, the molecular entities behind such phenomenon. Accessory information was also annotated, such as strains, mode of growth and experimental methods. The entities responsible for the annotated interactions and the interaction outcomes were classified into categories, as performed previously [17]. Briefly, interactions were classified into up- or downregulation (for gene and protein targets), stimulation or inhibition (for VM, VF, AI, and other targets), and indifference (for all target entity categories).

3.2.3 Network Reconstruction and Data Deposition

Data on *P. aeruginosa*-*C. albican*s interactions was reconstructed as networks using Cytoscape [18]. Different node colours and shapes were used to depict distinct source organisms and entity categories, respectively. All annotated data was made publicly available in the Inter Species CrossTalk Database (ISCTD) (www.ceb.uminho.pt/ISCTD) [17], which was previously built to deposit data on communication between microorganisms and was first loaded with information on the interactions between *P. aeruginosa* and *Staphylococcus aureus* obtained in a similar fashion as the present work.

3.3 Results and Discussion

3.3.1 Exploring the ISCTD

The ISCTD (www.ceb.uminho.pt/ISCTD) [17] now provides public access to the annotated information about *P. aeruginosa* and *C. albicans* interactions. Users can specify search preferences by selecting the direction of the interaction (*P. aeruginosa* > *C. albicans* or *C. albicans* > *P. aeruginosa*) and, optionally, the source and target entity categories. The respective data selection is shown in a table as an organized view of all interaction details, such as strains, modes of growth, experimental methods, and observations made by the expert curators. Links to PubMed records are always included so users can easily access the original publications. Users can further explore and narrow down the selected data by searching specific terms within the table, such as a specific mode of growth (e.g. “biofilm”), a VF (e.g. “pyoverdine”), or a gene (e.g. "*lasA*"). An example of a search focused on the effect of *P. aeruginosa* on *C. albicans*' genes in biofilms is illustrated in Figure 6

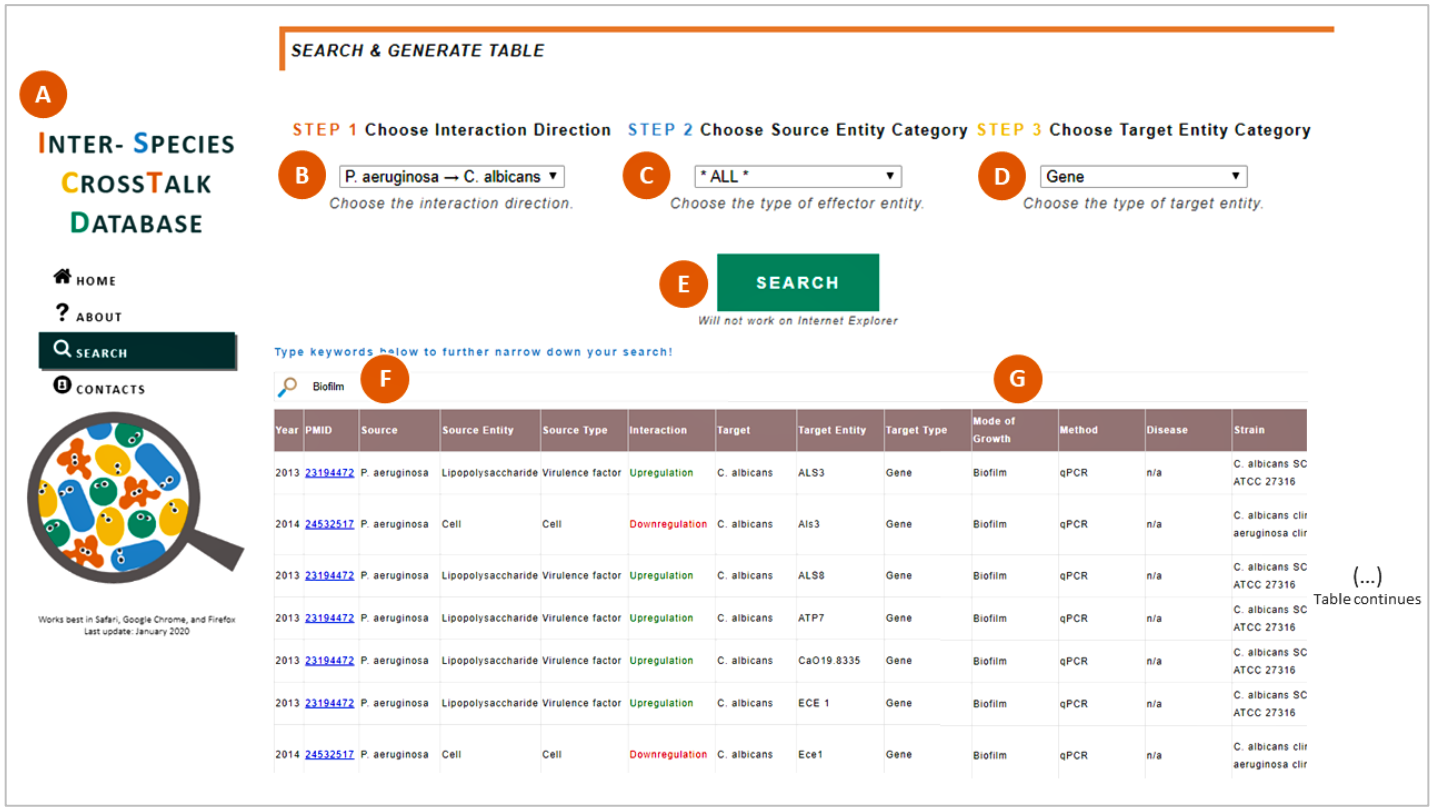

Figure 6. Searching within the ISCTD. (A) Navigation panel that allows users to jump within the webpage, including to the "Search" section. (B) Selection of the interaction direction of interest, here exemplified by the effect of *P. aeruginosa* on *C. albicans*. (C) Selection of the source entity category, which can be left unspecified by selecting the option "All". (D) Selection of the target entity category, here exemplified by "gene". (E) When clicking the "Search" button, a table is generated containing all the information annotated by the expert curators and sifted through the user's selection in (B–D). (F) Users can type keywords to further specify their search, here exemplified by "biofilm". (G) Table is narrowed down to show only interactions observed in biofilms.

3.3.2 Network Overview

The elaboration of the *P. aeruginosa*-*C. albicans* network comprised the analysis of 164 PubMed and 215 Web of Science documents (dating from June 1975 to July 2020), of which 60 were exclusive to the first and 108 to the latter. A total of 29 documents (dating from December 2004 until May 2020) were considered relevant. The annotation of these relevant studies outputted a total of 641 interactions between the two pathogens, in which 54% were related to the effect of *P. aeruginosa* on *C. albicans* and 46% to the opposite effect.

The most annotated type of interaction illustrating the effect of *P. aeruginosa* on *C. albicans* was upregulation followed by downregulation (Figure 7A), which reflects the great number of proteins and genes that are usually analysed in studies where methods such as Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) and RNA sequencing are used. These high-throughput studies also explain the great number of interactions annotated for proteins and genes as targets (Figure 7C). However, this does not translate to a majority of papers using these techniques. In fact, only 45% of annotated papers studying the effect of *P. aeruginosa* on *C. albicans* analysed protein or gene expression, and only 41% used these types of high-throughput methods. Regarding the source type, the effect of most interactions was reported for "cell" as the source entity (Figure 7B), which means that the molecular effector entity of the interaction was not studied in detail.

As for the effect of *C. albicans* on *P. aeruginosa*, the trend is very similar to the opposite direction. The most annotated interactions were downregulation and upregulation (Figure 8A), although a preponderance of downregulation is unveiled here, while the most annotated source and target types were cell and protein, respectively (Figure 8B/C), by the same reasons previously mentioned.

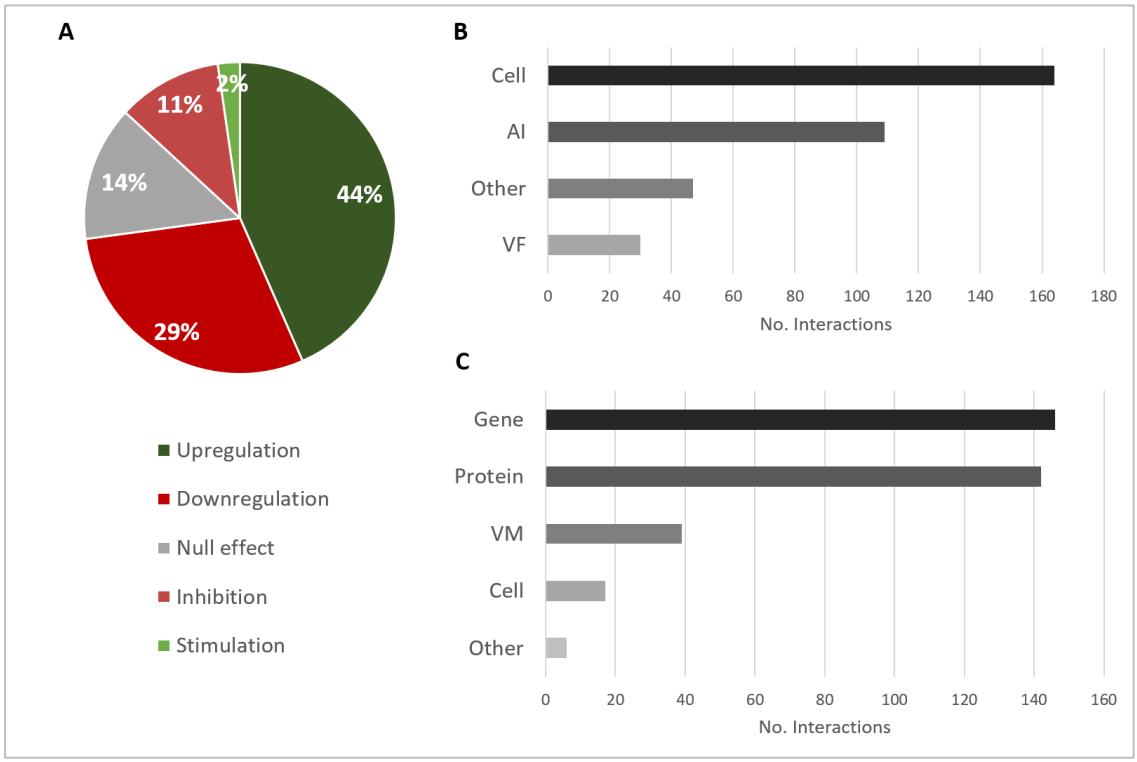

Figure 7. Overview of the types of interactions and entities annotated for the effects of *P. aeruginosa* on *C. albicans*. (A) Proportional data on the types of interactions; (B) Total number of interactions annotated for each source category; (C) Total number of interactions annotated for each target category. AI, autoinducer; VM, virulence mechanism; VF, virulence factor.

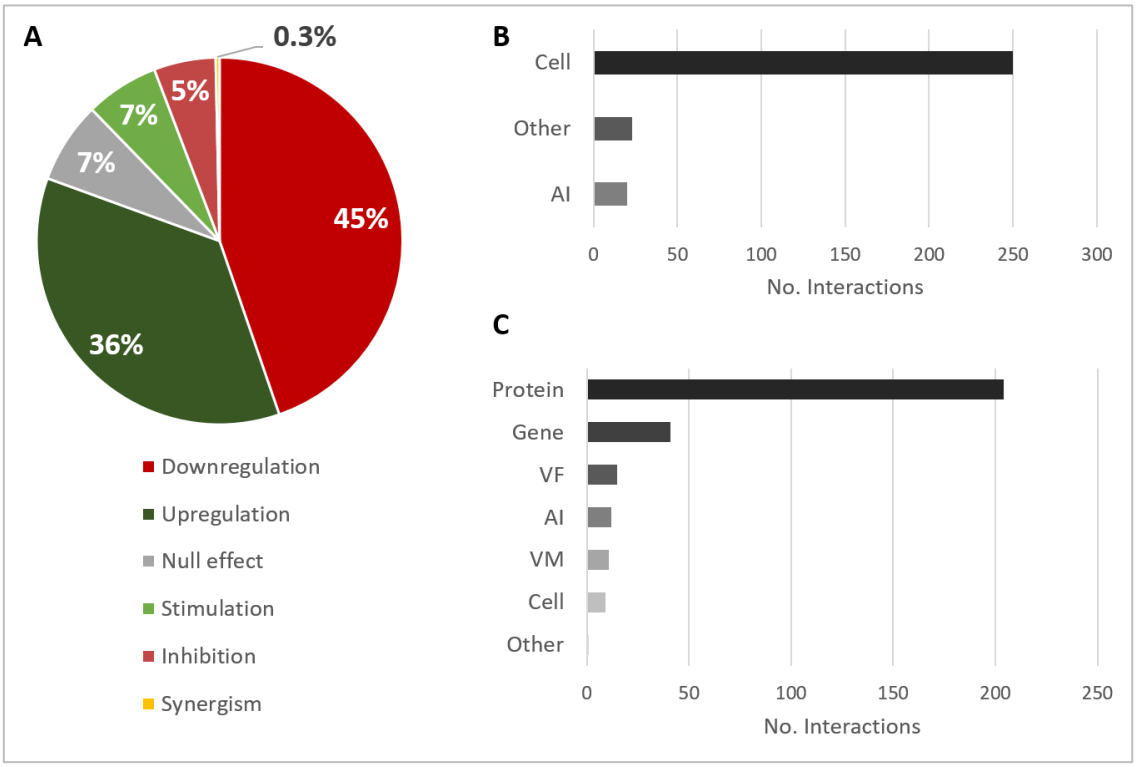

Figure 8. Overview of the types of interactions and entities annotated for the effects of *C. albicans* on *P. aeruginosa*. (A) Proportional data on the types of interactions; (B) Total number of interactions annotated for each source category; (C) Total number of interactions annotated for each target category. Legend: AI, autoinducer; VM, virulence mechanism; VF, virulence factor.

3.3.3 Effect of QS Molecular Players on *P. aeruginosa*-*C. albicans* Interactions

Four different AI from *P. aeruginosa* were annotated for their effect on *C. albicans*. For instance, 3-oxo-C12-HSL was annotated as inhibiting the hyphal form of the fungus [19,20], the secondary messenger cAMP [19], the cAMP synthesis-related enzyme Cyr1 [20], and biofilm formation, while stimulating *C. albicans* adhesion to mammalian cells [21] (Table 3). As previously mentioned, the morphogenesis of *C. albicans* is largely regulated through the cAMP/PKA pathway, meaning that 3-oxo-C12-HSL is able to inhibit yeast to hyphae transition by affecting it. Additionally, 3-oxo-C12-HSL was also shown to downregulate the expression of *ECE1*, *HWP1*, and *SAP5*, all hyphae-specific genes (Table 3) [20]. As stated, hyphae development by *C. albicans* is one of its most important VM, having an important role in the establishment of the infection process, which is hindered by 3-oxo-C12-HSL. Although this effect may be apparently detrimental to the infection, the reversion or maintenance of the fungus in the yeast form may enhance its dissemination capabilities and spread the infection to other areas of the host. However,

it has also been shown that the presence of 3-oxo-C12-HSL, at sub-growth and sub-hyphal inhibitory concentrations, favourably affects *C. albicans* when challenged with fluconazole by upregulation of genes known to be associated with antimicrobial resistance (e.g. *GAL102*, *MDR2*, *INO2*, *ADA2*) [22] (Table 3). Concerning other AI, C4-HSL, PQS, and its precursor, HHQ, were also annotated as interfering with biofilm formation on inert surfaces but to stimulate *C. albicans* adhesion to a cellular substratum [21] (Table 3). This switch from antagonistic to synergistic interactions is dependent on the host, which highlights the need for better understanding the role played by the latter in polymicrobial infections. PQS and HHQ have multifunctional roles in QS and iron uptake, playing a key role in coordinating virulence in *P. aeruginosa* [23]. Although the annotated effect of these molecules on *C. albicans* hyphae was null [24,25], they seem to interfere with biofilm formation (Table 3) [21,24]. Overall, there is a clear negative effect on the phenotypic switching and biofilm formation of *C. albicans* mediated through QS of *P. aeruginosa* when the host is not considered.

Regarding the two annotated AI of *C. albicans*, farnesol and tyrosol, the former seems to affect the production of AI from *P. aeruginosa*, namely PQS [26,27], HHQ, and C4-HSL [27] (Table 3). Farnesol is also involved in the inhibition of different VM and VF of this bacterium, such as adhesion, swarming motility, and haemolysin production [26,28,29] (Table 3). Distinct farnesol effects were also observed between planktonic and biofilm growth for pyocyanin production (Table 3). Tyrosol was also annotated as affecting the production of VF of *P. aeruginosa*, namely the inhibition of the production of haemolysin and proteases [29] (Table 3). These two exoenzymes greatly influence the pathogenicity of *P. aeruginosa*, contributing to infection establishment through elastin degradation and vascular permeability, respectively [30].

Overall, it is safe to say that QS in both pathogens is a key factor mediating their antagonistic relationship, with their capacity for infection establishment being one of their most affected traits.

Table 3. Effect of AI on *P. aeruginosa*-*C. albicans* interactions.

Source	AI	Interaction	Target	Ref.
P. aeruginosa	3-oxo-C12-HSL	Inhibition	Hyphae(p), cAMP(p), Cyr1(p), Biofilm(b)	[19–21]
		Stimulation	Adhesion(b)	[21]
		Downregulation	*ECE1*(p), *HWP1*(p), *SAP5*(p), *CDR1*(b), *MDR1*(b), *C6_02100W_A*(b), *CRH11*(b), *FBA1*(b), *IFR2*(b), *MNN12*(b), *PHHB*(b), *SOD5*(b), *TEL1*(b)	[19,20,22]
		Upregulation	*CDR2*(b), *AAF1*(b), *ADA2*(b), *ADH3*(b), *ALK2*(b), *ALS7*(b), *ARD*(b), *ATX1*(b), *AXL1*(b), *BCR1*(b), *C1_01130W_A*(b), *C1_01510W_A*(b), *C1_03990W_A*(b), *C1_04010C_A*(b), *C1_09210C_A*(b), *C2_01750C_A*(b), *C2_02920W_A*(b), *C2_03690C_A*(b), *C2_09880C_A*(b), *C3_00360W_A*(b), *C3_02630C_A*(b), *C3_03460C_A*(b), *C3_04330C_A*(b), *C3_05450C_A*(b), *C4_02740W_A*(b), *C4_03020W_A*(b), *C5_04030W_A*(b), *C6_00110C_A*(b), *C6_00290W_A*(b), *C6_00920W_A*(b), *C7_00770W_A*(b), *C7_04090C_A*(b), *CDR4*(b), *CR_00040C_A*(b), *CR_05860W_A*(b), *CR_06140W_A*(b), *CR_06960W_A*(b), *CR_07480W_A*(b), *CR_09100C_A*(b), *CR_10230W_A*(b), *CRZ2*(b), *CSH1*(b), *CUP9*(b), *EFG1*(b), *ERO1*(b), *GAL102*(b), *GOR1*(b), *GRP2*(b), *HAL9*(b), *HSP104*(b), *HSP78*(b), *HSP90*(b), *IFD6*(b), *INO2*(b), *ISA1*(b), *LPG20*(b), *MHP1*(b), *MOH1*(b), *NRG1*(b), *OPT3*(b), *PGA52*(b), *RFG1*(b), *RGS2*(b), *RME1*(b), *RPN4*(b), *SIS1*(b), *SNQ2*(b), *SRR1*(b), *STI1*(b), *UGT51C1*(b), *WOR4*(b), *YIM1*(b), *YOR1*(b), *ZCF1*(b), *ZCF39*(b)	[22]
	C4-HSL	Inhibition	Biofilm(b)	[21]
		Stimulation	Adhesion(b)	
	HHQ	Inhibition	Biofilm(b)	[21,24]
		Stimulation	Adhesion(b)	[21]
		Null effect	Hyphae(p), Adhesion(b)	[24]
	PQS	Inhibition	Biofilm(b)	[21]
		Stimulation	Adhesion(b)	
		Null effect	Hyphae(p)	[24,25]

Table 3. (continued)

Source	AI	Interaction	Target	Ref.
C. albicans	Farnesol	Inhibition	PQS(p), Adhesion(b), Swarming motility(p), Pyocyanin(p), Haemolysin(p), Cell(p)	[26,28,29]
		Downregulation	*pqsA*(p), *pqsR*(b)	[26]
		Null effect	Cell(p);(v), *pqsR*(b), Proteases(p)	[26,29,31]
		Upregulation	*pqsH*(b)	[27]
		Stimulation	HHQ(b), PQS(b), C4-HSL(b), Pyocyanin(b)	
	Tyrosol	Inhibition	Haemolysin(p), Proteases(p), Cell(p)	[29]

Legend: (p) planktonic; (b) biofilm; (v) *in vivo*.

3.3.4 Interaction Effects on Virulence Mechanisms and Virulence Factors

The influence of inter-species interactions on the expression of VM and VF can greatly affect the severity of the polymicrobial infection. Given their importance, two networks were constructed regarding the effects of one species on the virulence of the other. Concerning the effect of *P. aeruginosa* on *C. albicans*, hyphal development was the most annotated VM (Figure 9), for which the majority of interactions (75%) were inhibitory. Concerning the most reported source entities affecting hyphae, most annotated interactions reported effects of bacteria as a whole (annotated as "cell"), meaning that no molecular entity was identified/tested. LPS, rhamnolipids, and 3-oxo-C12-HSL were also annotated as inhibiting hyphal growth [19,25,32], while HHQ and PQS had no effect [24,25]. Biofilm formation by *C. albicans* was the second most annotated affected VM as a result of *P. aeruginosa* interaction. In this case, almost all interactions were inhibitory (93%) and caused by different source entities (Figure 9). No VF were annotated for *C. albicans*.

Regarding the effect of *C. albicans* on *P. aeruginosa*, swarming motility, adhesion, and biofilm formation were the most annotated VM (Figure 9). Swarming motility is inhibited in the presence of *C. albicans* due to farnesol [28] and ethanol [33]. The adhesion capability of *P. aeruginosa* was annotated as inhibited by farnesol [28] and stimulated by ethanol [33]. In the case of biofilm formation, 50% of interactions were of stimulation and the other 50% had a null effect. Concerning the source entities, these distinct biofilm effects were both annotated for the entity "cell", while only stimulation was annotated for ethanol (Figure 9).

Pyocyanin production by *P. aeruginosa* was the most annotated VF (Figure 9) and the effect of *C. albicans* on this molecule apparently depends on the mode of growth, with stimulation in biofilm growth and inhibition in planktonic growth. These differences are further explored in a later section. Other annotated VF include pyoverdine, proteases, rhamnolipids, and haemolysin (Figure 9). All interactions annotated for pyoverdine and rhamnolipids were stimulatory, with "cell" as the source entity and biofilm as the mode of growth for the first and both planktonic and biofilm as modes of growth for the latter. Both AI of *C. albicans*, farnesol and tyrosol, were annotated as inhibiting haemolysin in *P. aeruginosa* in planktonic cultures [29]. Regarding the production of proteases, tyrosol inhibited these enzymes, while a null effect was annotated for farnesol, in the planktonic mode of growth [29].

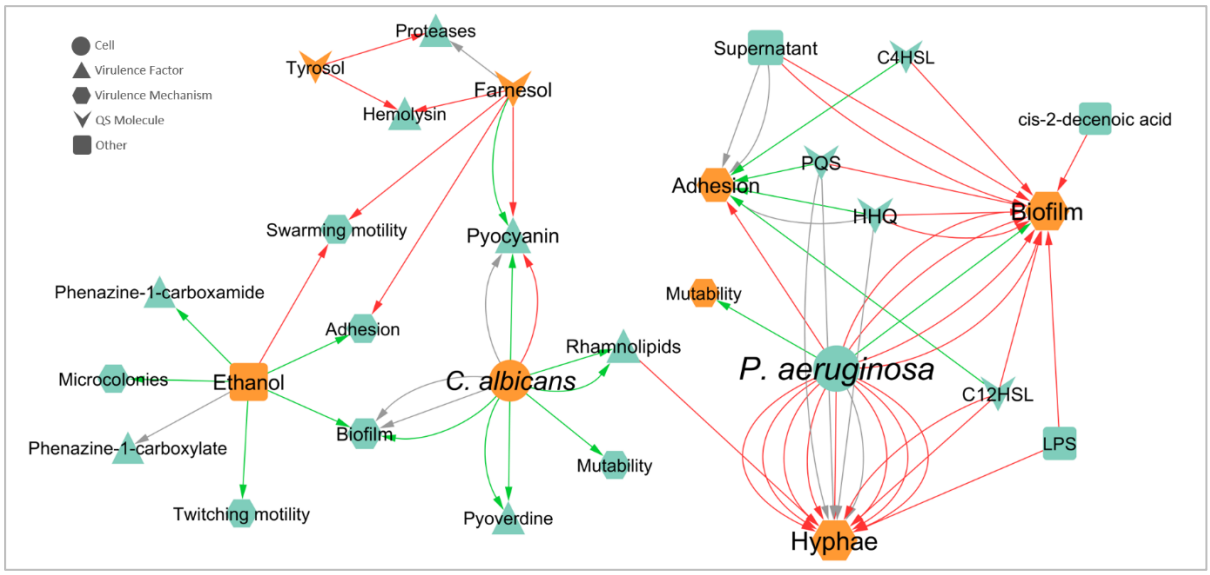

Figure 9. Network of the effects of *P. aeruginosa*-*C. albicans* interactions on VF and VM. Teal nodes, *P. aeruginosa*; orange nodes, *C. albicans*; green arrows, stimulation; grey arrows, null effect; red arrows: inhibition; node and node label sizes are directly proportional to the number of related (outward and inward) edges (interactions).

3.3.5 Interaction Effects on Gene and Protein Expression

Concerning the expression of genes and proteins as a result of microbial interaction, it was possible to annotate a total of 110 distinct genes differentially expressed in *C. albicans* due to the presence of *P. aeruginosa*, of which 83 were upregulated, 21 were downregulated, and 6 were both up and downregulated (Figure 10). These findings are correlated with the affected VM previously mentioned, especially hyphal growth. For instance, *HWP1* and *ECE1*, along with *ALS3*, all hyphae-specific genes, were downregulated in the presence of 3-oxo-C12-HSL [20] and of *P. aeruginosa* "cell" [34] and its supernatant [35]. These results are in accordance with the well-known inhibitory effect annotated for hyphal growth. However, there are some cases where some contradictory effects were annotated for other source entities, namely LPS upregulation of the previously mentioned genes [32]. This and other contrasting annotations are discussed in the next section.

With concern to protein expression, *C. albicans* differentially expressed 117 proteins, of which 59 were annotated as upregulated and 69 as downregulated (Figure 11). Some proteins are reported in more than one paper or even with different interaction types in the same paper; hence, there is a greater number of annotated interactions (Figure 11) in relation to the total number of different annotated proteins. Almost all interactions with proteins had "cell" as the source entity. Protein expression was also shown to vary depending on the time of maturation of the dual-species biofilm. For instance, proteins related with

adhesion and biofilm formation, namely Als1, Als2, Als3, and Pbr1, seem to be negatively affected by the presence of *P. aeruginosa* in a time dependent manner, being less expressed in later stages of biofilm development. The differences observed throughout time can be related with the interaction between both pathogens that probably is more pronounced with increased time of interaction, correlating with the lowering of the metabolic activity of *C. albicans* in the double consortia [36].

Three different proteins related to virulence in *C. albicans*, namely Tfp1, Ape2, and Bgl2, were annotated as upregulated in the presence of *P. aeruginosa* [37]. This upregulation could have a synergistic interaction with the VF of the bacterium, resulting in enhanced pathogenesis. All proteins of the cell wall were annotated as downregulated after interaction of the fungus with *P. aeruginosa.* This includes the cell wall proteins Crh11 and Ecm33, involved in cell wall assembly and regeneration, filamentation, and adherence to host cells, and also the hyphal cell wall proteins Rbt5, Hyr1, Ece1, and Rbe1 [36].

Regarding the effect of *C. albicans* on the gene expression of *P. aeruginosa*, 19 differentially expressed genes were annotated, of which 18 were downregulated and 1 was upregulated (Figure 12). It was very interesting to note that almost all interactions were of downregulation (90%). In fact, *pqsH* was the only gene of *P. aeruginosa* annotated as being upregulated in the presence of farnesol [26]. This QS-related gene is involved in the terminal step of the biosynthesis of quinolones by catalysing the hydroxylation of HHQ to PQS [38]. Most of the downregulated annotated genes belong to the *pvd* and *pch* gene families (Figure 12) in planktonic and *in vivo* conditions. Interestingly, the related protein PchD was upregulated in biofilm conditions [36,37]. These and other contrasting annotations are discussed in the next section.

Concerning the expression of proteins, *P. aeruginosa* differentially expressed 147 proteins due to the presence of *C. albicans.* A total of 92 of these proteins were upregulated and 86 were downregulated (Figure 13). The majority of the annotated proteins from *P. aeruginosa* related to virulence were upregulated due to the interaction with *C. albicans*. For instance, proteins related to siderophore biosynthesis and/or transport, namely ChtA, FptA, FpvA, PchD, FpvB, PvdA, PvdH, PvdF, and PvdQ, were all upregulated in biofilm settings [36,37]. Other upregulated proteins, namely OpdO, OpdP OpmH, Opr86, OprC, OprE, and OprQ, are involved in the transport of small molecules and antibiotic resistance [37]. HasA and HasR, two proteins related to heme uptake, were also upregulated as well as PilQ and XcpQ, which are proteins responsible for motility and attachment of the bacterium [36,37]. Proteins involved in cell wall and LPS synthesis, namely, GlmU, RmlA, and WbpA, were also annotated as upregulated. These findings reinforce the notion that *P. aeruginosa* becomes more virulent as consequence of the interaction with *C. albicans.*

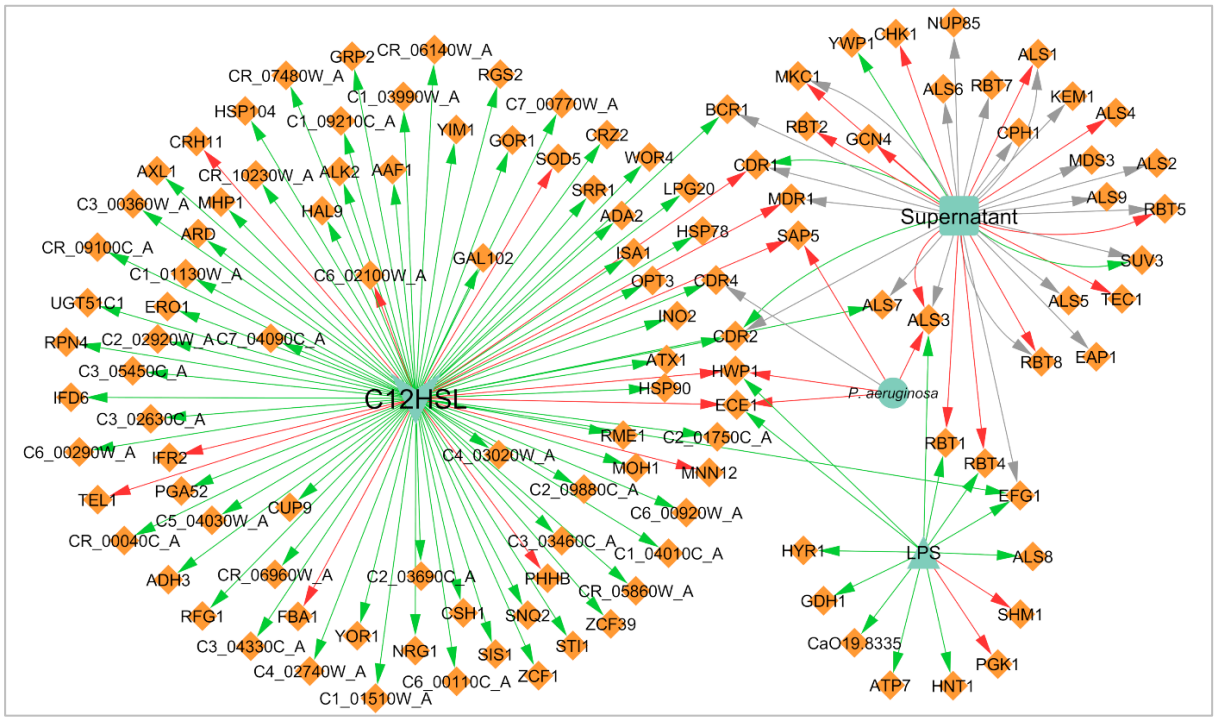

Figure 10. Network of the effects of *P. aeruginosa* on *C. albicans* gene expression. Legend: Teal nodes, *P. aeruginosa*; orange nodes, *C. albicans*; green arrows, upregulation; grey arrows, null effect; red arrows: downregulation; node and node label sizes are directly proportional to the number of related (outward and inward) edges (interactions).

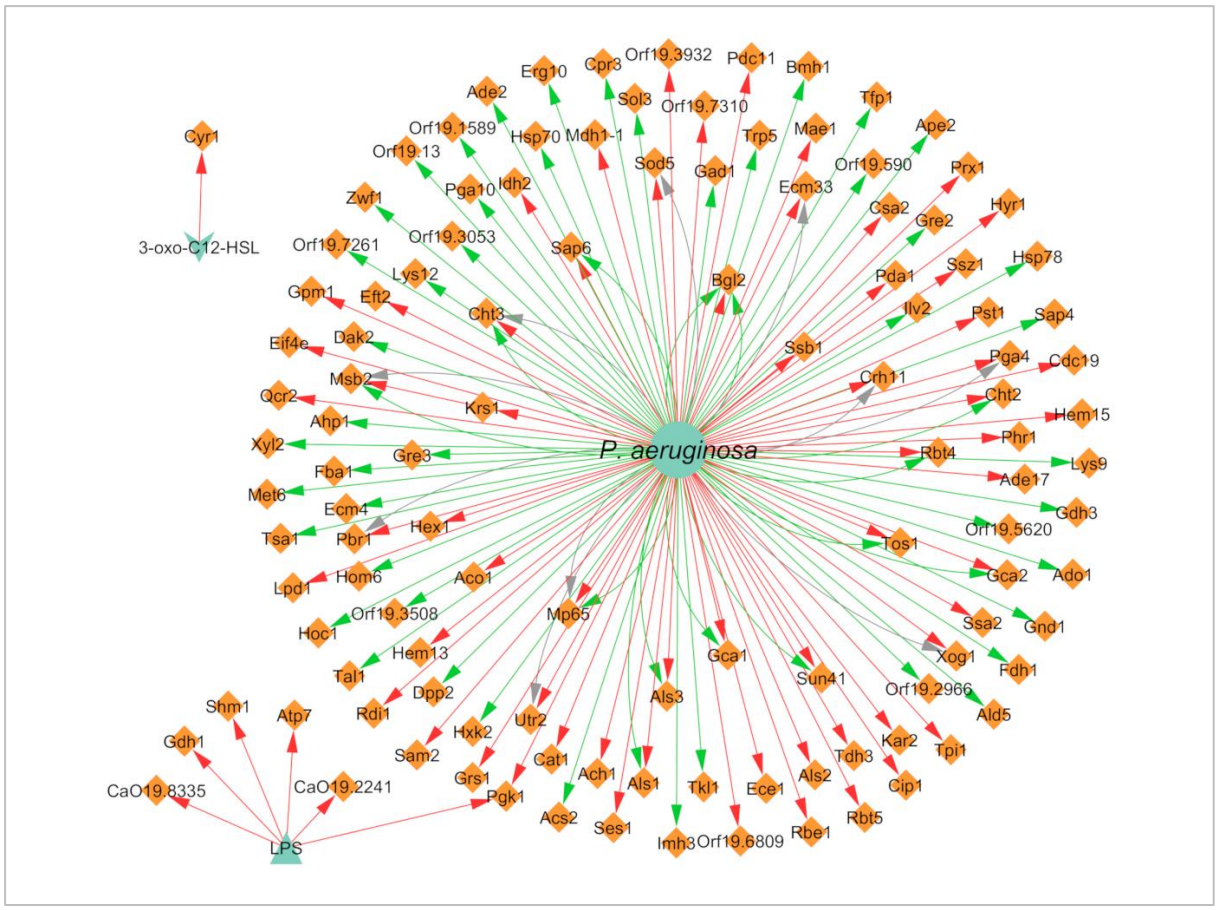

Figure 11. Network of the effects of *P. aeruginosa* on *C. albicans* protein expression. Legend: Teal nodes, *P. aeruginosa*; orange nodes, *C. albicans*; green arrows, upregulation; grey arrows, null effect; red arrows: downregulation; node and node label sizes are directly proportional to the number of related (outward and inward) edges (interactions).

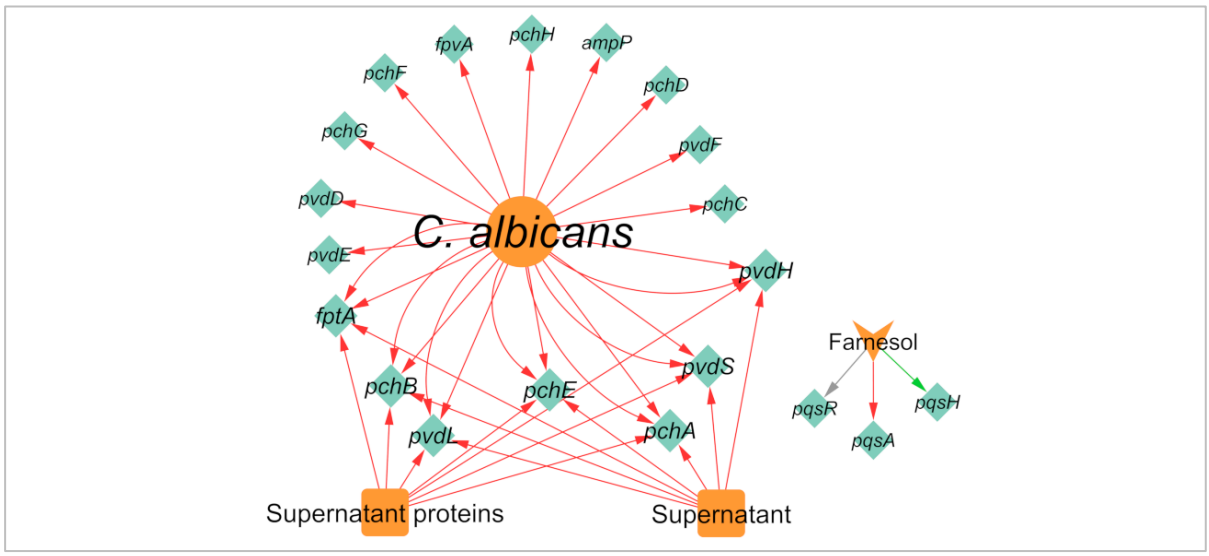

Figure 12. Network of the effects of *C. albicans* on *P. aeruginosa* gene expression. Legend: Teal nodes, *P. aeruginosa*; orange nodes, *C. albicans*; green arrows, upregulation; grey arrows, null effect; red arrows: downregulation; node and node label sizes are directly proportional to the number of related (outward and inward) edges (interactions).

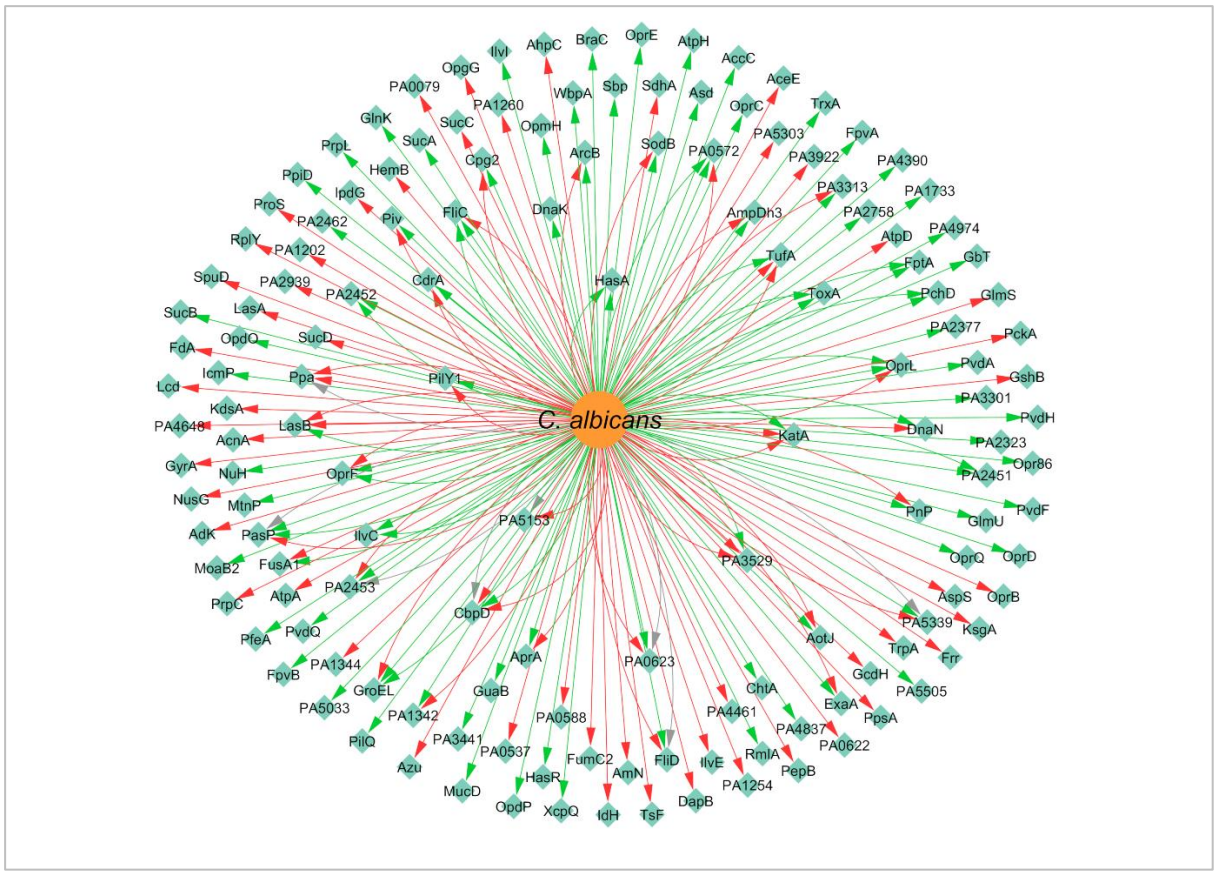

Figure 13. Network of the effects of *C. albicans* on *P. aeruginosa* protein expression. Legend: Teal nodes, *P. aeruginosa*; orange nodes, *C. albicans*; green arrows, upregulation; grey arrows, null effect; red arrows: downregulation; node and node label sizes are directly proportional to the number of related (outward and inward) edges (interactions).

3.3.6 Contrasting Annotations Illustrating the Complexity of Interspecies Study

Upon the analysis of all the gathered information, it was possible to discern some seemingly opposite effects annotated from the literature regarding *P. aeruginosa*-*C. albicans* interactions. All of these annotated interactions are outlined in Tables 4 and 5 for *P. aeruginosa* > *C. albicans* and *C. albicans* > *P. aeruginosa* interactions, respectively. These tables contain not only information on the interactions but also on the experimental conditions in which they were observed, as these define their comparability.

One of the more noticeable factors leading to contrasting observations is the period of time during which cells are co-cultivated or exposed to each other's molecular factors (e.g. AI, supernatants). For example, Purschke and colleagues showed the time-dependent differential expression of several *P. aeruginosa* and *C. albicans* proteins when the two microorganisms are grown together as mixed biofilms [36]. Many of these proteins are up- or downregulated dependent if cells are harvested at early or late time points (or vice-versa), which is corroborated by some similar observations in other publications (Table 4 and 5).

However, this is not always the case. For instance, Als3, a hyphae specific protein of *C. albicans*, appears to be upregulated at early and downregulated at later time-points when in contact with *P. aeruginosa* or its supernatant [34–36]. Still, *ALS3* was shown to be upregulated by LPS at 48 h [32] (Table 4). The effect of LPS is concentration and strain dependent [32], which could explain the differences observed.

Another important factor is the effector entity at play. Although most papers, as previously mentioned, use the whole bacterial cell to investigate its effect onto the other co-existing species, many studies use single molecular players to try to pinpoint the exact mechanisms behind the observed interactions. However, even when one molecular player seems to correlate with the effect of the whole cell, others sometimes seem to contradict it. For example, the gene *CDR1* encodes a drug efflux pump protein that is upregulated by the supernatant of *P. aeruginosa* PAO1 [35] but downregulated by 3-oxo-C12-HSL in *C. albicans* biofilms [22] (Table 4). Although 3-oxo-C12-HSL is extracellular, therefore usually present in *P. aeruginosa*'s supernatants, this contrasting observation is likely due to other metabolites present in the supernatant perceived as detrimental by *Candida*, which, therefore, activates efflux pumps to extrude them [35].

The type of media and, as consequence, the mode of growth are also key factors to take into consideration when analysing interspecies studies. Cugini et al. demonstrated that farnesol inhibits PQS in a liquid *P. aeruginosa* culture [26], but stimulates PQS production when *P. aeruginosa* is grown in solid media [27]. Virulence related genes, namely *fptA*, *fpvA*, *pchD*, *pvdF*, and *pvdH* were upregulated in *P. aeruginosa* biofilms [36,37] but downregulated in planktonic cultures and in an *in vivo* murine model of a gastro-intestinal (GI) infection [31] (Table 5). In the murine GI tract, *P. aeruginosa* is present in two populations: (i) in the gut lumen and (ii) adherent to the epithelium, in which only a subset may be in biofilms [31].

It is safe to say that the comparability of the observations made in interspecies studies is highly, if not totally, dependent on the experimental conditions, which should mimic, as far as possible, the real-like conditions of infections. The standardization of experiments and methodologies should be a priority in order to more quickly advance this research area.

Table 4. Contrasting *P. aeruginosa* > *C. albicans* interactions.

Target	Interaction	Source	Mode of growth	Experimental Conditions*		Method	Strains		Ref.
				Time (h)	Media		PA	CA	
Als1	Upregulation	Cell	Biofilm	1.5, 4.5, 6	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Downregulation			3, 24, 48					
ALS1	Downregulation	Supernatant		24 + 6 (treat.)		Microarray	HSL non-producing isolates (CF177, PAO1ΔQS)		[35]
Als3	Downregulation	Cell	Biofilm	6, 24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			1.5, 3, 4.5					
ALS3	Downregulation	Cell		24	TSB	RT-qPCR	Clinical isolate	Clinical isolate	[34]
		Supernatant		24 + 6 (treat.)	YNBNP	Microarray, RT-PCR	HSL producing (PAO1, CF144) and non-producing (CF177, PAO1ΔQS) isolates	SC5314	[35]
	Upregulation	LPS		48	YNB	RT-qPCR	ATCC 27316	SC5314	[32]
Atp7	Downregulation	LPS	Biofilm	18	YNB	2D-PAGE; MALDI-TOF	ATCC 27316	SC5314	[32]
ATP7	Upregulation			48		RT-qPCR			
Bgl2	Downregulation	Cell	Biofilm	3, 4.5, 6, 24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			1.5					

Table 4. (continued)

Target	Interaction	Source	Mode of growth	Experimental Conditions*		Method	Strains		Ref.
				Time (h)	Media		PA	CA	
Bgl2	Upregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
CDR1	Downregulation	3-oxo-C12-HSL	Biofilm	24 + 1 (treat.)	YNB + glucose	RT-qPCR	(n.a.)	SC5314	[22]
	Upregulation	Supernatant		24 + 6 (treat.)	YNBNP	Microarray	PAO1		[35]
Cht2	Downregulation	Cell	Biofilm	3, 4.5, 6, 24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	CAI4	[36]
	Upregulation			1.5					
Cht3	Downregulation	Cell	Biofilm	4.5, 6, 24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	CAI4	[36]
	Upregulation			3					
Ece1	Downregulation	Cell	Biofilm	1.5, 3, 4.5, 6	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
ECE1	Downregulation	Cell	Biofilm	24	TSB	RT-qPCR	Clinical isolate	Clinical isolate	[34]
		3-oxo-C12-HSL	Planktonic	8	YNBNP		PA14	SC5314	[20]
	Upregulation	LPS	Biofilm	48	YNB		ATCC 27316	SC5314	[32]
Fba1	Upregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
FBA1	Downregulation	3-oxo-C12-HSL			YNB + glucose	RNA-Seq.	(n.a.)	SC5314	[22]

Table 4. (continued)

Target	Interaction	Source	Mode of growth	Experimental Conditions*		Method	Strains		Ref.
				Time (h)	Media		PA	CA	
Gca1	Downregulation	Cell	Biofilm	3, 4.5, 6, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			24					
Gca2	Downregulation	Cell	Biofilm	3, 4.5, 6, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			24					
Gdh1	Downregulation	LPS	Biofilm	48	YNB	2D-PAGE; MALDI-TOF	ATCC 27316	SC5314	[32]
GDH1	Upregulation					RT-qPCR			
HWP1	Downregulation	Cell	Biofilm	24	TSB	RT-qPCR	Clinical isolate	Clinical isolate	[34]
		3-oxo-C12-HSL	Planktonic	8	YNBNP		PA14	SC5314	[20]
	Upregulation	LPS	Biofilm	48	YNB		ATCC 27316	SC5314	[32]
Hyr1	Downregulation	Cell	Biofilm	48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
HYR1	Upregulation	LPS			YNB	RT-qPCR	ATCC 27316		[32]
Mp65	Downregulation	Cell	Biofilm	48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			1.5, 3, 4.5, 6					
Msb2	Downregulation	Cell	Biofilm	6	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			3, 24, 48					

Table 4. (continued)

Target	Interaction	Source	Mode of growth	Experimental Conditions*		Method	Strains		Ref.
				Time (h)	Media		PA	CA	
RBT1	Downregulation	Supernatant	Biofilm	24 + 6 (treat.)	YNBNP	Microarray, RT-PCR	HSL producing (PAO1, CF144) and non-producing (CF177, PAO1ΔQS) isolates	SC5314	[35]
Rbt4	Downregulation	Cell	Biofilm	3, 4.5, 6, 24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			1.5					
RBT4	Upregulation	LPS		48	YNB	RT-qPCR	ATCC 27316		[32]
Sap6	Downregulation	Cell	Biofilm	24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			3, 4.5, 6					
Sun41	Downregulation	Cell	Biofilm	24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			3, 4.5, 6					
Tos1	Downregulation	Cell	Biofilm	24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			3, 4.5, 6					

Legend: PA – *P. aeruginosa*; CA – *C. albicans*; CF – Cystic Fibrosis; MALDI-TOF - Matrix-Assisted Laser Desorption/Ionization - Time of Flight; MS/MS – tandem Mass Spectrometry; qPCR - quantitative Polymerase Chain Reaction; RNA-Seq. – RNA Sequencing; RT-PCR - Reverse Transcription Polymerase Chain Reaction; 2D-PAGE - two-Dimensional PolyAcrylamide Gel Electrophoresis; YNBNP - Yeast Nitrogen Base N-acetyl-D-glucosamine Phosphate; TSB – Tryptic Soy Broth; treat. – treatment; n.a. – not applicable. * - all experiments were conducted at 37 °C.

Table 5. Contrasting *C. albicans* > *P. aeruginosa* interactions.

Target	Interaction	Source	Mode of growth	Experimental Conditions*		Method	Strains		Ref.
				Time (h)	Media		PA	CA	
AmpDh3	Downregulation	Cell	Biofilm	1.5, 4.5, 6, 24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			3					
AprA	Downregulation	Cell	Biofilm	1.5, 3, 4.5, 6, 24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF		CAI4	[37]
ArcB	Downregulation	Cell	Biofilm	1.5, 24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			3					
CbpD	Downregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
	Downregulation			1.5, 48	YNBNP	MALDI-TOF MS/MS		SC5314	[36]
	Upregulation			3, 6, 24					
CdrA	Downregulation	Cell	Biofilm	24,48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			3, 4.5, 6					
Cpg2	Downregulation	Cell	Biofilm	4.5	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			48					

Table 5. (continued)

Target	Interaction	Source	Mode of growth	Experimental Conditions*		Method	Strains		Ref.
				Time (h)	Media		PA	CA	
DnaN	Downregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
	Upregulation			48	YNBNP	MALDI-TOF MS/MS		SC5314	[36]
ExaA	Downregulation	Cell	Biofilm	24	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			48					
FliC	Upregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
	Upregulation			4.5	YNBNP	MALDI-TOF MS/MS		SC5314	[36]
	Downregulation			1.5, 3, 24,48					
FliD	Downregulation	Cell	Biofilm	1.5, 6, 24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			3					
FptA	Upregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]

Table 5. (continued)

Target	Interaction	Source	Mode of growth	Experimental Conditions*		Method	Strains		Ref.
				Time (h)	Media		PA	CA	
fptA	Downregulation	Cell	*In vivo*	(n.a.)	Virulence murine model	RNA-Seq.	PAO1	SC5314	[31]
			Planktonic	10 min	GGP + YPD	RT-qPCR			
		Supernatant	Planktonic	10 min	GGP				
		Supernatant proteins		10 min	GGP				
FpvA	Upregulation	Cell	Biofilm	48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
				24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF		CAI4	[37]
fpvA	Downregulation	Cell	*In vivo*	(n.a.)	Virulence murine model	RNA-Seq.		SC5314	[31]
FusA1	Downregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
	Upregulation			48	YNBNP	MALDI-TOF MS/MS		SC5314	[36]

Table 5. (continued)

Target	Interaction	Source	Mode of growth	Experimental Conditions*		Method	Strains		Ref.
				Time (h)	Media		PA	CA	
GroEL	Upregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
	Upregulation			6	YNBNP	MALDI-TOF MS/MS		SC5314	[36]
	Downregulation			1.5, 24, 48					
KatA	Downregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
	Downregulation			1.5, 4.5, 6, 24, 48	YNBNP	MALDI-TOF MS/MS		SC5314	[36]
	Upregulation			3					
LasB	Downregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
	Downregulation			1.5, 4.5, 6	YNBNP	MALDI-TOF MS/MS		SC5314	[36]
	Upregulation			3, 24					

Table 5. (continued)

Target	Interaction	Source	Mode of growth	Experimental Conditions*		Method	Strains		Ref.
				Time (h)	Media		PA		
OprF	Upregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
	Upregulation			3	YNBNP	MALDI-TOF MS/MS		SC5314	[36]
	Downregulation			1.5, 4.5, 6, 24, 48					
OprL	Upregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
	Upregulation			6	YNBNP	MALDI-TOF MS/MS		SC5314	[36]
	Downregulation			1.5, 4.5, 24, 48					
PA0572	Upregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
	Upregulation			3, 4.5, 6, 24, 48	YNBNP	MALDI-TOF MS/MS		SC5314	[36]
	Downregulation			1.5					
PA0623	Downregulation	Cell	Biofilm	24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			3, 6					

Table 5. (continued)

Target	Interaction	Source	Mode of growth	Experimental Conditions*		Method	Strains		Ref.
				Time (h)	Media		PA	CA	
PA1342	Downregulation	Cell	Biofilm	1.5, 4.5, 6, 24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			3					
PA2453	Downregulation	Cell	Biofilm	24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			1.5, 4.5, 6					
PA3313	Downregulation	Cell	Biofilm	24	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			48					
PA3529	Downregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
	Downregulation			24	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			48					
PA5339	Downregulation	Cell	Biofilm	1.5, 4.5, 6	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			48					
PasP	Upregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
	Upregulation			3, 24, 48	YNBNP	MALDI-TOF MS/MS		SC5314	[36]
	Downregulation			1.5, 4.5					

Table 5. (continued)

Target	Interaction	Source	Mode of growth	Experimental Conditions*		Method	Strains		Ref.
				Time (h)	Media		PA	CA	
PchD	Upregulation	Cell	Biofilm	1.5	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF		CAI4	[37]
pchD	Downregulation		*In vivo*	(n.a.)	Virulence murine model	RNA-Seq.		SC5314	[31]
PilY1	Downregulation	Cell	Biofilm	48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			24					
Piv	Downregulation	Cell	Biofilm	1.5, 3, 4.5, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			6, 24					
PnP (extracellular)	Downregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
PnP (intracellular)	Upregulation								
PQS	Stimulation	Farnesol	Planktonic	14	LB agar	TLC	PA14, PA14Δ*lasR*, PA14Δ*lasI*	(n.a.)	[27]

Table 5. (continued)

Target	Interaction	Source	Mode of growth	Experimental Conditions*		Method	Strains		Ref.
				Time (h)	Media		PA	CA	
PQS	Stimulation	Cell	Planktonic	24, 96 (stirred batch)	ASM	Bioluminescence (reporter strain PAO1 ΔpqsA CTX-lux::pqsA)	PAO1	SC5314	[39]
	Inhibition	Farnesol		6, 24	LB	TLC	PA14	(n.a.)	[26]
pqsH	Upregulation	Farnesol	Planktonic	14	LB agar	RT-qPCR	PA14Δ*lasR*+pUCP22, PA14Δ*lasR*+pPQSHPA 14Δ*pqsH*+pUCP22, PA14Δ*pqsH*+pPQSH	SC5314	[27]
pqsA	Downregulation	Farnesol	Planktonic	15 min	LB	RT-PCR	PA14	(n.a.)	[26]
PvdF	Upregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
pvdF	Downregulation		*In vivo*	(n.a.)	Virulence murine model	RNA-Seq.		SC5314	[31]
PvdH	Upregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]

Table 5. (continued)

Target	Interaction	Source	Mode of growth	Experimental Conditions*		Method	Strains		Ref.
				Time (h)	Media		PA	CA	
pvdH	Downregulation	Cell	*In vivo*	(n.a.)	Virulence murine model	RNA-Seq.	PAO1	SC5314	[31]
			Planktonic	10 min	GGP + YPD	RT-qPCR			
		Supernatant	Planktonic	10 min	GGP	RT-qPCR			
		Supernatant proteins							
Pyocyanin	Stimulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	HPLC	PAO1	CAI4	[37]
		Farnesol	Planktonic	14	LB agar	Chloroform extraction; Spectrophotometry	PA14Δ*lasR*, PA14Δ*lasI*	SC5314	[27]
	Inhibition	Cell	Planktonic	96 (aerobic batch and stirred batch)	ASM	Chloroform extraction; Spectrophotometry	PAO1	SC5314	[39]
		Farnesol	Planktonic	6, 24	LB		PA14, PAO1	(n.a.)	[26]
SodB	Downregulation	Cell	Biofilm	1.5, 4.5, 24, 48	YNBNP	MALDI-TOF MS/MS	PAO1	SC5314	[36]
	Upregulation			3					

Table 5. (continued)

Target	Interaction	Source	Mode of growth	Experimental Conditions*		Method	Strains		Ref.
				Time (h)	Media		PA	CA	
TufA	Downregulation	Cell	Biofilm	24	RPMI 1640 + L-glutamine + dextrose + uridine	2D-PAGE; MALDI-TOF	PAO1	CAI4	[37]
	Downregulation			1.5	YNBNP	MALDI-TOF MS/MS		SC5314	[36]
	Upregulation			48					

Legend: PA – *P. aeruginosa*; CA – *C. albicans*; MALDI-TOF - Matrix-Assisted Laser Desorption/Ionization - Time of Flight; MS/MS – tandem Mass Spectrometry; qPCR - quantitative Polymerase Chain Reaction; RNA-Seq. – RNA Sequencing; RT-PCR - Reverse Transcription Polymerase Chain Reaction; 2D-PAGE - two-Dimensional PolyAcrylamide Gel Electrophoresis; ASM - Artificial Sputum Medium; GGP - Glucose-Glycerol-Peptone; RPMI - Roswell Park Memorial Institute Medium; YNBNP - Yeast Nitrogen Base N-acetyl-D-glucosamine Phosphate; n.a. – not applicable. * - all experiments were conducted at 37 °C.

3.3.7 Alternative Approaches in *P. aeruginosa*-*C. albicans* Biofilm Control: QS Inhibition

Biofilm is known as the preferred mode of growth of most microorganisms, including bacterial and fungal pathogens. These consortia are typically resilient with dynamic structures, giving their microbial constituents a broad range of advantages, as previously mentioned. In real infection scenarios, microorganisms are usually found in the biofilm form and these consortia can easily turn an infection into a chronic condition [40]. Thus, although planktonic testing is practical and informative, studies involving biofilms can better mimic a real-life infection scenario and allow a better comprehension of the microbial behaviour under these situations. Concerning the annotated information in this work, although the number of annotated interactions was higher for biofilms (83%), this only reflects the types of methods being used. Actually, the number of studies using planktonic or biofilm as the mode of growth was similar (18 *vs* 17, respectively), which shows that biofilm studies are still lacking in order to get a real perspective on these inter-species interactions.

Given the biofilm problematic, along with the ever-rising antimicrobial resistance, the need for alternative therapies is in high demand. For example, antivirulence agents carry advantages like circumvention of antibiotic resistance, by targeting VF rather than bacterial growth [41], and a high number of putative virulent targets [42]. Considering that QS is the main regulator of virulence in both bacteria and fungi, the use of quorum quenching (QQ) compounds that inhibit specific QS mechanisms related to virulence can be a promising strategy to modulate it [43]. Additionally, QQ compounds are probably less likely to induce resistance in cases where their targets are located extracellularly [44]. Notwithstanding all the advantages, target selection in this approach is of critical importance given the existence of redundancy and alternative regulatory pathways, which may compensate for a given disturbance, and the complexity of the outcomes of inter-species interactions, which can make an apparently detrimental effect on the pathogen lead to an opposite desired effect in the infection as a whole. For example, the interference with the QS system of one pathogen can potentially facilitate the pathogenicity of the other co-infecting species. Moreover, effective antivirulence therapy would probably entail combinations with other agents, such as other antivirulence drugs or even antibiotics, to increase antimicrobial effectiveness in polymicrobial communities [17]. Other factors to take into account when designing anti-QS approaches are the negative impacts on the host and its microflora, the possibility of bacteremia/sepsis as a consequence of the biofilm disruption, altered immune and inflammatory responses, and resistance development for intracellular targets [45].

As far as it was possible to check, there are no studies regarding the use of anti-QS or anti-virulence compounds against polymicrobial biofilms of *P. aeruginosa* and *C. albicans*. However, some interesting works have been published recently for each pathogen alone. Works prior to 2017 for *P. aeruginosa* and to 2018 for *C. albicans* can be consulted in the publications by Pérez-Pérez et al. (2017) and Grainha et al. (2018), respectively. Particularly, some focus has been given to anti-QS agents targeting the major regulator PqsR. This QS system of *P. aeruginosa*, related to the AI PQS and to HHQ, plays a major role in biofilm formation and inflammation, being crucial in chronic infection establishment. Such agents include synthetic compounds, such as quinolone- and quinazolinone-based derivatives, that compete and/or antagonize this QS system, but also natural compounds [48]. Concerning their impact on co-infection scenarios, not much information was annotated for the effect of this system and its AI on *C. albicans*. HHQ was annotated as inhibiting *C. albicans* biofilm formation in a concentration dependent-manner [24], which means that disruption of HHQ action could be detrimental for *P. aeruginosa* but positive for the fungus.

The idea of using the AI themselves to control virulence and biofilm formation has also been tested. There are many studies using exogenous farnesol to control biofilms of *C. albicans* [47]. A recent study used both farnesol and tyrosol to control *C. albicans* biofilms in hopes of using them as an adjuvant in oral hygiene [49]. In fact, farnesol was annotated as affecting the production of AI from *P. aeruginosa*, namely PQS, HHQ, and C4-HSL, and their related genes, depending on the mode of growth. The upregulation of the expression of AI and of the VF pyocyanin by farnesol in *P. aeruginosa* in biofilm settings [27] has to be evaluated concerning the effect of this molecule if used to treat mixed consortia.

3.4 Conclusions

It is well known that *P. aeruginosa* and *C. albicans* coexist in many infections, but, unfortunately, the mechanisms of bacterial-fungal interactions remained unclear. A better understanding of the social behaviour in the consortium and how microbes change their virulence as a result of established interactions is clinically relevant, being crucial to comprehend the infection process while simultaneously guiding new effective therapeutic strategies. Bacteria and fungi influence each other directly or indirectly in different ways and this work clearly illustrated the amount and complexity of the mechanisms behind such interactions. Importantly, it was interesting to observe that the interaction type was dependent on experimental conditions, namely the microbial mode of growth.

This work allowed the deposition of all available information on the scientific literature regarding *P. aeruginosa* and *C. albicans* interactions on an existing database (www.ceb.uminho.pt/ISCTD) which will

assist research on this topic by cutting on effort and time spent tracking down and analysing the scientific literature. As future work, a continuous update of this knowledge database will be performed, with additional information of new discoveries and new microorganisms.

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CHAPTER 4

Tailoring an antimicrobial coating using a polymicrobial interkingdom biofilm model

The presence of ETT in patients under invasive ventilation is a major risk factor for developing VAP since the tube acts as an entry point of microorganisms through the airway, leading often to biofilm establishment. VAP is described as having polymicrobial and biofilm-mediated nature, with different microorganisms co-existing together. Biofilm preventive measures have been considered the best option to control such infections. Thus, several strategies have been conducted to endow ETT with antimicrobial properties. Among them, surface modification using antimicrobial coatings to limit microbial colonization and biofilm formation is one of the strategies that has been increasingly studied to decrease VAP incidence. Accordingly, the goal of the present work was to exploit and tailor an effective antimicrobial strategy addressing the VAP polymicrobial nature, by combining antimicrobial compounds targeting both bacteria and fungi. Several different antimicrobials were screened to functionalize PVC surfaces using a pDA-based approach. This initial screening pointed out ciprofloxacin (CIP) and amphotericin B (AmB) as the compounds retaining the most promising antimicrobial performance after their immobilization onto PVC. Moreover, their co-immobilization onto PVC surfaces successfully inhibited, up to 24 h, single, dual-, and triple-species biofilm formation encompassing important VAP-related pathogens: *P. aeruginosa, S. aureus* and *C. albicans*. Furthermore, this antimicrobials combination did not exhibit cytotoxicity against lung epithelial cells. These promising outcomes highlight the feasibility of this coating to be applied in ETT to prevent polymicrobial interkingdom biofilm formation.

4.1 Brief Introduction

VAP is a common infection in ICU and in mechanically ventilated patients that arises after endotracheal intubation [1]. This nosocomial infection is particularly worrying because it is associated with prolonged hospitalization, high morbidity and mortality rates, as well as increased healthcare costs [2]. The use of the ETT is the main risk factor for VAP development [3], acting as an airway entry point for microorganisms through microbial adherence and multiplication on the trachea [1], giving rise to biofilms that provide a significant source of bacterial inoculation of the lungs. An important feature of VAP pathogenesis is their association with biofilms and several studies have demonstrated that most of them are composed of polymicrobial communities [4–6]. A wide range of microorganisms is often involved in VAP, with bacteria being the most predominant. *P. aeruginosa* and *S. aureus* are the most frequently reported Gram-negative and Gram-positive bacteria, respectively [7–10]. Additionally, there is an increasing recognition that a considerable part of nosocomial pneumonia may be also associated with viruses and fungi in immunocompromised patients [11,12]. For instance, *Candida* spp, especially *C. albicans*, has been found as part of the array of microorganisms isolated from the respiratory tract of patients diagnosed with VAP [7,9,10,13]. The co-existence of these pathogens is devastating in a variety of human-associated infections, including lung infections [14,15], because of their propensity to develop biofilms highly resilient to conventional antimicrobial therapy [16–20]. Therefore, it is urgent to develop effective therapeutic strategies targeting this particular trait of infections [21–23]. Understanding the impact of microorganisms in VAP and their interactions is essential to unveil infection pathogenesis. Co-infection studies involving bacterial and fungal species, such as *P. aeruginosa* and *S. aureus* with *C. albicans*, have emerged in the last two decades [17,24–28].

Given the recognized role of ETT in VAP development, one promising approach is to prevent ETT colonization through the modification of the surface materials used to design these medical devices [29]. Thus, the aim of the current study was, using dopamine chemistry to functionalize PVC, which has been widely used in the design of ETT and whose intrinsic properties are propitious to the establishment of infection, to leverage antimicrobial features towards *P. aeruginosa*, *S. aureus* and *C. albicans*. After identification of compounds retaining their antimicrobial activity after immobilization onto PVC, a subsequent screening testing different concentrations and antimicrobial combinations was performed to establish coating compositions giving rise to superior performance against all species without cytotoxicity to mammalian cells.

4.2 Materials and Methods

4.2.1 Microbial strains and culture conditions

P. aeruginosa PAO1, *S. aureus* American Type Culture Collection (ATCC) 25923 and *C. albicans* SC5314 were used throughout this work. All strains were stored at -80 ± 2 °C in broth medium with 20% (v/v) glycerol. Before each assay, bacterial and fungal strains were subcultured from frozen stock preparations, onto Tryptic Soy Agar (TSA) or Sabouraud Dextrose Agar (SDA), respectively. TSA and SDA plates were prepared from Tryptic Soy Broth (TSB; Liofilchem) or Sabouraud Dextrose Broth (SDB; Liofilchem), respectively, supplemented with 2% (w/v) agar (Liofilchem). The agar plates were then incubated at 37 °C for 18-24 h.
To prepare microbial suspensions, some colonies were retrieved from the agar plates and incubated in TBS or SDB at 37 °C, overnight under agitation (120 rpm). Cells were then harvested by centrifugation (9000 g, 5 min) and washed in saline solution (0.9% w/v, NaCl) or phosphate-buffered saline (PBS, pH 7.4). The concentration of bacterial suspensions was finally adjusted by measuring the absorbance at 620 nm (EZ Read 800 Plus, Biochrom) and estimated by previously established standard curves while yeast cells were enumerated by microscopy using a Neubauer counting chamber.

4.2.2 Antimicrobials

Several antimicrobial compounds, including potential alternatives to antibiotics, were tested (Table 6). These compounds were chosen since they have already been reported with antimicrobial effect against at least one of the species under study. The rationale was to characterize their effectiveness to be used further in the design of the antimicrobial PVC surfaces.

Table 6. Antimicrobial compounds selected to be tested in this work.

Class	Compound
Natural compounds	Carvacrol Chlorogenic acid Farnesol Linalool Salicylic acid
Antimicrobial peptide	Camel (KWKLFKKIGAVLKVL-NH_2)
Enzyme	Acylase I
Synthetic drugs	Amphotericin B Ciprofloxacin Gentamicin

All antimicrobial compounds were purchased from Sigma (Sigma-Aldrich, USA) with the exception of camel which was kindly provided by Doctor Wojciech Kamysz from the Faculty of Pharmacy, Medical University of Gdansk, Poland. For the functionalization assays, almost all of the compounds were directly dissolved in the work solutions. The only exception was AmB whose stock solutions were prepared using dimethyl sulfoxide (DMSO; Fisher Chemicals). Storage was performed according to the manufacturer's instructions. To obtain the work solutions of carvacrol, farnesol, and linalool, DMSO was added to improve the solubility of these agents.

4.2.3 PVC preparation and functionalization

Unplasticized sheets of PVC (Goodfellow GmbH, United Kingdom) with 1 mm thickness were cut into 1 cm × 1 cm squares. Before surface modification, PVC coupons were subjected to a cleaning process to remove all impurities and traces of grease. For that, samples were placed in a commercial ammonia solution (2%) and sonicated for 5 min, being then thoroughly washed with distilled water. The PVC samples were subsequently sterilized with ethanol (70%) for 30 min, washed with sterile distilled water, and afterwards exposed to ultraviolet irradiation for 1 h. Finally, they were dried at room temperature.

PVC functionalization was performed using a pDA-based coating strategy [30] following a 1-step approach. Briefly, dopamine (Sigma, 2 mg/mL) was dissolved together with the antimicrobials in 10 mM bicine buffer (Sigma, pH 8.5) and the PVC coupons were immediately placed in this solution to obtain the different

coatings. After overnight incubation, at room temperature and constant agitation at approximately 60 rpm, the modified coupons were rinsed with sterile ultrapure water and air-dried before further utilization.

4.2.4 Antimicrobial performance of modified surfaces

4.2.4.1 Contact-killing and leaching properties

The antimicrobial activity of the compounds was evaluated after immobilization onto PVC as previously described [30]. Briefly, microbial suspensions were adjusted to a concentration of approximately 10^6 colony-forming units (CFU) per mL and 20 μL of each were added on top of each surface and incubated under static conditions at 37 °C, until the drop was dried. Coupons were then placed on SDA or TSA plates (for *C. albicans* or bacterial strains, respectively), with the face exposed to microbial suspension in contact with the agar and incubated at 37 °C for 72 h. Microbial growth was then assessed and tabulated as "+" for growth and "-" for no visible growth. To evaluate the leaching of antimicrobials from the modified surfaces, a qualitative method previously described was used [30]. Surfaces were placed on top of TSA plates previously streaked with a microbial suspension adjusted to 10^8 CFU/mL and incubated for 72 h at 37 °C. Afterwards, TSA plates were inspected for the presence or absence of an inhibition zone as an indication of antimicrobial release from the surfaces. Two independent assays were performed in duplicate for each condition except for the cases where no inhibitory activity was detected in contact-killing and leaching assays.

4.2.4.2 Single-species biofilm inhibition activity

The anti-biofilm potential of the modified surfaces was evaluated by enumerating the number of viable cells adhered to the surfaces. Microbial suspensions were prepared from overnight cultures adjusted to a final concentration of 10^6 CFU/mL in RPMI or TSB for *C. albicans* or bacteria, respectively. Un- and modified PVC coupons were placed into the wells of a 24-well microtiter plate and covered with 1 mL of each microbial suspension. Plates were incubated at 37 °C, under static conditions and cells were allowed to adhere on the surfaces for 24 h. The coupons were then washed twice with 1 mL of PBS or NaCl for *C. albicans* or bacterial samples, respectively. They were placed in new wells filled with 1 mL of saline solution or PBS to detach the adhered cells from the surfaces using an ultrasonic bath (Sonicor SC-52, Sonicor Instruments) operating at 50 kHz, for 6 min (parameters previously optimized) or by scraping, in the case of *C. albicans*. Afterwards,

the resulting microbial solutions were collected, vortexed to disrupt possible cell aggregates, 10-fold serial diluted and plated into SDA or TSA plates that were incubated overnight at 37 °C under aerobic conditions before enumeration. Three independent assays with three replicates for each condition were performed.

4.2.4.3 Mixed-species biofilm inhibition activity

The antimicrobial activity of the modified surfaces was also evaluated against polymicrobial consortia. Bacterial and fungal suspensions were prepared as previously described at a concentration of approximately 10^6 CFU/mL. For dual and triple-species adhesion, inoculums were mixed at proportions of 1:1 and 1:1:1, respectively, and then placed onto 24-well plates containing un- and modified PVC surfaces. TSB was used for dual-species biofilms formed by *S. aureus* and *P. aeruginosa* while RPMI was used in all consortia encompassing *C. albicans*, including the triple one. Plates were incubated at 37 °C for 24 h, under static conditions. After that, the liquid content of the plates was discarded, and the PVC coupons plus 24 h-old biofilms were washed with saline solution or PBS according to the microorganism within the biofilms. The adhered cells were detached from the surfaces accordingly to the microorganisms involved following the sonication and scraping processes abovementioned. Serial 10-fold dilutions of the biofilm-cell suspensions were performed and plated onto agar plates with selective growth media. Pseudomonas isolation agar (PIA, Sigma), Mannitol Salt Agar (MSA, Liofilchem) and SDA supplemented with gentamicin (GM) (60 mg/L) were the selective media used to discriminate *P. aeruginosa*, *S. aureus* and *C. albicans*, respectively. TSA supplemented with AmB (10 mg/mL) was used to discriminate *C. albicans* in dual-species biofilms. The plates were incubated overnight at 37 °C under aerobic conditions before enumeration. Three independent assays with three replicates for each condition were performed.

4.2.4.3.1 Evaluation of mixed-species biofilms by fluorescence microscopy

To obtain a more detailed investigation regarding the antimicrobial activity of the surfaces, the presence of cells adhered on PVC coupons was inspected by microscopy using DAPI staining (Invitrogen™, USA). After biofilm formation, the cells adhered to the coupons were washed and fixed as previously described [31]. Briefly, the coupons were left to dry for 1 h at 37 °C. Afterwards, 20 µL of methanol (Fisher Scientific, UK, 100%) was added on top of each surface for 10 min. After that time, the excess methanol was removed and 20 µL of paraformaldehyde (Sigma, 4%) was added on each surface and left to dry for 15 min. Then, the

excess of paraformaldehyde was removed and 20 µL of ethanol (Fisher Scientific, 50%) was added on top of each surface and left to dry for 15 min. Lastly, the excess of ethanol was removed, and the surfaces were allowed to completely dry before staining. For microscopy visualization, the cells were stained with 20 µL of DAPI (30 µg/mL) for 10 min. Then, the excess dye was removed and after drying, the cells were visualized under the epifluorescence microscope (Olympus, BX51). At least two independent assays were performed in duplicate for each consortium and several images per coupon were collected.

4.2.5 Surface characterization

4.2.5.1 Surface morphology, chemical composition, and roughness

To have insights into the morphological aspects of the surfaces, un- and modified PVC were analysed by scanning electron microscopy (SEM). Before observation, samples were covered with a very thin film of Au-Pd (80–20 weight %), 5 nm, in a high-resolution sputter coater and observed with an Ultra-high resolution Field Emission Gun Scanning Electron Microscopy (FEG-SEM), NOVA 200 Nano SEM FEI Company. Topographic images were performed with a secondary electron detector at an acceleration voltage of 10 kV. The chemical composition of the surfaces was also investigated through Energy Dispersive Spectroscopy (EDS), using an EDAX Si (Li) detector, with an acceleration voltage of 15 kV.

Surface roughness was evaluated using atomic force microscopy (AFM). AFM measurements were performed at room temperature using a Nanoscope III Multimode Atomic Force Microscope (Digital Instruments) operating in tapping mode. Scan rates were set at 1 Hz and the scanning area per sample was fixed at 10 × 10 µm. Surface roughness analyses were conducted using Gwyddion software.

4.2.5.2 Surface hydrophobicity parameters

The wettability parameters were determined using the sessile drop contact angle method. Contact angles were measured on an automated contact angle device (OCA 15 Plus, Dataphysics, Germany) that allows image acquisition and data analysis. Measurements were performed at room temperature, using 3 µL drops of water. Three independent assays with three replicates for each condition were performed.

4.2.5.3 AmB and CIP release profile

To determine AmB and CIP release profiles, functionalized PVC coupons were placed into the wells of a 24-well microtiter plate (Orange Scientific, USA) and covered with 1mL of PBS. The plate was then constantly agitated at 120 rpm and 37 °C. After 24 h, all PBS was collected and refreshed. The amount of released AmB or CIP was then determined by ultraviolet-visible spectroscopy (UV–Vis), measuring the absorbance at 335 or 270 nm in the case of AmB or CIP, respectively. The absorbance values were then converted to concentration values using calibration curves previously established. Two independent assays in triplicate were performed.

4.2.6 Cytotoxicity assay

Cytotoxicity analysis of the coatings was performed using lung epithelial cells A549, according to ISO 10993-5:2006. Lung cells were grown in Dulbecco-modified eagle medium (DMEM, Gibco) supplemented with 10% of fetal bovine serum (FBS, Gibco) and 1% antibiotic (ZellShield™, Biochrom) at 37 °C and 5% CO_2. Once achieved the confluence, cells were detached using trypsin (Sigma), and 1 mL of a cell suspension adjusted to approximately 1×10^5 cells/mL was added to each well of a 96-well microtiter plate. In parallel, unmodified and modified surfaces were inserted in 24-well plates filled with 1 mL of DMEM per well. Both plates were then incubated for 24 h at 37 °C and 5% CO_2. Afterwards, the supernatant was removed and 100 μL of the medium, which was in contact with the surfaces, were added to the adhered cells. Fresh DMEM was also added to cells as a positive control. The plate was then incubated for further 24 h at 37 °C, 5% CO_2, and after that period, 20 μL of MTS (3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium) inner salt (Promega) were added in each well. After 1 h of incubation at 37 °C, 5% CO_2 in the dark, the absorbance of the resulting solution was measured at 490 nm. The percentage of cell viability was calculated by the ratio between the cell metabolic activity in the presence of modified surfaces and the control (cell growth in DMEM). Two independent experiments in triplicate were performed.

4.2.7 Statistical analysis

Results were presented as mean ± standard deviation (SD). Statistical analysis was performed by using the Prism software package (GraphPad Software version 9.0.0). One-way ANOVA tests were performed, and means were compared by applying Tukey's multiple comparison test. In all the analyses performed, the confidence interval used was 95%.

4.3 Results

4.3.1 Antimicrobial performance of the modified surfaces

4.3.1.1 Contact-killing and leaching properties

As in this study it was intended to tailor a coating able to prevent microbial attachment to PVC surfaces but also to the surrounding environment in the bulk phase, the contact-killing and antimicrobial-releasing features were analysed jointly to choose the best compounds. Table 7 summarizes the antimicrobial activity of the selected compounds after immobilization onto PVC, namely their ability to impede microbial growth, as well as the qualitative release of those antimicrobials from the surfaces.

Table 7. Contact-killing and leaching properties of functionalized PVC with different antimicrobial compounds at different concentrations. Visible growth was used as an indication of antimicrobial activity being tabulated as "+" for microbial growth and "–" for no growth. The antimicrobial release was evaluated by the presence (P) or absence (A) of an inhibition zone. Some conditions that were not tested were listed as "NT". CA: *C. albicans*; SA: *S. aureus*; PA: *P. aeruginosa*.

Antimicrobial compound	Concentration	Microbial growth			Inhibition zone		
		CA	SA	PA	CA	SA	PA
Acylase I	1 mg/mL	+	+	+	A	A	A
Amphotericin B	1 mg/mL	–	NT	NT	P	NT	NT
	0.5 mg/mL	–	NT	NT	P	NT	NT
	0.25 mg/mL	–	NT	NT	P	NT	NT
Camel	1 mg/mL	+	+	+	A	A	A
	1 mg/mL	+	+	+	A	A	A

Table 7. (continued)

Antimicrobial compound	Concentration	Microbial growth			Inhibition zone		
		CA	SA	PA	CA	SA	PA
Carvacrol	10 µL/mL	–	–	–	P/A	P/A	A
	5 µL/mL	–	–/+	–	A	A	A
Chlorogenic acid	2 mg/mL	+	+	+	A	A	A
	1 mg/mL	+	+	+	A	A	A
Ciprofloxacin	1 mg/mL	+	–	–	A	P	P
	0.5 mg/mL	+	–	–	A	P	P
	0.25 mg/mL	+	–	–	A	P	P
Farnesol	5 mg/mL	–/+	–	–/+	A	P	A
	1 mg/mL	+	–	+	A	P	A
Gentamicin	2 mg/mL	+	–	–	A	P	P
	1 mg/mL	+	–/+	–	A	P/A	P
Linalool	100 µL/mL	–	–	–	A	A	A
	50 µL/mL	–	–	–/+	A	A	A
Salicylic acid	5 mg/mL	+	+	+	A	A	A
	1 mg/mL	+	+	+	A	A	A

This initial screening showed that the surfaces functionalized with acylase I, camel, chlorogenic acid and salicylic acid did not exhibit antimicrobial effects against *C. albicans, S. aureus,* and *P. aeruginosa* (Table 7). The PVC coupons functionalized with carvacrol showed antimicrobial activity against all species as evidenced by their contact-killing activity. However, this compound was not considered for further tests because solubility problems difficult to overcome were found with its use.

Results showed that the immobilization of AmB did not compromise its antifungal activity, as shown by its contact-killing activity against *C. albicans* since no growth was noticed for any of the concentrations tested. Inhibition zones were also observed, indicating that AmB was released from the surfaces.

Functionalized surfaces with the antibiotic CIP exhibit antibacterial activity against both bacteria, whatever the concentration tested. Furthermore, the amount of CIP released from each of the PVC surfaces was also effective in inhibiting *P. aeruginosa* and *S. aureus* growth since inhibition zones were observed for all concentrations used in PVC functionalization.

Regarding farnesol, this compound was efficient against *S. aureus* as evidenced by its contact-killing and leaching activities. Although some antimicrobial activity was detected against *C. albicans* and *P. aeruginosa* when using the highest concentration, no inhibition zone was observed in the leaching assay.

GM maintained its antibacterial activity after immobilization on PVC surfaces, especially for the highest concentration tested, as evidenced by its contact-killing and leaching activities against *S. aureus* and *P. aeruginosa*.

The bioactive compound linalool exhibited antimicrobial activity against all species under investigation when used to coat PVC surfaces as shown by contact-killing activity, nevertheless, no inhibition zone was observed in the leaching assay.

Overall, these data determined the exclusion of some compounds (acylase I, camel, carvacrol, chlorogenic acid, linalool, and salicylic acid) and even of some concentrations of the remaining compounds for further investigation since not all exhibited the desired antimicrobial activities. GM was also excluded because, even though it is effective at the highest concentration, CIP, the other antibiotic, showed better performance against bacterial strains at lowest concentrations.

Hence, as AmB, CIP, and farnesol exhibited promising contact-killing and leaching traits, they were selected to pursue the work. The highest concentrations of AmB and CIP (1 mg/mL) were also disregarded because the lower concentrations tested gave rise to similar antimicrobial activity.

At this point, it is important to note that the concentrations presented in Table 7 are those that were initially used in the immobilization process, and the effective concentration retaining on the PVC surfaces will certainly be much lower. To facilitate the interpretation of the results, these initial concentrations will be shown in the following data presentations. The concentration that remains on the surface and that can be later released will be addressed and studied in more detail later in this work.

4.3.1.2 Single-species biofilm inhibition activity

The antimicrobial activity of the modified surfaces with the selected compounds (AmB, CIP, and farnesol), at different concentrations, was further investigated against single-species biofilms of *C. albicans, S. aureus,* and *P. aeruginosa*. For that, the number of viable cells adhered on the surfaces was enumerated after an exposure time of 24 h (Figure 14). Results showed that, in general, all microbial cells were able to adhere to PVC (unmodified) control surfaces. Additionally, the deposition of pDA did not affect the adhesion of all species, since the number of adhered cells was almost the same when compared to bare PVC. When it comes to the antimicrobial activity of the selected compounds, it was possible to conclude that the previous antimicrobial traits (Table 7) were preserved in the biofilm model. Surfaces functionalized with AmB were effective against *C. albicans,* especially when AmB was immobilized at concentrations between 0.5 and 0.1 mg/mL, as evidenced by the number of viable cells lower than the limit detection. Such effect was not so notorious for a concentration of 0.05 mg/mL as only approximately 1 log CFU/mL reduction in the number of viable cells was noticed. AmB did not exhibit any effect against both *S. aureus* and *P. aeruginosa*.

CIP inhibited both *S. aureus* and *P. aeruginosa* single-species biofilm formation. In the case of *S. aureus,* this effect was more noticeable at a concentration of immobilization of 0.5 mg/mL, which lowered the number of viable cells to below the detection limit. For the immobilization of CIP at a concentration of 0.25 mg/mL, only a reduction of approximately 2 log CFU/mL was achieved. Surfaces functionalized with this antibiotic were more efficient in impairing biofilm formation of *P. aeruginosa,* as the viable cells detected were under the limit detection until a concentration of immobilization of 0.25 mg/mL. Even for the lowest concentration tested, only slight growth of this bacterium could be observed, corresponding to a reduction of approximately 4.8 log CFU/mL when compared to the growth on PVC without modification. CIP coatings did not show any effect against *C. albicans* biofilm formation.

PVC surfaces functionalized with farnesol significantly reduced the number of *S. aureus* cells able to adhere to PVC surfaces. This effect was more pronounced when a concentration of 5 mg/mL was used for its immobilization, reaching a reduction of 4.7 log CFU/mL compared to the growth of this bacterium on unmodified PVC. The immobilization of this agent did not exhibit any effect against both *C. albicans* and *P. aeruginosa* single-species biofilm formation for all concentrations tested.

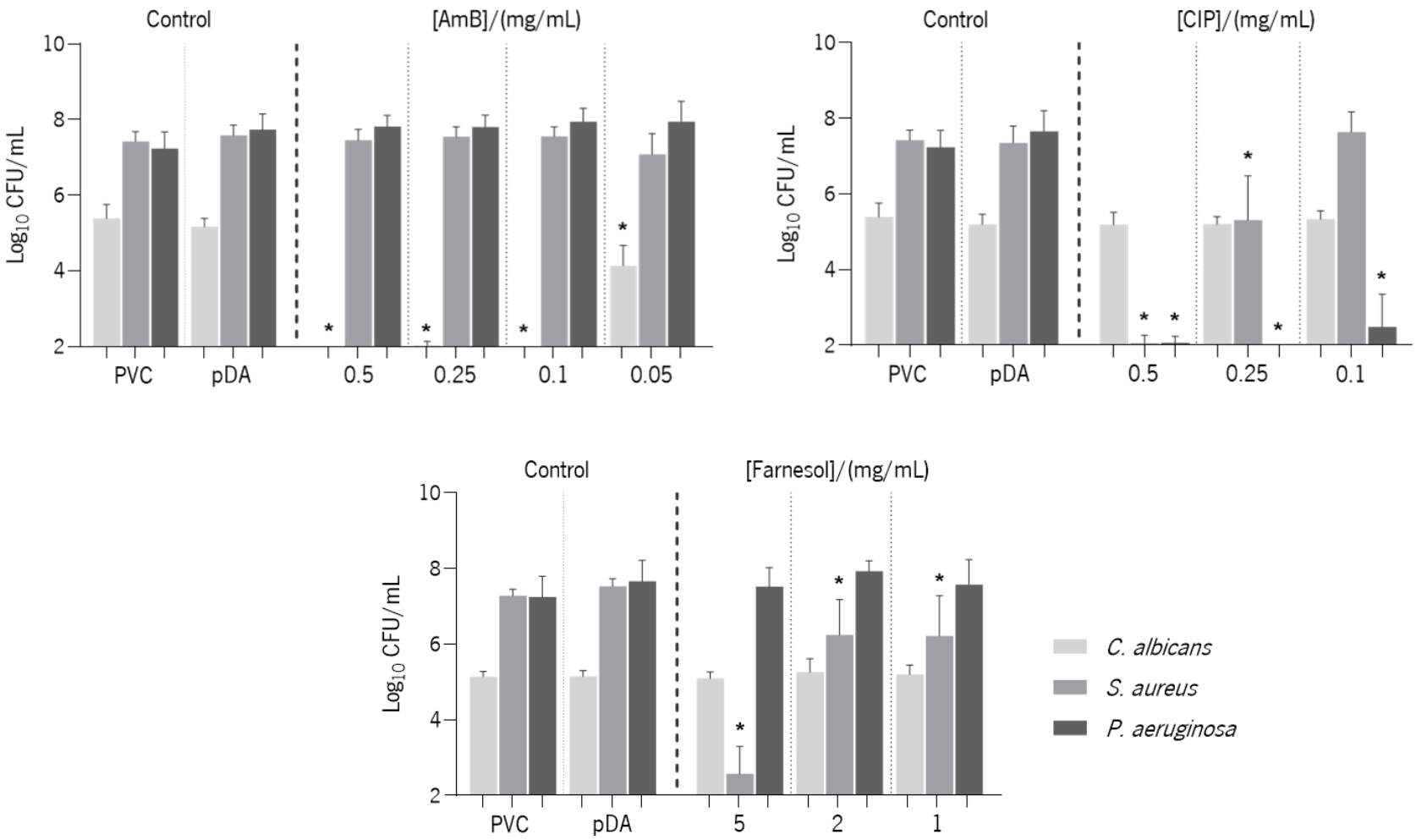

Figure 14. Antimicrobial performance of PVC surfaces functionalized with AmB, CIP, and farnesol at different concentrations against *C. albicans*, *S. aureus,* and *P. aeruginosa* single-species biofilm, after a contact of 24 h. PVC was included as a control while pDA was used for comparison purposes. Significant differences were found for (*) $p < 0.0001$, when reductions were observed compared to cells adhered to PVC. The minimum value of the Y axis on the graphs was defined as 2 since it corresponds to the detection limit of CFU counting (log 2 CFU/mL).

To obtain a wide spectrum coating strategy to be applied in the context of VAP and targeting its polymicrobial nature, the combinatorial effect of AmB, CIP, and farnesol, immobilized at different concentrations, was also tested. The antimicrobial activity was evaluated using dual and triple combinations of these compounds.

Results (Figure 15) showed that co-immobilization of 0.1 mg/mL of AmB with 0.5 mg/mL of CIP yielded surfaces with antimicrobial activity against all microorganisms tested, as evidenced by the great reduction in the number of adhered cells (3.1 log CFU/mL for *C. albicans*, 4.3 log CFU/mL in the case of *S. aureus* and 5.2 log CFU/mL for *P. aeruginosa*) comparing to PVC surface control. Reducing CIP concentration to 0.25 mg/mL compromised its antimicrobial activity against *S. aureus* as the reduction in the number of adhered cells was much lower (1.5 log CFU/mL). Taking into account that the single immobilization of CIP at this concentration was able to reduce *S. aureus* biofilm formation in 5.4 log CFU/mL (Figure 14), results suggest that there was a slight loss of CIP activity when the compounds were combined. All the combinations with AmB and CIP tested were very effective against *C. albicans* and *P. aeruginosa*.

After combining AmB and farnesol on PVC surfaces, the compounds maintained their antimicrobial activity against *C. albicans* and *S. aureus,* respectively. However, results suggest that there was a slight loss of AmB activity with this combination. When AmB was used alone at 0.25 and 0.1 mg/mL, the number of adhered cells was under the limit detection. On the other hand, when these same concentrations of AmB were combined with farnesol, a reduction of 2.3 log and 3.7 log CFU/mL, respectively, was achieved in *C. albicans*. Also, farnesol activity seems to be affected when combined with 0.25 mg/mL of AmB translating into a slight increase in the number of *S. aureus* adhered cells.

The results obtained for the combination of CIP and farnesol revealed no inhibitory activity between these agents. When the higher concentrations were tested (0.5 and 5 mg/mL for CIP and farnesol, respectively), the number of *P. aeruginosa* and *S. aureus* cells remained near the limit detection, as observed when CIP was tested alone. Farnesol did not compromise the activity of the antibiotic once a great reduction in the number of *P. aeruginosa* adhered cells was also observed when 0.25 mg/mL was tested. Regarding *S. aureus*, great reductions in its adhesion were found when 0.5 mg/mL of CIP and 5 mg/mL of farnesol were used in the combinations. When it comes to the combination of CIP and farnesol at 0.25 and 1 mg/mL, respectively, results showed that the number of *S. aureus* adhered cells was around 3.3 log CFU/mL. This drug combination proved to be more efficient than when tested individually. *S. aureus* achieved 5.3 log CFU/mL in PVC surfaces with CIP at 0.25 mg/mL and 6.2 log CFU/mL in functionalized surfaces with 1 mg/mL of farnesol (Figure 14), suggesting a synergistic effect between CIP and farnesol.

Concerning the triple combination of AmB, CIP, and farnesol at the selected concentrations of 0.1, 0.5, and 1 mg/mL, respectively, the adhesion of *C. albicans* was significantly inhibited as no fungal cells were detected. Additionally, a reduction of 4.4 and 5 log CFU/mL was achieved for *S. aureus* and *P. aeruginosa*, respectively, when this combination was applied to functionalize PVC surfaces. Besides this great achievement elicited by the triple combination, the results were similar to those obtained with the dual combination of AmB and CIP at the same concentrations, bringing up that the inclusion of farnesol in the combination had not any antimicrobial advantage. This evidence, together with the fact that the use of two compounds results in a more simple and economical approach, settled the selection of the dual combination of the antifungal with the antibiotic for further investigations.

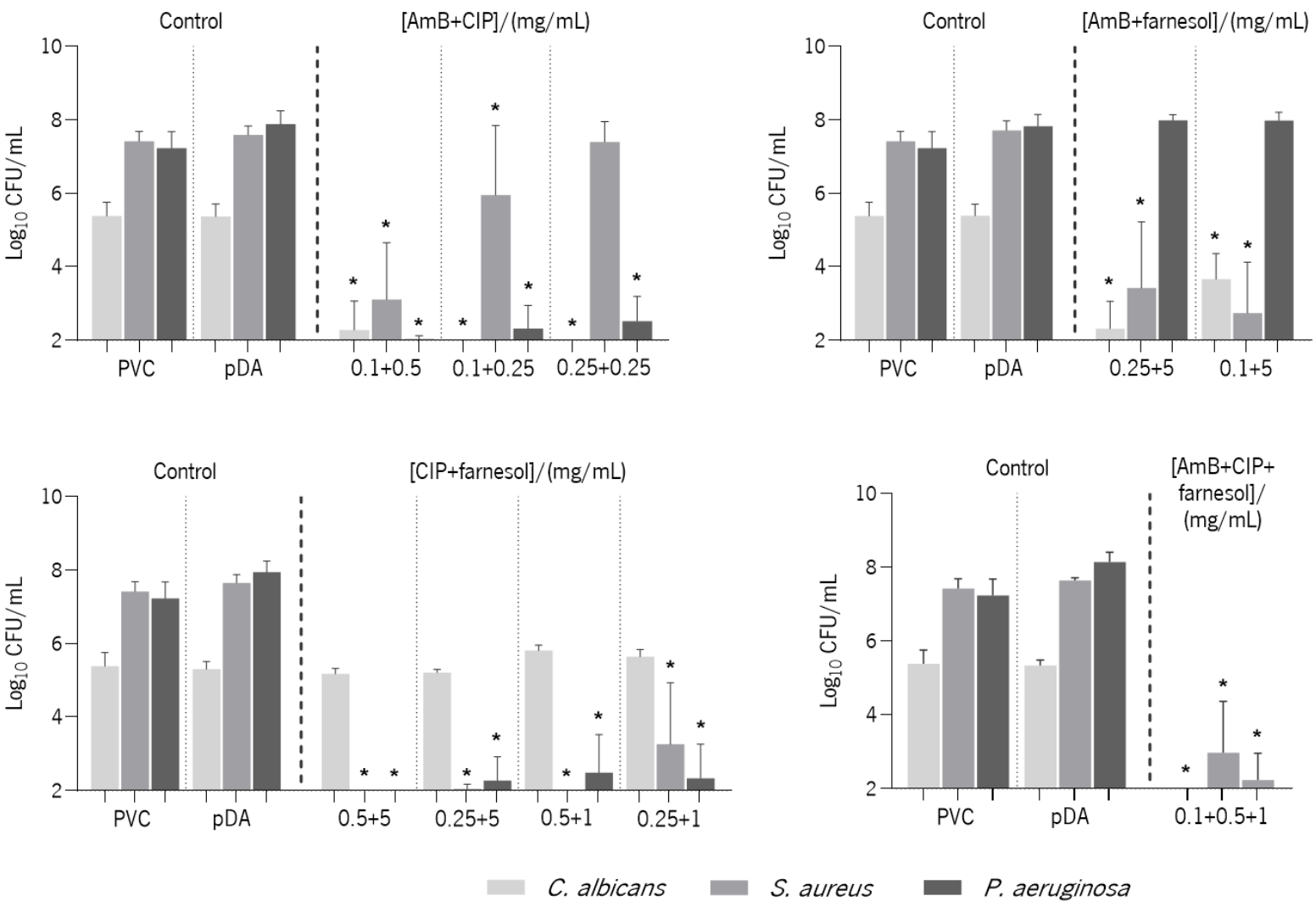

Figure 15. Antimicrobial performance of functionalized PVC surfaces using different combinations of antimicrobial concentrations against *C. albicans*, *S. aureus,* and *P. aeruginosa* single-species biofilm, after a contact of 24 h. PVC was included as a control while pDA was used for comparison purposes. Significant differences were found for (*) $p < 0.0001$, when reductions were observed compared to cells adhered to PVC. The minimum value of the Y axis on the graphs was defined as 2 since it corresponds to the detection limit of CFU counting (log 2 CFU/mL).

4.3.2 Surface characterization

Once established CIP and AmB (0.1 and 0.5 mg/mL, respectively) as the combination rendering the best anti-biofilm properties, some parameters were inspected in order to have knowledge about the physical-chemical properties of the un- and modified PVC surfaces. For comparison purposes, surfaces modified with AmB and CIP individually were also characterised.

4.3.2.1 Surface morphology, roughness, and chemical composition

Morphological analysis of the un- and modified PVC surfaces was performed by SEM (Figure 16). Results showed that bare PVC exhibited high homogeneity with smooth and neat surfaces. Additionally, some microspores on PVC were observed, probably being a consequence of the evaporation of the solvent during its production [32]. The success of pDA deposition on PVC surfaces was suggested by the absence of the micropores in addition to the presence of particles resulting from the self-polymerization of pDA in the bulk solution [33]. This effect has been previously reported in other studies that used dopamine chemistry for surface modifications [30]. Further functionalization with AmB resulted in surfaces less homogenous with more and higher agglomerates across the entire surface. This can be explained by the behaviour of AmB which can self-assemble in aggregation states in aqueous media due to its chemical structure [34]. PVC functionalization with CIP resulted in a similar morphology as compared to pDA.
The images obtained during AFM analysis are shown in Figure 17 and these were used to determine the average roughness of the surfaces (Figure 18) using Gwyddion software. Results showed that pDA did not introduce significant changes in PVC surface roughness as well as when CIP was used. On the other hand, functionalization with AmB caused a significant increase in the surface roughness. Nevertheless, this latter trait was not observed when AmB and CIP were used together since PVC surfaces co-functionalized with both antimicrobials showed similar roughness values to pDA or CIP. These results suggest that the aggregates observed by SEM on PVC surfaces co-functionalized with both AmB and CIP are not as large as those formed on surfaces coated with only AmB.

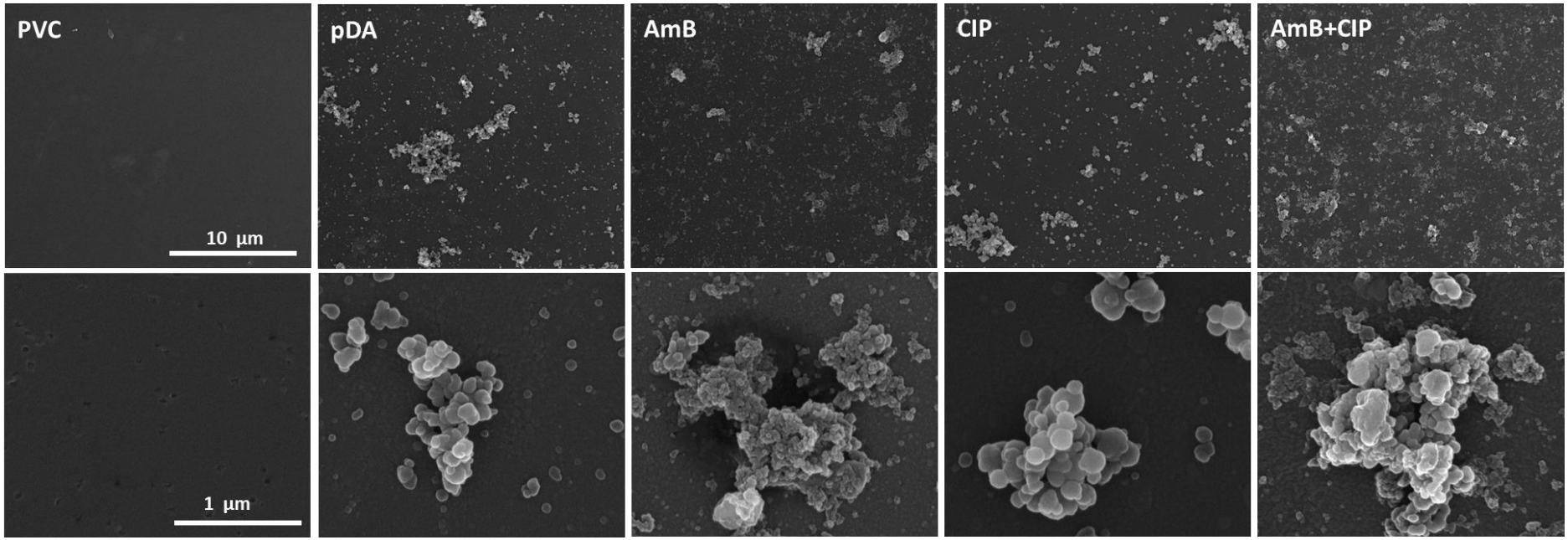

Figure 16. Surface morphology characterization. Representative images of SEM analysis of PVC surfaces before and after AmB and CIP immobilization. The scale bar in the upper and lower column indicates 10 and 1 µm, respectively.

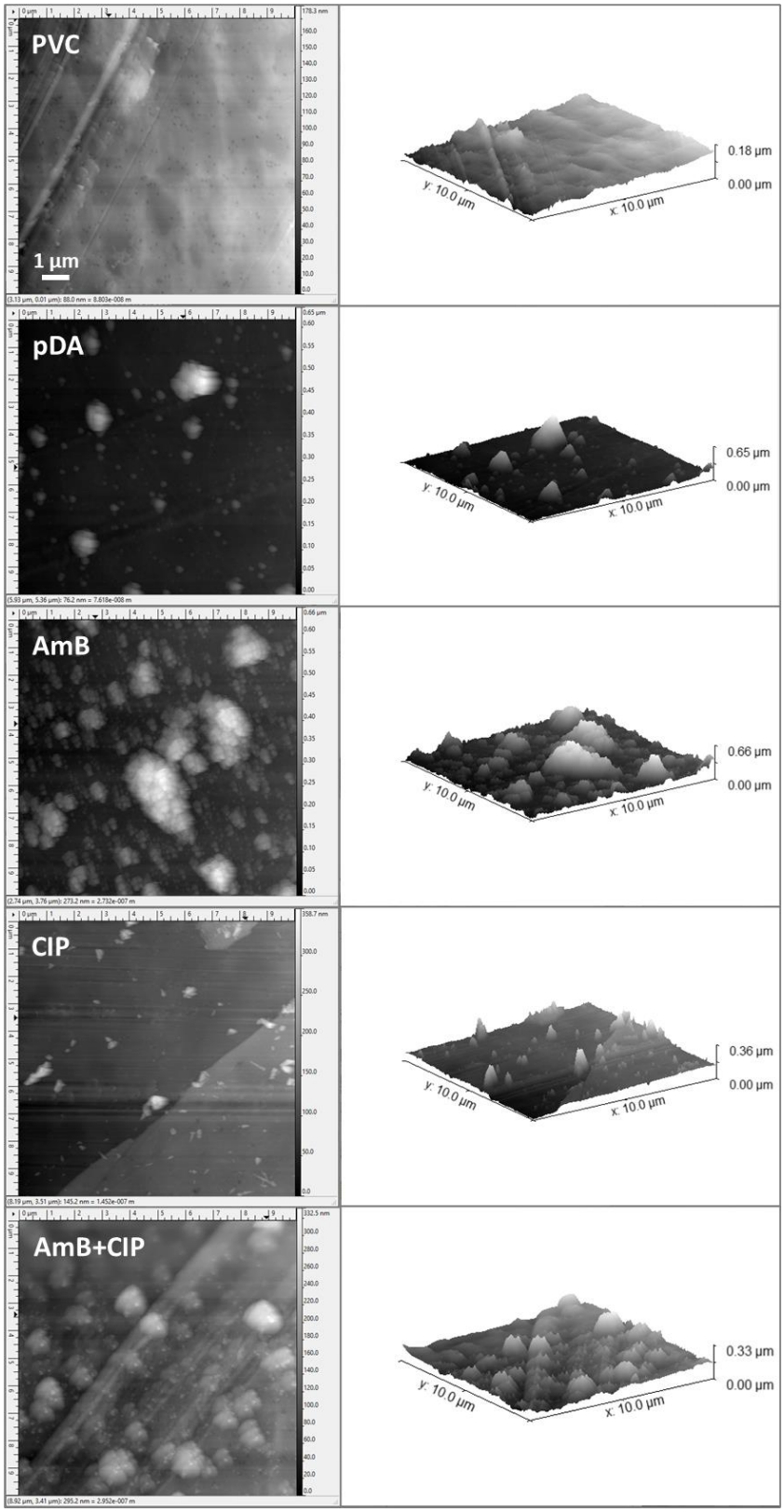

Figure 17. Representative AFM images of un- and modified PVC surfaces. The images on the right correspond to the 3D representation of the images on the left. The scale bar indicates 1 µm.

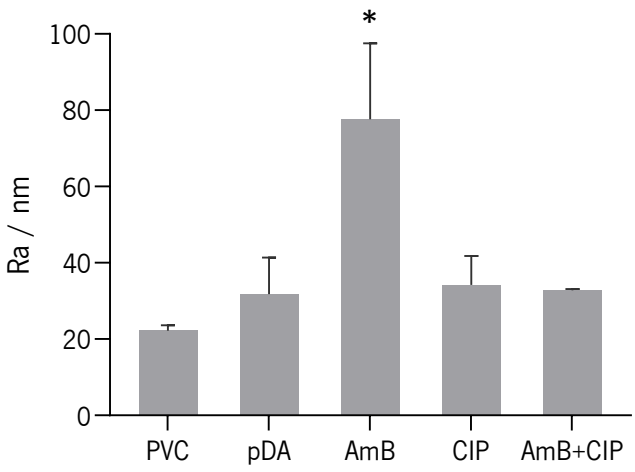

Figure 18. Average roughness (Ra) of un- and modified PVC surfaces. Significant differences were found for (*) $p < 0.05$, compared to PVC.

PVC surfaces were analysed in terms of chemical composition using EDS to confirm each modification step (Table 8). This polymer is composed of Carbon (C), Chlorine (Cl) and Hydrogen (H). Therefore, results showed high concentrations for C as well as Cl, whereas Oxygen (O) and Nitrogen (N) could not be detected. The deposition of pDA onto PVC could be confirmed by the decrease of Cl as well as the increase in N content. Functionalization of PVC surfaces using AmB, CIP, or both antimicrobial compounds together revealed similar patterns in terms of chemical composition. In all cases, an increase in the N content accompanied by a decrease of Cl, as compared to PVC untreated surfaces was also observed. A slight increase in the O content in all pDA-based coated surfaces was observed. Similar chemical structures can be explained by the sampling depth achieved by the EDS. Indeed, it is higher than 50 nm, which is the maximal thickness of pDA coatings [35], meaning thus that PVC is the major contributor to the chemical signature detected by EDS analysis.

Table 8. EDS quantification of atomic compositions of PVC surfaces, before and after AmB and/or CIP immobilization.

Surface	C (%)	O (%)	Cl (%)	N (%)
PVC	73.27	2.06	24.67	0.00
pDA	73.42	3.47	21.33	1.77
AmB	73.88	3.41	22.01	0.70
CIP	72.35	3.34	21.66	2.64
AmB+CIP	73.52	3.13	21.87	1.47

4.3.2.2 Surface hydrophobicity parameters

Surface hydrophobicity parameters of PVC and different modified surfaces were investigated by measuring the water contact angles (Figure 19). A significant decrease in the water contact angle values was observed for all coated surfaces when compared to bare PVC. Results showed that PVC is a hydrophobic surface, evidenced by the water contact angle of approximately 92.2°. pDA coating rendered the PVC surfaces with hydrophilic properties as evidenced by the decrease of water contact angle to 52.8°. This feature is attributed to dopamine since its polymerization introduces new hydrophilic functional groups, especially the catechol and amine groups, on the PVC surface. This observation on materials functionalized with pDA has been previously reported [30,36]. The surfaces functionalized with AmB and CIP separately and in combination also displayed this hydrophilic characteristic. Still, it is important to note that AmB immobilization on PVC rendered the most hydrophilic surfaces with values of water contact angle around 31.5° which may be attributed to the high roughness of that surfaces and the polyol subunit of AmB (which contains multiple –OH groups), which is capable of hydrogen bonding with water [37]. Additionally, co-functionalized surfaces with AmB and CIP reveal estimated values of contact angles between those determined for the compounds when used separately onto PVC surfaces, which suggests a contribution of both agents to the final wettability behaviour of these surfaces.

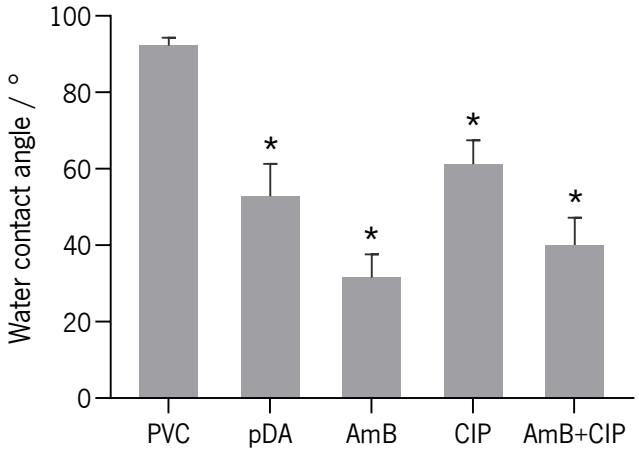

Figure 19. Values of contact angles of un- and modified PVC surfaces. Significant differences were found for (*) $p < 0.0001$, compared to PVC.

4.3.2.3 AmB and CIP release profile

Since the amount of effective agents retained on the surface is expected to be at a much lower concentration than those used initially in the immobilization solution, it was intended to assess the profile of AmB and CIP release from the surfaces after antimicrobial functionalization. For that, PVC surfaces were functionalized with AmB and CIP, separately, to detect and quantify each one of the antimicrobial compounds. Results, presented in Table 9, show that it was not possible to determine the amount of AmB both at 24 and 48 h, meaning that the amount of the antifungal released is probably lower than the limit detection of the apparatus. The amount of CIP released from the surfaces after 24 h was estimated at 1.68 μg/mL. After 48 h, no amount of CIP was detected probably due to the amount of released compound was under the limit detection of the apparatus.

Table 9. Quantification of AmB and CIP released from PVC surfaces after 24 and 48 h of initial incubation. Results are expressed in [μg/mL]. ND: not detected.

Antimicrobial	Released amount	
	24 h	48 h
AmB	ND	ND
CIP	1.68 ± 0.19	ND

4.3.3 Mixed-species biofilm inhibition activity

The effectiveness of the co-functionalized surfaces with AmB and CIP (0.1 and 0.5 mg/mL, respectively) was further evaluated against mixed-species biofilms (i.e. dual- and triple-species biofilms). Results demonstrated that, in general, the inhibitory effect of AmB and CIP previously observed for single species-biofilms was maintained when the compounds were tested individually and in combination against the dual- and triple-species 24 h-old biofilms (Figure 20). Concerning the dual-species biofilms comprising *C. albicans* and *S. aureus*, the number of CFU found for all tested conditions (control and functionalized surfaces) was similar to those obtained for single-species counterparts (Figure 14 and 15). There was even a slight decrease in the number of cells of both *C. albicans* and *S. aureus* adhered on the co-functionalized surfaces (AmB+CIP) when compared to the respective single-species biofilms. Therefore, it was possible to conclude that the eventual interactions established within the consortium did not affect the outcome of the antimicrobial performance of the surfaces when challenged by *C. albicans* and *S. aureus* simultaneously.

Regarding the dual-species biofilms formed by *C. albicans* and *P. aeruginosa*, the number of CFU for all surfaces tested was also almost the same as compared to the respective parts in single-species biofilms. Results suggest that both species coexist without any effect observed on the growth between them for the time and surfaces tested. Additionally, the antimicrobial activity of the functionalized surfaces was also maintained. There was also a slight increase in the reduction of cells of both species adhered on the co-functionalized surfaces (AmB+CIP), compared to respective single-species biofilms, being the CFU counts under the limit of detection.

Regarding *S. aureus* and *P. aeruginosa* in dual-species biofilms, results showed that the capability of *S. aureus* to grow on bare PVC and surfaces coated with pDA and AmB was affected by the presence of *P. aeruginosa.* The number of *S. aureus* adhered cells on PVC control typically achieves more than 7 log CFU/mL, however, in the presence of *P. aeruginosa*, its growth decreased to 4.6 log CFU/mL under the same conditions. Also, for surfaces functionalized with pDA and AmB, its growth decreased by 1.8 log CFU/mL in both cases, when comparing dual *versus* single-species biofilms. In the case of *P. aeruginosa*, no differences in terms of CFU counts were observed, in the presence of *S. aureus*. Regarding the surfaces functionalized with CIP and co-functionalized with AmB and CIP, the reductions observed for both bacteria in single-species biofilms were maintained when the surfaces were tested against both bacteria at the same time. The findings observed in

this consortium reflect some inhibitory effect of *P. aeruginosa* on *S. aureus,* which translates into their loss of ability to grow on solid media and consequently the decrease in CFU counts.

To increase, even more, the complexity of our consortia, the co-adhesion of all the 3 microorganisms was also evaluated. The adhesion extent of *C. albicans*, *S. aureus,* and *P. aeruginosa* to PVC control and pDA surfaces was similar to single-species biofilms. The antimicrobial activity of the surfaces coated with AmB was preserved for *C. albicans* with no effect against *S. aureus* and *P. aeruginosa.* Interestingly, the loss of *S. aureus* capability to grow on PVC and surfaces functionalized with pDA and AmB, observed when this bacterium was grown with *P. aeruginosa*, was not detected when *S. aureus* integrated the triple-species biofilm. Concerning functional surfaces with CIP, the antimicrobial activity was maintained against *S. aureus* and *P. aeruginosa* without significant effect on fungal growth, as expected. Additionally, results showed that the surfaces co-functionalized with AmB and CIP were able to significantly inhibit all microorganisms in the triple-species biofilms. Overall, it was possible to conclude that the interactions established in this triple consortium did not affect the outcome of the antimicrobial performance of the surfaces when challenged by all species at the same time.

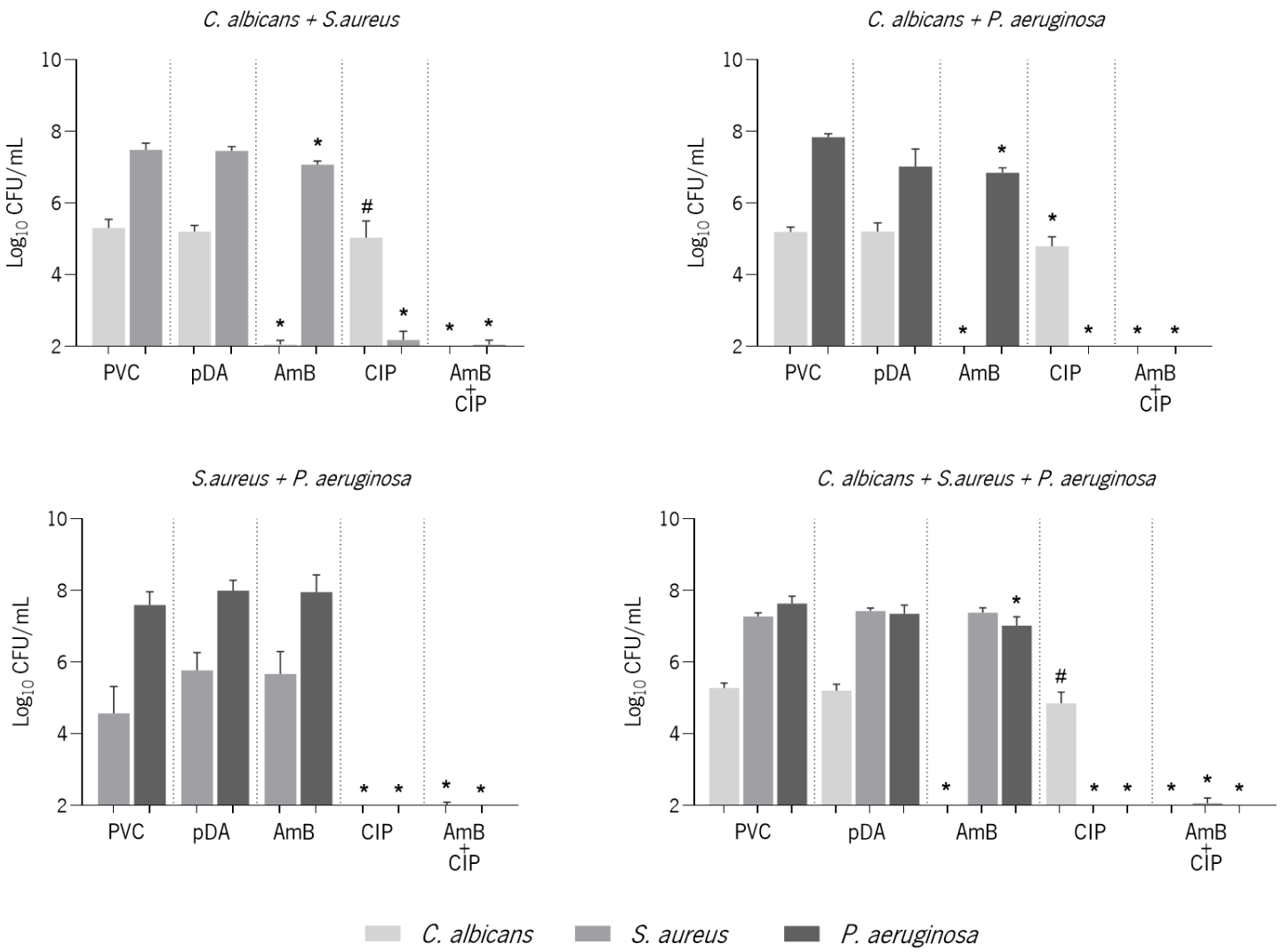

Figure 20. Antimicrobial performance of PVC surfaces functionalized with AmB (0.1 mg/mL) and/or CIP (0.5 mg/mL) against mixed-species biofilms, after a contact of 24 h. PVC was used as a control while pDA was included for comparison purposes. Significant differences were found for (*) $p < 0.0001$ and for (#) $p < 0.05$, when reductions were observed compared to cells adhered to PVC. The minimum value of the Y axis on the graphs was defined as 2 since it corresponds to the detection limit of CFU counting (log 2 CFU/mL).

To complement the previously established antimicrobial activity of the modified surfaces against mixed-species biofilms, an *in situ* evaluation was performed by fluorescence microscopy to inspect the presence of adhered cells. DAPI is a cell-permeable fluorescent compound that can stain the DNA of eukaryotic and prokaryotic cells. Therefore, its application allowed the differentiation of each microorganism based on their distinctive morphological characteristics. *C. albicans* exhibit different morphotypes at different stages: yeast, pseudohyphae, and hyphae [38]. The presence of hyphae allows the identification of the fungus and even when *C. albicans* presents the oval form, it can be distinguished from the bacterial cells because of their size: around 3-4 μm in diameter. *S. aureus* is spherically shaped and exhibits approximately 0.5-1.5 μm in diameter [38], and *P. aeruginosa* exhibits a rod-shaped about 1–5 μm long and 0.5–1.0 μm wide [39].

The observation of the microscopic images (Figure 21) pointed out a significant reduction in the number of cells adhered to the modified surfaces, compared to the control surfaces (bare PVC). However, for some conditions, in which no cultivable cells were detected (Figure 20), some cells were observed under the microscope. Although this discrepancy does not predict the viability of cells, these results should be considered.

In the case of *C. albicans* for all consortia tested, no cells were detected by CFU enumeration on surfaces functionalized with AmB alone or combined with CIP. However, some fungal cells could be observed for these conditions, when evaluated by fluorescence microscopy. Nevertheless, it is important to verify that these cells had lost their ability to form hyphae, as compared to control surfaces.

Concerning *S. aureus*, some cells were observed adhered to all modified surfaces, even in dual-species biofilms with *P. aeruginosa,* in which no CFU had been detected. In the case of *P. aeruginosa*, this was not observed on surfaces functionalized with CIP in the dual consortia with *C. albicans* or in the triple-species biofilm. Nevertheless, *P. aeruginosa* cells were visualized on surfaces modified with CIP when this bacterium was grown in the presence of *S. aureus.* In the case of surfaces co-functionalized with AmB and CIP, they were not able to completely prevent *P. aeruginosa* growth in all consortia studied.

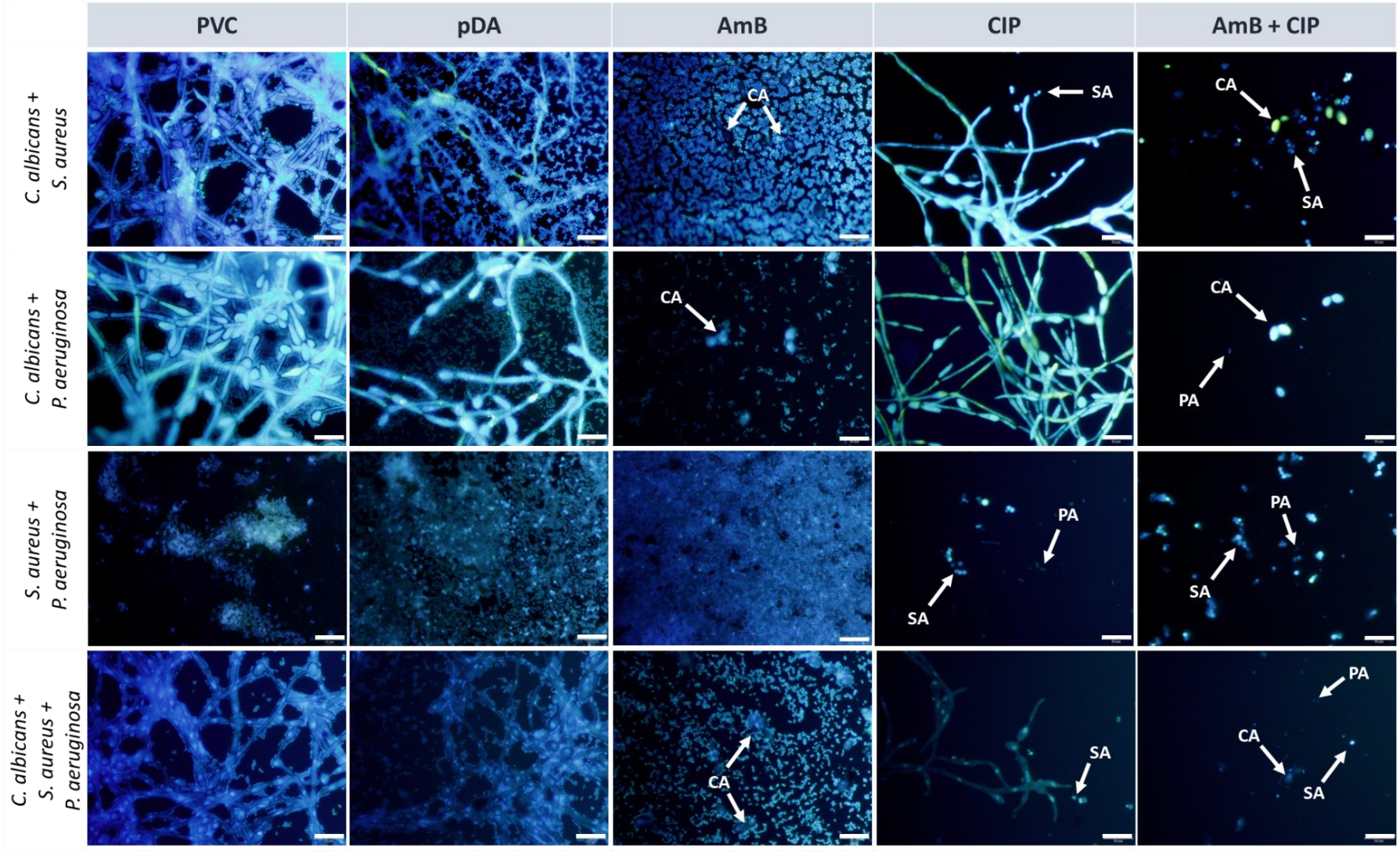

Figure 21. Representative fluorescent DAPI stained images of different consortia obtained after adhesion for 24 h on un- and modified PVC surfaces. The scale bars indicate 10 µm. CA: *C. albicans*; SA: *S. aureus*; PA: *P. aeruginosa*.

4.3.4 Cytotoxicity assay

An important point to strengthen the applicability of coating strategies relies on guaranteeing its non-cytotoxicity on human cells. Therefore, to predict the effects of the functional coatings on mammalian cells, the toxicity of the released compounds from the best antimicrobial coating strategy tested (co-immobilization of AmB and CIP at the concentrations of 0.1 and 0.5 mg/mL, respectively) was evaluated on lung epithelial cells and compared to PVC control surfaces. Results presented in Figure 22 showed that PVC surfaces before and after pDA coating did not exhibit any cytotoxicity effect. Further functionalization with AmB and CIP also did not compromise cells' metabolic activity, evidencing no toxicity to lung epithelial cells.

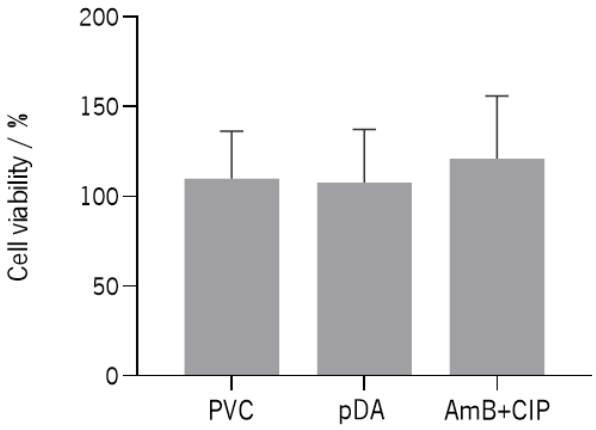

Figure 22. Viability of lung epithelial cells after indirect contact with un- and modified PVC surfaces.

4.4 Discussion

The use of the ETT is vital in some clinical situations when MV is required to provide proper ventilation. Even though MV is a life-saving procedure, it is also associated with some complications, presenting risks for patients, including the development of infection [40]. Indeed, the use of these devices is recognized as the major risk factor for the establishment of VAP [3]. Additionally, it is recognized that VAP is biofilm-mediated, exhibiting a polymicrobial nature with bacterial and fungal species often found associated with this infection [10,13].

The main purpose of the present work was to develop a broad-spectrum antimicrobial coating for further ETT application and, thus, reduce VAP occurrence. For that, the potential of different compounds to be used in PVC functionalization was inspected by assessing their antibacterial and/or antifungal activity. Firstly, the selected compounds were immobilized onto PVC surfaces, using a pDA-based approach. After an initial screening regarding its antimicrobial activity, the efficacy of its co-immobilization was also assessed to identify the best combination and the concentrations displaying the best performance.

The recent increase in bacterial tolerance and antibiotic resistance has encouraged the scientific community to research novel therapeutic approaches to treat microbial infections. Alternatives include the use of AMPs) QS inhibitors, enzymes, bacteriophage therapy and natural products with antimicrobial properties [41–43]. Particularly, the antimicrobial properties of natural products, such as essential oils and their components have been widely explored. Notably, essential oils are known for their antibacterial, antifungal, and insecticidal activities. Data reported that Gram-positive bacteria are more susceptible to the phenolic components of essential oils than Gram-negative bacteria since they act as membrane permeabilizers [44]. In this work, components from essential oils, namely, carvacrol, farnesol and linalool were tested to evaluate their potential to be used in surface functionalization.

Carvacrol, a phenolic monoterpenoid, is a constituent of essential oils produced by numerous aromatic plants and spices. This compound has shown antimicrobial and anti-biofilm activities against fungi and a wide range of Gram-positive and Gram-negative bacteria [45]. Moreover, carvacrol lends itself to being combined with nanomaterials [46], thus providing an opportunity for preventing biofilm-associated infections by developing anti-infective materials.

Farnesol belongs to the class of organic compounds known as sesquiterpenoids and is produced from isoprene compounds in both plants and animals. This component from essential oils is also known as a naturally occurring QS molecule of *C. albicans* that acts as a repressor of yeast morphological transition to hyphae [47,48]. Some studies have demonstrated its ability to affect a number of fungal and bacterial species, including *C. albicans, S. aureus* and *P. aeruginosa* [49–52]. Farnesol has been successfully used in a number of applications, including surface modification [53,54] .

Linalool is a terpene alcohol with a floral aroma that is commonly found as a component of the essential oils of several aromatic plants [55,56]. Its antimicrobial activity has been reported, in specific, against *C. albicans* and *P. aeruginosa* [57–59].

Chlorogenic acid and salicylic acid, two phenolic compounds that occur naturally, were also considered candidates to be applied to PVC. Chlorogenic acid is a phenolic compound widely found in fruits and vegetables. This compound acts against microbial cells by significantly increasing the outer and plasma

membrane permeability, resulting in the loss of barrier function. Chlorogenic acid exhibit antimicrobial activity against bacteria, including *S. aureus* [60–62]. Salicylic acid is an active secondary metabolite that occurs in bacteria, fungi, and plants. Their antimicrobial activity has been investigated against different bacterial species including *S. aureus* and *P. aeruginosa* [63–65]. This compound was recently used for the functionalization of a graphene surface [66].

The AMP camel and the acylase I enzyme were also included to evaluate their potential to be used on surface functionalization. Camel is a 15-residue hybrid peptide derived from the sequences of two insect peptides, cecropin A (isolated from the larvae of the silk moth *Hyalophora cecropia*) and melittin (isolated from honey bee venom). This hybrid peptide is more active than the native molecules and also lacks the undesirable haemolytic properties of melittin [67]. Some studies have reported promising *in vitro* activities of Camel and its analogues against anaerobic bacteria [68] and staphylococcal skin infections [69]. Acylase I belongs to the aminoacylase family of enzymes, and it is reported as a quorum-quenching enzyme interfering with the QS process in *P. aeruginosa* through AHL signals' hydrolysis. This enzyme has already been successfully applied in the coating of silicone catheters [70].

In addition to the previously mentioned compounds, synthetic drugs with known effects against the species under study were included to assess their effectiveness to be used for PVC coatings. Specifically, AmB, an antifungal agent used to treat fungal infections, including candidiasis, was chosen as a model antifungal agent. The antibiotics CIP and GM, that are used to treat a number of bacterial infections including those caused by *P. aeruginosa* and *S. aureus,* were also considered. All of them are on the World Health Organization's List of Essential Medicines. The World Health Organization classifies AmB, CIP and GM as critically important for human medicine. Moreover, their use has been explored in surface modification [36,71–74].

In the present study, it was intended to tailor a coating able to prevent microbial attachment to PVC surfaces but also to the surrounding environment in the bulk phase. This can be achieved with contact-killing and antimicrobial-releasing features. Moreover, although some antimicrobial targets are not intracellular and, thus, their immobilization should not compromise their antimicrobial action, others, that act intracellularly, need to be released from the surfaces to ensure their action. For instance, the fluoroquinolone antibiotic CIP must be internalized by the bacterium, since it acts by causing DNA damage by inhibiting DNA topoisomerases [75]. Therefore, to exhibit antibacterial activity, this compound requires to be released from the surfaces. GM also needs to be internalized by bacteria since its mechanism of action involves the inhibition of bacterial protein synthesis by binding to 30S ribosomes [76]. Taking together these requirements, and since it was intended to combine the most promising compounds, only

the coatings that displayed both contact-killing properties and antimicrobial leaching from the surfaces were considered for further studies.

Dopamine chemistry was used for antimicrobial immobilization and further release from the PVC surfaces. Previous works had demonstrated the potential of pDA-based approaches to rendering different types of material surfaces with antimicrobial properties, using different antimicrobials [30,36,73]. The present study confirmed that pDA chemistry allowed to effectively immobilize different antimicrobial compounds (i.e., AmB, carvacrol, CIP, farnesol, GM, and linalool) onto PVC surfaces. Moreover, the immobilization of AmB, CIP, farnesol, and GM resulted in antimicrobial and releasing surfaces. The coatings containing AmB impede the adhesion of *C. albicans* on PVC while CIP and GM immobilization inhibit simultaneously the adhesion of *S. aureus* and *P. aeruginosa*. Since both CIP and GM are antibiotics, CIP was chosen to pursue the investigations because it was more efficient against both bacterial strains.

Previous studies had demonstrated that AmB was successfully immobilized using this approach [36,72]. Additionally, other authors have also reported dopamine to coat surfaces with antibiotics, including CIP, vancomycin, and even polymyxins [73,77,78].

Regarding the anti-biofilm features of the generated surfaces, antimicrobial immobilization using a pDA-based strategy engendered coated PVC surfaces able to prevent both bacterial and fungal growth at different antimicrobial concentrations. This suggests its ability to prevent microbial contamination from the ETT surface if applied in a clinical context. When the antimicrobial compounds were mixed with dopamine to obtain a wide-spread coating, in general, its antimicrobial activity was maintained and the generated surfaces were able to resist the adhesion of *C. albicans*, *S. aureus,* and *P. aeruginosa* single-species biofilms. Antibiotic combinations with different modes of action can synergize for successful treatment allowing to use lower concentrations of drugs [79]. In this study, since both CIP and farnesol displayed antibacterial properties against *S. aureus*, it was hypothesized that CIP co-immobilized with farnesol would be more effective to prevent *S. aureus* biofilms compared to the applications of these compounds separately. This effect was reported in a previous study showing that the incorporation of farnesol significantly increases the efficacy of CIP against *P. aeruginosa* biofilms when both compounds were encapsulated in liposomes [80]. Other studies also demonstrated synergy between farnesol and vancomycin in the treatment of *S. aureus* [53] and between farnesol and GM [50], suggesting the potential of the application for farnesol as an adjuvant therapeutic agent for the prevention of biofilm-related infections. In the present work, this behaviour was also observed: farnesol apparently exhibits a synergetic effect with CIP against *S. aureus*. However, when these compounds were mixed with AmB to create a broad-spectrum surface, similar results were achieved to those obtained for surfaces co-functionalized

with AmB and CIP. Apparently, the synergism previously displayed between CIP and farnesol was not observed in the presence of AmB. This evidence, together with the point that the use of two compounds results in a more simple and economical approach, pave the way to the selection of the dual combination of 0.1 of AmB with 0.5 mg/mL of CIP as the best to pursue further research.

As previously mentioned, VAP presents a biofilm and polymicrobial nature. Currently, the treatment of this kind of infection poses serious concerns in the clinic arena. The combination of antimicrobials could be the option to counteract this complex challenge. Combined therapies of antibiotics with antifungal drugs, simultaneously or sequentially, are commonly adopted in clinical practice, although without full knowledge of the outcomes [81,82]. In this work, it was demonstrated that co-immobilization of AmB and CIP onto PVC did not affect the antimicrobial activity of the surfaces when challenged by single-species biofilms of *C. albicans*, *S. aureus* and *P. aeruginosa,* highlighting the potential application of this combination. Going further in the study of the efficacy of PVC surfaces co-functionalized with AmB and CIP, their capability to impair the adhesion of mixed-species biofilms was also assessed. The obtained results revealed that the selected coating was also efficient in preventing the adhesion of dual-species biofilms and even the triple one. This highlights the potential of this antimicrobial combination to render material surfaces with broad-spectrum antimicrobial activity.

To complement the aforementioned antimicrobial activity of modified surfaces against mixed-species biofilms, the *in situ* microscopic evaluation of adhered cells pointed out that, some cells were observed on the coupons. Once in most cases no cultivable cells were detected after 24 h of contact with the factionalized surfaces, the outcomes derived from the microscopic observations alerted to the need to validate the performance of the functionalized surfaces over time, aspect that will be researched and presented in the next chapter.

The physicochemical characterization of un- and modified PVC surfaces allowed to conclude that PVC was successfully coated with dopamine and subsequently functionalized with AmB and CIP. Additionally, the coatings imparted PVC surfaces with an altered homogeneously scattered morphology and varied wettability but similar smooth topography (low roughness) compared to bare PVC. AmB introduces the higher variabilities in terms of roughness and wettability. Indeed, AmB deposition rendered the most hydrophilic surfaces which can be related to the high roughness of those surfaces and the capability of AmB to perform hydrogen bonding in water. This hydrophilic nature observed for AmB-coated surfaces was previously reported [71].

It was also intended to determine the release profile of the antimicrobial compounds from the surfaces over time. Although the concentration of CIP was estimated at 24 h the antibiotic released from 24 to 48

h was not possible to quantify. For AmB, it could not be detected at any time-point analysed (24 and 48 h). Taking together these findings with the results of leaching properties, it was possible to infer that the release of AmB occurs at least until 24 h but at low concentrations probably below the limit detection of the apparatus. It is also important to note that the released concentration of this antifungal agent is enough to efficiently impede *C. albicans* growth as the amount of released CIP from the surfaces was efficient also to prevent bacterial growth on the surfaces until 24 h. It is also important to note that the concentration of both antimicrobial agents is much lower than those initially used for immobilization, as previously postulated.

Taking together the previous observations regarding the release of CIP and AmB with those observed by epifluorescence microscopy raises the question about the long-term performance of the surfaces.

Another important point that should be addressed in the design of coatings strategies to be applied in indwelling devices relies on guaranteeing no cytotoxicity. The generated surfaces in this work did not exhibit toxicity against lung epithelial cells, strengthening their promising character for human application.

In conclusion, overall results demonstrated the high potential of functional PVC surfaces to be applied on the ETT design since it displayed excellent antimicrobial activity by preventing polymicrobial interkingdom biofilm formation for a short-term application (until 24 h).

4.5 References

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CHAPTER 5

Long-term efficacy of functionalized PVC surfaces in co-infection scenarios

Anti-infective biomaterials have evolved in the last decades as a worthwhile solution to tackle device-related infections. Particularly, in the case of VAP, the modification of the ETT to reduce biofilm formation has been established itself as a very promising solution in the prevention of this infection. Yet, despite being effective for short-term application, most strategies lack the ability to prevent infections for medium and long-term extension. The long-term antibiofilm activity is the key narrowing in the advancement of modification technologies (e.g.: antimicrobial coatings). Some of the current antibiofilm coatings are short-lived, and microbial adhesion on freshly coated surfaces is not predictive of their long-term antibiofilm conduct. Thus, further approaches are needed to prevent biofilm formation and generate long-term activity against polymicrobial consortia. In this work, the antimicrobial performance of modified PVC surfaces was challenged for long-term applications focusing on polymicrobial inter-kingdom biofilms. Although the antimicrobial action of the co-functionalized PVC with AmB and CIP was strain-dependent, data revealed a high performance of the coatings against *C. albicans*, *S. aureus,* and *P. aeruginosa* in the triple consortia until 48 h. Longer application times show reduced efficacy after 72 h against *S. aureus* and after 5 days against both *P. aeruginosa* and *S. aureus,* but they still demonstrated inhibition activity against *C. albicans*. Bacterial cells recovered from the functionalized surfaces at the end of 5 days revealed an altered CIP tolerance profile that may be associated with CIP exposure. In the case of *S. aureus* this behaviour also seems to result from interaction with *P. aeruginosa*. However, the application of a single dose of CIP helped to boost the functionality of PVC surfaces, improving their broad-spectrum efficacy for long-term applications (until 5 days).

5.1 Brief Introduction

Since MV can be required for shorter periods, of a few hours, until more extended applications of several days of intubation, this premise should be considered when designing antimicrobial strategies for ETT applications. Some of the current studies involving the design of anti-infective materials demonstrate good anti-biofilm activity for short-term applications but their ability to prevent infections for longer applications was not tested. Indeed, some studies have demonstrated good applicability for shorter periods [1–5], however, nothing can be anticipated regarding their long-term antibiofilm behaviour. Indeed, in certain situations, some short-term antibiofilm coatings may instead function as microbial reservoirs over the longer term [6,7], which may compromise their use in healthcare. Moreover, the design of coatings for VAP must address the polymicrobial nature of this kind of infection [8], as argued previously. In effect, some studies carried out antimicrobial strategies only against bacterial strains as single-species biofilms and some even only considered one single species to demonstrate its effectiveness [9–14].

Driven by these gaps related to current investigations, the aim of this study was to challenge the performance of the antimicrobial coating settled in Chapter 4 for long-term applications (up to 5 days) targeting inter-kingdom polymicrobial biofilms.

Since the use of antimicrobials can have impact in the evolution and dissemination of resistance and tolerance mechanisms, this issue was also appraised in this study.

5.2 Material and Methods

5.2.1 Microbial strains and culture conditions

P. aeruginosa PAO1, *S. aureus* ATCC 25923 and *C. albicans* SC5314 were used throughout this work. All strains were preserved and cultured as described in section 4.2.1.

5.2.2 Antimicrobials

AmB and CIP were used throughout this work. Aliquots of AmB were prepared using DMSO. CIP was dissolved in sterile distilled water with HCl (0.2 M) except in functionalization assays, in which CIP was directly dissolved in the work solutions. Storage was performed according to the manufacturer's instructions.

5.2.3 Polyvinyl chloride preparation and functionalization

PVC preparation and functionalization were performed as described in section 4.2.3.

5.2.4 Antimicrobial performance of the modified surfaces against mixed-species biofilms

The antimicrobial performance of modified PVC surfaces was evaluated against polymicrobial communities as described in section 4.2.4.3. After biofilm formation, plates were incubated at 37 °C for 24, 48, 72, or 120 h, depending on the assay. For the experiments with incubation times longer than 24 h, the bulk liquid was removed and replaced by fresh medium every 24 h. After that, cells adhered to surfaces were collected and analyzed as described in section 4.2.4.3.

5.2.5 Determination of minimal inhibitory and bactericidal/ fungicidal concentrations

The minimal inhibitory (MIC) and bactericidal (MBC)/fungicidal (MFC) concentrations of CIP and AmB, respectively, were determined using the microdilution method, with minor modifications. Briefly, the wells of sterile 96-well round-bottom microtiter plates (Orange, USA) were filled with 100 μL of MHB or RPMI with increasing concentrations of CIP or AmB, respectively, to which 100 μL of each microbial inoculum (adjusted to a final concentration of 5×10^5 CFU/mL) were added. For MBC and MFC determination using a triple-species suspension, the inoculums were mixed at 1:1:1, after being adjusted to the final concentration of 5×10^5 CFU/mL (in RPMI). The plates were afterwards incubated at 37 °C for 24 h. In this experiment, two controls were used: culture media without bacteria, as a negative control, and bacterial suspension growth without antimicrobials, as a positive control. Moreover, culture media with increasing concentrations of antimicrobials without cells were also performed to guarantee that any increase in the absorbance of media was only caused by microbial growth. The MIC was obtained by measuring the absorbance at 620 nm (A620nm), in which clear wells (A620nm – A620nm of negative control) were evidence of microbial growth inhibition. MBC/MFC determination was performed by adding a droplet of 10 μL from each well with no visible growth on agar plates. MHA and SDA were used for bacterial strains or *C. albicans*, respectively, in single-species suspensions. To discriminate the cells from triple-suspensions, PIA, MSA and SDA supplemented with gentamicin (60 mg/L) were used to isolate *P. aeruginosa*, *S. aureus* and *C. albicans*, respectively. The lowest concentration that yielded no colony growth after 24 h at 37 °C was identified as the MBC/MFC. Three independent assays with three replicates for each condition were performed.

5.2.6 Evaluation of modified surfaces' ability to induce microbial resistance

To assess the eventual development of resistance towards the antimicrobials immobilized on PVC surfaces, microbial cells were allowed to attach to the functionalized surfaces for 5 days, and at the end of which they were harvested and their susceptibility to CIP determined. Briefly, after 5 days of incubation with the triple-species consortia, the coupons were washed twice with PBS to remove free-floating cells and transferred to new wells filled with 1 mL of PBS. Adhered cells were removed by ultrasonic bath followed by scrapping and used for MIC and MBC determination. To accomplish it, 100 μL of the resulting microbial suspension were placed in 96-well round bottom microtiter plates which had been filled with increasing concentrations of CIP diluted in RPMI. The plates were incubated for 24 h aerobically, at 37 °C. MIC and MBC were determined as previously described. For comparison, microbial cells attached to PVC and pDA were used as control samples. Five independent assays with three replicates were performed.

5.2.7 Susceptibility of adhered cells to CIP chemotherapy

The susceptibility of some cells that adhered to surfaces co-functionalized with CIP and AmB to CIP chemotherapy was evaluated. For that, triple-species adhesion for 48 h was performed as aforementioned. After that, microbial suspensions were removed, and 1 mL of CIP (0.5 μg/mL, in RPMI) was added to each well. The plates were incubated for 24 h at 37 °C, under agitation. CIP solution was then removed and replaced with fresh medium for more 48 h. At the end of the 5 days, the adhesion of cells on the surfaces was evaluated by CFU enumerating as aforementioned. Four independent experiments in triplicate were performed.

5.2.8 Statistical analysis

Results were presented as mean ± standard deviation (SD). Statistical analysis was performed by using the Prism software package (GraphPad Software version 9.0.0). One-way ANOVA tests were performed, and means were compared by applying Tukey's multiple comparison test. In all the analyses performed, the confidence interval used was 95%.

5.3 Results

5.3.1 Antimicrobial performance of modified surfaces against mixed-species biofilms

Considering the notable results in terms of anti-biofilm activity achieved until 24 h, shown in Chapter 4, it was intended to boost the worth of the previously tailored coating. For that, the long-term efficacy of the co-functionalized PVC was evaluated a assessing its antimicrobial performance for 5 days using polymicrobial consortia (Figure 23).

In the case of the *C. albicans* and *S. aureus* dual-species biofilms, the antimicrobial performance of the surfaces coated with AmB was maintained against *C. albicans* (Figure 23) Functionalized surfaces with CIP were not as efficient in preventing *S. aureus* growth for 5 days (Figure 23) as achieved at 24 h (Figure 20), as only 1.8 log CFU/mL reduction was noticed at 5 days conversely to 5.3 log CFU/mL reduction observed at 24 h (compared to respective PVC controls). For the surfaces co-functionalized with AmB and CIP, although the number of *C. albicans* cells remains lower than the control (approximately 3.6 log CFU/mL less), an increase in its growth was also observed at the end of 5 days comparing the same condition at 24 h. This loss of antimicrobial effectiveness was more pronounced in the case of *S. aureus* since there was only a reduction of about 0.9 log CFU/mL compared to the control.

Regarding *C. albicans* and *P. aeruginosa* dual-species biofilms, the noticeable antimicrobial performance of the surfaces functionalized with individual AmB and CIP, observed at 24 h, was preserved after 5 days. On the other hand, the co-functionalized surfaces (AmB+CIP) were not as efficient to prevent microbial growth as at 24 h. *C. albicans* and *P. aeruginosa* were able to recover their growth reaching 3.9 and 5.7 log CFU/mL, respectively, at the end of the 5 days.

Regarding *S. aureus* and *P. aeruginosa* dual-species biofilms, an increase in the number of cells adhered to the surfaces coated with CIP was observed compared to 24 h results. It was more pronounced for *S. aureus*, which achieves 6.7 log CFU/mL, while *P. aeruginosa* growth reaches approximately 4.5 log CFU/mL.

When testing the triple-species biofilms, the antimicrobial activity of the modified surfaces with AmB was maintained, preventing *C. albicans* growth after 5 days. In the case of surfaces functionalized with CIP, some growth was observed for both bacteria. Indeed, *P. aeruginosa* has reached 4.7 log CFU/mL, however, some antimicrobial activity was still verified since the growth of this bacteria on these surfaces was 2 log CFU/mL lower compared with the PVC control. Concerning *S. aureus*, results showed the adhesion of 4.8 log CFU/mL after 5 days. The growth of this bacterium on PVC was very low as in the case of pDA surfaces, strengthening the previously postulated idea that *P. aeruginosa* exerts an inhibitory

effect on *S. aureus* growth, effect that takes more time to be noticed when *S. aureus* develops in triple-species biofilms. Unexpectedly, that inhibitory effect was not noticeable when the surfaces are functionalized with CIP.

It is also important to note that microbial growth, in general, presents more variability at the end of 5 days compared to 24 h data.

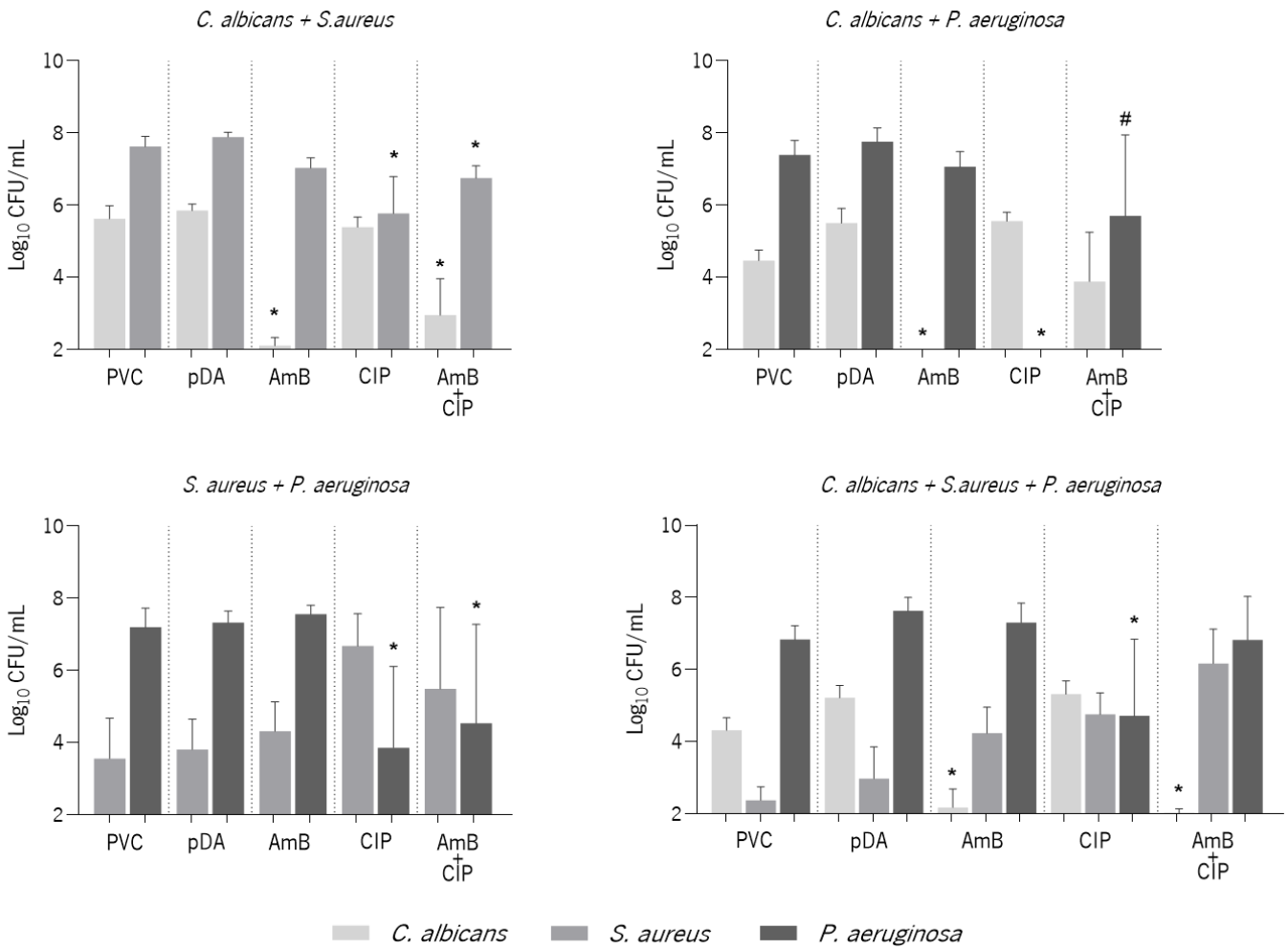

Figure 23. Antimicrobial performance of functionalized surfaces against mixed-specie biofilms after 5 days of incubation. PVC was included as a control while pDA was used for comparison purposes. Significant differences were found for (*) $p < 0.0001$ and for (#) $p < 0.05$, when reductions were observed compared to cells adhered to PVC. The minimum value of the Y axis on the graphs was defined as 2 since it corresponds to the detection limit of CFU counting (log 2 CFU/mL).

Results obtained by microscopy observation (Chapter 4) and those obtained by CFU counting after 5 days of microbial adhesion suggest that some of the cells remaining on the PVC coupons, observed by microscopy at 24 h, were viable although not cultivable but able to grow when fresh media was added for a longer period. On the other hand, the microbial adhesion observed at the end of 5 days may also be due to the existence of some viable cells attached to the walls of the microtiter plates containing the coupons that can detach over time, adhere to the surfaces and initiate biofilm formation.

Since the ultimate goal is to develop surfaces able to tackle the adhesion of all the microbial species under study, the triple-species adhesion to surfaces over the 5 days was instigated in more detail. Taking into account that at the end of that time, the co-functionalized surfaces only showed clear antimicrobial effect against *C. albicans*, it was intended to understand, in the 5-days frame, until what period of time the effectiveness of the coatings manifested itself for all species at the same time. For that, the antimicrobial performance of the surfaces was inspected at 48 and 72 h (Figure 24). Data allowed to conclude that all modified surfaces (i.e. with AmB, CIP and AmB+CIP) were efficient at 48 h. Functionalized surfaces with AmB were able to prevent the adhesion and growth of *C. albicans*, while the surfaces coated with CIP were effective by reducing the adhesion of *S. aureus* and *P. aeruginosa*, 3 and 6 log CFU/mL respectively, when compared with the PVC control. The co-functionalized surfaces were also efficient in preventing microbial growth, especially for *C. albicans* and *P. aeruginosa*, being more modest for *S. aureus* as only around 2.8 log CFU/mL reduction was noticed. Regarding the results at 72 h, the efficacy of the surfaces was similar to those observed at 48 h except for the co-functionalized surfaces (AmB+CIP) against *S. aureus*, since this bacterium achieved 6.7 log CFU/mL.

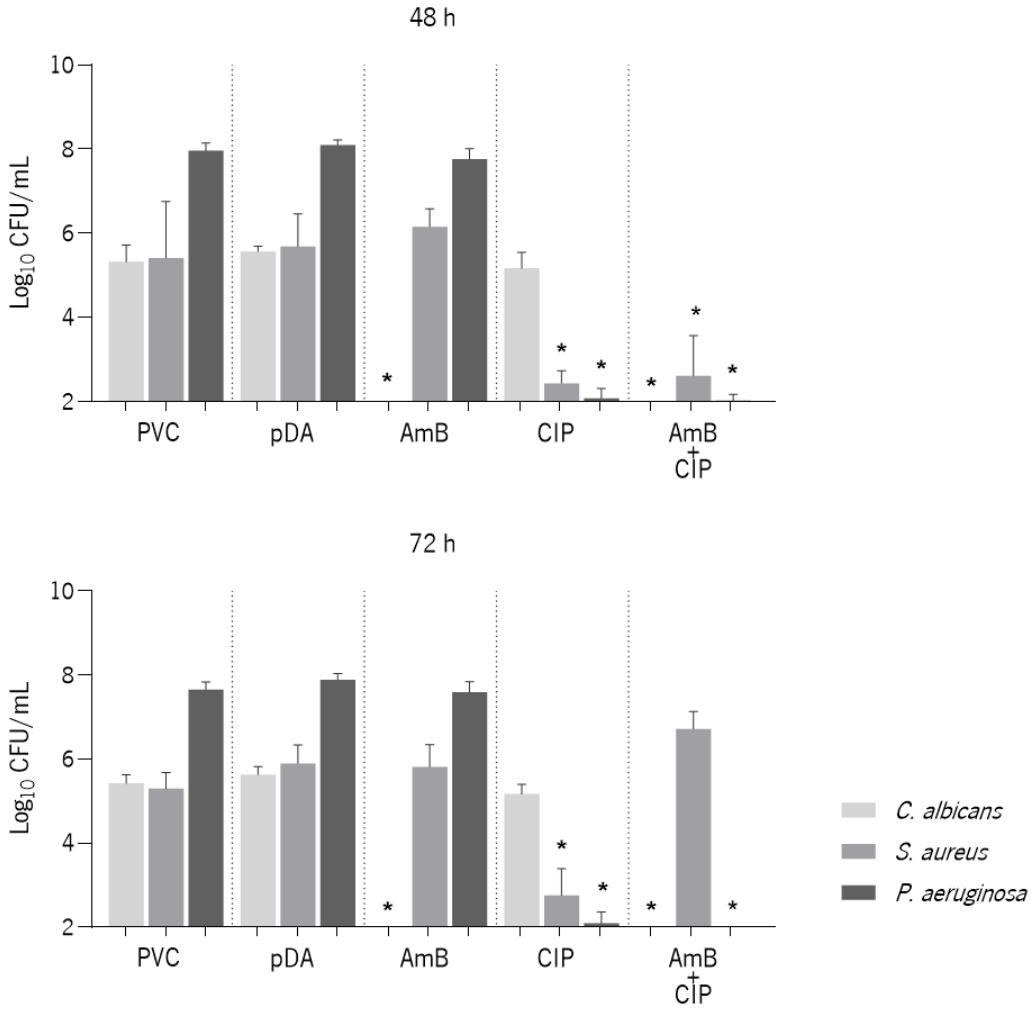

Figure 24. Antimicrobial performance of the modified surfaces against triple-specie biofilms after a contact of 48 and 72 h. PVC was included as control while pDA was used for comparison purposes. Significant differences were found for (*) $p < 0.0001$, when reductions were observed compared to cells adhered to PVC. The minimum value of the Y axis on the graphs was defined as 2 since it corresponds to the detection limit of CFU counting (log 2 CFU/mL).

5.3.2 Susceptibility pattern of planktonic and adhered cells to modified surfaces

Once identified the time point at which the coating is not able to prevent simultaneously the adhesion of all species, the possible development of resistance by the cells that managed to still adhered on the surfaces for up to 5 days was assessed. The susceptibility of planktonic cells was also evaluated for comparison purposes.

The values of MIC and MFC of AmB towards *C. albicans* and the MIC and MBC of CIP for *S. aureus* and *P. aeruginosa*, grown separately, were determined. For comparison purposes, the MBC and MFC were also evaluated when the microorganisms were placed together.

Results showed that the susceptibility of *S. aureus* and *P. aeruginosa* adhered cells to CIP was not influenced by the presence of cells from the other strain since equal MBC values were determined for single and triple-species consortia (Table 10). Regarding *C. albicans,* MFC values are slightly lower when the fungal cells developed in the triple consortia. This suggests that the microbial interactions established with bacterial cells induced some susceptibility of *C. albicans* to the AmB action.

In the case of triple-species consortia, the value of MIC could not be determined since CIP does not inhibit *C. albicans* growth and AmB does not inhibit *S. aureus* or *P. aeruginosa* growth.

Table 10. MIC and MBC values of AmB and CIP against fungal and bacterial cells, respectively. MBC/ MFC values were determined for of each one of the microorganisms (single consortia) and then when the species were placed together (triple consortia). MIC and MBC values are expressed in μg/mL. The MIC values that were not possible to be determined (triple consortia) were listed as "ND".

Condition	*C. albicans*		*S. aureus*		*P. aeruginosa*	
	MIC	MFC	MIC	MBC	MIC	MBC
Single consortia	0.25	0.25-0.5	0.5	1	0.25	0.25
Triple consortia	ND	0.125-0.25	ND	1	ND	0.25

Since *S. aureus* and *P. aeruginosa* cells were founded after 5 days of contact to the functionalized surfaces with CIP and co-functionalized with AmB+CIP, the potential development of resistance towards CIP was assessed. For that, the cells adhered to PVC and to the several modified surfaces were collected after 5 days of exposure and the MBC of CIP determined (Table 11). Results showed that the MBC values exhibited variability as evidenced by the range of values obtained, especially for *S. aureus*. Such results may be attributed to the presence or not of all members in the consortia during the 5 days of exposure. For instance, in the case of *S. aureus* cells adhered on modified surfaces with CIP, the variability was particularly noted since the lowest values of MBC were found when no *P. aeruginosa* was detected by CFU enumeration after 5 days. When both bacteria were detected, the MBC values for *S. aureus* were higher, raising the hypothesis that the variabilities could be related to microbial interactions. Indeed, an increased tolerance of *S. aureus* to CIP in the presence of *P. aeruginosa* was previously reported [15]. Additionally, it can also be due to the initial cells concentration that was not adjusted to a defined value since the whole cells were immediately used after being recovered from the surfaces.

Data also highlighted that both *S. aureus* and *P. aeruginosa* cells recovered from surfaces functionalized with AmB exhibited a similar susceptibility pattern as compared to cells in contact with the control surfaces (PVC and pDA), i.e., with no antimicrobials immobilized, which is indicative of no tolerance development. On the other hand, microbial cells recovered from surfaces functionalized with the antibiotic, alone or combined with AmB, presented higher values of MBC, an indication of tolerance development towards CIP. For *S. aureus*, a 2-fold and a 4-fold increase on MBC was found for cells recovered from surfaces functionalized with CIP or AmB+CIP, respectively. Concerning *P. aeruginosa*, a 4-fold and a 2-fold increase on MBC was observed for cells recovered from surfaces modified with CIP and AmB+CIP, respectively, when compared to the cells from PVC.

Table 11. MBC values of CIP against *S. aureus* and *P. aeruginosa* cells recovered after 5 days of exposure to different surfaces. MBC values are expressed in μg/mL.

Surface	*S. aureus*	*P. aeruginosa*
PVC	4-16	0.5-1
pDA	4-16	0.5-1
AmB	8-16	0.5-1
CIP	1-32	2-4
AmB+CIP	16-64	1-2

5.3.3 Susceptibility of adhered cells to CIP chemotherapy

Since the co-functionalized surfaces were not able to prevent the adhesion of all species at 72 h (Figure 24), the possibility of complementing the use of these surfaces with an additional treatment at 48 h, aiming to lengthen their efficacy throughout the 5 days was tested. Besides, with this strategy it was also intended to prevent the development of tolerance towards CIP as observed for bacterial cells recovered at the end of the 5 days from CIP and AmB+CIP functionalized surfaces (Table 11). Therefore, an additional treatment with 0.5 μg/mL of CIP was applied 48 h after initiating biofilm formation. This concentration was selected based on the results of the susceptibility pattern (Table 10) being the lowest MIC value of the antibiotic able to simultaneously inhibit both bacteria. After 24 h of treatment application, the CIP solution was withdrawn and replaced with fresh medium. At the end of the 5 days, the antimicrobial efficacy of the surfaces was evaluated by CFU enumeration. Figure 25 disclosed that the single application of CIP improved the behaviour of the surfaces within the 5-days frame. Besides the

anti-adhesive effect for *C. albicans* previously observed, the surfaces co-functionalized with AmB and CIP were also efficient in preventing *P. aeruginosa* adhesion and growth until 5 days, when CIP was applied at 48 h. In the case of *S. aureus*, although some variability was noticed, there are still some cells that were able to resist, even after being subjected to CIP additional treatment. Nevertheless, its growth was compromised, and this bacterium lost some capability to grow when compared to results obtained without the CIP application (Figure 23) (3.6 and 6.2 log CFU/mL, respectively). It is important to note that the application of 0.5 µg/mL CIP at 48 h on PVC and pDA surfaces did not have a significant effect on the adhered cells when compared to pDA and PVC at day 5 and without CIP application (Figure 23). These findings suggest a synergistic effect between modified surfaces and antibiotic therapy.

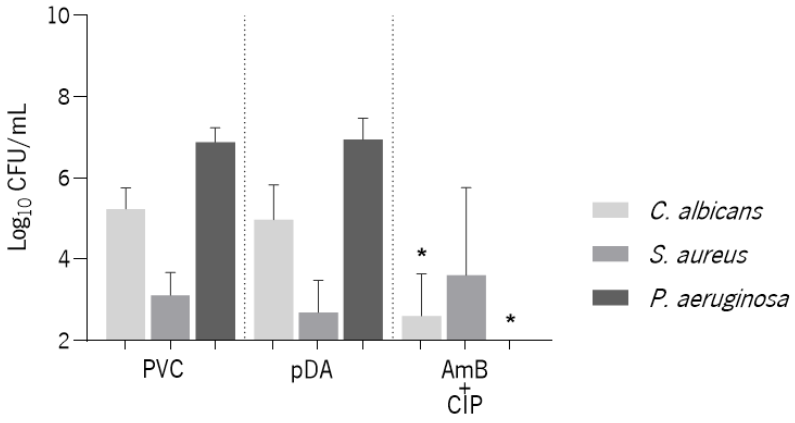

Figure 25. Antimicrobial performance of co-functional PVC surfaces against triple-species biofilms after 5 days of incubation with an application of 0.5 ug/mL CIP at 48 h (for 24 h). PVC was included as a control while pDA was used for comparison purposes. Significant differences were found for (*) $p < 0.0001$, when reductions were observed compared to cells adhered to PVC. The minimum value of the Y axis on the graphs was defined as 2 since it corresponds to the detection limit of CFU counting (log 2 CFU/mL).

5.4 Discussion

One important feature to reinvigorate the applicability of anti-infective biomaterials is their ability to prevent infections for long-term applications. This particularity has been neglected in some current investigations [1,4,5]. The capability of modified materials to generate long-term activity against polymicrobial inter-kingdom biofilms is particularly required. Most of the recent studies related to materials modification still neglected this important aspect and some of them only consider one single species to demonstrate its efficacy [11–13]. In this study, it was intended to fully address these traits to better tailor an antimicrobial coating strategy.

The potential of the co-functionalized PVC surfaces to resist the adhesion of mixed-species biofilms (i.e. dual and triple-species) was inspected and discussed in Chapter 4. The results revealed that the previously selected compounds (AmB and CIP co-immobilized at 0.1 and 0.5 mg/mL) were very efficient in preventing the adhesion and formation of dual-species biofilms and even the triple one up to 24 h. Also in Chapter 4, the microscopic observations and the released profile of the antimicrobial compounds from the surfaces raised some doubts regarding the performance of these coatings for longer applications. Thus, to gain knowledge helping to clarify that worries, a long-term study assessing the feasibility of the previous optimized coatings was performed. Accordingly, the antimicrobial ability of the functionalized PVC was challenged for 5 days against mixed-species biofilms. Results revealed that, after that period, some cells recovered their growth in dual-species biofilms. In the triple-species biofilms, after 5 days, the surfaces were only efficient in impairing fungal attachment. The loss of effectiveness of the surfaces for longer applications might be due to the presence of tolerant cells able to resist the action of the initial amount of antimicrobials released from the surfaces, suggesting that the very small number of cells that had been detected by CFU (in the case of *S. aureus*) were then able to grow. Additionally, the possibility of some cells, not detected by CFU after 24 h of adhesion, are in a VBNC state may also count towards the observed effect. Indeed, the VBNC state is known in *S. aureus* and can be induced due to external stresses like the use of antibiotics [16,17]. Furthermore, *S. aureus* can also exhibit this VBNC state in the presence of *P. aeruginosa*, a bacterial strategy adopted to survive in dual-species biofilms [18]. This cellular state of VBNC was also reported for *P. aeruginosa* [19]. Another reason helping to explain these results can be the presence of viable cells attached to the walls of the microtiter plates containing the coupons which initially didn't adhere onto the PVC but, due to the exhaustion of antimicrobial compounds from the surfaces, can then later attach to PVC and proliferate.

To gain knowledge that helps to understand these results, the susceptibility pattern of the cells in the triple consortia that manage to adhere on functional surfaces was evaluated. After 5 days, the bacterial cells remaining on the PVC surfaces were recovered, and the MBC of CIP was determined. MFC was not determined as the use of AmB to functionalize PVC rendered surfaces with antifungal effectiveness in the 5 days' period. Unmodified PVC and pDA were included as control surfaces. Results showed that both *S. aureus* and *P. aeruginosa* cells recovered from surfaces functionalized with AmB do not exhibit tolerance development to CIP. On the other hand, both *S. aureus* and *P. aeruginosa* cells that were able to adhere on surfaces functionalized with CIP and AmB+CIP seem to have developed tolerance towards CIP. The development of resistance by bacterial cells after exposure to antibiotics is well elucidated. This phenomenon has been documented for *P. aeruginosa* after exposure to CIP. Su and colleagues reported

a multistage process by which *P. aeruginosa* acquires drug resistance following exposure to CIP at different levels ranging from 0.5 to 8 fold the initial MIC [20]. Other studies have also reported the development of resistance to CIP by *P. aeruginosa* when exposed to both inhibitory and sub-inhibitory concentrations [21,22]. In the case of *S. aureus*, this bacterium is known for its high-resistance frequency to different classes of antibiotics, including CIP [23]. Additionally, in this work, the increased tolerance to CIP seems to be higher when *P. aeruginosa* also appears with *S. aureus* on modified surfaces containing CIP. This behaviour of *S. aureus* in the presence of *P. aeruginosa* was previously reported [15]. Taking all together, it was possible to postulate that the increased tolerance noticed for *S. aureus* might be the corollary of the combination of several factors. To overcome these findings, measures should be considered to guarantee the complete eradication of cells.

Going further with an in-deep analysis regarding the effectiveness of the co-functionalized PVC surfaces with AmB and CIP for all species over time, their antimicrobial performance was inspected at increasing times of 24 h. It was noted that the co-functionalized PVC surfaces were very efficient in preventing the adhesion of all species until 48 h. After 72 h of initial inoculation, the antimicrobial activity of the modified PVC surfaces was maintained against *C. albicans* and *P. aeruginosa* but not for *S. aureus*. Attempting to enhance the use of the modified surfaces as long as possible and avoid the development of CIP tolerance, a strategy encompassing an additional treatment to complement the action of the coatings was plotted. For that, CIP at 0.5 µg/mL was applied at 48 h for 24 h and the ability of the surfaces to impair adhesion was newly inspected after 5 days. CIP application at 48 h combined with the action of the functionalized surfaces apparently displayed synergistic activity as *P. aeruginosa* attachment was inhibited in addition to *C. albicans*. For *S. aureus,* although eradication was not achieved, the growth of this bacterium was affected mainly when compared to the growth achieved after 5 days without CIP application This approach can be reproducible in a real scenario with the administration of an antibiotic. In this study, a single application of CIP was tested but an antibiotic prescription includes taking several dosages periodically meaning that better results could be achieved in a clinical situation requiring MV for long term. Moreover, the role of the immune system cannot be disregarded as it also acts in the infection process. This latter premise underlines the importance of strengthening the feasibility of the here proposed functionalized PVC surfaces by considering the immune system as an important player in the process of fighting infection associated with devices, aspect that will be one of the next steps in our research.

Overall, our results showed high performance of the co-functionalized PVC surfaces (AmB+CIP) against *C. albicans*, *S. aureus,* and *P. aeruginosa* evolving as triple consortia until 48 h. Furthermore, the

application of a single dose of CIP chemotherapy enhanced the antimicrobial functionality of the PVC-coated surfaces, improving their broad-spectrum efficacy for long-term applications (until 5 days).

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CHAPTER 6

Concluding remarks and future work

This chapter describes the main findings and conclusions drawn from the work performed under the subject of this PhD project as well as some suggestions for future research.

6.1 Conclusions

Due to the growing use of medical devices to support or restore human body function, the incidence of BAI has also steadily increased. Despite the efforts currently used in the clinic to prevent these infections, it remains an unsolved clinical challenge. As reviewed in Chapter 2, the ETT is a medical device that greatly contributes to the development of a specific BAI named VAP, and for which antimicrobial coatings have been widely explored. Several strategies achieving considerable advances and encouraging results have been proposed in the last years; however, some gaps in these approaches have been acknowledged as narrow-spectrum antimicrobial efficacy and absence or unproved long-term antimicrobial activity.

Attempting to overcome those issues, the main goal of this PhD project was to engineer an effective wide-spectrum antimicrobial coating for ETT able to prevent microbial adhesion and biofilm formation, while ensuring no toxicity towards mammalian cells. Given that polymicrobial nature is an important aspect of VAP, it was also intended to assess the performance of the coating against polymicrobial consortia and thus infer whether the interactions taking place in these communities contribute to the (in)success of the coating. Furthermore, a deeper analysis and network reconstruction of existing data on inter-species interactions between *P. aeruginosa* and *C. albicans* was envisioned to better comprehend this consortium.

The inspection of the microbial interactions between *P. aeruginosa* and *C. albicans*, as detailed in Chapter 3, allowed the identification of a vast set of interactions and different behaviours reported for this consortium, including contradictory mechanisms of interactions. This information provided insights of how established interactions are undoubtedly associated with different aspects, importantly the surrounding environment, which includes external stresses to which microorganisms are exposed. This phenomenon is also quite clear in the interactions between *P. aeruginosa* and *S. aureus*, as discussed in Chapter 2.

Considering these dilemmas, it is clear the need for careful and meticulous analysis when we are designing therapies to tackle mixed-species biofilms, and this becomes even more critical the more complex the consortium under study is. In fact, there has been increasing attention in this regard over the years and the number of studies that include mixed-species biofilms has also been expanding; however, the majority of the investigations are focused on dual-species biofilms, with studies concerning triple-species interactions, as performed in this work, being scarce.

In Chapter 4, a preliminary screening using a group of different antimicrobial compounds was performed by determining the contact-killing and leaching properties patterns against all species under study. After that, the most promising agents, which displayed the best antimicrobial activity after immobilization on

PVC, were selected to pursue the investigation. Prior to co-immobilization, their single immobilization was optimized in terms of antimicrobial concentration. Dopamine chemistry was successfully applied to immobilize different antimicrobial compounds on PVC surfaces without compromising their action. Moreover, the coatings containing CIP displayed good inhibition activity against *P. aeruginosa* and *S. aureus* single-species biofilms, while those containing AmB showed good activity by preventing *C. albicans* biofilm formation. Co-immobilization of these agents rendered PVC surfaces with broad-spectrum antimicrobial activity able to resist the adhesion of single, dual, and even triple-species biofilms composed of *P. aeruginosa*, *C. albicans*, and *S. aureus*. This immobilization strategy also resulted in a functional PVC surface with biocompatible features, with no toxicity demonstrated against lung epithelial cells. Furthermore, a physicochemical characterization of the generated surfaces showed that the immobilization process rendered them with different properties, regarding their morphology, roughness, and wettability attributes, and confirmed the deposition of pDA and subsequent immobilization of CIP and/or AmB onto PVC.

Further results regarding the long-term stability of the modified surfaces predicts their good performance in real-life scenarios since they were able to prevent microbial adhesion and biofilm formation up to 48 h, using the triple-species biofilm model. For longer applications periods, exogenous antimicrobial treatment helped to ensure the success of the functionalized PVC. More specifically, the application of a single dose of CIP chemotherapy appeared to synergise with the functional PVC surfaces improving their broad-spectrum efficacy up to 5 days (Chapter 5).

Overall, the work conducted throughout this PhD project reassures that mussel-inspired surface modification is a simple and useful approach in the research of antimicrobial surfaces by allowing the co-immobilization of an antibiotic with an antifungal to develop a functional coating. PVC was successfully imparted with both anti-adhesive and antimicrobial properties against bacteria and a fungus grown as single, dual, and triple species biofilms and showed no cytotoxicity. Accordingly, this strategy proved to be promising to be further applied in ETT design and consequently be used in the fight against VAP.

6.2 Future work

Although the findings in this thesis have highlighted the great potential of the co-immobilization of CIP and AmB onto PVC to prevent VAP, additional studies should be conducted to confirm the applicability of the developed coatings.

When inserted in the body, the ETT comes in contact with biological fluids, mainly saliva. Thus, it would be appropriate to evaluate the stability of the coatings and their effectiveness after being exposed to these biological fluids. In order to boost the applicability of the modified surface, it would be profitable to assess its effectiveness against other microbial species and also consider the use of clinical isolates. Future studies using dynamic infection models are also suitable to estimate the performance of the coatings *in vivo* since they better mimic a real infection situation. It would also be appropriate to check the application of the coating on endotracheal tubes and find out whether the antimicrobial effect is maintained, hence predicting its performance *in vivo*. Although *in vitro* studies of both anti-adhesive and anti-biofilm activities have been performed in this project, the anti-infective potential of the functional coating needs to be evaluated *in vivo* to confirm its clinical applicability. As such, animal and clinical studies are warranted as a last step to determine whether coated ETTs can significantly reduce the risk of VAP.

To obtain a more robust chemical characterization of the functionalized surfaces, it would be useful to use complementary techniques to the ones used in this work, such as X-ray photoelectron spectroscopy. While the purpose of this project was to establish a proof of concept of the direct immobilization of AmB and CIP using pDA, further work can be done to improve the conjugation efficacy and improve the density of the surface-bound antimicrobials. This can be a useful strategy to extend the useful life of the coating, without resorting to additional treatments. Also, using nanocarrier-entrapped drugs, such as in liposomes, instead of free antimicrobial agents could be an alternative to obtain a sustained and controlled drug release over time.

To overcome the limitations encountered in the determination of the amount of compound released over time, probably due to the detection limit of the device used, the use of more sensitive techniques, such as high-performance liquid chromatography, could be a feasible alternative. Moreover, it would also be interesting to use an antibiotic of a different class as an additional treatment to improve the performance against *S. aureus*, since it a small number of these bacterial cells were observed after 5 days with the additional application of CIP. Since bacterial cells such as *P. aeruginosa* and *S. aureus* have a remarkable capacity to resist antibiotics, it is common in clinical practices to use combined therapies to reduce the risk of development of drug resistance and improve clinical outcome. Likewise, in this case, it would be appropriate to screen for an antimicrobial that demonstrates synergy with the surface. It would also be

suitable to evaluate the susceptibility profile of *S. aureus* cells that were able to withstand on the surfaces even when CIP was additionally applied at 48 h. Finally, it would also be interesting to check for possible differences at a molecular level (e.g. expression of genes associated to resistance and virulence). This could result in an interesting contribution, giving clues on how to better adapt the therapy to be applied and guarantee its success.

In terms of polymicrobial interactions and the effect they produce at the therapeutic level, it would be important to examine in more detail what underlies the changes during co-infections. Since, in this study, these effects were only evaluated at the cellular level, it would be important to verify what happens at the molecular level by performing gene expression analysis using PCR techniques and/or RNA sequencing. Further characterization of the intricate interaction between these pathogens at the molecular level is warranted and could allow us to better understand the triple interactions and verify how behaviour differs when we analyse single and dual versus triple consortia. Ultimately, this analysis may aid in designing novel therapeutic strategies to combat fungal–bacterial polymicrobial infection.