Universidade do Minho
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outubro de 2015

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e co-orientação da
Doutora Maria Elisa Rodrigues
e da
Doutora Susana Lopes

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Polymicrobial interactions in infections: the case *Pseudomonas aeruginosa* and *Candida albicans* in ventilator-associated pneumonia

Interações polimicrobianas em infeções: o caso *Pseudomonas aeruginosa* e *Candida albicans* na pneumonia associada à ventilação

Orientadora: Professora Doutora Mariana Henriques

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Aos meus pais. À minha irmã.

A ti, Ricardo.

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"Ninguém escapa ao sonho de voar,
de ultrapassar os limites do espaço onde nasceu,
de ver novos lugares e novas gentes.
Mas saber ver em cada coisa, em cada pessoa,
aquele algo que a define como especial,
um objecto singular, um amigo, - é fundamental.
Navegar é preciso, reconhecer o valor das coisas e das pessoas,
é mais preciso ainda"

Antoine de Saint-Exupéry

Após esta árdua mas enriquecedora experiência é gratificante pensar que os objectivos propostos foram alcançados, constituindo por tal motivo e acima de tudo, uma grande satisfação, não pela dissertação em si, mas pelo pequeno contributo que espero ter dado para a investigação na área da saúde. No entanto, e como nada teria sido possível sem a ajuda de determinadas pessoas, há contributos de natureza diversa que não podem e nem devem deixar de ser realçados. Por essa razão, dedico esta secção como forma de expressar algumas palavras de agradecimento e profundo reconhecimento, em particular:

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"Há gente que fica na história, da história da gente"

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…que os dias felizes sejam mais longos e só nossos

Abstract

Ventilator-associated pneumonia (VAP) is one of the most common nosocomial pneumonia among intensive care units contributing to high rates of morbidity and mortality. Polymicrobial biofilm infections with *Pseudomonas aeruginosa* and *Candida albicans* have being recently reported in VAP. Their antimicrobial resistance profiles represent a serious impact on the treatment of the disease by reducing the effective therapies and affecting the state of health of patients. As such, the present work aimed to provide novel insights concerning the characterization of single- and dual-species biofilms phenotype involving *P. aeruginosa* and *C. albicans* under the presence of different antimicrobials agents. Planktonic and biofilm assays were performed using *P. aeruginosa* PAO1, *C. albicans* SC5314 and four clinically important antimicrobials: amphotericin B (AmB), tobramycin (ToB), colistin (CoL) and polymyxin B (PolyB) as single or in combination. The quantitative methods (CFUs enumeration and biomass quantification) and qualitative methods (SEM, PNA FISH analysis and LIVE/DEAD staining) were used to study the single- and dual-species biofilms. The results showed that the incubation time did not influence biofilm formed by both pathogens in the overall consortia. Concerning single antimicrobials use, *P. aeruginosa* was the pathogen more sensitive to most tested antimicrobials and their effect showed to be concentration and time dependent. For antimicrobial combinations it was demonstrated that most formulations presented synergistic effect in *P. aeruginosa,* both in single and in mixed planktonic cultures. Specially, when applied to biofilms only AmB/PolyB and ToB/PolyB combinations (particularly with PolyB at high concentrations: 256 mg/L), promoted a significant reduction in the number of cultivable cells of both strains entrapped in single- and in dual-species biofilms. However, PNA FISH analysis and LIVE/DEAD staining showed that both strains are still existent and viable in presence of these antimicrobial combinations. In conclusion, different antimicrobial therapies used in this work did not display any effectiveness in the treatment of polymicrobial infections associated to VAP. Nevertheless, certain antimicrobial combinations tested in this work are essentials to future studies in order to better clarify the clinical dosage concentrations. Despite of the aforementioned concentrations being toxicity for humans they present strong potency to be used in future novel methodologies for VAP therapy.

Resumo

A pneumonia associada a ventilação (PAV) é uma das pneumonias nosocomiais mais comuns nas unidades de cuidados intensivos contribuindo para elevadas taxas de morbidade e mortalidade. As infeções polimicrobianas associadas a biofilmes causadas por *Pseudomonas aeruginosa* e *Candida albicans* têm sido recentemente relatadas na PAV. Os seus perfis de resistência antimicrobiana representam um sério impacto no tratamento da doença, devido à diminuição da eficácia das terapias afetando assim o estado de saúde dos pacientes. Desta forma, o presente trabalho teve como objetivo aumentar o conhecimento relativo à caracterização fenotípica de biofilmes simples e duplos envolvendo *P. aeruginosa* e *C. albicans* na presença de diferentes agentes antimicrobianos. Para tal, foram realizados ensaios planctônicos e de biofilmes utilizando *P. aeruginosa* PAO1, *C. albicans* SC5314 e quatro agentes antimicrobianos de relevância clínica: anfotericina B (AmB), tobramicina (ToB), colistina (COL) e polimixina B (PolyB), com utilização única ou em combinação. Os métodos quantitativos (enumeração de unidades formadoras de colónias e quantificação de biomassa) e métodos qualitativos (visualização em microscópio eletrónico de varrimento, análise PNA-FISH e coloração LIVE/ DEAD) foram realizados com o objetivo de estudar os biofilmes simples e duplos. Os resultados mostraram que o tempo de incubação não influenciou a formação de biofilme por parte de ambos os agentes patogénicos no consórcio global. Relativamente ao uso de um único agente antimicrobiano, a bactéria *P. aeruginosa* foi o agente patogénico mais sensível aos antimicrobianos testados revelando um efeito dependente da concentração e do tempo. Para combinações de antimicrobianos foi demonstrado que a maioria das formulações apresentaram efeito sinérgico em *P. aeruginosa*, tanto em culturas planctónicas simples como mistas. Especialmente, quando aplicados em biofilmes, apenas as combinações AmB / PolyB e ToB / PolyB (particularmente com PolyB a elevadas concentrações: 256 mg/L), promoveram uma redução significativa no número de células viáveis e cultiváveis em ambas as estripes quer em biofilmes simples ou duplos. No entanto, a análise PNA FISH e a coloração LIVE/DEAD mostrou que ambas as estripes se encontravam presentes e viáveis na presença destas combinações antimicrobianas. Em conclusão, as diferentes terapias antimicrobianas usadas neste trabalho não apresentaram grande eficácia no tratamento de infeções polimicrobianas associados à PAV. No entanto, certas combinações antimicrobianas, já testadas neste trabalho, são essenciais para estudos futuros, a fim de melhor clarificar as concentrações de dosagem clínicas. Apesar das concentrações acima referidas serem tóxicas para os seres humanos estas apresentam forte potencial para serem usadas em futuras metodologias na terapia PAV.

Outline and Aims

The present thesis reports the work performed at the Biofilm Group in LIBRO-Laboratório de Investigação em Biofilmes Rosário Oliveira, Centre of Biological Engineering (University of Minho, Braga, Portugal).

The main goal of this work was focus on the characterization of single- and dual-species (*P. aeruginosa* or/and *C. albicans*) biofilms phenotype associated to ventilator associated pneumonia (VAP) infection, particularly under the presence of different antimicrobial agents. In order to achieve this goal, several aspects were studied throughout this work, namely: the ability of both strains to develop biofilm (single- and dual-species) and the assessment of the susceptibility to different antimicrobial agents (single and combinations of antimicrobials) of both strains in planktonic cultures. Lastly, the effect of antimicrobials (single or in combination) in the ability to form biofilms of both strains and their antimicrobial resistance profiles were evaluated.

This thesis is structured in five chapters. Chapter I. summarizes the state-of-the art, carefully reviews relevant aspects concerning VAP, emphasizing the particular case of polymicrobial interactions, *P. aeruginosa* and *C. albicans* in VAP infections. The interactions between *P. aeruginosa* and *C. albicans* recently reported in VAP setting and the significance of polymicrobial biofilms characteristics on VAP antimicrobial resistance for worsening VAP infection are also outlined in this chapter. Chapter II describes the methodologies and techniques throughout this work. The rationale beyond methodologies employment is discussed therein. The Chapter III reported the different results obtained in the present work focus on the capacity of microorganisms to develop biofilm in the absence or presence of single or antimicrobial combinations. Chapter IV provides the discussion of this thesis, presented the hypothesis has been demonstrated by the new research and then show how the field's knowledge has been changed by the addition of this new data. Lastly, Chapter V finalizes the thesis by presenting general conclusions of the work performed and proposes future research lines are also addressed to improve knowledge in the field.

Contents

Abbreviations and Acronyms

AmB- Amphotericin B
ANOVA- Analysis of variance
AprA- Alkaline protease
ATS- American Thoracic Society
BAL- Bronchoalveolar lavage
CFF- Cystic Fibrosis Foundation
CFUs- Colony forming units
CIP- Ciprofloxacin
CoL- Colistin
CV- Crystal violet
DAPI- 4`, 6-diamidino-2-phenylindole
DNA- Deoxyribonucleic acid
ECM- Extracellular matrix
ECOOFs- Epidemiological cut-off values
eDNA- Extracellular deoxyribonucleic acid
ESBL- Extended spectrum beta-lactamase
ESCMID- European Society for Clinical Microbiology and Infectious Diseases
ET- Endotraqueal tube
EUCAST- European Committee on Antimicrobial Susceptibility Testing
ExoA- Exotoxin A
FIC- Fractional inhibitory concentration
FOH- Farnesol
gent- Gentamicin
ICU- Intensive care units
LPSS- Lipopolysaccharide
MBC- Minimum bacterial concentration
MBEC- Minimum biofilm eradication concentration
MBIC- Minimum biofilm inhibitory concentration
MDR- Multidrug-resistant
Merp- Meropenem
MFC- Minimum fungicidal concentration
MIC- Minimum inhibitory concentration
MMC- Minimum microbicidal concentration
MRSA- Methicillin-resistant S. aureus
MSSA- Methicillin-sensitive Staphylococcus aureus
MV- Mechanical ventilation
NaCl- Sodium chloride
NI- Nosocomial infection
NP- Nosocomial pneumonia
OD- optical density
PD- Pharmacodynamic

PIA- Pseudomonas Isolation Agar
PK- Pharmacokinetic
PlcH- Phospholipase C
PNA FISH - Peptide nucleic acid fluorescence in situ hybridization
PolyB- Polymyxin B
PS- Polystyrene
PYO- Pyocyanin
QS- Quorum sensing
RNA- Ribonucleic acid
ROS- Reactive oxygen species
rRNA- Ribosomal ribonucleic acid
RTI- Respiratory tract infection
SDA- Sabouraud Dextrose Agar
SDB- Sabouraud Dextrose Broth
SDs- Standard deviations
SEM- Scanning electron microscopy
T3SS- Type III secretion system
TA- Toxin/Antitoxin
ToB- Tobramycin
TSA- Tryptic Soy Agar
TSB- Tryptic Soy Broth
UP- Ultrapure
v- Volum
VAP- Ventilator-associated pneumonia
VAT- Ventilator-associated tracheobronchitis
VBNC- Viable but not cultivable
WHO- World Health Organization
wt- Weight
YPD- Yeast extract peptone dextrose
3OC12HSL- 3-oxo-C12-homoserine lacton

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CHAPTER I

INTRODUCTION

1.1. Contextualization

According to World Health Organization (WHO), a nosocomial infection (NI) is defined as "an infection occurring in a patient in a hospital or other healthcare facility, in whom the infection was not present or it was incubating at the time of admission. This include infections acquired in the hospital but appearing after discharge and also occupational infections among staff of the facility"[1]. NIs are a major source of morbidity and mortality, with prolonged hospitalization leading to increased costs in health care sector. NIs incidence in developed countries varies between 5 - 10 %, whereas it is reported values up to 25 % in developing countries[2][3][4][5]. Furthermore, the highest mortality rates occur after nosocomial pneumonia (NP) with a mortality rate of NIs up to 33 %[3] .

When patients are admitted in intensive care units (ICU), the NIs occurring in ICU are defined as infections that occur after 48 - 72 h of hospital admission. NI is the major complication in patients who are hospitalized in the ICU[6]. NIs rates in ICU are almost 5 - 10 times higher than NIs in other departments with an incidence rate of 15 - 40 %[7].

According to investigation conducted by WHO in 4 Regions (Europe, Eastern Mediterranean, South-East Asia and Western Pacific) infected patients consist of 5 - 10 % hospitalized patients and 8.7 % of those patients exhibit NIs. ICU infections correspond to 25 % of all NIs[7][8] and consequently overall mortality rate of NIs in ICU patients varies between 10 - 80 %[9].

The major factors associated to the increased NIs' rate in ICU are documented in the literature, such as: staying in ICU more than 48 h, presence of invasive devices such as mechanical ventilation (MV), use of urinary catheter and central line, prophylaxis of stress ulcer and trauma[10]. In addition, the long-term and irrational use of antimicrobials also leads to the development of resistant strains of pathogens. The existence of such resistant strains of pathogens is responsible for chronic diseases and long-term hospitalization. For the increased NI´ rate also accounts steroids and immunosuppressive therapies, an increased number of invasive procedures, the malnutrition and advanced age of patients as well as the leniency of hospital staff and infection control committee in maintaining sterile conditions[8][11].

The most common type of NI comes from the respiratory tract infections (RTIs) with 64.7 % of incidence and NP associated with 2 - 3 fold increase in mortality rates. Urinary tract infections, surgical site infections and blood-stream infections are also very frequent sites to the onset of NIs in ICU[8][11][12]. Ventilator-associated pneumonia (VAP), one of the major clinical problems, is one of the most common infection in adults ICU wherein 10 to 20 % of patients receive MV support[13]. Patients admitted with acute respiratory failure undergoing MV are

prone to develop NIs due to the high invasive device usage (tracheostomy tube and endotracheal intubation)[14]. In this way, the tracheostomy or endotraqueal tube (ET) create problems in defense mechanism of the respiratory tract and the risk of cross transmission of pathogens while handling and manipulating the ventilator associated devices is higher[8].

So, the invasive MV is a risk factor for the increase of NP[15][16], being the VAP one of the most severe public health problem[17].

1.2. Ventilator-associated pneumonia

The pathogenesis of lower RTI frequently begins with tracheal colonization from the oropharynx by leakage around the ET cuff, which may progress to ventilator-associated tracheobronchitis (VAT) and in certain patients to VAP- Figure 1.1[18]. Several studies reported controversies concerning the definition of VAT and its distinction from VAP. However, recent data suggest that VAT is an important risk factor for VAP[18][19].

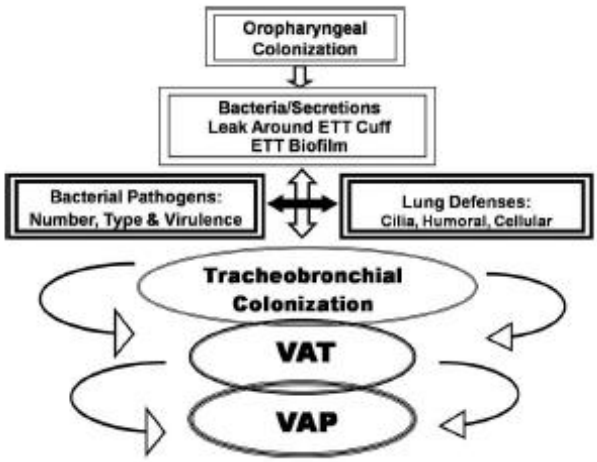


Figure 1.1- Pathogenesis of bacterial lower RTI and the disease progresses to its most severely state VAP. Image reprinted, from [18].

VAP represents one of the most common NP in patients who need MV to assist or control respiration, and it generally occurs 48 – 72 h or thereafter following MV (tracheostomy or endotracheal intubation)[20][21][22][23]. Nevertheless, it can also be conceptually defined as a lung parenchyma inflammation caused by pathogens that were still incubating or not present at the time as MV was started[23][24][25][26].

Based on the timing of onset, it is considered as early-onset VAP if it occurs during the first 4 days of MV. Frequently, it is less severe associated with a better prognosis, and mainly caused by community pathogens with a positive pattern of antimicrobial sensitivity (e.g.

Streptococcus pneumoniae, *Haemophilus influenzae* and anaerobes of the oral cavity). Then, the late-onset VAP develops 5 or more days after initiation of MV and it is caused by multidrug-resistant (MDR) pathogens (e.g., *Staphylococcus aureus, Pseudomonas aeruginosa*, *Enterobacteriaceae* and *Acinetobacter baumannii*) selected by exposure to broad-spectrum antimicrobials. It is associated with increased morbidity and mortality, prolonged ICU stay and longer duration of MV, with excessive health-care costs per event[23][27][28][29][30]. Nevertheless, not all the studies consider early- and late-onset VAP within the similar time range frame (Table 1.1)[17].

Table 1.1- Different definitions of early and late-onset VAP. Table adapted, from [17]

Citation	Early-onset VAP	Late-onset VAP
[31]	≤ 4 - 7 days	> 7 days
[32]	≤ 7 days	> 7 days
[33]	≤ 3 - 5 days	> 3 - 5 days

VAP is a potentially lethal infection and represents one of the most common problem among mechanically- ventilated patients in ICU contributing to approximately half of all cases of NP[11][34]. It is the second most common NI in the ICU and the most common in mechanically ventilated patients affecting 9 - 27 % of all mechanically ventilated patients[27][35]. This risk is between 3 and 10 times higher compared to patients who do not receive MV[26][34][36].

Recently reports from surveillance data show that the pooled rate ranges from 0 – 4.4 cases of VAP per 1.000 ventilator days and depending on the underlying population, type of ICU and surveillance method[37]. In addition, the risk of acquiring VAP is high during the first 5 days of MV (3 %) and the average of duration between intubation and development of VAP is about 3.3 days[34][38]. Between the 5th and the 10th day after ventilation, this risk declines to 2 % and 1 % per day thereafter, respectively[23][39].

VAP is one of the major factors contributing to morbidity and mortality in the ICU[22][38][40][41][42]. However the VAP mortality incidence is controversial (earlier studies placed the attributable mortality for VAP at between 33 – 50 %), with several recent studies reporting as being significant and between 1 to 1.5 %[23][41][42]. Furthermore, VAP significantly increases the length of stay in the ICU, the duration of MV, hospital stay and the healthcare costs[43][44][45]. This pathology is also responsible for more than half of the prescribed antimicrobials in the ICU[23] and the cost to the health care system has been estimated to range from $10,000 to $13,000 USD per case of VAP[46][47].

Due to all the implications for the individual patient (morbidity and mortality) and the cost to the healthcare system, it is necessary to explore preventive measures (Table 1.2) along with VAP early diagnosis and effective management, in order to reduce these parameters, as well as to prevent the onset of the disease[48][49][50][51].

Table 1.2- Strategies for VAP preventation. Table adapted, from [50]

• Non-invasive positive-pressure ventilation
• Semi-recumbent position to decrease aspiration of oropharyngeal secretions
• Oral hygiene with chlorhexidine
• Specialized ETs (subglottic secretion drainage; silver coated)

1.2.1. Risk factors

The presence of an ET implies the impairment of the mucociliary clearance of secretions, the pooling of subglottic secretions around the cuff and it is considered an important risk factor for the development of VAP, mainly due to the development of a biofilm laden with bacteria within the ET tube[52]. Few studies have demonstrated that the predisposing risk factors for the development of the VAP are numerous and are divided into three groups related with the host, the hospitalization procedure and the antimicrobial therapy (Table 1.3)[34][53][54][55][56].

Table 1.3- Risk factors of VAP. Table adapted, from[17]

Related with host	Related with hospitalization process	Related with drug therapy
• Advanced age	• Bronchoscopy	• Antacids [c]
• Burns	• Emergency intubation [b]	• Excessive sedation
• Chronic or preexisting pulmonary disease (tuberculosis, chronic obstructive pulmonary disease [b], bronchiolitis)	• High frequency of antibiotic resistance in the hospital unit where the patient is hospitalized [a]	• Antibiotic therapy in the previous 90 days [a]
• Cigarette smoking	• Enteral nutrition	• H2-receptor antagonists
• Coma	• Endotracheal intubation	• Intravenous sedatives [b]
• Gastric colonization	• Gastric aspiration [b]	• Immunosuppressive drugs (corticosteroids) [a]
• Immunosuppressive disease [a]	• Frequent changes of the ventilator circuit	• Neuromuscular blockers
• Impaired consciousness	• Hospitalization ≥ 5 days [a]	• Prior exposure to antibiotics, particularly to third-generation cephalosporins
• Male gender	• Long-term hospital and ICU length of stay	• Proton pump inhibitors
• Malnutrition	• Long-term intubation	• Stress ulcer prophylaxis
• Neurological/neuromuscular disease	• Multiple central venous lines [b]	• Red blood cells transfusions (immunomodulatory effects)
• Organ failure	• MV	
• Oropharynx colonization	• Nasogastric tube	
• Post-operative acute respiratory failure	• Transportation from ICU to other hospital sites	
• Post-surgical	• Re-intubation [c]	
• Post- traumatic	• Supine body position	
• Septicemia	• Thoracic surgery	
• Sinusitis	• Tracheostomy [c]	
• Trauma [c]		
• Underlying disease, and its severity		

[a] Risk factor for MDR pathogens
[b] Specific risk factor of early-onset VAP
[c] Specific risk factor of late-onset VAP

In addition, there are several sources of VAP pathogens that can be categorized as exogenous and endogenous relative to the patient. While the exogenous sources are commonly from aerosols of the contaminated air, medical devices (humidifier, ventilator circuit, catheter and bronchoscope), health professionals and other patients; oral, pharyngeal and gastric flora of the patient, are some fonts that symbolize the endogenous sources[57][58].

1.2.2. Diagnosis of Ventilator-associated pneumonia

In general, the clinical diagnosis of VAP involves a combination of the clinical symptoms/signs, chest radiography, and microbiological data[59]. Despite several clinical methods have been recommended, none of the methods have the accurate sensitivity or specificity to exactly identify this disease when compared to the demonstration of pneumonia on histological samples obtained by either biopsy or necropsy[60]. Clinical symptoms and signs include variations in sputum or tracheal secretions in terms of purulence, color and/or increasing production; cough; temperature > 38 °C or < 36 °C; rales or bronchial breath sounds on examination and worsening oxygenation. Non-specific indicators of infection are obtained through laboratory findings including leukocytosis (> 12×10^9 leukocytes/L) or leukopenia (< 4.0×10^9 leukocytes/L). Lastly, the development of new infiltrates or the presence of persistent and/or worsening infiltrate on chest radiography are signs for VAP presence[61][62].

So, in the absence of a reference standard and the poor reliability of clinical criteria, the clinician needs to balance all factors, including the overall clinical status of the patient to take a treatment decision. In addition, respiratory tract sampling should be routinely conducted when there is a clinical suspicion of VAP via non-bronchoscopic or bronchoscopic techniques. Bronchoscopic sampling includes bronchoalveolar lavage (BAL) or protected specimen brush demonstrating superiority in relation to non-bronchoscopic techniques that include endotracheal aspirates and mini-BAL[63].

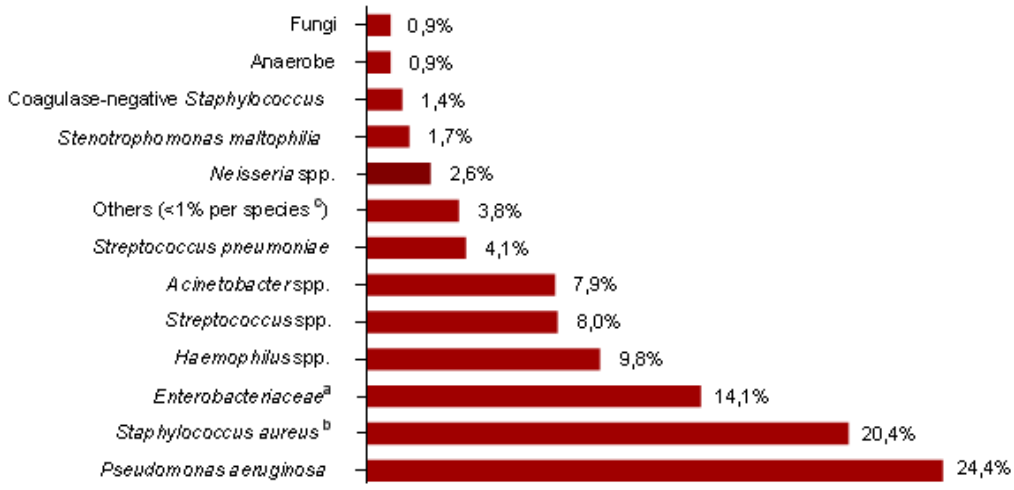
1.2.3. Pathogenesis of Ventilator-associated pneumonia

There is a variety of microorganisms that cause VAP, including bacteria, fungi and viruses; and this disease may be due to a single pathogen or can have polymicrobial origin[34][64]. However, the fungi and virus are present in low incidence only when the immune system of the patients is weakened, increasing the magnitude of this health problem[31][34].

The composition of pathogens that causes VAP typically depends on the duration of MV[65][66].

In general, early-VAP (typically occurring in less than 5 days after MV) is caused by pathogens that are sensitive to antimicrobials *(Haemophilus influenzae*, *Streptococcus pneumoniae*, methicillin-sensitive *Staphylococcus aureus* (MSSA) or *Enterobacteriaceae*) whereas late-onset VAP (occurring after 5 days of MV) is caused by MDR microorganisms, which are more difficult to treat and it encompasses MDR bacteria, such as *P. aeruginosa*, *Acinetobacter* species, methicillin-resistant *S. aureus* (MRSA) and extended-spectrum beta-lactamase producing bacteria (ESBL))[27][67][68][69][70][71].

Furthermore, the patients with and without risk factors for VAP infection with MDR microorganisms and the type of ICU population (i.e. medical, surgical and trauma) could also be associated with specific pathogens as the causative VAP agent[65][66][69][72][73]. Nevertheless, gram-negative bacilli are frequently involved in the pathogenesis of VAP accounting for 60 % of all VAP cases. Figure 1.2 summarizes the typical pathogens causing VAP[65]



[a] *Klebsiella* spp. (15.6%), *Escherichia coli*(24%), *Proteus* spp. (22.3%), *Enterobacter* spp. (18.8%), *Serratia* spp. (12.1%), *Citrobacter* spp. (5.0%), *Hafnia alvei*(2.1%)
[b] *S.aureus* methicillin resistant (55.7%), *S.aureus* methicillin sensitive (44.3%)
[c] Includes *Corynebacterium* spp, *Moraxella* spp. and *Enterococcus* spp.

Figure 1.2- Pathogens causing VAP, together with their frequencies. Figure adapted, from [51].

1.3. Monomicrobial and polymicrobial infections in Ventilator-associated pneumonia

Although mostly VAP infections are initiated by a single pathogen or virulence factor-monomicrobial origin, currently the incidence of a complex milieu of microorganisms - polymicrobial origin is increasing[23][62][64][68]. These consortia of microorganisms typically

coexist as combinations of highly structured communities of bacteria, viruses, protozoans, and fungi attached to biotic and abiotic surfaces, such as mechanical ventilator, in VAP infections. Their architectures are facilitated by specific inter-microbial and microbial-host interactions[74][75][76]. The existence of mixed species population can contribute to a shift and decrease in the host immunity, promoting the colonization and subsequent infection by opportunistic pathogens that exploit unique niches in the polymicrobial environment[77].In addition, it has been reported that polymicrobial infections can change significantly the treatment and patient outcome, leading to that the necessary criteria for characterizing diagnosing and treatment these infections are still not well defined[78][79].

Pseudomonas aeruginosa and *Candida albicans* are example pathogens that have been reported in infections associated to VAP.

1.3.1. *P. aeruginosa*

P. aeruginosa is a motile non-fermentative gram-negative bacilli belonging to the family Pseudomonadaceae. It is an opportunistic pathogen considered to be one of the main causes of NI in immunocompromised patients[80][81] and it is associated with late-onset VAP[38].

Throughout the history, *P. aeruginosa* received several names based on its characteristic blue-green coloration observed in culture. In 1850, Sédillot had reported for the first time the discoloration of surgical wound dressings associated with a transferable agent[81]. Fordos extracted the pigment responsible for the blue coloration in 1860, and in 1862 Lucke was the first to associate this infectious microorganisms to the rod-shaped morphology[82][83]. In 1882, Carle Gessard, a chemist and bacteriologist from Paris-France, discovered *P. aeruginosa* performing an experiment that identified the growth of this microbe in cutaneous wounds of two patients with bluish-green pus[84]. *P. aeruginosa* (Bacillus pyocyaneus) was reported as the causative pathogen of blue-green purulence in the wounds of patients has reported in several additional studies between 1889 and 1894[85]. The ability of invasion and dissemination of *P. aeruginosa* leading to severe acute and chronic infections was recognized by Freeman in 1916[86]. In 1960s, *P. aeruginosa* emerged as an important human pathogen[87]. The complete sequencing of a wild-type *P. aeruginosa* (PAO1) strain, achieved in 2000, has provided access to useful information about its pathogenicity and potential for resistance[88].

Its adaptability to different environments is associated with the high proportion of predicted regulatory genes in *P. aeruginosa* genome in comparison to all other sequenced bacterial genomes[88][89]. *P. aeruginosa* is a non-fastidious microorganisms having a broad range of growth substrate and minimal nutrient requirements[90]. Moreover, it is tolerant to

high temperatures ≈50 °C and it is able of growing under aerobic as well as anaerobic conditions[91].

P. aeruginosa stands out as a threatening microorganism capable of causing severe invasive disease and trigger persisting infections nearly impossible to eradicate. Its pathogenesis is mediated by multiple bacterial virulence factors that facilitate adhesion and/or disrupter of host cell signaling pathways[92].

Several virulence mechanisms, as initial role in motility and adhesion to the epithelium, have been described for *P. aeruginosa* infecting airway epithelia. These are probably associated with subsequent tissue damage, invasion, and dissemination of this pathogen[93][94][95][96]. There are some virulence determinants that contribute to the development of the disease: type III secretion system (such as T3SS with four effector cytotoxins : ExoS, ExoT, ExoU and ExoY), type IV secretion system, quorum sensing (QS) and biofilm formation, lipopolysaccharide (LPSS) (such as Lipid A and O-polysaccharide), proteases (such as elastase and alkaline protease (AprA)), alginate, pyocyanin (PYO), exotoxin A (ExoA), flagellum and type IV pili and oxidant generation in the airspace[97][98][99][100][101].

Furthermore, *P. aeruginosa* is commonly found to be the first or second major pathogen causing VAP (22.8 %)[102][103] and it is the most frequently isolated gram-negative microorganism both at early- and late-VAP[104]. VAP caused by *P. aeruginosa* is associated to high morbidity and mortality in ICU and it is related with increased length of ICU stay and high treatment costs[105].

1.3.2. *C. albicans*

C. albicans is typically a harmless eukaryotic commensal yeast that is member of the family Sccharomycetaceae[106]. It is an opportunistic fungal pathogen in humans and therefore it is easily found in indwelling medical devices (such as mechanical ventilator) mostly associated with biofilms[107][108][109][110][111][112].

Historically, *C. albicans* was discovery in year 400 B.C. by Greek physician, Hippocrates that identified a microbial infection caused by this pathogen[113]. But till late twentieth century it was not studied like any other model microorganisms[114][115][116]. In the 1970s and 1980s, some laboratories started working on *C. albicans*, and in the 1990s, a large number of yeast laboratories changed to study different aspects of *C. albicans* promoting the initiation of genome sequencing of the SC5314 strain in 1996. The completion and availability of genome sequence of *C. albicans* SC5314 occurred in 2004 allowing start rigorous research activities and expand the knowledge of this important pathogen[116][117][118].

C. albicans is a dimorphic species that can grow as yeast or filamentous forms, and it is one of the only two *Candida* species capable of forming true hyphae[119][120]. Transitions between the two phenotypes can be induced in complex media, chemically defined media and serum and can be favored by temperatures above 35 °C and a pH ranging between 6.5 and 7.0 or slightly alkaline[121][122][123][124].

It is predominantly diploid, but it may present aneuploidy since it exhibits a high degree of genome plasticity and reveals frequent losses of heterozygosity, as well as, gross chromosomal rearrangements. Though reproduction is predominantly clonal and under stressful conditions *C. albicans* can also utilize a parasexual cycle[125]. The ability of *C. albicans* to infect different niches at various anatomic sites of the host and express infection-associated genes is an essential step for the establishment of an infection with success[126]. The pathogenicity will depend on the several of virulence factors and fitness attributes presented by *C. albicans*: morphological transition between yeast and hyphal forms, expression of adhesins and invasins on the cell surface, contact sensing and thigmotropism, formation of biofilms, phenotypic switching and secretion of hydrolytic enzymes[126][127][128][129][130]. In addition, fitness attributes include rapid adaptation to fluctuations in environmental pH, metabolic flexibility, powerful nutrient acquisition systems and robust stress response machineries[130]. Studies about virulence factors of *Candida* species have shown that *C. albicans* is the most pathogenic microorganism in the *Candida* group[131][132] and it has the highest levels of virulence factors, which contributes to increased severity and persistence of infections and consequently difficult for eradication[133].

Although, generally, the etiology of VAP is typically bacterial, fungal airway colonization (such as by *C. albicans*) is a frequent finding in patients submitted to MV[134][135][136][137]. Even if *Candida* species infections are rare causes of VAP, mostly of these occur in immunocompromised patients[31][134]. Recent studies reported that critically ill patients with VAP, exhibit pulmonary *C. albicans* colonization in 57 % of these patients and means an independent risk factor for MDR super-infection associated with an increased risk of NP, prolonged length of ICU and mortality[134][138][139].

1.3.3. *P. aeruginosa*- *C. albicans* polymicrobial infection

Rarely the microorganisms exist as single-species planktonic forms. Instead, they are frequently found in complex polymicrobial communities attached to biotic and abiotic sites, known as biofilms[140].

Currently, the bacterial-fungal interactions are one example of polymicrobial infections growing with directly and indirectly influence of each other in several manners. The virulence of bacteria[141] or fungi[142] can also be altered within the polymicrobial consortium: for instance, bacterial factors can influence fungal growth or even its physiology[143]. Also, fungal factors can regulate bacterial behavior and survival[143]. In addition to antagonistic interactions (an association between two microorganisms that is detrimental to at least one of them and that is caused by the release of metabolites or cell components[144]), it is possible to occur beneficial interactions in mixed environments where different species are able to provide protection for each other against an attacking immune response or antimicrobial agent. Thus, these bacterial-fungal interplay contributes to the worsening health condition of the patient (Figure 1.3)[143].

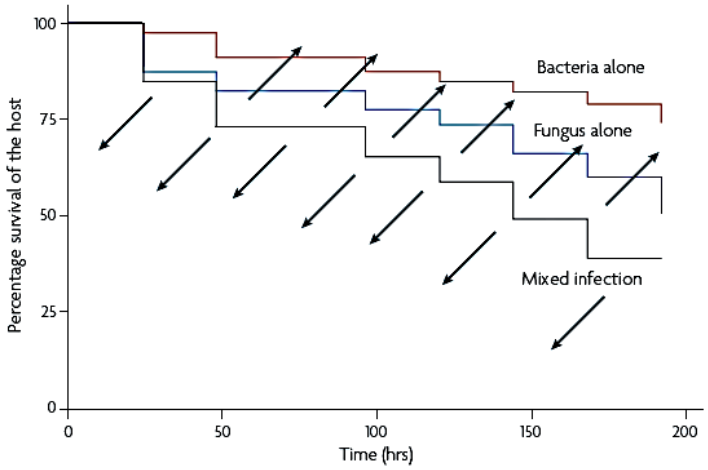


Figure 1.3- Survival curves for polymicrobial infections. Image reprinted, from [445].

The particular association case of *P. aeruginosa* and *C. albicans* in ICU is frequently exhibited although their importance to human health is not yet completely understood[145][146]. Mixed species biofilms involving *C. albicans* and several pathogenic bacteria such as *P. aeruginosa* on the surface of medical devices (e.g. mechanical ventilator) and in some susceptible sites of the human body are critical for the development of infectious disorders[147].

VAP is the most commonly NI and approximately 25 % of all cases occur due to *P. aeruginosa*[65]. *C. albicans* is also highly represented in this niche[148][149]. So, the occurrence of *P. aeruginosa* and *C. albicans* in the respiratory tract not only increases the risk of developing VAP[138] but also interferes with VAP-associated mortality[150].

The environmental conditions are important factors to determinate the outcome of wide spectrum of interactions between *P. aeruginosa* and *C. albicans* (Figure 1.4).

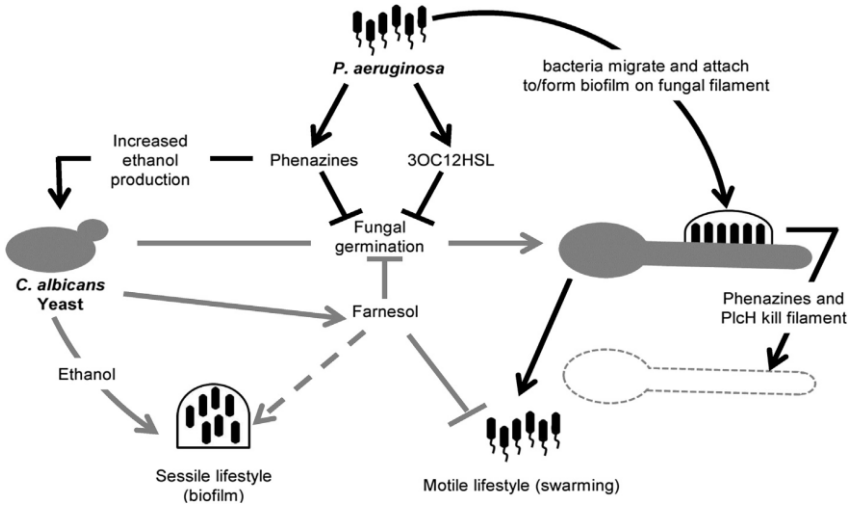


Figure 1.4- Interactions between *P. aeruginosa* and *C. albicans*. Image reprinted, from [468].

The ability of the bacteria to distinguish different fungal morphotypes is likely due to the specificity in the cell wall surface proteins such as[152][153]: 1) the mannoproteins located along the surface of filaments that promote the favorable acid-base conditions[154][155]; and 2) the *P. aeruginosa* chitin binding protein[156].

After attachment, *P. aeruginosa* are able to form a biofilm along *C. albicans* filaments but not on yeast cells[151]. This biofilm formation induces death of the fungal cell, caused by the action of two pseudomonal virulence factors: 1) a secreted haemolytic phospholipase C (PlcH) that degrades phosphatidylcholine and 2) redoxactive phenazines, which create highly toxic reactive oxygen species (ROS)[151][157]. Also, biofilm formation is essential for fungal killing in liquid cocultures[151]. The restriction in space during growth on agar plates leads to the production of phenazines by *P. aeruginosa* which induces toxicity for both forms of *C. albicans*, yeast and hyphae[157]. *In vitro* antagonism interactions are observed between *P. aeruginosa* and *C. albicans* in chronic infections, such as when bacteria encounter hyphae[158][159].

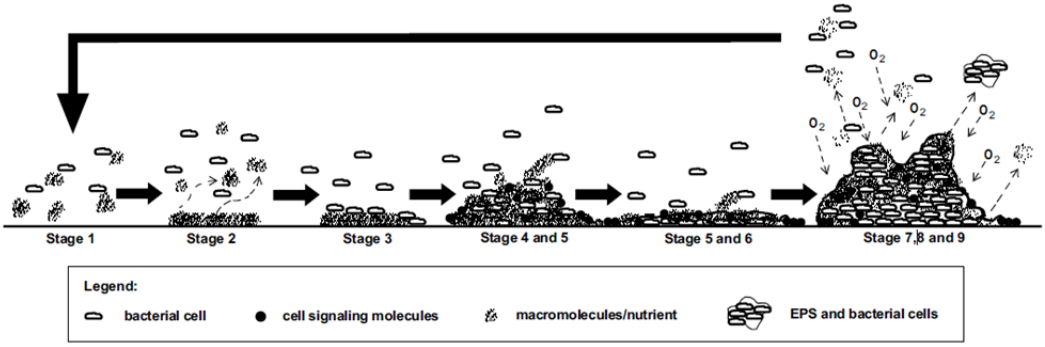
P. aeruginosa mutants defective in flagellar motility promote a poor fungal colonization and a reduced capacity of killing. The bacterial growth is not capable of killing unless the mutant defective occurs in type IV pili, capable of forming large biofilms being able of killing defect[151]. Additionally, *P. aeruginosa* produces various enzymes and small molecules including PlcH, phenazines and the QS molecule 3-oxo-C12-homoserine lacton (3OC12HSL), which have effect in the biology and survival of *C. albicans*. PlcH is a secreted lethal enzyme once degrades phospholipids of eukaryotic cells, namely fungi[160][161][162][163]. For this reason, a mutant defective in PlcH production is attenuated in killing fungal filaments[151]. *P. aeruginosa* also secretes small molecules such as redox-active phenazines that inhibit filamentation and biofilm development in *C. albicans*[164][165]. At low concentrations, phenazine methosulfate (an analog of *P. aeruginosa* 5MPCA) and PYO suppress filamentation and simultaneously increases

the fermentative metabolism and glycolysis modifying the Krebs cycle and affecting the production of metabolites associated with amino acid metabolism[164][165]. Furthermore, recent studies indicate that the extent of metabolic inhibition is directly associated with the extent of inhibition of morphology within the consortium[164]. Like phenazines, the *P. aeruginosa* QS molecule, 3OC12HSL, inhibits fungal germination and stimulates the filament-to-yeast transition through a different pathway where the adenylate cyclase presents as the direct target[166][167][168].

C. albicans also secretes a QS molecule, farnesol (FOH), which regulates its own morphology repressing hyphal growth regardless of the conditions that normally induces the filamentation (such as serum and 37 °C)[169]. Like PYO, the presence of FOH is also associated to changes in *C. albicans* metabolic pathways[170] and consequently changes in fungal metabolism, which indirectly affects other microorganisms in the consortium. Furthermore, FOH (which has limited structural similarity to 3OC12HSL) also represses the filamentation and promotes the filament-to-yeast transition through the inhibition of adenylate cyclase[166][169][171][172][173][174]. FOH also inhibits the production of the *Pseudomonas* quinolone, signal that positively regulates phenazine synthesis, thereby reducing phenazine production[141]. On the other hand, it can restore levels of butyryl homoserine lactone, which in turn activates the LasT-regulated components capable of inducing phenazine biosynthesis[175]. These data show that *C. albicans* may contribute for the regulation of *P. aeruginosa* virulence pathways, once FOH also inhibits bacterial swarming (rapid and coordinated movement of bacterial cells across a surface) motility. Thus, high FOH levels are associated with bacterial switch from a motile to sessile lifestyle[168].

1.4. Biofilms in Ventilator-associated pneumonia

Biofilms are the most prevalent growth form of microorganisms and are commonly defined as structurally complex communities of cells attached to an inert (e.g. medical devices such as mechanical ventilator) or living (e.g. lung tissue) surface and embedded in an extracellular matrix (ECM) that is composed by substances produced by microbial cells[176][177][178][179][180][181][182][183][184]. The model for biofilm formation (Figure 1.5) is extremely complex and so far, nine distinct stages have been identified.



Stage 1	• Surface pre-conditioning • Utilizes molecules which are already coated onto the adhesion surface or sequesters macromolecules from the surrounding bulk liquid
Stage 2	• Planktonic cells are transported through the bulk liquid and begin to deposit onto the adhesion surface
Stage 3	• Planktonic cells begin to adsorb onto the adhesion surface
Stage 4	• Cells that were reversibly adsorbed onto the adhesion surface will leave by the process of desorption
Stage 5	• Remaining cells will begin to irreversibly adsorb onto the adhesion surface • Cell-to-cell signalling will commence • EPS production is initiated
Stage 6	• Cell-to-cell signalling molecules will be synthesised • Oxygen and nutrients are transported by convection and diffusion through the bulk liquid surrounding the biofilm
Stage 7	• Molecule transport into and around the biofilm begins • Microbial cells with the biofilm begin to replicate and grow
Stage 8	• Cells within the biofilm begin to metabolise surrounding substrate • Various products are transported out of the biofilm • Concurrent cell growth, replication and EPS synthesis processes on-going
Stage 9	• Detachment, sloughing and erosion of the biofilm may occur • Detached cells disperse as planktonic cells and the cycle begins again

Figure 1.5- The nine stages of biofilm formation. Image adopted, from [469].

In the presence of changing environmental conditions this structure provides an advantageous strategy for survival and adaptation of microorganisms: 1) defense and protection from the hostile host environment; 2) preferential colonization in areas that are rich in nutrients; 3) benefits associated with cooperativity as part of a community, and 4) for some species it is their normal default growth mode[178][182][185][186].

The presence of biofilms in medical devices has some major consequences such chronic infections. It is estimated that up to 80 % of all infections worldwide are biofilm-related being almost impossible to eradicate given the inherent resistance to antimicrobial agents[187][188].

The mucus accumulation, the impairment of host defense mechanisms and the introduction of pathogens in sterile airways are favorable conditions for the development of

biofilms on the distal part of the ET in mechanically ventilated patients[189][190][191][192][193][194]. Besides, there is correlation between the presence of a biofilm on the ET and the development of VAP[195][189][196]. However, although VAP development, the biofilm formation on ET also promotes (partial) ET obstruction[190][197][198], subglottic stenosis[199] and bronchopulmonary.

It is important to refer that *P. aeruginosa* plays an important role in the development of VAP. Several studies have been frequently identified *P. aeruginosa* in ET biofilms. Additionally, it was already suggested that *C. albicans* is also one of the causative agents of VAP biofilms[31][58][62][68][189][190][192][194][200][201][202][203][204][205][206][207][208][209][210].Nonetheless, the precise mechanism of *Candida* species in the development of VAP is not well understood once it is hard to distinguish between harmless colonization of *Candida* species in the airways and the development of an infection associated to its presence[23][211].

The formation of VAP biofilms on ET may be associated with a polymicrobial nature. A model for the development of ET biofilms was suggested based on literature data (Figure 1.6).

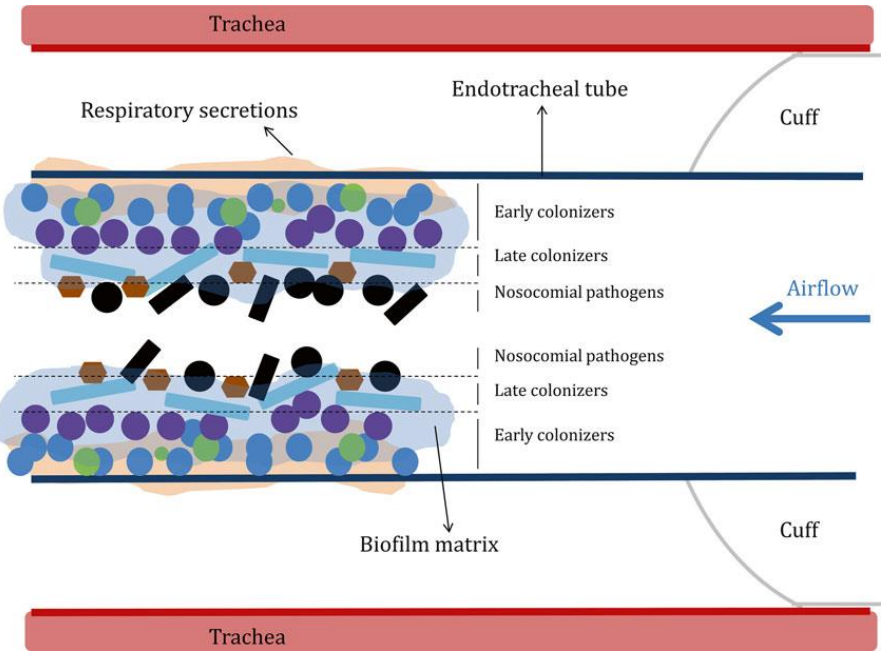


Figure 1.6- Model of biofilm formation on the distal end of ET. Image reprinted, from [470].

At first, the leakage occurs at the ET cuff and the nasopharyngeal secretions (containing oral bacteria) leach out and it goes into the trachea and accumulates at the distal end of the ET. Then, the bacterial adhesion is favored by ET surface and due to nutrients availability in the secretions[192][202][212]. In fact, ET represents a source for the development of biofilms by bacteria present in nasopharyngeal secretions and it is predominantly composed of respiratory secretions[202] and water (90 – 95 %)[210]. The typical oral flora bacteria are generally present in the respiratory secretions and responsible for initial adhesion to the ET[213]. The

coaggregation between primary colonizers with a variety of oral bacteria enables them to start colonization initiating the formation of biofilms[192][213][214]. Other bacteria are attracted by the primary colonizers[215] and subsequently the latest colonizers attract others acting as a switch between early and late colonizers[213][216][217][218][219]. Finally, nosocomial pathogens adhere onto the biofilm formed by the oral bacteria, where the interactions between oral bacteria are mediated by receptors and adhesins[213]. The coaggregation of diverse microbial species enhances the virulent characteristics of certain bacteria as well as increases their tolerance to antimicrobials[203].

Overall, biofilm formation of oral microorganisms on ET represents a favorable environment for the adherence of potential respiratory pathogens such as *P. aeruginosa*[203][204].

1.5. Ventilator-associated pneumonia antimicrobial treatment

Selecting the appropriate antimicrobial and a therapy initiated as early as possible is critical to reduce VAP's associated mortality[23][220][221]. However, this depends on the duration of MV, whereas late-onset VAP (> 4 days) can be treated with broad-spectrum antimicrobials; early-onset VAP (≤ 4 days) requires a limited spectrum antimicrobials[23].

The choice of empiric therapy is influenced by important factors, including institutional or unit-specific antibiograms and patient risk factors. Prior cultures or colonization data, duration of MV, prior exposure to other antimicrobials and severity of the illness are essential information to guide optimal dosage of initial empiric therapy[23][50]. Although, there is no universal regimen for VAP treatment, some recommended therapies for VAP treatment stand out. The most common antimicrobial treatments in early-onset VAP are[23][222][223][224]:

- The second or third generation cephalosporin (e. g., ceftriaxone: 2 g daily; cefuroxime: 1.5 g every 8 h; cefuroxime: 1.5 g every 8 h);
- Fluoroquinolones (e. g., levofloxacin: 750 mg daily; moxifloxacin: 400 mg daily);
- Aminopenicillin + beta-lactamase inhibitor (e. g. ampicillin + sulbactam: 3 g every 8 h);
- Ertapenem (1 g daily).

For late-onset VAP the following are used:

- Cephalosporin (e. g., cefepime: 1–2 g every 8 h; ceftazidime 2 g every 8 h);
- Carbepenem (e. g., imipenem + cilastin: 500 mg every 6 h or 1 g every 8 h; meropenem: 1 g every 8 h);

- Beta-lactam/beta-lactamase inhibitor (e. g., piperacillin + tazobactam: 4.5 g every 6 h) + aminoglycoside(e. g., amikacin: 20 mg/kg/day; gentamicin: 7 mg/kg/day; tobramycin: 7 mg/kg/day);
- Antipseudomonal fluoroquinolone (e. g., ciprofloxacin 400 mg every 8 h; levofloxacin 750 mg daily) + coverage for MRSA (e. g., vancomycin: 15 mg/kg every 12 h);
- Linezolid (600 mg every 12 h).

It is important refer that optimal dosage includes adjusting for hepatic and renal failure. Trough levels for vancomycin (15 – 20 mcg/ml), amikacin (< 5 mcg/ml), gentamicin (< 1 mcg/ml) and tobramycin (< 1 mcg/ml) should be measured frequently to avoid untoward systemic side effects. All recommended doses are for intravenous infusion. Usual duration of therapy is 8 days unless treatment is for MDR microorganisms, in which case treatment will be for 14 days. As such, several options, which includes aminoglycosides, antipseudomonal carbapenems, antipseudomonal cephalosporins, fluoroquinolones, antipseudomonal penicillins + b-lactamase inhibitors, aztreonam, fosfomycin and also polymyxins are antimicrobial therapies used in pseudomonal coverage[225]. The polymyxins are being increasingly used as rescue therapy for infections due to the few novel antimicrobial agents[226] and an increasing incidence of infections caused by MDR gram-negative microorganisms such as *P. aeruginosa*[227]. These antibiotics are classified into four major groups based upon their intracellular target and their mechanism of action (Figure 1.7)[228].

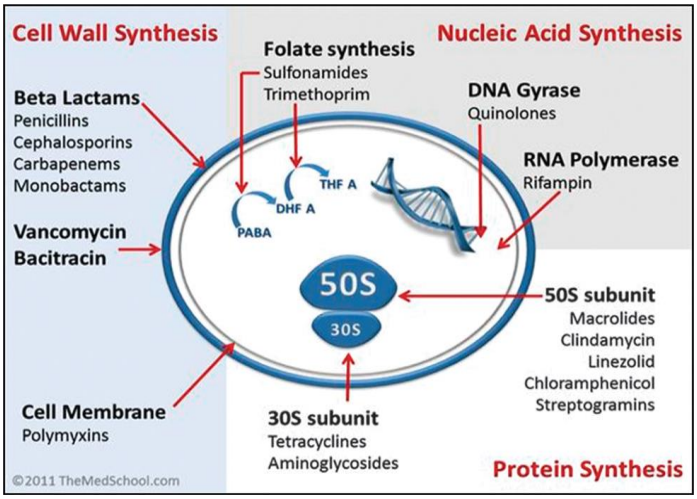


Figure 1.7- Classification of antibiotics by mechanism of action: cell wall synthesis inhibition (e.g. penicillin and derivatives, cephalosporins, carbapenems and glycopeptides) [471][472][473] **; cell membrane disruption (e.g. the family of polycationic peptide antibiotics called polymyxins)** [474][475][476]**; nucleic acid synthesis inhibition (e.g. quinolones, rifampicin and sulphonamides)** [477][478][479][480]**; protein synthesis inhibition by targeting the ribosomal-RNA (rRNA) rich surfaces of ribosomes (e.g. tetracycline, aminoglycosides, chloramphenicol and macrolides)** [481][482][483]**. Image reprinted, from** [228]**.**

The treatment of fungi in VAP, comprises several antifungals classes including azoles (such as fluconazole and voriconazole) and equinocandines (such as caspofungin)[229]. However in *C. albicans* infections associated to VAP, the antifungal therapy is not considered as a therapeutic option, since this pathogen is frequently found only in pulmonary secretions of critically ill patients. In this respect the *C. albicans* colonization may not play an important pathogenic role in the context of VAP[134][211]. Additionally, the European Society for Clinical Microbiology and Infectious Diseases (ESCMID) corroborates this idea, recommending that isolation of *C. albicans* and other *Candida* species from pulmonary secretions rarely requires treatment with antifungal therapy, once the presence of these pathogens represents usually the colonization of the airways, rather than pneumonia in immunocompetent patients[134][211][230].

Relatively to amphotericin B, a polyene antifungal, has been reported the gold standard in cases of serious and invasive *Candida* infections. Its potential is due to its remarkably low level of resistance amongst fungal species and its fungicidal mechanisms that account for broad-spectrum coverage[231]. These antifungal agents are classified according to their targets and mechanism of action in the antifungal therapy (Figure 1.8)[232].

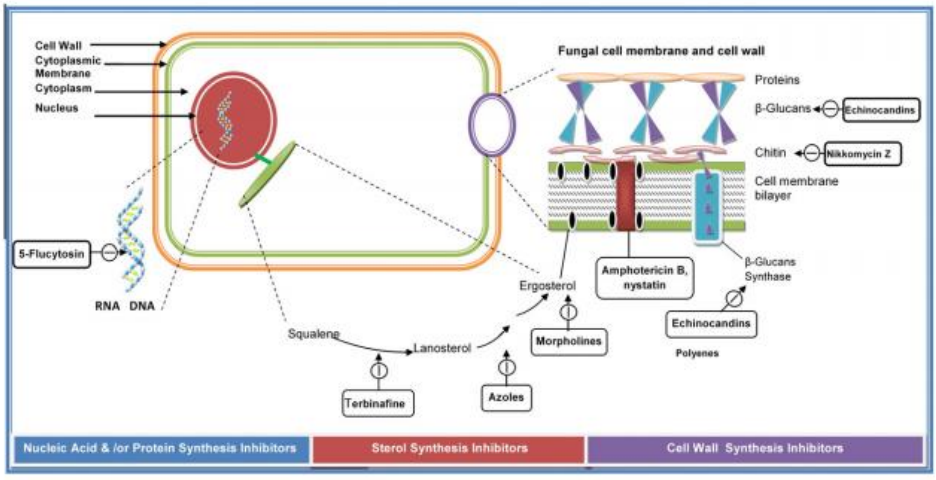


Figure 1.8- Classification of antifungals by mechanism of action: fungal ergosterol synthesis inhibitors (e.g. azoles); ergosterol disruptors (e.g. polyenes); nucleic acid synthesis inhibitor (e.g. flucytosine) and glucan synthesis inhibitors (e.g. echinocandins)[232]. Image reprinted, from [232].

The antimicrobial agents (antibiotics and antifungals) are also classified according to their pharmacokinetic/pharmacodynamic (PK/PD) index that corresponds to quantitative relationship between a PK parameter and a microbiological parameter. The three main PK/PD indexes are: the time during which the concentration of the drug was over the minimum inhibitory concentration (MIC) (T > MIC), the peak concentration and MIC ratio (Cmax/MIC) and the ratio of the 24 - h area under the concentration-time curve divided by the MIC

(AUC/MIC). PK/PD index provide information about the antimicrobial efficacy depending on the activity pattern of each antimicrobial[233].

Three major patterns of antimicrobial activity have been described: 1) *Antimicrobials with concentration-dependent killing along with prolonged persistent effects*. Cmax/MIC or the AUC/MIC ratios are PK/PD indexes for these antimicrobials, once the prolonged persistent effects protect against re-growth when active antimicrobials concentration decreases to below the MIC. This pattern has been described for all of the aminoglycosides, fluoroquinolones, polymyxins, daptomycin, metronidazole, echinocandins and polyenes[234][235][236]; 2) *Antimicrobials with time-dependent killing and absence or very short persistent effects*. The duration of time that active antimicrobial concentrations exceeded the MIC is the best PK/PD index associated with efficacy therapies. In general, it is expressed as the percentage of the dosing interval and it is only considered the fraction of antimicrobial not bound to proteins. This behavior is characteristic for a wide number of antimicrobials including b-lactam antimicrobials, such as penicillins, cephalosporins, carbapenems, monobactams and flucytosine[234][236]; 3) *Antimicrobials with concentration-independent killing and prolonged persistent effects*. The best PK/PD indexes for these drugs are Cmax/MIC or the AUC/MIC. As in the first case, it shows the prolonged persistent effects that protect against re-growth when active antimicrobial concentration decreases to below MIC. Tetracyclines, tigecycline, macrolides, azithromycin, clindamycin, linezolid and other oxazolidinones, chloramphenicol, trimethoprim, sulfonamides, vancomycin and azoles are examples for this pattern[234][235][236].

1.5.1. Combination therapy

The importance of optimizing therapies for *Pseudomonas* species infections is a critical and imminent measure due their prevalent mortality rates comparatively with all of the other pathogens[11][237][238]. *P. aeruginosa* ability to simultaneously express several mechanisms of resistance increases the challenge of successfully treating it[239][240]. There is evidence supporting that the initial use of combination therapy is more effective than monotherapy for severe infections with gram-negative bacteria (e.g. *P. aeruginosa*), such VAP in the existing environment of MDR microorganisms. This happens because of the broad empiric coverage provided by two antimicrobial agents with different spectra of activity[241][242][243][244][245][246][247][248][249]. A multicenter, retrospective study reported that in cases of VAP associated to *P. aeruginosa*, rates of appropriate empiric therapy were higher in patients who were prescribed combination therapy than in those on monotherapy[250].

However, many NIs, such as VAP, are associated to microbial biofilms and the persistence of chronic infections is attributed to the persistence of polymicrobial biofilms (e.g. bacterial-fungal origin)[251][252]. However, in most situations, traditional therapies are generally targeted at individual causative agents, not considering the polymicrobial cause or addressed to each members of microbial consortium. Yet, the standard treatment regimen employed for polymicrobial infections involves two or more antimicrobials (combination therapies) thus, promoting the effectiveness of antimicrobial therapies[251][253][254]. The use of antibiotics and antifungals simultaneously or sequentially, for prophylactic and therapeutic purposes, is a common clinical practice in severe infections in response to the emergence of resistance to the host immune system response and to antimicrobial therapy[255].

A combination therapy, applying anti-biofilm antimicrobials with traditional antibiotics to target cell growth, could be a better alternative to control biofilm-related infectious diseases such as VAP. In such combination therapy, the anti-biofilm drugs will promote planktonic growth, thus removing the additional community level resistance provided by biofilms and facilitating the targeting of pathogens at the cellular level by traditional antibiotics[256].

Nonetheless, it is essential to observe a synergistic effect between two antimicrobials *in vitro* against the pathogen responsible for the infection. The synergism may be associated with a significantly better outcome achieved with a combination, which is not synergistic for this pathogen. So, the synergistic activity of antimicrobials cannot be assumed and should be tested prior to treatment with a combination regimen[257]. The explanation which supports the initial use of combination therapy for infections with gram-negative bacteria is based on the following reasons: (1) to broaden the empiric coverage provided by two antimicrobial agents with different spectra of activity (to ensure that the pathogen is adequately covered by at least one of the two components of the regimen), (2) to exploit the synergy observed *in vitro* between two antimicrobial agents compared to one (and hence improve clinical outcomes), or (3) to prevent or delay the emergence of resistance during antimicrobial therapy[258][259][260]. Despite the intuitive appeal for use of these new approaches, strong evidences had proven that the use of two antimicrobials to treat infections with gram-negative bacteria is almost lacking, and may even be harmful for patient health. The addition of a second antimicrobial agent to treat a gram-negative microorganism that is susceptible to a single agent may actually lead to increased adverse effects including drug toxicity, costs healthcare and also antimicrobial resistance[261][262].

Particularizing for *P. aeruginosa- C. albicans* infections in VAP, a combination therapy with antibiotics and antifungals is not considered, since *Candida* species colonization of respiratory-tract is not recognized as requiring antifungal treatment[230].

Although, the combination therapy is not used in clinical practice given the limitations associated, this has shown a high potential for treatment of VAP in future. As such, the optimization of this therapy, namely randomized controlled trials with robust study designs, are required to ensure successful treatment of these infections[263].

1.6. Antimicrobial resistance

As already mentioned, the occurrence of *P. aeruginosa* and *C. albicans* in the RTI not only increases the risk of developing VAP but also brings high morbidity and mortality rates in ill patients. According to WHO, the antimicrobial resistance of these microorganisms is one of the main sources associated with this phenomenon, leading to ineffective treatment which results in persistence and spreading of infections. These infections show as an expanding problem in medical field[89][264][265][266][267].

This antimicrobial resistance is the cause for microorganisms to fail to respond to standard antimicrobials, which relies on extending the duration of treatment and consequently increases of the health care costs[267]. Despite of administration of appropriate doses of antimicrobials for a specific duration of time, the high levels of resistance developed by several microbial strains promoted survival of the microorganisms[267]. The treatment failure occurs due to the antimicrobial resistance but also due to the suppressed immune function, poor/deprived antimicrobial bioavailability or increased rate of antimicrobial metabolism[267].

However, the understanding of numerous factors associated to the antimicrobial resistance such as: the increase of intrinsically resistant species; the accumulation of mutations that cause resistance; the swap of mobile resistance components and the extensive use of antimicrobials for treatment of VAP infections, is necessary to stand actual trends[98][268].

Antimicrobial resistance can be classified as follows:

- Primary resistance: occurs when the microorganism has never encountered the antimicrobial of interest in a certain host[267];
- Secondary resistance: also known as “acquired resistance”; describes the resistance that only arises in a microorganism after an exposure to the antimicrobial agent[227][269]. It may further be classified as: 1) *Intrinsic resistance*; refers to the non-sensitivity of all microorganisms of a single-species to certain common first-line

antimicrobials, which are used for treatment of diseases based on the clinical evidence of the patient. It is also known as MDR[227]; 2) *Extensive resistance*; defines the ability of microorganisms to withstand the inhibitory effects of at least one or two most effective antimicrobials. Also mentioned as extensively drug resistant- XDR, this seemed to arise in patients after they have undergone a treatment with first line antimicrobials[270][271].

- Clinical Resistance, defined when the infecting microorganism is inhibited by a concentration of an antimicrobial agent, which associated with a high likelihood of therapeutic failure or reappearance of infections due to impaired host immune function. In other words, the pathogen is inhibited by an antimicrobial concentration that is higher than could be safely achieved with normal dosing[227].

Generally, the antimicrobial agent acts on the microorganism by inhibiting a metabolic pathway like nucleotide synthesis that leads to the inhibition of deoxyribonucleic acid (DNA)/ ribonucleic acid (RNA) synthesis and protein synthesis, disruption of the cell membrane or by competing with the substrate of any enzyme involved in cell wall synthesis (e.g., chitin synthase)[272]. So, the microorganisms have evolved in terms of a multitude of resistance mechanisms in order to overcome the effectiveness of antimicrobials, surviving and persisting in sites of infections (Figure 1.9).

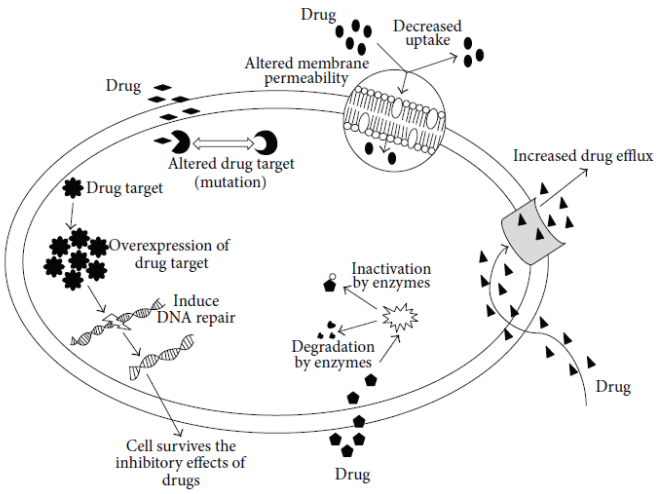


Figure 1.9- Mechanisms of MDR. Image reprinted, from [267].

1.6.1. Resistance mechanisms associated to biofilms

Cells in a mature biofilm (sessile cells) are phenotypically and physiologically different from non-adhered (planktonic cells) cells. One of the characteristics of sessile cells relies on the requirement of much higher concentrations of antimicrobial agents to kill these cells in compared with the planktonic one[188]. Biofilms exhibit greater antimicrobial resistance in comparison with planktonic cells (up to 10 - 1000 fold more) and this could be the explanation for frequent therapeutic failure of antimicrobials against biofilm infections[273][183][274][275][276][277][278].

A complex and multifactorial mechanism is involved in biofilm resistance to antimicrobials agents (Figure 1.10).

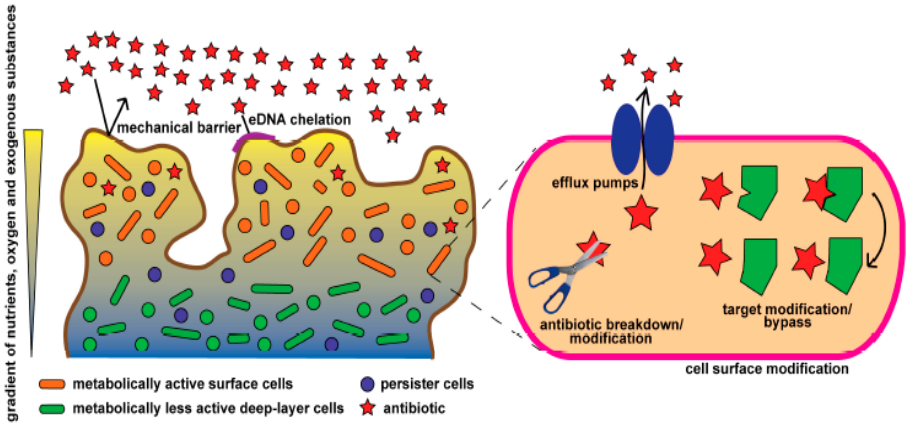


Figure 1.10- Representation of the resistance mechanisms of biofilms. Image reprinted, from [256].

The mechanisms of biofilm resistance to antimicrobials agents include:

- Failure of antibiotics to penetrate ECM biofilm: the ECM function as a structure providing protection to the cells in the biofilm acting as a barrier, and is considered one of the causes associated to antimicrobial resistance, where the antimicrobial agents may be prevented from penetrating the biofilm. These antimicrobials can be prevented from penetrating if they bind to components of the biofilm matrix or to microorganism membranes[279][280]. Positively charged antimicrobials that bind to negatively charged biofilm matrix polymers delay their penetration through biofilm. Additionally, biofilm protect themselves from antimicrobials through meeting with retention places on medical devices. Furthermore, the selection of resistant microorganism increases due to the high density of microorganisms in biofilms under pressure from antimicrobials by enhancing horizontal gene transfer and the frequency

of mutation[281]. Recently studies have reported the role of biofilm polysaccharides in the protection of the bacterial and fungal biofilm against antimicrobials[282]. Retardation of the antimicrobial penetration rate can also induce expression of genes that mediate resistance within the biofilm[283].

- Slow growth rate: multiple microcolonies in the biofilm induce a metabolically heterogeneous microorganism population[185][284]. In certain environments, local diffusion gradients are created for promoting anoxic and acidic zones in the interior of the biofilm[285]. In general, zones that are nutrient-deficient can develop stationary phase-like dormant cells, which may be responsible for antimicrobials resistance of the biofilm[279][286]. In addition, it is thought that limited penetration of nutrients rather than restricted access for antimicrobials contribute to the general resistance seen in biofilms[252].
- Altered metabolism: cells with various genotypes and phenotypes coexist within the biofilm population. Thus, this causes expression of distinct metabolic pathways based on the local environmental circumstances in the biofilm. Studies have shown that biofilms are heterogeneous structures with three chemical patterns that correspond to gradient of antimicrobials with differences in concentration from outside to inside the biofilm. The metabolic activity of microorganisms is higher in the external part of the biofilm and lower in the internal part leading to a reduced susceptibility to antimicrobials[279][287][288]. The metabolic intermediates pattern indicates a greater aqueous phase concentration between the boundary of the biofilm[289]. The limited oxygen and nutrient penetration due to their consumption by microorganisms present are factors causing this difference of physiologic activity[279]. In a biofilm, the antimicrobial tolerance can occur through nutrient deprivation, which causes slow bacterial growth or starvation[290][291]. Numerous antimicrobials target mechanisms occurring in the growth of microorganisms (such as replication, transcription, translation and cell wall synthesis). The increase of the antimicrobials tolerance will occur in biofilm with low metabolic activity located in the internal part of biofilms and not in the external part, thus compromising the antimicrobial penetration[292].
- Persister cells: after prolonged exposure (or overdose) to antimicrobials, some microorganisms exhibit a small percentage of the viable cell population, which are denoted by persister cells. This is a small subpopulation of microorganisms that has entered in a slow-growing or starving state, temporarily stopping the replication for the survival of the community[293][294]. Persister cells survive to doses of antimicrobials that generally kill normal cells. The reduced metabolic rates of these cells make them

more resistant to antimicrobials compared to the active metabolic ones- exponential growth-phase microorganisms. Once antimicrobials must work on growing cells to destroy them, the hibernating cells can outlast the antimicrobials and contribute to the persistent infections principally in sites where immune components are limited, such as in biofilms[293]. It seems that biofilms work as protective habitat for persisters cells[295][296][297][298] and typically these cells present the down regulation of energy-producing and biosynthetic functions exhibiting enhanced toxin/antitoxin (TA) systems induced by starvation or DNA damage. In biofilms, several TA systems have been associated with high numbers of multidrug tolerant persister cells. However this tolerance is restricted to specific antimicrobials and TA. Therefore, this demonstrates that persister cells may be produced by multiple pathways[299]. However, persister cells can resuscitate and even revert to a growing state following antimicrobial therapy, thereby repopulating the biofilm and initiating a relapse of the infection. Additionally, persister cells do not produce offspring resistance to the antimicrobial agent and are capable of growing in the presence of the antimicrobial while maintaining the same MIC. The main evidences of the existence of persistent cells in biofilms are: a) presence of biphasic dimension in biofilms; b) expression of persistence gene; c) use of bacteriostatic antimicrobials inhibit the growth of sensitive cells which contribute to persistent cell growth and preservation of biofilm; d) when therapy is withdrawing the therapy of the biofilm reshapes[290][300].

- Oxygen gradients: the oxygen tension in the depth of the biofilm is low, such as been found in *P. aeruginosa*. Hypoxia promotes antimicrobial resistance by altering the composition of multidrug efflux pumps[301]. Efflux pumps are protein structures that are able to expel compounds such as antimicrobials. The presence of these pumps can be permanent (constitutive expression) or intermittent (expression can be induced) and these structures may be specific to a substrate or similar compounds and may be associated with MDR[302][303]. The oxygen limitation accounts for 70 % (depending on the antimicrobial) or more of all the antimicrobial tolerance in *P. aeruginosa* biofilms grown *in vitro* for 48 h, where most of the cells occupied an oxygen-limited stationary phase[304]. In addition, the anaerobic environment within biofilms will most likely affect aminoglycoside antimicrobial activity due to the downregulation of energy metabolism genes[305] and by triggering changes in gene expression[306].

Other mechanisms may compromise the antimicrobial treatment of biofilm-induced disease[307] such as:

- Antimicrobial resistance determinants in biofilm;

- β-Lactamases: substances from neighboring biofilm cells may protect against antimicrobials; subpopulations in biofilm- cells that are metabolic active in the biofilm and also important for development of antimicrobials resistance but that depend on the involvement of different genes;
- DNA: ECM contains large amounts of extracellular DNA (eDNA). Although, sub inhibitory concentrations of DNA in biofilms resulting in protection of the microorganisms from antimicrobials. The reduced biofilm activity to positively charged antimicrobials may also be related to the cation chelating properties of eDNA[308];
- Proteins: some are preferentially expressed in biofilm. Biofilm development manly their growth and maturation is accompanied by a progressive increase in protein production related to antimicrobial resistance and virulence[309];
- Stress: biofilm resistance may simply reflect biofilm cells responding to stress (scarcity of nutrients, excess of waste products, hypoxia, and antimicrobials), which may induce mutations in biofilms. Oxidative compounds in the biofilm are also thought to favor the overexpression of some efflux proteins that are involved in the extrusion of antimicrobials from microorganism, thereby causing antimicrobial resistance[310];
- Sub-MIC: it is likely that some cells in biofilm are exposed to sub-MIC levels of antimicrobials during therapy due to falling concentrations by dilution or diffusion gradients for antimicrobials in biofilm. Sub- MIC of antimicrobials can induce mutagenesis, which confers resistance[311];
- Swarming: the microorganisms with the ability to swarm reflect a social multicellular behavior inducing antimicrobial resistance[312];
- Mutation: the biofilm growth mode can lead to oxidative stress, which may cause enhanced mutability in biofilms[313][314]. Mutations in cells include alteration of antimicrobial targets, increases in the expression of drug efflux pumps, and reduction in the permeability of the cells due to alterations in the outer membrane and in the action of modifying enzymes[282];
- QS: it is a biofilm-specific mechanism, which regulates several factors that contribute to biofilm formation and persistence, once inhibits the penetration of some antimicrobials into the biofilm[292];
- Genetic transfer: the biofilm might be ideal for horizontal gene transfer[315] and this can make biofilm a reservoir for antimicrobial resistance genes. The transfer of DNA occurs within the biofilm community by three major mechanisms: transformation, transduction and conjugation[316]

CHAPTER II

METHODOLOGY

2.1. Microorganisms and Culture Conditions

2.1.1. Microorganisms

In order to create a polymicrobial environment, such as the one present in VAP, all assays were performed with two different reference microorganisms, a yeast strain of *C. albicans* (SC5314) and a bacterial strain of *P. aeruginosa* (PAO1).

2.1.2. Microorganisms preservation

Sabouraud Dextrose Broth (SDB; Liofilchem®, Italy) medium was used for *C. albicans* and Tryptic Soy Broth (TSB; Liofilchem®, Italy) medium for *P. aeruginosa*. Both strains were properly stored at -80 °C ± 2 °C in criovials containing the respective growth medium supplemented with 20 % (v/v) glycerol.

Prior to each experiment, bacterial cells were thawed from the frozen stock solutions and subcultured on Tryptic Soy Agar (TSA; Liofilchem®,Italy) plates. On the other hand, the yeast cells were thawed from frozen stock solution and subcultured on Sabouraud Dextrose Agar (SDA; Liofilchem®,Italy) plates. Both plates were incubated aerobically at 37 °C for 18 - 24 h in static conditions. Afterwards, both agar plates were storage at 4 °C for a maximum period of two weeks.

2.1.3. Culture media and buffers

Pure liquid culture of *P. aeruginosa* was grown in TSB, while *C. albicans* was grown in SDB. In order to distinguish the different microorganisms, several media were prepared as following:

- Tryptic Soy Agar (corresponding to TSB supplemented with 1.2 % wt/v agar) was used as non-selective culture medium for *P. aeruginosa*;
- Sabouraud Dextrose Agar (corresponding to SDB supplemented with 2 % wt/v agar) was used as non-selective growth medium for *C. albicans*;
- Pseudomonas Isolation Agar (PIA; Sigma- Aldrich, St. Louis, MO, USA) was used as selective growth medium for specific isolation of *P. aeruginosa* from mixed cultures of *P. aeruginosa* and *C. albicans*;

- SDA supplemented with 30 mg/L gentamicin (gent; Sigma- Aldrich, St. Louis, MO, USA) was used as selective culture medium for specific isolation of *C. albicans* from mixed cultures of *P. aeruginosa* and *C. albicans*.

All growth culture media were sterilized in a steam autoclave at 121 °C for 20 min, at 1 atm, immediately after addition of all components.

Furthermore, RPMI 1640 medium (Gibco® by life technologies ™, Grand Island, NY, USA) adjusted to pH 7.0 was used to prepare antimicrobial agents working solutions, planktonic and biofilm assays. This culture medium was sterilized by membrane filtration process using a 0.22 µm and stored at 4 °C.

Unless otherwise stated, sterilized distilled water was used in all rinsing steps. To prepare the stock solutions of all antimicrobial agents it was used ultrapure (UP) sterile water.

2.1.4. Preparation of microbial suspensions

In order to prepare the microbial suspensions for each assay, 3 to 4 colonies of *P. aeruginosa* and *C. albicans* (from TSA and SDA plates, respectively) were collected and inoculated in 15 mL of TSB and SDB, respectively for 12 - 18 h at 37 °C in a horizontal shaker with a constant agitation of 120 rpm.

Subsequently, both suspensions were harvested by centrifugation at 3000 g for 10 min at 4 °C to recover the cells. The recovered cells were washed twice with sterilized distilled water and re-suspended in 5 mL of RPMI 1640 medium for further analysis.

2.1.5. Antimicrobials agents

Three clinically relevant antibiotics (tobramycin, meropenem and ciprofloxacin), a clinically important antifungal agent (amphotericin B) and two different antimicrobials peptides (colistin and polymyxin B), were used throughout this study as stated below:

- Tobramycin (ToB), a narrow-spectrum antimicrobial from aminoglycosides drug class[317];
- Meropenem (Merp), an ultra-broad-spectrum antimicrobial from carbapenem class[317];
- Ciprofloxacin (CIP), a broad-spectrum antimicrobial from fluoroquinolones drug class[317];

- Colistin (CoL) and polymyxin B (PolyB) a relatively narrow-spectrum antimicrobials (activity against most gram-negative aerobic bacilli), from polymyxins drug class[317];
- Amphotericin B (AmB), a broad-spectrum antimicrobial from polyene drug class[318].

While antibiotics and antimicrobial peptides were employed in the current study due to their use in health clinics, aiming to treat infections caused by *P. aeruginosa,* the antifungal was used due to its relevance in cases of serious and invasive *Candida* infections.

All antimicrobials were purchased from Sigma-Aldrich, with exception of PolyB from Biochrom GmbH, Berlin, Germany and Merp from USP®, RockVille, MD.

Stock solutions of all antimicrobials were prepared in UP sterile water in a concentration of 5000 mg/L. ToB and AmB were diluted in UP sterile water preparing a solution of 1000 mg/L. All stock solutions were stored in the freezer (-20 °C) and used for preparing the adequate dilutions for further assays. For susceptibility testing, antimicrobials were serially two-fold diluted in RPMI 1640 medium.

2.2. Planktonic assays

Determination of minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC)

In this experiment, the MIC for both strains and the MMC for *P. aeruginosa* (MBC-minimum bacterial concentration) and *C. albicans* (MFC-minimum fungicidal concentration), were determined in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards[319].

The antimicrobial work concentrations (diluted antimicrobials in RPMI 1640 medium) ranged from:

- 0.031 to 16 mg/L for AmB;
- 0.031 to 512 mg/L for ToB and Merp;
- 0.031 to 1024 mg/L for CoL, PolyB and CIP.

All antimicrobial agents' concentrations were used to determine the MIC of both microorganisms throughout this study, under single and in mixed planktonic cultures.

Briefly, the optical density (OD) at 640 nm (OD_{640}) of bacterial suspension was measured and the yeast suspensions were enumerated using a neubauer counting chamber. A

cellular concentration of 1×10^6 cells/mL was prepared for both strains. Then, in each well of a sterile 96-well round-bottom polystyrene (PS) microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium), 100 µl of antimicrobials in double concentration of the final concentration desired were dispensed in the wells. Then, each well containing 100 µL of diluted antibiotic agent in RPMI 1640 medium, a volume of 100 µl of each cell suspension in RPMI 1640 medium (in case of mixed culture was added 50 µl of *P. aeruginosa* inoculum and 50 µl of *C. albicans* inoculum) was added. In one of the wells, only 200 µL of RPMI 1640 medium was added for the negative control and for the positive control was added 100 µL of RPMI 1640 medium and 100 µL of each desired cell culture. Therefore, the final inoculum concentration was adjusted to 5×10^5 cells/mL for *P. aeruginosa* and 5×10^5 cells/mL for *C. albicans*. All 96-well round-bottom plates were incubated at 37 °C for 24 h in static conditions.

After incubation, the MIC was obtained by reading the OD_{640} of the planktonic cell fraction. Alternatively, for some cultures, MICs were obtained by visual observation of the turbidity gradient. This turbidity shows the microorganisms capacity of growing as a planktonic population in the presence of antimicrobials. The minimum concentration where growth inhibition occurs is equivalent to the MIC value for most microorganisms[273][320].It is important to refer that after antimicrobials exposure and prior OD_{640} readings, the content of the wells (planktonic suspension) was transferred to new plates, in order to prevent interference by biofilm-cells in the bottom of the wells.

The enumeration of colony forming units (CFUs) was performed for determination of the MMC values (corresponding to the lowest concentration of an antimicrobial that had resulted on 99 % killing of planktonic microorganism.). Though, 10 µL of culture medium was collected from the wells of the microdilution trays after incubation and were plated in normal and selective agar plates, according to the microorganism in study (section 2.1.3). The lowest antimicrobial concentration that yielded no colony growth after 12 - 24 h at 37 °C in static conditions was documented as the MMC.

Checkerboard microdilution assay

In order to determine the interaction between the antimicrobials, one of the two most common methods of synergy testing - the checkerboard microdilution assay - was performed. There has been interest in the use of synergy testing to provide to the clinicians the suitable antimicrobial combinations. This is extremely important in order to expand the spectrum of activity of individual agents. Mostly, it has been employed for patients with infections associated *P. aeruginosa*[317].

The checkerboard microdilution assay was performed with two agents combined from different antimicrobial classes in serial two-fold increasing concentrations.

In this study, the combinations of antimicrobials and their concentrations range were tested as follow described:

- 0.016 to 4 mg/L AmB and 0.016 to 1024 mg/L PolyB or CoL for *C. albicans* culture and mixed culture (*P. aeruginosa* + *C. albicans*);
- 0.016 to 128 mg/L ToB and 0.016 to 512 mg/L PolyB or CoL for *P. aeruginosa* culture and mixed culture (*P. aeruginosa* + *C. albicans*);
- 0.016 to 4 mg/L AmB and 0.016 to 128 mg/L ToB for *C. albicans* culture, *P. aeruginosa* culture and mixed culture (*P. aeruginosa* + *C. albicans*).

In order to evaluate the potential synergy, the fractional inhibitory concentration (FIC) was calculated by comparing the MIC of each agent alone (mentioned in previous section) with the MIC of the agent in combination as shown in Figure 2.11. The methodology used to determine the MIC of antimicrobial combinations described in previous section, with exception that in each well was dispensed 50 µL of each antimicrobials agents, make up a final volume of 100 µL. Each antimicrobial agent was prepared four-fold above of the desired final concentration.

$$FIC = \frac{MIC\ A_{\ comb\ A/B}}{MIC_{\ agent\ A}} + \frac{MIC\ B_{\ comb\ A/B}}{MIC_{\ agent\ B}}$$

$$FIC = \frac{2}{16} + \frac{2}{16} = 0.25$$

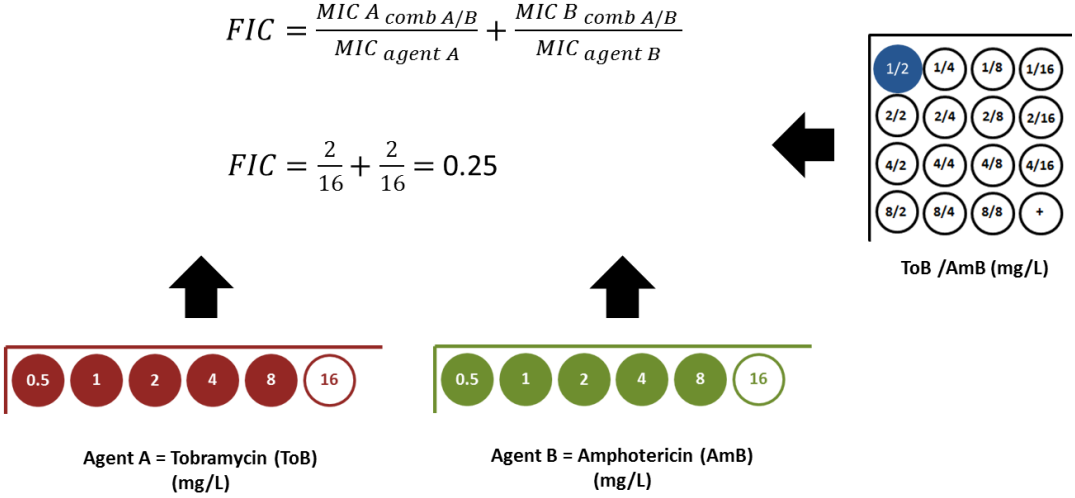


Figure 2.11- Calculation of the FIC. For ToB and AmB combination, the FIC value of 0.25 is considered as synergistic. Image adapted from[317].

The synergy was defined as a four-fold reduction in the MIC of the agents alone compared with the MIC in combination. The breakpoint is to be interpreted based on the

Table 2.1. Although the interpretation of FIC may vary from study to study, however synergy remains the same.

Table 2.1- Comparison of interpretative criteria FIC. Table adapted from[317]

FIC	Interpretation 1	Interpretation 2
≤0.5	Synergistic	Synergistic
>0.5 – 1.0	Non-synergistic	Additive
1 – 4		Indifferent
>4	Antagonistic	Antagonistic

2.3. Biofilm assays

2.3.1. Biofilm formation

For biofilm formation, as mentioned previously in section 2.1.4, 3 to 4 colonies of each strain was ressuspended in 15 mL of TSB medium (*P. aeruginosa)* and SDB medium (*C. albicans*) at 37 °C and 120 rpm.

After cell incubation (12 - 18 h) the cultures were centrifuged at 3000 g for 10 min at 4 °C and washed twice with sterilized distilled water.

Then, bacteria were ressuspended and diluted in RPMI 1640 medium to achieve a final concentration of 1 x 10^7 CFU/mL measured by ELISA microtiter plate reader with a wavelength of 640 nm (Sunrise-Basic Tecan, Austria). Additionally, yeast cells were enumerated using a neubauer counting chamber and were ressuspended to a final concentration of 1 × 10^7 cells/mL in RPMI 1640 medium.

Dual-species biofilms encompassing the *P. aeruginosa* and *C. albicans* were evaluated in order to investigate the interplay of both pathogens on VAP infections.

The following conditions were tested:

- Single-species biofilm composed by *C. albicans;*
- Single-species biofilm composed by *P. aeruginosa;*
- Dual-species biofilm composed by *P. aeruginosa* and *C. albicans.*

The methodology used to grow the biofilms was based on the microtiter plate test developed by Stepanovic *et al*.[321]. Biofilm formation assays were performed in sterile 96-well PS microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). Each well was filled with 200 µL of each cell suspension, except to develop dual-species biofilms, where 50 % of cell

suspension of each microorganism (*P. aeruginosa* and *C. albicans)* was transferred into the same well of 96-well plates. Negative controls consisted of wells filled with 200 µl of culture medium only (RPMI 1640 medium). All plates were incubated aerobically on a horizontal shaker with a constant agitation (120 rpm) at 37 °C for 24 h and 48 h for biofilm formation. To form and maintain 48 h biofilms, 100 µl of culture medium was removed and an equal volume of fresh medium was added, after 24 h. After biofilm formation, all planktonic fractions in the wells were removed, and the wells were washed twice with sterilized distilled water to remove the non-adherent cells. Then, the results were assessed using different methods (see section 2.3.3).

2.3.2. Biofilm cells susceptibility tests to antimicrobial agents

In order to assess biofilm cells susceptibility, antimicrobial agents were added after 24 h of biofilm formation. Several concentrations of the antimicrobial agents were prepared in RPMI 1640 medium.

2.3.2.1. Effect of single antimicrobials

Biofilms (single- and dual-species) were formed after 24 h, as previously described in section 2.3.1. Afterwards, 100 µl of culture medium was removed and an equal volume of the respective antimicrobial concentration (1x, 2x and 4x the MIC of AmB, ToB, CoL and PolyB) was added to wells that were incubated for further 24 h at 37 °C in static conditions. In the positive control, 100 µl of RPMI 1640 medium was added, without cells.

After each incubation time, the culture medium was aspirated and the wells were washed twice with sterilized distilled water. The results were assessed using the CFUs enumeration method as described in section 2.3.3.1

In order to evaluate the therapeutic potential (antimicrobials effect along time), the antimicrobials concentration and methodology used follows the same as stated above, altering exclusively the time of incubation. Thus, the assay was repeated every 12 h up to 48 h.

Every 12 h, the culture medium was aspirated and the corresponding wells were washed twice. Further analysis of the CFUs enumeration was performed. To the remaining wells, 100 µl of culture medium was removed and an equal volume of fresh RPMI 1640 medium (in positive control) or antimicrobial agents was added.

2.3.2.2. Effect of the combination antimicrobials'

After performing the checkerboard microdilution assay, the following antimicrobial combinations were selected to be applied to the single- and dual-species biofilms:

- AmB, ToB and PolyB (0.25 + 0.016 + 1 mg/L);
- ToB and CoL (1 + 0.016 mg/L);
- ToB and PoLyB (1 + 0.016 mg/L), (0.016 + 256 mg/L), (0.016 + 32 mg/L), (0.016 + 16 mg/L) and (0.016 + 8 mg/L);
- AmB and CoL (1 + 0.063 mg/L);
- AmB and PoLyB (1 + 2 mg/L), (0.016 + 256 mg/L), (0.016 + 32 mg/L), (0.016 + 16 mg/L) and (0.016 + 8 mg/L);
- AmB and ToB (1 + 0.063 mg/L).

These combinations were selected based on the existence of synergism between the antimicrobial agents against the planktonic cells. The combinations showing the lowest concentration were selected.

Furthermore, this assay was performed according to the methodology mentioned in section 2.3.2.1, changing solely the amount of antimicrobial agent from 100 µl to 50 µl of each antimicrobial agent in the respective aforementioned concentrations, except for AmB, ToB and PolyB combination where it was added 33,3 µl of each antimicrobial agent.

Kinetic effect

This assay was performed as already described in section 2.3.2.1. However, it is important to note that after 24 h-old pre- established dual-species biofilms, 50 µl of each concentration of antimicrobials was added in the selected wells, resulting the following combinations:

- AmB, ToB and PolyB (0.25 + 0.016 + 1 mg/L);
- ToB and PoLyB (0.016 + 256 mg/L), (0.016 + 16 mg/L) and (0.016 + 8 mg/L);
- AmB and PoLyB (0.016 + 256 mg/L), (0.016 + 16 mg/L) and (0.016 + 8 mg/L).

The 24 h kinetic study was performed by sampling every 2 h up to 24 h after exposure to different concentrations of antimicrobials agents aforementioned. The analysis of the dual-species biofilms at different time points was performed by CFUs enumeration.

Post antimicrobial effect

This assay was performed as described in *Kinetic effect* section. All antimicrobial combinations used were the same, though exception made to the AmB and CoL combination (1 + 0.063 mg/L) combination, which was not tested in this assay. Also, this assay was only applied to dual-species biofilms.

This assay was performed every 24 h up to 120 h. It considers two cycles of 48 h each. Immediately after 24 h of pre-formation of biofilms and analysis by CFUs enumeration, 50 µl of each concentration of the antimicrobial combinations mentioned above was added to all wells. The plates were incubated at 37 °C for further 24 h in static conditions. After 24 h, the culture medium of wells was aspirated and the wells were washed twice with sterilized distilled water. Further analysis of these time points was done by the CFUs enumeration. To the remaining wells, 200 µl of fresh RPMI 1640 medium was added. Afterwards, the plates were incubated aerobically on a horizontal shaker with a constant agitation (120 rpm), at 37 °C, for another 24 h.

The results were analyzed once more by CFUs enumeration and this cycle was repeated again.

2.3.3. Biofilm analysis

CFUs enumeration, crystal violet (CV) method and scanning electron microscopy (SEM) were used to analyze the biofilms. To quantify the number of cultivable cells present in the biofilms, the CFUs were estimated. CV was carried out to quantify the total biofilm biomass by absorbance reading at 570 nm. SEM was conducted for structure observation of the biofilm formation.

2.3.3.1. CFUs enumeration

To determine the number of CFUs, 200 µl of sterilized distilled water was added to each well and the adherent cells were detached from the surface by scrapping. The triplicates

were pulled in the same container. The total cell suspension was vortexed (to disrupt the biofilm matrix on the case of the biofilm formation assays). Subsequently, it was done serial decimal dilutions (usually up to 10^{-7}) of the inoculum in sterilized distilled water and plated for CFUs counting. CFUs enumeration was estimated using the micro drop technique, in which droplets of 10 µL of each dilution were placed on agar plates and allowed to dry. The 96-well plates were incubated at 37 °C in aerobic and static conditions. *P. aeruginosa* was incubated overnight and *C. albicans* for 18 - 24 h, to enable the counting of colonies.

The results were then expressed as the number of CFUs per unit area (Log_{10} CFU/cm^2).

2.3.3.2. CV staining

The 96-well plates containing the biofilms were left to air dry for 30 min, and then 200 µL per well of pure methanol (Valente e Ribeira Lda, Belas) (100 % v/v) was added to each well in order to fix the biofilm- cells for 15 min. Following this, the liquid phase was removed and plates were left to dry for 5 min at room temperature until they were completely dehydrated.

Afterwards, biofilms were stained with 200 µL of 1 % (wt/v) CV (Gram colour-staining set for microscopy; Pro-Lab Diagnostics, UK) per well, which remained in contact with the cells for approximately 5 min at room temperature.

Then, CV was aspirated and the wells were washed twice with tap water to remove excess of stain and dried at room temperature for approximately 20 min until complete drying. Then, 200 µl of acetic acid (Fisher Scientific, UK) (33 % v/v) was added to each well in order to solubilize the CV bound to the adherent cells.

The quantitative analysis of biofilm production was performed by measuring the OD at 570 nm (OD_{570}), in each well, using an ELISA microtiter plate reader.

If the OD was higher than 1.0, the sample was diluted with acetic acid (33 % v/v).

2.3.3.3. SEM

Prior to SEM observations, the biofilms attached to wells were gradually dehydrated by immersion in ethanol (Farlab Comércio e Representações Lda, Fânzeres, Portugal) solutions with increasing concentrations (20, 40, 50, 70, 90 % v/v) for 10 min and at last 100 % for 20 min. Subsequently, the wells-attached biofilms were transferred to the desiccator for complete dying. After this step, the walls of the wells were sputter-coated with gold and mounted on aluminum stubs with carbon tape. The examination of the surface structural

conformation of the biofilms was performed with a Leo SEM (Cambridge, FEI Company, USA). SEM observations were documented by the acquisition of photomicrograph.

2.4. PNA FISH (Peptide nucleic acid fluorescence in situ hybridization) analysis

PNA FISH enable the detection and discrimination between bacterial populations. It also allows observing the *in situ* localization, distribution and arrangement of bacterial cells within the consortia, without biofilm disruption. In this way, it leads to a better understanding of the real bacterial interactions occurring in the polymicrobial biofilms[322].

A classic FISH procedure contains three main steps: fixation, hybridization and washing, as demonstrated in Figure 2.2.

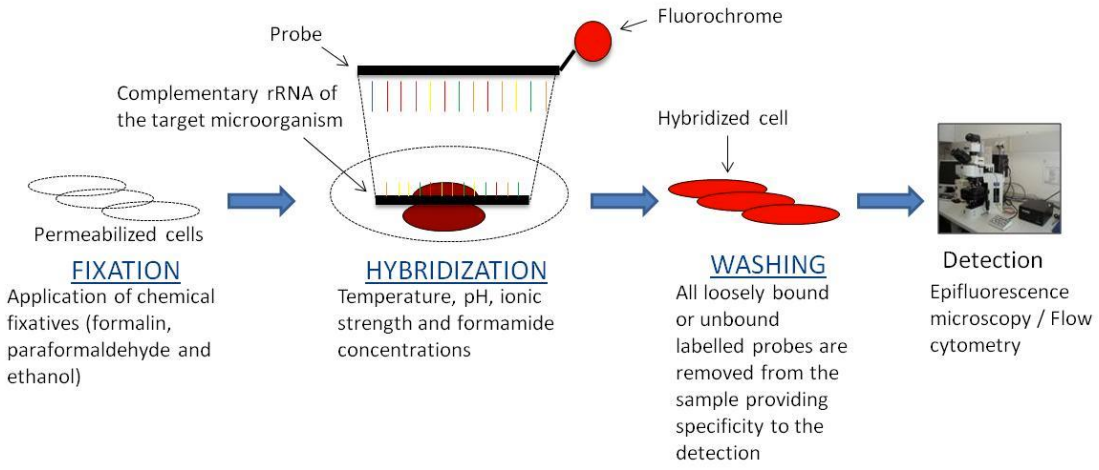


Figure 2.2- Schematic representation of FISH methodology. Image reprinted, from[323].

Firstly, the sample is fixed using an aqueous solution of chemical fixatives, such as formalin, paraformaldehyde and/or ethanol. Then, the bacterial sample is hybridized with a fluorescently labeled probe that is complementary to the 16S rRNA in the cells. After incubation, the probe has to be removed by a washing step, to provide specificity to the detection[324][325][326]. Samples can be visualized using fluorescent or laser scanning microscopy, where it is possible to identify the microorganisms, reveal their precise location in a three-dimensional community, retaining cells morphology and integrity[326][327].

Biofilm formation on PS coupons

In order to apply PNA FISH analysis to biofilms, they have to be formed in PS coupons (1 × 1 cm) placed in the bottom of the wells of sterile 24-well PS plates (Orange Scientific, Braine-l'Alleud, Belgium).

Prior to this step, PS surfaces were immersed in a commercial detergent (Sonasol, Henkel Ibérica Portugal, Bobadela, Portugal), during 3 min, washed three times in UP sterile water and soaked for 3 h.

To promote biofilm formation on coupons, biofilm inoculum of *P. aeruginosa* and *C. albicans* were prepared as described in section 2.1.4. Afterwards, the biofilms were dispensed 24-well plates containing the coupons. In case of single-species biofilms, these were dispensed at 1 mL of each strain. In dual-species biofilms (*P. aeruginosa* and *C. albicans)* were dispensed 500 µL of each of the two strains. These biofilms were formed on such coupons for 24 h or 48 h at 37 °C under agitation (120 rpm).

After 24 h and 48 h of biofilm formation, the PS surfaces of positive controls were washed twice with 1 mL UP sterile water and allowed to dry (~60 °C) for 15 min. The biofilm was fixed with methanol (Valente e Ribeira Lda, Belas) (100 % v/v) for 20 min. This initial step of fixing the biofilm (with methanol) is essential to avoid the detachment of cells during the hybridization procedure[328].

However, this method was also applied to the assay with antimicrobial combinations in 24 h-old pre-established biofilms described in section 2.3.2.2. only for dual-species biofilms. So, after 24 h-old pre- established biofilms, it was removed 500 µl of culture and added the combinations of antimicrobials agents in accordance with the methodology referred in the present section. Biofilms were maintained for further 24 h at 37 °C in order to evaluate the therapeutic action of the antimicrobials. The respective washes and methanol addition to dual-species biofilms were performed.

Fixed biofilms were stored at 4 °C for a maximum of 48 h before the PNA FISH method.

PNA FISH applied to biofilms

After performing the biofilms fixation, a PNA FISH assay was evaluated on dual-species biofilms encompassing *P. aeruginosa* and *C. albicans*. The yeast strain was identified by counterstaining the samples with 4`, 6 –diamidino–2 -phenylindole (DAPI; Sigma- Aldrich, St.

Louis, MO, USA) staining at the end of the hybridization procedure. The probe used in this assay was designated Paer565[329] in order to label *P. aeruginosa.*

After biofilms development on PS coupons, 30 µl of each solution of 4 % (wt/v) paraformaldehyde followed by 50 % (v/v) ethanol was dispensed in the PS coupons for 10 min each and allowed to air dry. This step allows the fixation of the cells and increases the permeabilization of the cell membrane to the subsequent hybridization allowing the labeled oligonucleotide probes to diffuse to their intracellular rRNA target molecules.

Subsequently, 20 µl of hybridization solution containing the probes mixture at 200 nM were dispensed on the coupons, which were finally covered with coverslips and incubated in the dark for 1 h at 65 °C. Soon after hybridization, PS coupons were carefully removed and were immersed for 30 min in 24-well plates containing 1 mL per well of a pre-warmed (65 °C) washing solution composed of 5 mM Tris Base, 15 mM sodium chloride (NaCl) and 1 % (v/v) Triton X-100 (all from Sigma- Aldrich, St. Louis, MO, USA). The PS coupons were removed from the 24-well plates and allowed to air dry in the dark before counterstaining with DAPI. For yeast cells, each coupon was covered with 20 µL of DAPI (40 µg/mL) for 5 min at room temperature in the dark. Immediately afterwards, it was performed the observation in the fluorescence microscope, where the negative controls were assessed for each experiment, without any probe added to the hybridization solution.

For microscopic visualization, it was used a fluorescence microscope (Olympus BX51, Perafita, Portugal), a DAPI filter (BP 365 - 370, FT 400, LP 421) with $\lambda_{excitation}$ = 365 - 370 nm and the signaling molecule of the PNA probe (BP 530 - 550, FT 570, LP 591, for Alexa 594).

2.5. LIVE DEAD staining

The traditional colony counts or membrane procedures has come to prove methods with many weaknesses, rising questions about the validity of heterotrophic bacteria plates counts and coliform as indicators[330].

The enumeration of CFUs only includes cultivable cells which are able to initiate cell division at a sufficient rate to form colonies and they are very sensitive to culture conditions (temperature, media, incubation time[331] and responses) may require from 24 h to more than 1 week[330].

In order to evaluate the efficacy of the antimicrobial agents on cell viability, as an alternative method to plate counts to enumerate viable cells, the Live/Dead *Bac*Light Bacterial Viability Kit (Molecular Probes, Leiden, Netherlands), was performed.

After washing the biofilms formed (as described as section 2.4.) on PS coupons, they were placed in sterile 24-well PS plates.

After 24- and 48 h-old pre-established dual-species biofilms, 500 µl of culture medium were removed and 250 µl of each antimicrobial combination was added to the wells to assess the therapeutic action the following combinations of antimicrobials:

- ToB and PoLyB (0.016 + 256 mg/L), (0.016 + 16 mg/L) and (0.016 + 8 mg/L);
- AmB and PoLyB (0.016 + 256 mg/L), (0.016 + 16 mg/L) and (0.016 + 8 mg/L).

The 24-well plates were maintained for further 24 h at 37 °C. Both the positive control coupons of 24 h-old pre- established biofilm and biofilms development PS coupons allowed the evaluation of the therapeutic action of the antimicrobial combinations were subsequently removed from each well and immersed in a new plate containing 1 mL UP sterile water in order to remove non-adherent and weakly adherent cells.

Finally, PS coupons were stained with 100 µL of diluted component A (SYTO 9) (3 µl/ml) and 100 µl of diluted component B (Propidium Iodide) (3 µl/ml) for 15 min in the dark at 27 °C.

To observe the stained microorganisms, an Olympus BX51 (Perafita, Portugal) microscope fitted with fluorescence illumination was used. The optical filter combination consisted of 470 to 490 in combination with 530 to 550 excitation filters. The range of total cells for each field was between 20 - 200 cells/field.

2.6. Statistical analysis

All quantitative assays were performed in triplicates in three independent assays.

The data was statistically analyzed using GraphPad Prism version 6.0. Results are expressed as the average ± standard error of the average (SD). One-way analysis of variance (ANOVA) was used for statistical significance values of the groups for biofilm quantification and number of cultivable biofilm-cells. Following, comparisons were performed using Turkey's test. All tests were performed with a 95 % confidence level, $p$value<0.05 was considered statistically significant.

CHAPTER III

RESULTS

3.1. Single- and dual-species biofilms (24 and 48 h) phenotype

The comparison of 24 h- and 48 h-old biofilms formed by two strains of VAP related microorganisms, *P. aeruginosa* PAO1 and *C. albicans* SC5314, is shown in Figure 3.1.

In general, biofilm phenotypic characterization showed that both species, in single- and dual-species, exhibited similar values ($p>0.05$) of cultivable cells at both time points (24 and 48 h) (Figure 3.1 A).

From the analysis of biomass assay (Figure 3.1 B), it is shown that *P. aeruginosa* single-species biofilm present similar values at 24 and 48 h, whereas *C. albicans* biomass is significantly disturbed after 48 h of incubation ($p<0.05$). This observation is also noticed when both species *(P. aeruginosa* + *C. albicans)* are in the same consortia, with the overall biomass decreasing to half of the biomass produced at 24 h.

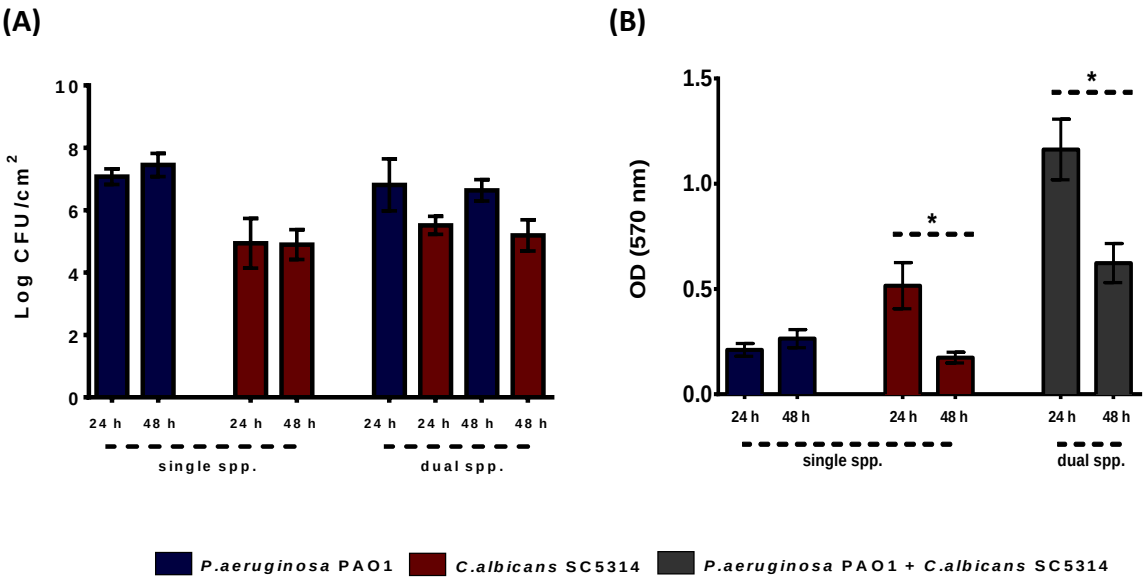


Figure 3.1- Comparison of 24 h- and 48 h-old single- and dual-species biofilms by *P. aeruginosa* PAO1 and *C. albicans* SC5314 in terms of: (A) number of cultivable cells and (B) biomass quantification. Cultivable cells are expressed as log CFU per cm^2 and biomass quantification is proportional to OD_{570}. Bars represent the average of three independent assays ± standard deviations (SDs). Symbol (*) indicates statistically different reduction values between 24 h and 48 h for each strain (* $p<0.05$).

Concerning single-species biofilms, *P. aeruginosa* was the microorganism that showed higher number of cultivable cells (7,08 ± 0,25 log CFU/cm^2 for 24 h and 7,45 ± 0,37 log CFU/cm^2 for 48 h), though producing the smallest amount of biomass. The results of CFUs enumeration for this species in dual-species biofilms is quite similar (6,81 ± 0,83 log CFU/cm^2 for 24 h and 6,64 ± 0,34 log CFU/cm^2 for 48 h), with *P. aeruginosa* as the predominant population in the

consortia both at 24 h (97,1 %) and at 48 h (95,3 %) (data not shown). Regarding biomass quantification, dual-species biofilms (*P. aeruginosa* + *C. albicans)* could form greater biomass than the sum of the effect of both species individually at both time points (24 h and 48 h).

These biofilms were also examined by SEM (Figure 3.2), which allowed observing an increase in bacterial cell numbers and a multilayer structure in *P. aeruginosa* single-specie biofilm after 48 h (Figure 3.2 B).

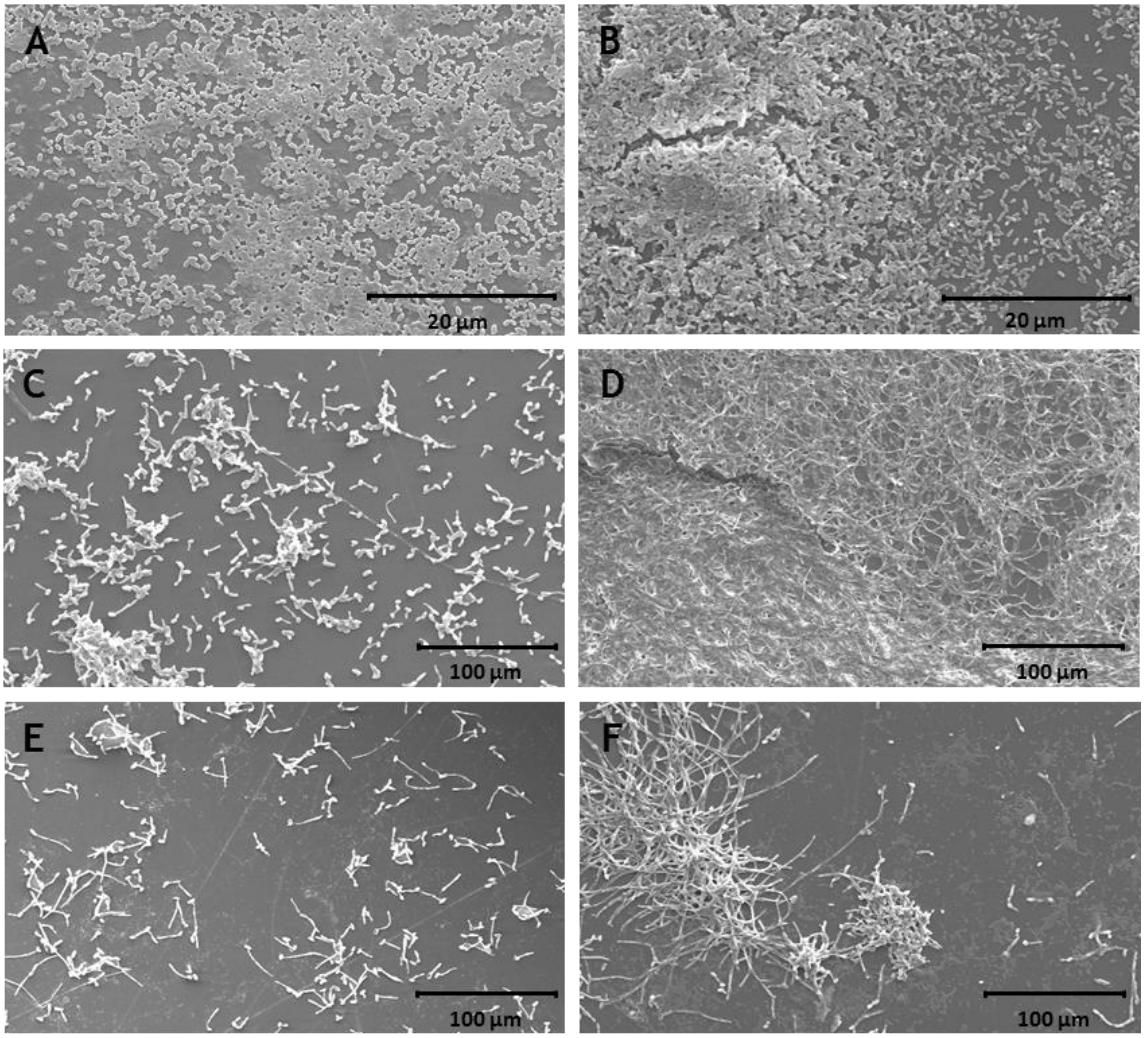


Figure 3.2- SEM images of 24 h- and 48 h-old biofilms developed by *P. aeruginosa* PAO1 and *C. albicans* SC5314. (A;B) *P. aeruginosa* biofilms; (C;D) *C. albicans* biofilms and (E;F) *P. aeruginosa* and *C. albicans* dual-species biofilms. Left column represent 24 h-old biofilms whereas in the right column are represented 48 h-old biofilms.

Comparing the phenotypic changes between 24 h and 48 h in the single- specie biofilm of *C. albicans* (Figure 3.2 C and D, respectively), it was also observed an increase of yeast cells and thickness of biofilm. However, after 48 h, a yeast-hyphal cells transition was found, which promoted a dense and highly organized structure with long and intermingled hyphae and a

crack in *C. albicans* single-specie biofilm. Dual-species biofilms were apparently dominated by *P. aeruginosa* cells, which it was observed for both time points (24 h and 48 h).

3.2. Effect of single antimicrobials

3.2.1. Susceptibility of planktonic populations

The MIC and the MMC values obtained in this study are summarized in Table 3.1.

In general, most antimicrobials were effective against planktonic growth in *P. aeruginosa* at low concentrations (MIC ranged between 0.0625 and 8 mg/L), with the exception of AmB, even being necessary to use concentrations higher than 16 mg/L. In planktonic cells of *C. albicans*, AmB was the most effective antimicrobial at lower concentrations (0.25 mg/L), with all other antimicrobials able to inhibit planktonic growth *C. albicans* but at extremely higher concentrations (MIC ranged between 256 and ≥ 1024 mg/L). In planktonic mixed cultures of both strains (*P. aeruginosa* + *C. albicans*) concentrations equal to or even higher than those used to inhibit the planktonic growth of single populations (*P. aeruginosa* or *C. albicans*) were necessary.

With regard to the determination of the MMC, it was shown that for all antimicrobials the MMC values were significantly higher when compared to MIC. Similarly to the aforementioned results, all antimicrobials were able to kill planktonic cells of *P. aeruginosa* at relatively low concentrations (ranging MMC between 0.125 and 8 mg/L).Whilst higher concentrations were necessary to kill *C. albicans* planktonic cells (MMC ranging between 256 and ≥1024 mg/L). These values were maintained when both species are in the same culture. However, MMC was 8 times higher in the case of AmB in *C. albicans* (0.25 to 2 mg/L) and for ToB in *P. aeruginosa* (4 to 32 mg/L) respectively.

Planktonic cells of *C. albicans,* both in single as in mixed cultures, were significantly more resistant to most antimicrobials tested, with a high number of antimicrobials (5 a total of six) being effective at concentrations between 256 and ≥1024 mg/L..

Based on the antimicrobial activity results, MIC values of antimicrobials were chosen for the following single- and dual-species biofilm susceptibility assays.

Table 3.1- MIC and MMC of 6 clinically-relevant antimicrobial agents (AmB; ToB; CoL; PolyB; CIP; Merp) against single and mixed planktonic populations involving *P. aeruginosa* PAO1 and *C. albicans* SC5314 (CA)

	AmB		ToB		CoL		PolyB		CIP		Merp	
	MIC*	MMC*	MIC*	MMC*	MIC*	MMC*	MIC*	MMC*	MIC*	MMC*	MIC*	MMC*
***P. aeruginosa* PAO1**	≥ 16	≥ 16	2	4	2	8	2	4	0.0625	0.125	1	2
***C. albicans* SC 5314**	0.25	0.25	≥ 512	≥ 512	512-1024	1024	256	256	≥ 1024	≥ 1024	≥ 512	≥5 12
Mixed culture (PAO1+ CA)	≥ 16	**PAO1** ≥ 16 **CA** 2	≥ 64	**PAO1** 32 **CA** ≥64	1024	**PAO1** 8 **CA** 1024	512	**PAO1** 4 **CA** 512	≥ 1024	**PAO1** 0.125-0.25 **CA** ≥1024	≥ 512	**PAO1** 2 **CA** ≥512

*MIC and MMC values are expressed in mg/L

3.2.2. Effect on biofilms

In order to evaluate the effect of the most interesting antimicrobial agents of the previous assay (section 3.2.1.): AmB, ToB, CoL and PolyB. Biofilms were allowed to develop for 24 h under the conditions mentioned in Chapter II (section 2.3.2.1), and were then grown, for additional 24 h, with increasing concentrations (1x, 2x and 4x the MIC) of each antimicrobial agent (Figure 3.3). For this purpose, the lowest MIC obtained for single-species populations was considered. For instance, AmB had the lowest MIC of 0.25 mg/L (in *C. albicans*) whereas the remaining agents presented minimum MIC of 2 mg/L in *P. aeruginosa.*

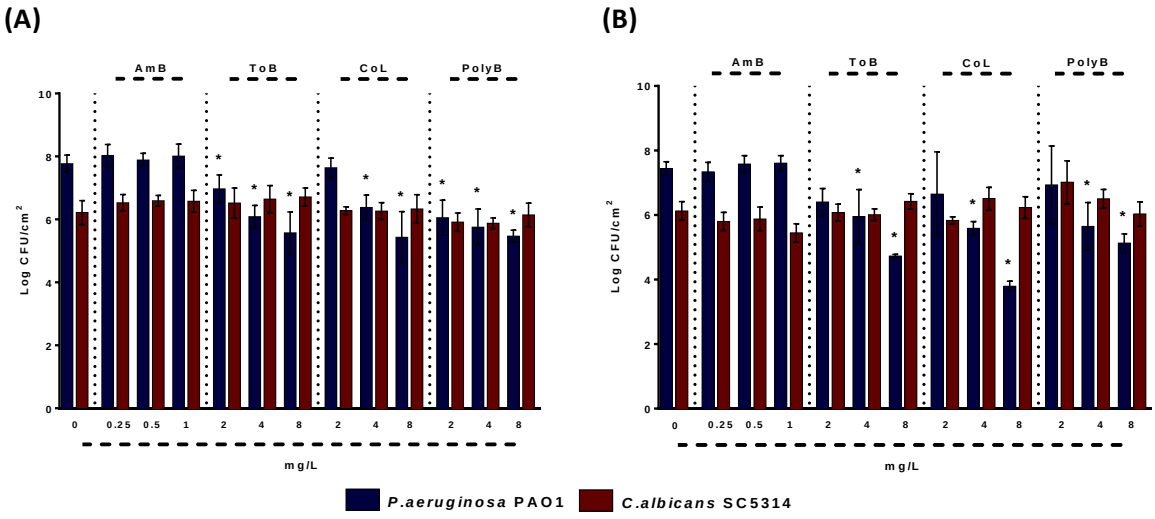


Figure 3.3- Effect of increasing concentrations of AmB, ToB, CoL and PolyB in single- (A) and dual-species pre-established biofilms (B) formed by *P. aeruginosa* PAO1 and *C. albicans* SC5314. Values are expessed as log CFU per cm^2. Bars represent the average of three independent assays ± standard deviations (SDs). Symbol (*) indicates statistically different reduction values between positive control (0 mg/L) and different antimicrobials concentrations (* p<0.05).

As it can be observed in Figure 3.3, AmB did not promote any reduction in cell number of single- (Figure 3.3 A) and dual-species biofilms (Figure 3.3 B) when compared with control biofilms ($p>0.05$). By contrary, ToB presented a significant reduction, which was concentration-dependent, in *P. aeruginosa* cells in single- and dual-species consortia. The presence of 2 mg/L (MIC) of ToB only had an effect in single-species biofilm of *P. aeruginosa* ($p<0.05$), but the presence of 4 mg/L and 8 mg/L (2x and 4x MIC respectively) promoted a significant reduction in both single- and dual-species *P. aeruginosa* biofilms ($p<0.05$).

Concerning the CoL, at 2 mg/L (MIC), it did not cause any significant reduction in the number of single- and dual-species biofilms-entrapped cells for both strains ($p>0.05$), but the application of the highest CoL concentrations: 4 mg/L and 8 mg/L (2x and 4x MIC respectively)

presented a reduction in the number of single- and dual-species biofilms-entrapped cells only for *P. aeruginosa* in comparison with control biofilms ($p<0.05$).

PolyB had a similar activity as ToB, with all concentrations (2 mg/L, 4 mg/L and 8 mg/L) reducing *P. aeruginosa* single-species biofilms, but only with the highest concentrations significantly disturbing this species in dual-species consortia ($p<0.05$) comparatively with untreated controls.

In summary, any antimicrobial agent was effective against *C. albicans* biofilms. By contrary, *P. aeruginosa* biofilms could be disturbed by the presence of ToB, CoL and PolyB and reductions were concentration-dependent used. Reductions in *P. aeruginosa* cell numbers were estimated between 1 and 3.5 log, with the highest reductions observed for dual-species biofilms. The exception was observed for AmB, which was not effective to treat these biofilms.

Another assay was performed for the evaluation of the therapeutic potential (antimicrobial's effect along time) of AmB, ToB, CoL and PolyB in 24 h-old pre-established single- and dual-species biofilms (Figure 3.4.), which was determined by the same procedure described in the section 2.3.2.1, however following the effect (in terms of cultivable cell number) of each antimicrobial for each 12 h until 48 h.

Once again, *C. albicans* were resistant to most antimicrobial agents, with no significant changes or even punctual but small reductions occurring. For instance, AmB could only reduce ($p<0.05$) *C. albicans* cell number after 48 h at the highest concentration tested (1 mg/L), when in dual-species biofilms (Figure 3.4 B).

The therapeutic potential of ToB is supposedly time-dependent for *P. aeruginosa*, leading to increasing and statistically significant reductions over time. However, these antimicrobials only promoted the reduction in number of cultivable biofilm-entrapped cells in *P. aeruginosa* strain in comparison with untreated biofilms ($p<0.05$) (Figure 3.4 C and D).

Even so, the greatest therapeutic potential was demonstrated for all concentrations of ToB: 2 mg/L, 4 mg/L and 8 mg/L (1x, 2x, 4x MIC respectively) mainly after 36 h and 48 h of the ToB exposure for *P. aeruginosa* strain in single- and dual-species biofilms (Figure 3.4 C and D).

Regarding CoL, significant but punctual reductions in *P. aeruginosa* cell number was observed, in particular for single-species biofilms (Figure 3.4 E). Conversely, a gradual disturbance in the *P. aeruginosa* community in dual-species biofilms is visible after 36 h of exposure to CoL, becoming apparently time-dependent after this time point (Figure 3.4 F). Once again, the CoL agent only demonstrated therapeutic potential in *P. aeruginosa* strain biofilms in comparison with biofilm's controls ($p<0.05$).

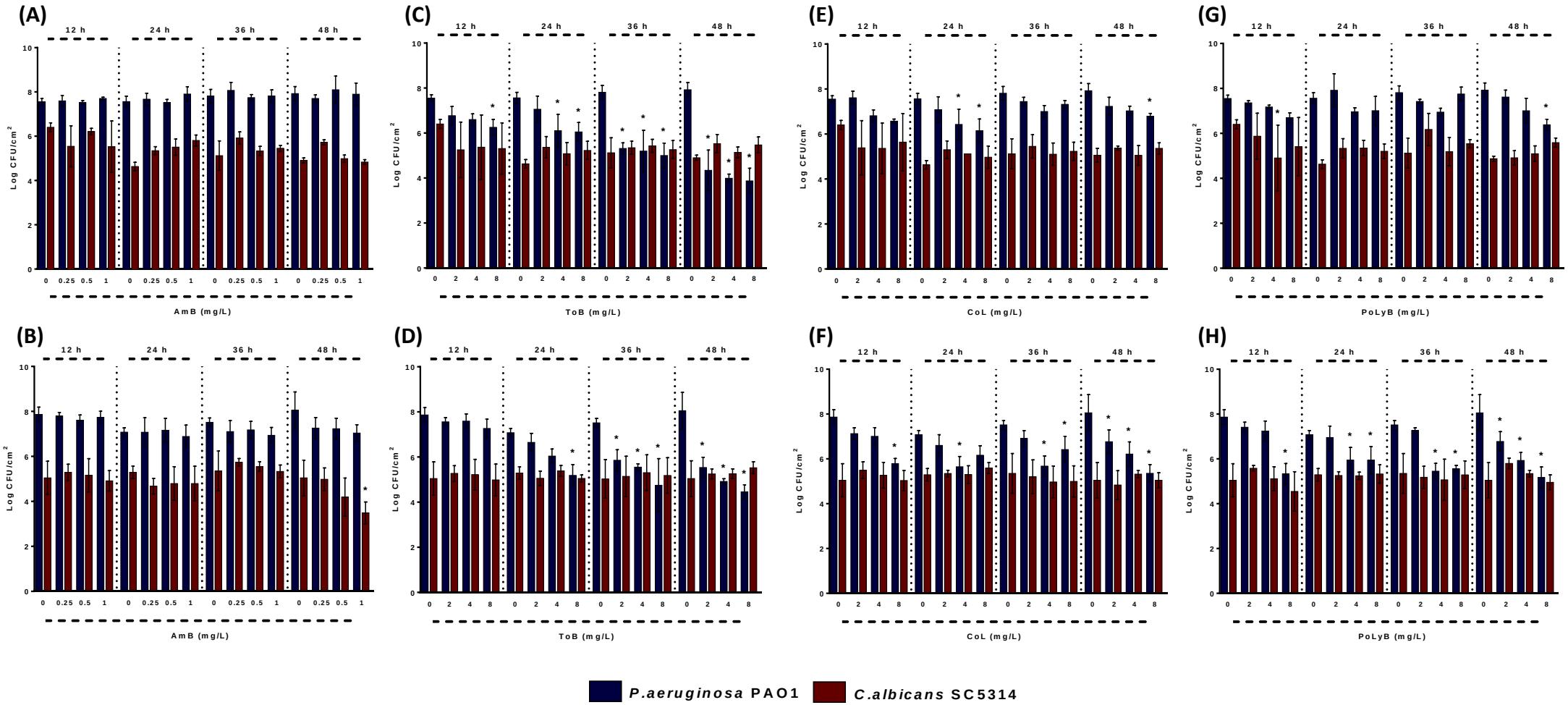


Figure 3.4- Therapeutic potential of increasing concentrations of AmB (A,B), ToB (C,D), CoL (E,F) and PolyB (G,H) in single- and dual-species pre-established biofilms encompassing *P. aeruginosa* PAO1 and *C. albicans* SC5314. On top row, single-species biofilms are represented and dual-species consortia are shown at the bottom row. Values are expessed as log CFU per cm^2. Bars represent the average of three independent assays ± standard deviations (SDs). Symbol (*) indicates statistically different reduction values between positive control (0 mg/L) and different concentrations for each antimicrobial (* p<0.05).

Concentrations of 4 mg/L and 8 mg/L of CoL (2x and 4x MIC respectively) were the concentrations strongly reducing the number of cultivable cells for *P. aeruginosa* in single- and dual-species biofilms. However this therapeutic potential shows a nonlinear behavior for the aforementioned concentrations, since for the same concentration of CoL is possible to observe different reduction degrees.

The presence of PolyB promoted a therapeutic potential dependent of the time factor for *P. aeruginosa* strain in single- and dual-species biofilms, in comparison with biofilm's controls ($p<0.05$) (Figure 3.4 G and H). This was particularly observed for dual-species biofilms, in which *P. aeruginosa* suffered a gradual reduction in cell number even from 12 h of antimicrobial exposure. In relation to *C. albicans* strain it was only found a therapeutic potential in the presence of 4 mg/L of PolyB (2x MIC) in single-species biofilms after 12 h of the exposure ($p<0.05$) when compared with biofilms developed in the absence of PolyB (Figure 3.4 G). Therefore, it is not possible to establish a relationship of dependency with the time.

In general, mostly antimicrobial agents' therapeutic potantial strongly depends of concentration and time factor.

3.3. Effect of the combination antimicrobials'

Based on these previous results and since these demonstrated that the effect of the single antimicrobials was not in accordance to the expected, the next step was to combine the different antimicrobial agents and evaluate the effect in single- and dual-species biofilms involving *P. aeruginosa* and *C. albicans*.

3.3.1. Susceptibility of planktonic populations

In order to evaluate the predictive effects in single and mixed cultures of both species the MIC value was initially determined for two of the antimicrobials agents combined and subsequently FIC value was calculated, according with the methodology from Saiman L.[317] (see section 2.2).

The range of concentrations of the different antimicrobial combinations used in this study, which allowed the inhibition of both planktonic strains (MIC), is summarized in Table 3.2. The colors indicated in Table 3.2 allow an indication of the predictive potential synergy of the range of concentrations of antimicrobial agents' combinations used.

Table 3.2- Range of MIC and interpretation of the FIC of antimicrobial agent combinations (involving AmB, ToB, CoL and PolyB) against single and planktonic mixed populations involving *P. aeruginosa* PAO1 and *C. albicans* SC5314 (CA)

	MIC* of combinations									
	AmB	CoL	AmB	PolyB	ToB	CoL	ToB	PolyB	AmB	ToB
C. albicans SC 5314	2 - 4	1 - 4	1 - 2	1- 4					1 - 2	0.5 - 1
P. aeruginosa PAO1					0.016 - 2	0.016 – 2	0.016 - 2	0.016 - 2	0.016	1
Mixed culture (PAO1+ CA)	1 - 4	0.063 - 4	0.016 - 4	1 – 256	1 - 4	0.063 – 2	≥ 512 (FIC> 8,5)		0.016 - 0.031	256

*MIC value are expressed in mg/L

Synergistic effect | Additve effect | Indifferent effect | Antagonistic effect

First, *C. albicans* susceptibility towards combinations of AmB with all other antimicrobial agents (ToB, CoL and PolyB) was determined. In fact it was verified that the range of concentrations achieved against *C. albicans* was relatively low (MIC range between 0.5 and 4 mg/L). In all tested combinations the value of FIC, determined theoretically, demonstrated antagonistic effect on the *C. albicans* strain. For *P. aeruginosa*, ToB was combined with all other antimicrobial agents (AmB, CoL and PolyB). Also for the *P. aeruginosa* strain, the range of concentrations achieved was relatively low (MIC range between 0.016 and 2 mg/L). However, the value of FIC showed a synergistic effect against *P. aeruginosa* strain.

In relation to the planktonic mixed cultures of both strains (*P. aeruginosa* + *C. albicans)* all possible combinations involving all antimicrobial agents in use in the present study were tested. As observed, the range of concentrations also presented low values for most antimicrobial combinations (MIC range between 0.016 and 4 mg/L). Some exceptions were noticed, namely for AmB/PolyB and AmB/ToB combinations, wherein the range of concentrations of PolyB and ToB had reached a value of 256 mg/L. In addition, it was not possible to determine the value of MIC for the antimicrobial combination ToB/PolyB, even using concentrations of 512 mg/L for each antimicrobial agent. This antimicrobial combination showed antagonistic effect against planktonic mixed culture (*P. aeruginosa* + *C. albicans).* For the remaining antimicrobial combinations tested, a synergistic effect was found against planktonic mixed cultures according to the FIC value determined theoretically.

In general, the combination of antimicrobial agents presented a synergistic effect in *P. aeruginosa* single planktonic cells and planktonic mixed cultures. The opposite was found for *C. albicans* single planktonic cells were an antagonistic effect was observed.

Based on the FIC values of the combinations of antimicrobial agents, where the combinations at lower concentrations and the ones presenting synergistic effect were chosen, the best combinations were selected in order to perform the susceptibility assays on single- and dual-species biofilms for both strains.

3.3.2. Effect on biofilms

The susceptibility of the 24 h-old pre-established single- and dual-species biofilms, concerning number of cultivable cells can be observed in Figures 3.5 and 3.7, respectively. Biomass quantification is presented in Figures 3.6 and 3.8, for single- and dual-species biofilm, respectively.

In general, none of the antimicrobial combinations agents demonstrated any reduction in *C. albicans* in single-species biofilms (Figure 3.5) for cultivable cells. The only exception was

for ToB/CoL (1 mg/L and 0.016 mg/L, respectively), which provided a small reduction in cultivable cells for *C. albicans* in single-species biofilms when compared with no-treated biofilms ($p<0.05$). Nonetheless, it was found a reduction of cultivable cells in *P. aeruginosa* in single-species biofilm only for ToB/PolyB (1 mg/L and 0.016 mg/L, respectively), ToB/CoL (1 mg/L and 0.016 mg/L, respectively), ToB/PolyB (0.016 mg/L and ratios of 256, 32 or 16 mg/L, respectively) and AmB/PolyB (0.016 mg/L and ratios of 256, 32 or 16 mg/L, respectively) combinations when compared with biofilms in absence of antimicrobials ($p<0.05$) (Figure 3.5).

For all combinations mentioned, the reduction of cultivable cells in biofilms of *P. aeruginosa* is due to the action of ToB (1mg/L) or PolyB (256 mg/L, 32 mg/L or 16 mg/L). Moreover the combinations with PolyB at 256 mg/L (AmB/PolyB (0.016 mg/L and 256 mg/L, respectively) and ToB/Poly B (0.016 mg/L and 256 mg/L, respectively)), are the best formulations promoting a total reduction in the number of cultivable cells of *P. aeruginosa* in single-specie biofilm.

Concerning the biomass quantification in single-species biofilms (Figure 3.6), the ToB/CoL (1 mg/L and 0.016 mg/L, respectively) and ToB/PolyB (1 mg/L and 0.016 mg/L, respectively) combinations showed a reduction in biomass for *C. albicans* strain in comparison with the controls ($p<0.05$). Nevertheless, for *P. aeruginosa* strain it was found that all combinations of antimicrobial agents demonstrated a reduction in the biomass values in comparison with control biofilms ($p<0.05$). The biomass reduction is mainly due to the action of the antimicrobial agent used at higher concentrations.

Regarding dual-species biofilms, the reduction of the number of cultivable cells of *C. albicans* occurs in the presence of combinations of antimicrobials agents: AmB/ToB (1 mg/L and 0.063 mg/L, respectively), AmB/CoL (1 mg/L and 0.063 mg/L, respectively), AmB/ PolyB (1 mg/L and 2 mg/L, respectively), AmB/PolyB (0.016 mg/L and 256 mg/L, respectively) and ToB/Poly B (0.016 mg/L and 256 mg/L, respectively) ($p<0.05$) (Figure 3.7). The combinations of AmB/PolyB (0.016 mg/L and 256 mg/L, respectively) and ToB/Poly B (0.016 mg/L and 256 mg/L, respectively) are the best formulations, since they promote a total reduction in the number of cultivable *C. albicans* cells entrapped in dual-species biofilms in comparison with control biofilms ($p<0.05$). *P. aeruginosa* cells in dual-species biofilms (Figure 3.7) have shown a reduction in the number of cultivable cells for

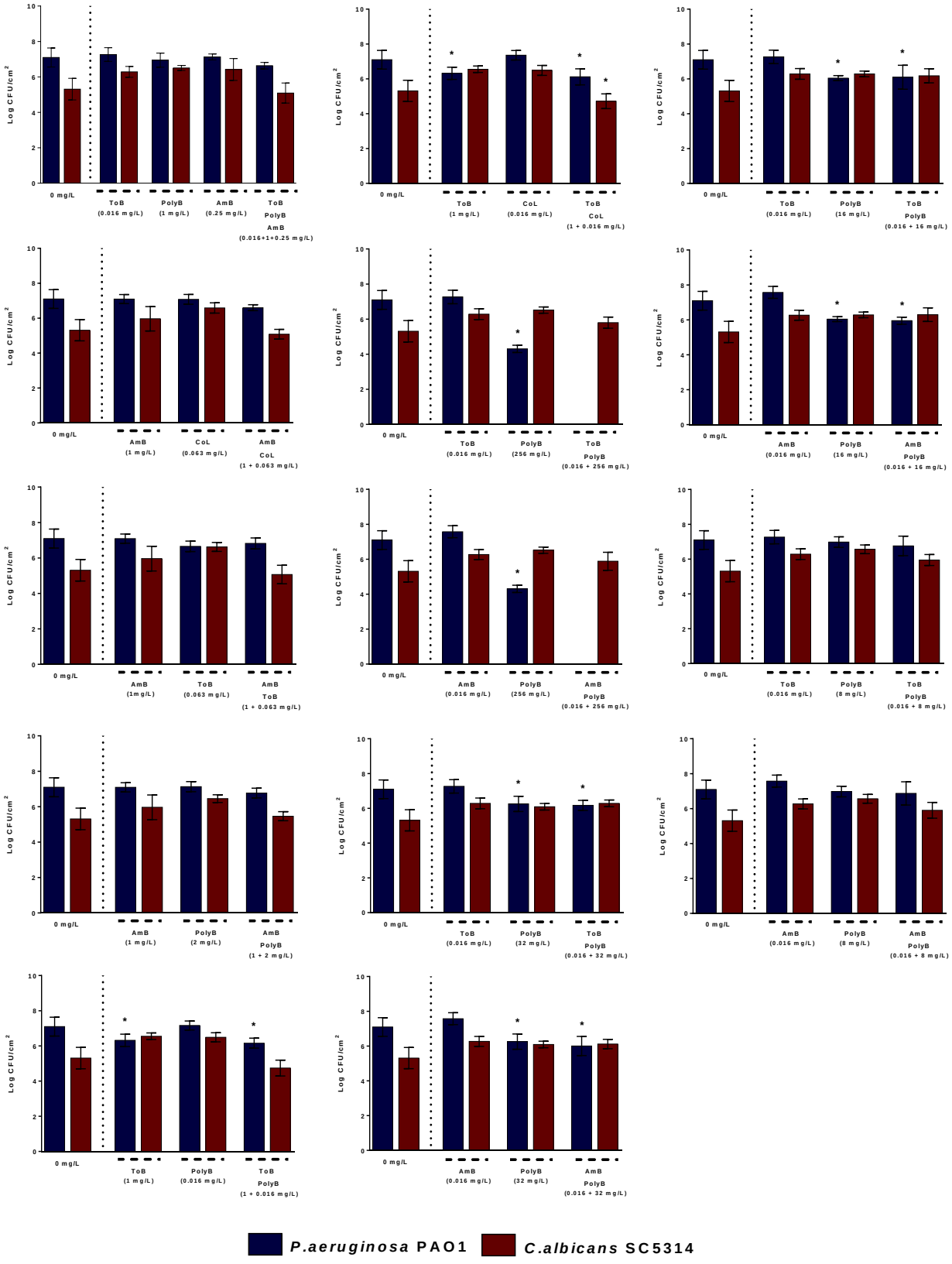


Figure 3.5- Number of cultivable cells per cm^2 present in single-species biofilms in 24 h- old pre-established biofilms for addition 24 h in the presence of antimicrobial agent combinations (involving AmB, ToB, CoL and PolyB) against *P. aeruginosa* PAO1 and *C. albicans* SC5314. Bars represent the average of three independent assays ± standard deviations (SDs). Symbol indicate statistically different reduction values between positive control (0 mg/L) and different antimicrobials concentrations combined for each strain (* p<0.05).

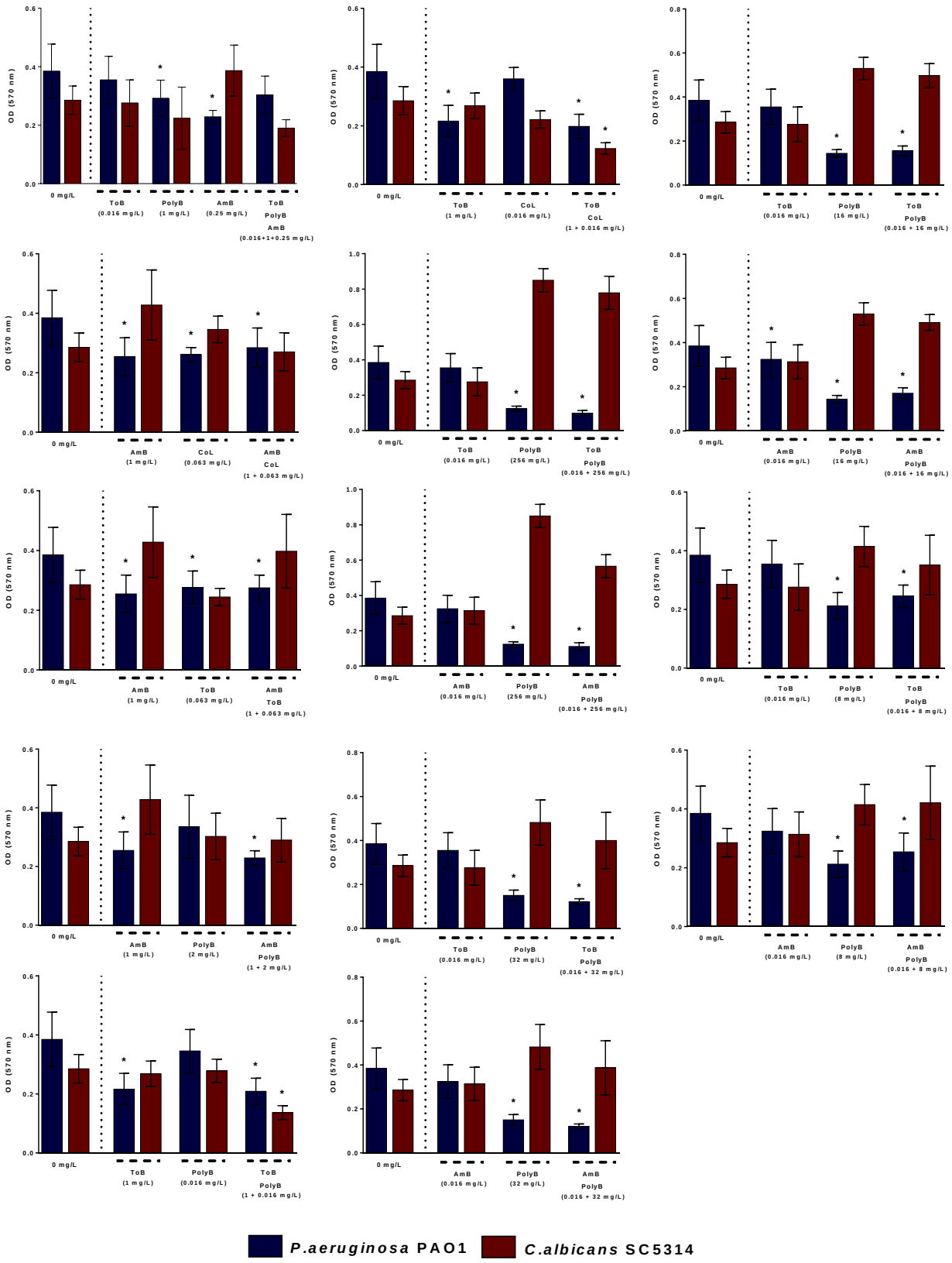


Figure 3.6- Biomass quantification (OD570) present in single-species biofilms in 24 h- old pre-established biofilms for addition 24 h in the presence of antimicrobial agent combinations (involving AmB, ToB, CoL and PolyB) against *P. aeruginosa* PAO1 and *C. albicans* SC5314. Bars represent the average of three independent assays ± standard deviations (SDs). Symbol indicate statistically different reduction values between positive control (0 mg/L) and different antimicrobials concentrations combined for each strain (* p<0.05).

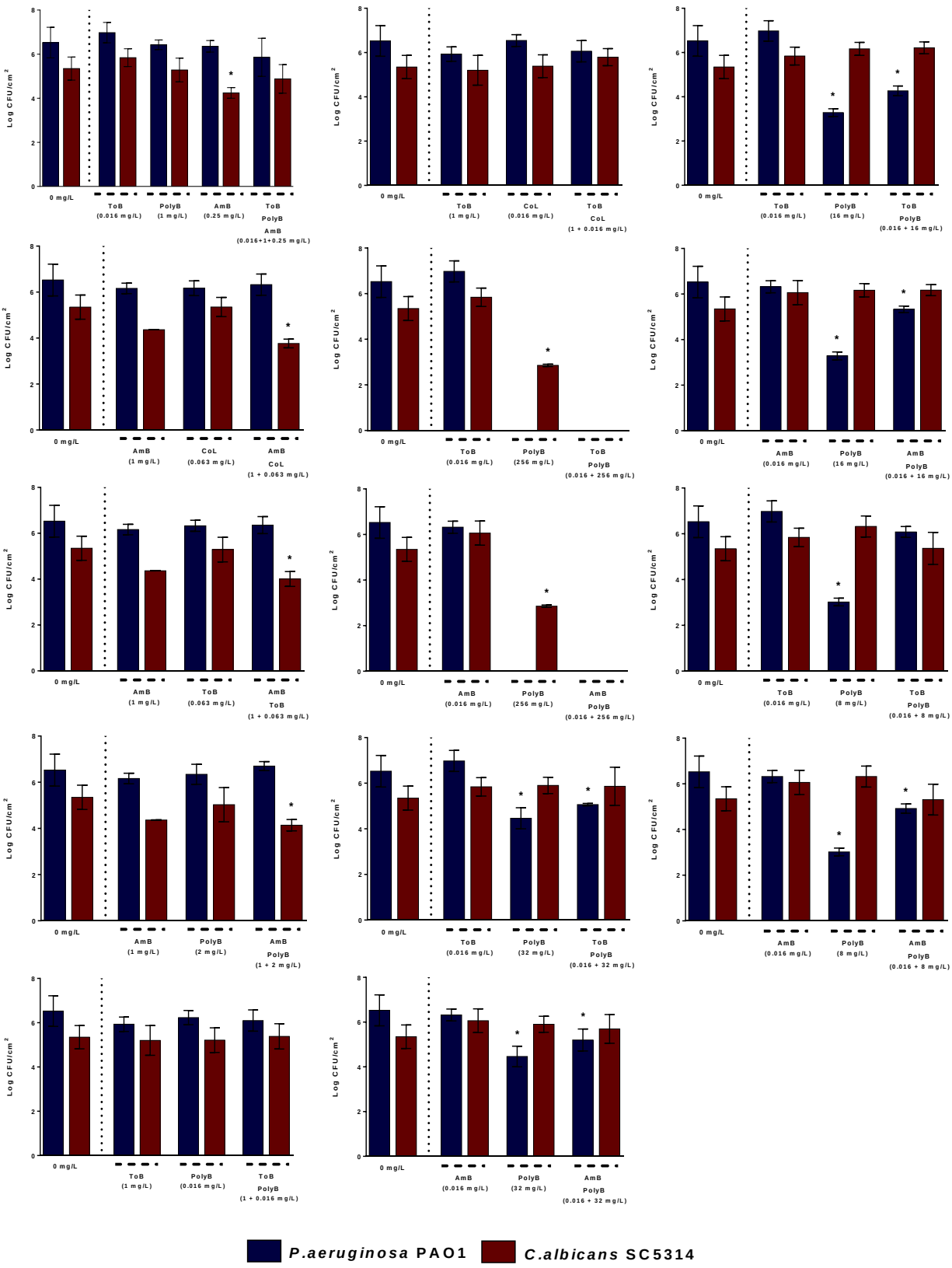


Figure 3.7- Number of cultivable cells per cm^2 present in dual-species biofilms in 24 h- old pre-established biofilms for addition 24 h in the presence of antimicrobial agent combinations (involving AmB, ToB, CoL and PolyB) against *P. aeruginosa* PAO1 and *C. albicans* SC5314. Bars represent the average of three independent assays ± standard deviations (SDs). Symbol indicate statistically different reduction values between positive control (0 mg/L) and different antimicrobials concentrations combined for each strain (* p<0.05).

combinations ToB/ PolyB (0.016 mg/L and ratios of 256 mg/L, 32 mg/L, 16 mg/L or 8 mg/L, respectively) and AmB/PolyB (0.016 mg/L and ratios of 256 mg/L, 32 mg/L, 16 mg/L or 8 mg/L, respectively) in comparison with controls ($p<0.05$).Also again, the combinations of AmB/PolyB (0.016 mg/L and 256 mg/L respectively) and ToB/PolyB (0.016 mg/L and 256 mg/L, respectively) are the best formulations leading to a total reduction in the number of cultivable cells of *P. aeruginosa* in dual-species biofilms.

Concerning biomass quantification of dual-species biofilms, combination of AmB/PolyB and ToB/PolyB at different concentrations (0.016 mg/L and ratios of 256 mg/L, 32 mg/L, 16 mg/L or 8 mg/L, respectively) and AmB/PolyB (1 mg/L and 2 mg/L respectively), exhibited a reduction of biomass values when compared with biofilms without the presence of antimicrobials ($p < 0.05$) (Figure 3.8). This reduction is mainly due to the action of the antimicrobial agent present at a higher concentration.

In general, the better therapeutic efficacy was seen in dual-species biofilms (*P. aeruginosa* + *C. albicans*) when compared with single-species biofilms.

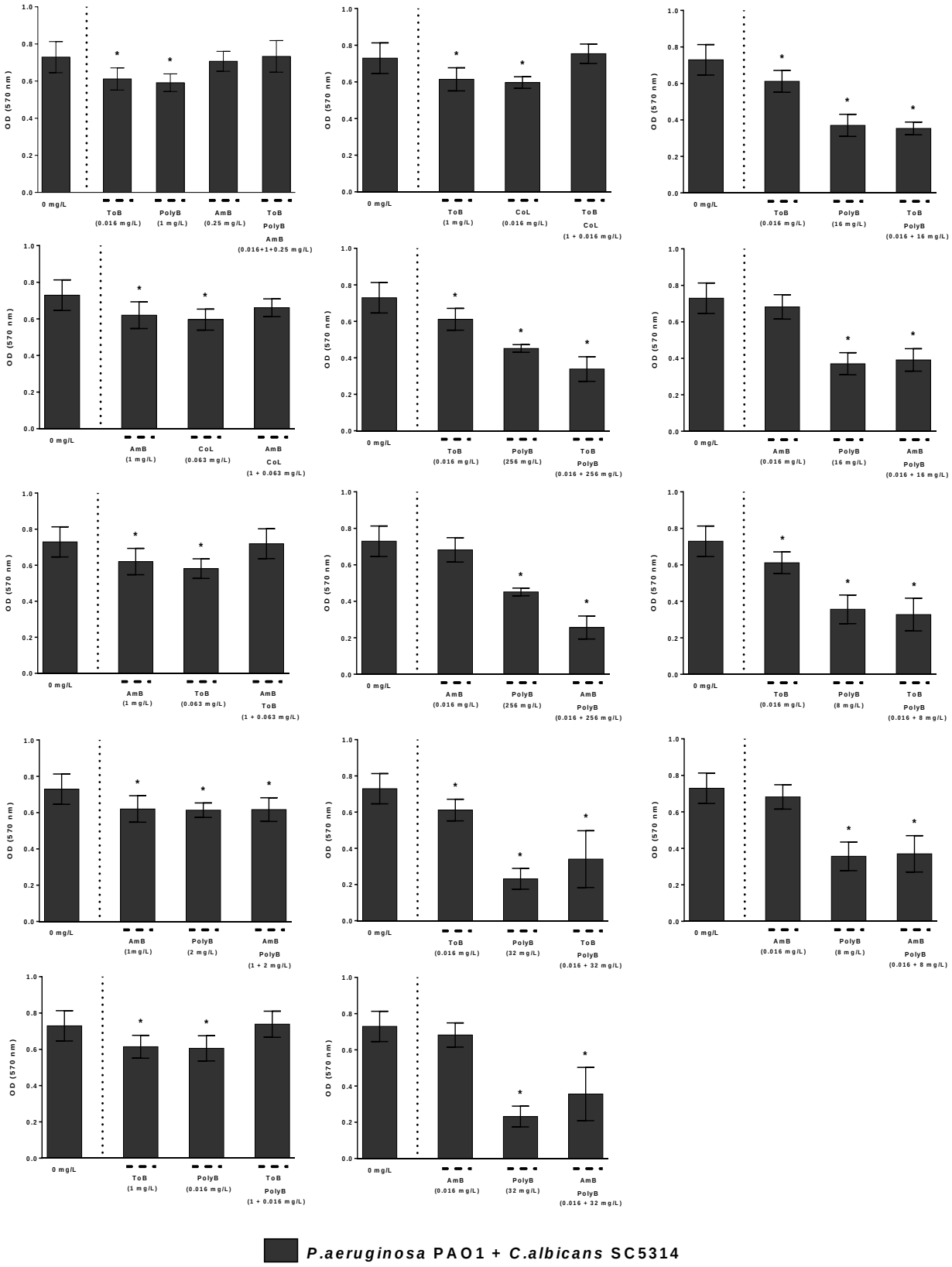


Figure 3.8- Biomass quantification (OD_{570}) present in dual-species biofilms in 24 h- old pre-established biofilms for addition 24 h in the presence of antimicrobial agent combinations (involving AmB, ToB, CoL and PolyB) against *P. aeruginosa* PAO1 and *C. albicans* SC5314. Bars represent the average of three independent assays ± standard deviations (SDs). Symbol indicate statistically different reduction values between positive control (0 mg/L) and different antimicrobials concentrations combined for each strain (* p<0.05).

PNA FISH analysis

PNA FISH assay was performed to confirm the results previously obtained in section 3.1 and previous section Moreover, PNA FISH analysis allowed visualizing the location and distribution of these microorganisms within the dual-species biofilms. PNA FISH methodology involved the use of a previously designed red-labeled probe to detect *P. aeruginosa* and the blue stain DAPI to discriminate *C. albicans* within the biofilms.

In Figure 3.9, it is possible to observe the distribution and composition of single- and dual-species biofilms of *P. aeruginosa* and *C. albicans* after growth for 24 h and 48 h without the effect of antimicrobial agents.

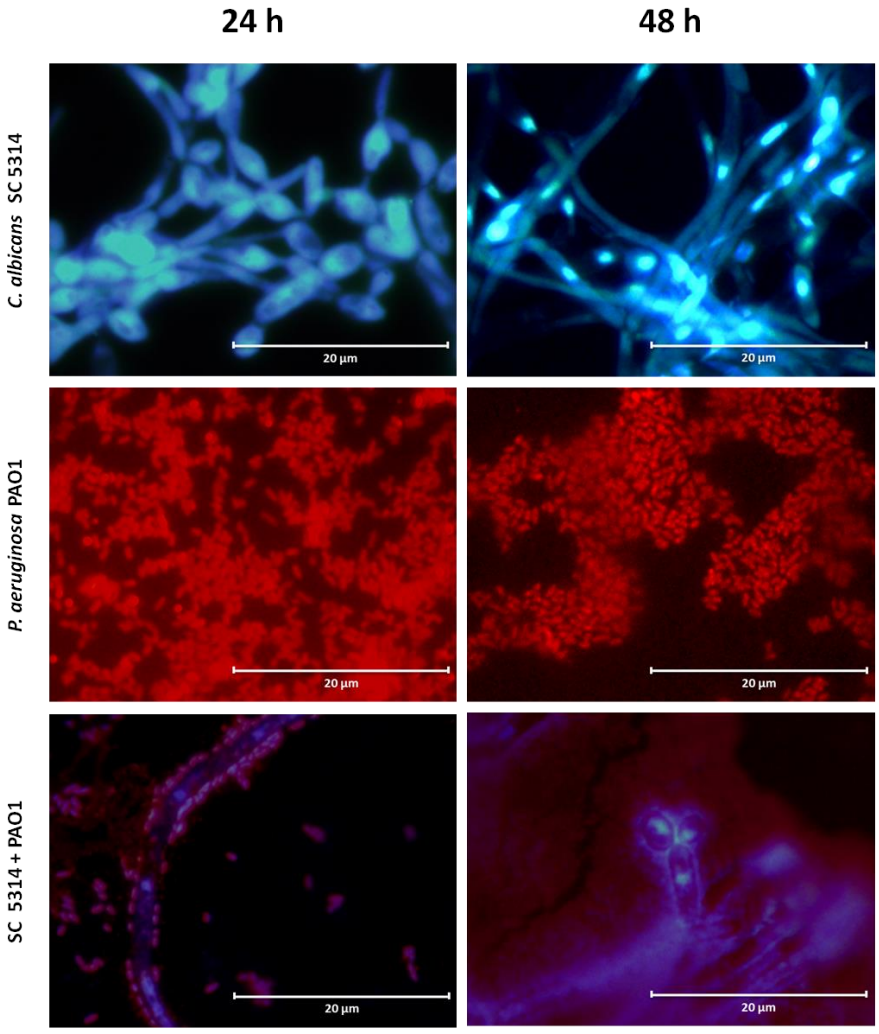


Figure 3.9- PNA-FISH applied to single- and dual-species biofilms involving *P. aeruginosa* PAO1 (red bacterial cells) and *C. albicans* SC5314 (blue yeast cells) grown for 24 h and 48 h. On top and middle rows, the different channels enable to visualize the species involved in the biofilms, according with the fluorochromes used (Alexa fluor 594, red: *P. aeruginosa* and DAPI, blue: *C. albicans*, respectively). The bands superposition discriminating both strains involving in dual-species biofilm is represented on the bottom row.

Then, these biofilms were allowed to grow for additional 24 h in the presence of the same combination of antimicrobial agents used in previous section-Figure 3.10. Exception made for combinations AmB or ToB with PolyB at 0.016 mg/L and 32 mg/L respectively, which was not tested in this assay.

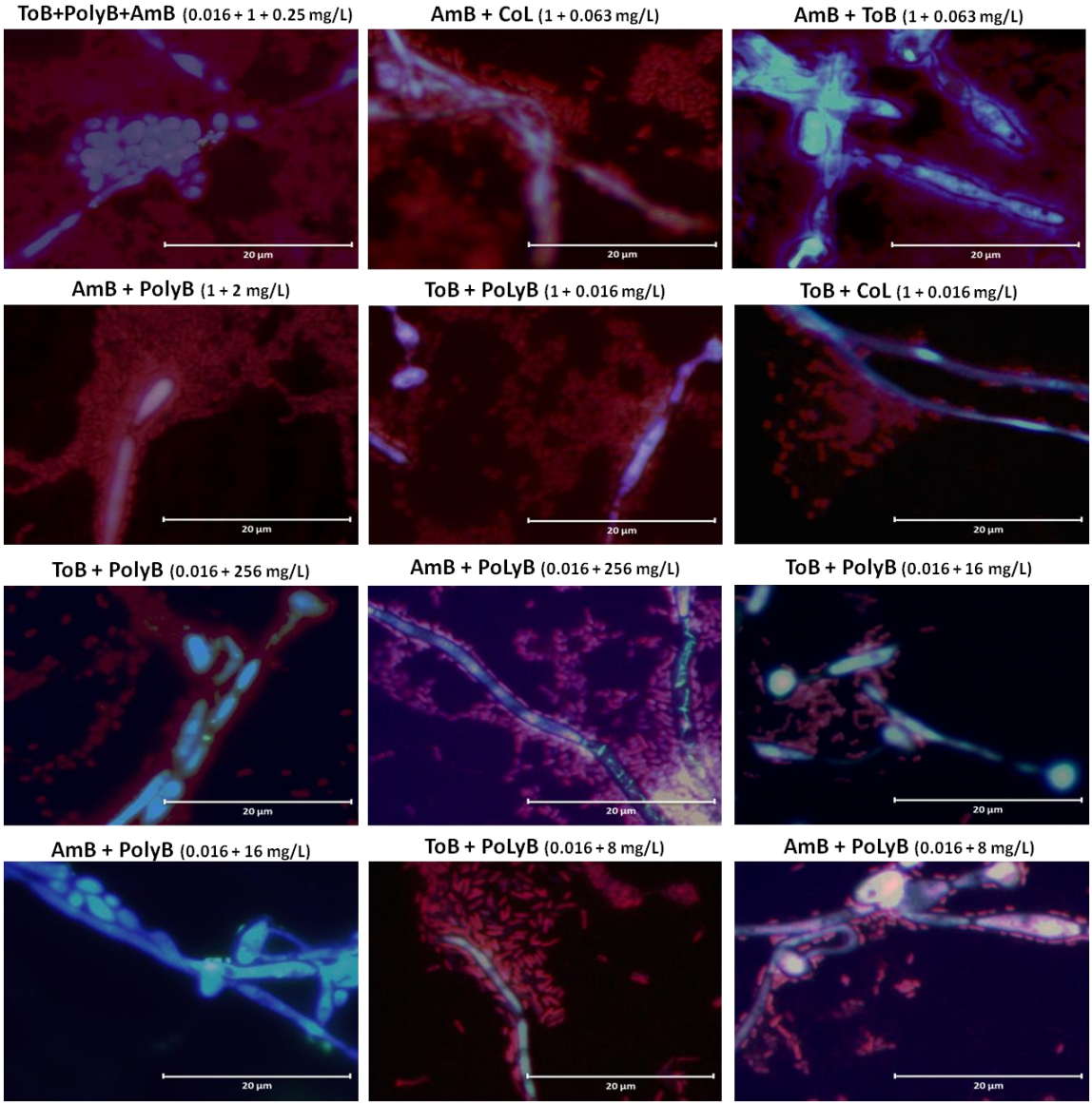


Figure 3.10- PNA-FISH applied to 24 h-old pre-established dual-species *P. aeruginosa* PAO1 (red bacterial cells) and *C. albicans* SC5314 (blue yeast cells) biofilms after treatment with antimicrobial agent combinations (involving AmB, ToB, CoL and PolyB). Images resulted from the bands superposition of the two channels used to visualize the fluorochromes used (Alexa fluor 594, red: *P. aeruginosa* and DAPI, blue: *C. albicans*).

Regarding *C. albicans* biofilms, an increase of the yeast-hyphal transition was shown from 24 h to 48 h (Figure 3.9), whereas *P. aeruginosa* cells tended to agglomerate over time , when in both single- and dual-species biofilms (Figure 3.9).

In dual-species biofilms, an increase of structural complexity of biofilm composed of a larger number of bacterial cells and hyphae yeasts was shown predominantly between both periods (24 h and 48 h). However, in 24 h and 48 h, *P. aeruginosa* cells were found in abundance and predominately located around the hyphae, such as a protection mode (Figure 3.9).

A similar behavior was shown for all tested antimicrobial combinations applied in dual-species biofilms (Figure 3.10), with *C. albicans* cells being surrounded by *P. aeruginosa* and without any treatment causing a significant disturbance in cell numbers. This observation suggests, therefore, that no effective therapy was achieved with the used antimicrobial combinations.

As previously observed, AmB/PolyB and ToB/PolyB combinations were the best formulations for treatment, mainly of the dual-species biofilms involving *P. aeruginosa* and *C. albicans*, leading to the total reduction in cultivable cells of both strains for certain concentrations.

In order to assess the viability of 24 h-old pre-established dual-species biofilms and to know when this will actually totally reduce and whether it will be reversible or not, more assays (LIVE DEAD staining, kinetic effect and post antimicrobial effect) using the AmB/PolyB (0.016 mg/L and ratios of 8 or 16 or 256 mg/L, of PolyB, respectively) and ToB/PolyB (0.016 mg/L and ratios of 8 or 16 or 256 mg/L, of PolyB, respectively) combinations were performed.

LIVE DEAD staining

To assess the viability of 24 h-old pre-established dual-species biofilms, these were allowed to grow for additional 24 h in the presence of the antimicrobial combinations: AmB/PolyB (0.016 mg/L and ratios of 8 or 16 or 256 mg/L, of PolyB, respectively) and ToB/PolyB (0.016 mg/L and ratios of 8 or 16 or 256 mg/L of Poly B, respectively). The Live/Dead *Bac*Light Bacterial Viability Kit was employed to assess bacterial viability after antimicrobial treatment, as shown in Figure 3.11. No treated biofilms were used as control.

Figure 3.11 shows that the bacterial cells were in abundance comparatively to *C. albicans* and presented a green color, meaning that they are viable, after antimicrobial

treatment and independently to the AmB/PolyB and ToB/PolyB concentrations employed to treat the dual-species biofilms. Once again, it was observed that *P. aeruginosa* was the predominant microorganism within the biofilm and it is located preferably around the hyphae yeasts.

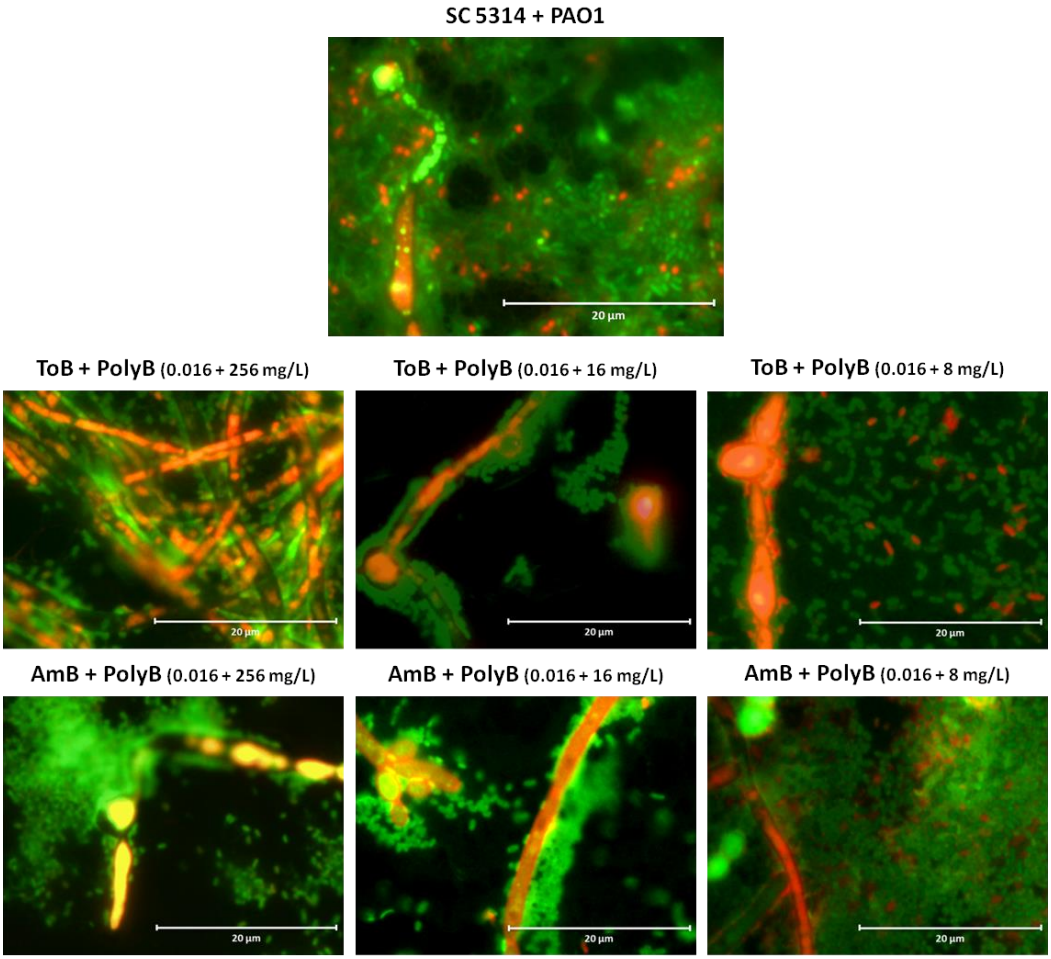


Figure 3.11- Live/Dead BacLight Bacterial Viability Kit applied to 24 h- old pre-established dual-species *P. aeruginosa* PAO1 (green bacterial cells) and *C. albicans* SC5314 (orange yeast cell) biofilms after treatment with some antimicrobial agent combinations (involving AmB, ToB, CoL and PolyB).

Kinetic effect

To assess the time-kill curves of dual-species biofilms *(P. aeruginosa* + *C. albicans),* 24 h-old pre-established biofilms were allowed to grow for additional 24 h in the presence of AmB/PolyB (0.016 mg/L and ratios of 8 or 16 or 256 mg/L, of PolyB, respectively) and ToB/PolyB (0.016 mg/L and ratios of 8 or 16 or 256 mg/L, of PolyB, respectively) combinations (Figure 3.12).

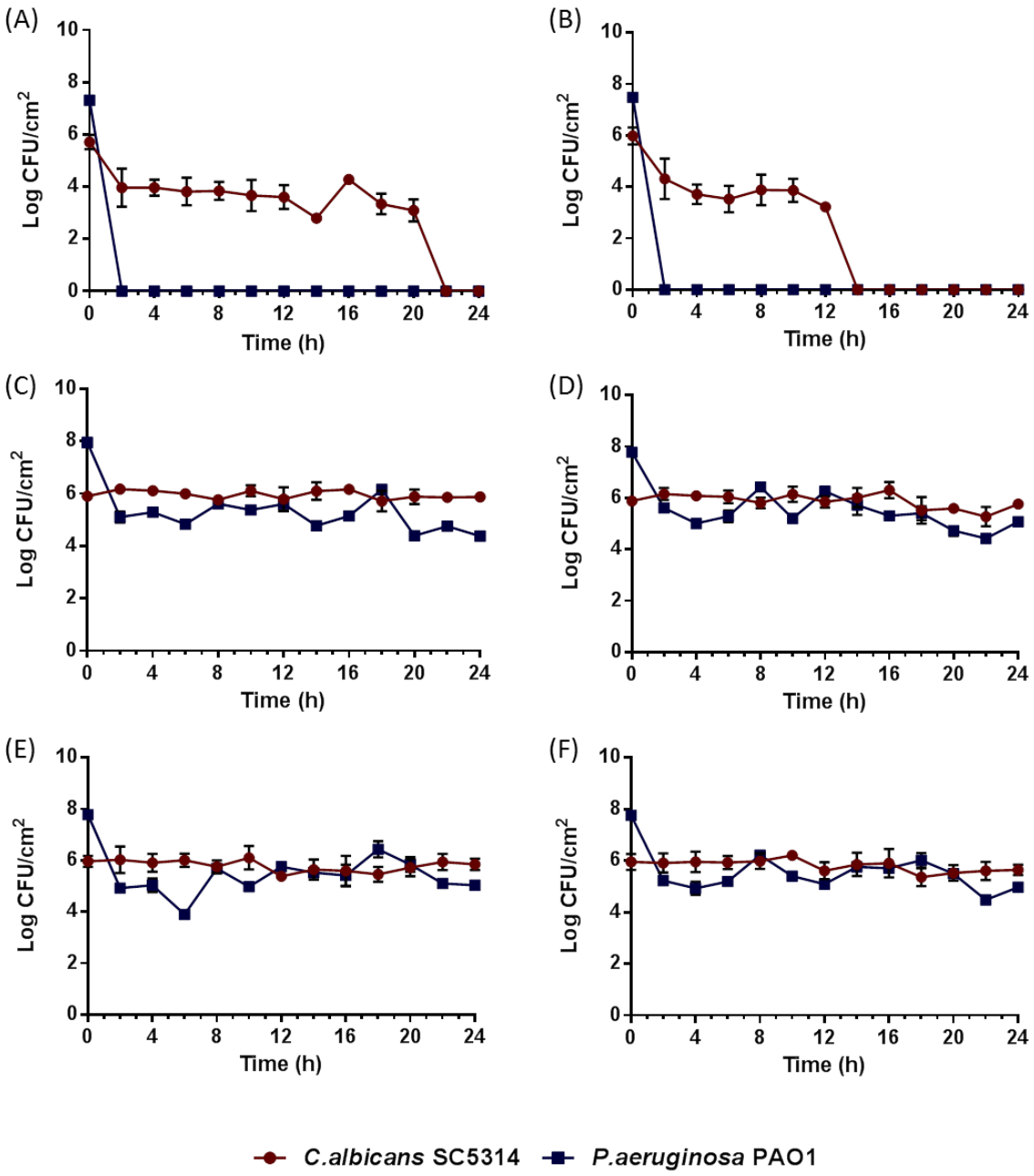


Figure 3.12- Time kill curves obtained for 24h-old pre-established dual-species biofilms (blue line: *P. aeruginosa* PAO1 and red line: *C. albicans* SC5314) after treatment with ToB/PolyB (A,C,E) and AmB/PolyB (B,D,F) combinations involving PolyB at 256 mg/L (A,B), 16mg/L (C,D) and 8mg/L (E,F) assessed in number of cultivable cells. Bars represent the average of three independent assays ± standard deviations (SDs).

Through the analysis of Figure 3.12 A and B, it is possible to identify the combinations with PolyB at a concentration of 256 mg/L as those with better efficacy in reducing the number of cultivable cells for both strains. In *P. aeruginosa* strain, a total reduction in the number of cultivable cells was found immediately after adding the combinations AmB/PolyB (0.016 mg/L and 256 mg/L, respectively) and ToB/PolyB (0.016 mg/L and 256 mg/L, respectively). For *C. albicans*, cell number reduction occurred gradually over 24 h. For the ToB/PolyB (0.06 mg/L

and 256 mg/L, respectively) combination (Figure 3.12 A), in the first 2h it was possible to identify a reduction of the number of cultivable *C. albicans* cells in a 2 log order. After 2 h, the number of cultivable cells remained constant, with insignificant variations, up to 20 h. After 20 h, a complete inhibition in the number of *C. albicans* cells was observed. For the combination AmB/PolyB (0.06 mg/L and 256 mg/L, respectively) (Figure 3.12 B), a reduction of the number of cultivable cells in the order of 2 log was found for the first 4 h in *C. albicans* cells. After 4 h, a constant number of cultivable cells were observed up to 12 h. However, after 12 h the number of cultivable cells decreased dramatically, leading to the total reduction in the number of cultivable cells for the *C. albicans* strain.

For concentrations of PolyB at 16 and 8 mg/L in both combinations (ToB/PolyB (Figure 3.12 C and E, respectively) and AmB/PolyB (Figure 3.12 D and F, respectively)), no significant disturbances were observed over time, with CFUs presenting constant values (~ 6 log CFU/cm^2) for *C. albicans* strain. For *P. aeruginosa* strain, for the first 2 h, there was a decrease of the number of cultivable cells in the order of 3 log. Yet, after 2 h, the CFUs enumeration (~ 5 log CFU/cm^2) presented a linear and constant behavior over 24 h.

Post antimicrobial effect

To evaluate the recovery of the cultivable capacity of cells entrapped in the 24 h-old pre-established dual-species biofilms, bacterial and yeast cells were grown in interleaved cycles in absence and presence of combinations of AmB/PolyB (0.016 mg/L and ratios of 8 or 16 or 256 mg/L, of PolyB, respectively) and ToB/PolyB (0.016 mg/L and ratios of 8 or 16 or 256 mg/L, of PolyB, respectively) over time as shown in Figure 3.13.

In Figure 3.13, it is possible to observe that ToB/PolyB and AmB/PolyB combinations with PolyB at a concentration of 16 (Figure 3.13 C and D) and 8 mg/L (Figure 3.13 E and F) did not show any effect in reducing the number of cultivable cells for both strains. Regardless of the absence or presence of these antimicrobial agent combinations, the number of cultivable cells (~ 6 log CFU/cm^2 for *C. albicans* strain and ~ 7 log CFU/cm^2 for *P. aeruginosa* strain) in dual-species biofilms presented a linear and constant behavior over 120 h for both strains.

However, for the combinations with PolyB at 256 mg/L, both strains exhibit a non-linear behavior over time (Figure 3.13 A and B). These values are in accordance with the interleaved cycle (presence or absence of combination ToB/PolyB (0.016 mg/L and 256 mg/L,

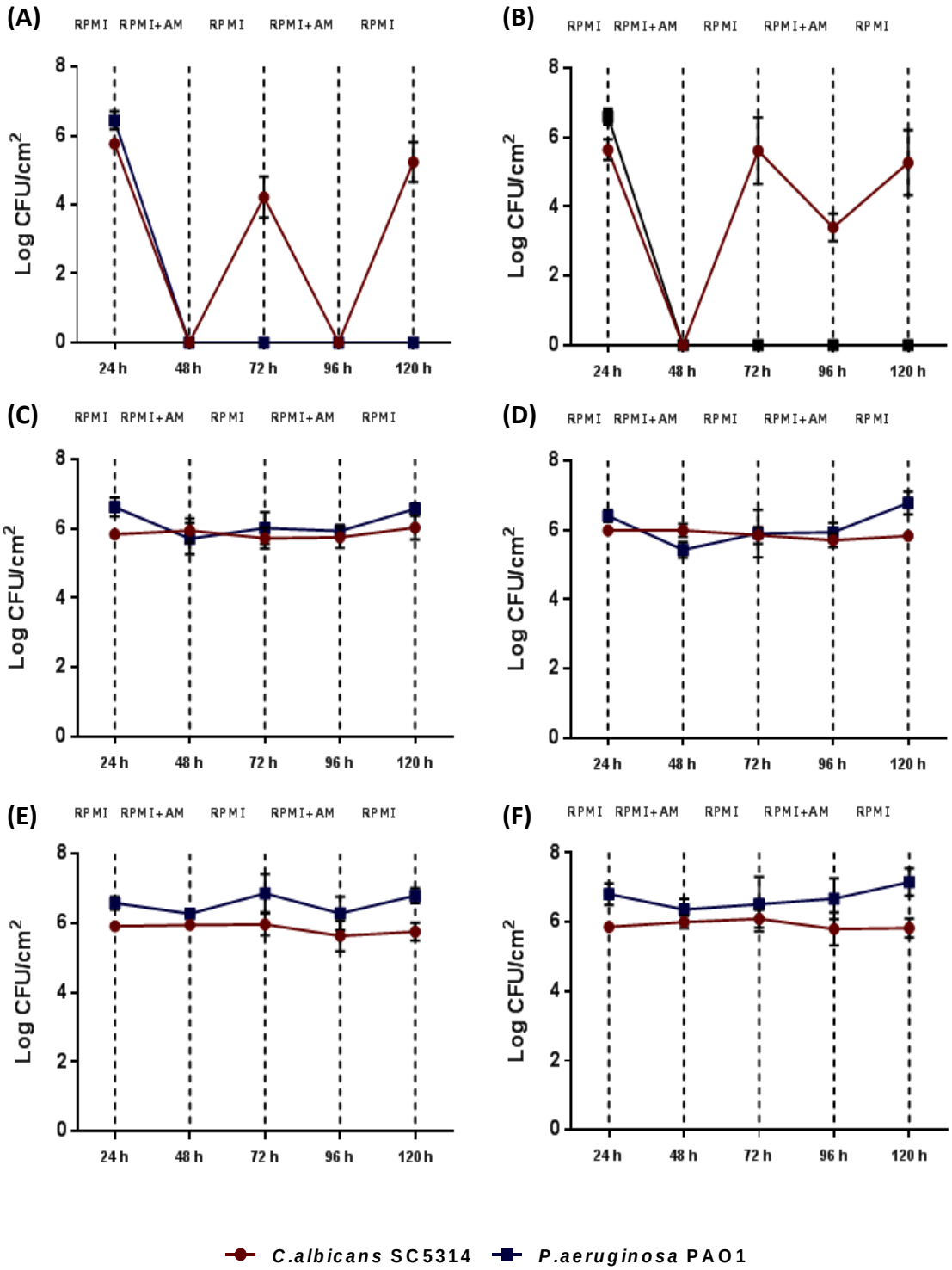


Figure 3.13- Post antimicrobial effects obtained for 24 h-old pre-established dual-species biofilms (blue line: *P. aeruginosa* PAO1 and red line: *C. albicans* SC5314) assessed in number of cultivable cells. Biofilms were evaluated in two cycles : presence and absence of ToB/PolyB (A,C,E) and AmB/PolyB (B,D,F) combinations involving PolyB at 256 mg/L (A,B), 16mg/L (C,D) and 8mg/L (E,F). Bars represent the average of three independent assays ± standard deviations (SDs).

respectively) (Figure 3.13 A) and AmB/ PolyB (0.016 mg/L and 256 mg/L, respectively) (Figure 3.13 B).

P. aeruginosa strain, after 24 h of biofilm development, showed a high number of cultivable cells (~ 7 log CFU/cm^2). However, after the first contact cycle with the antimicrobial combinations there was an overall reduction in the number of cultivable *P. aeruginosa* cells entrapped in dual-species biofilms. Then, it was found that regardless the interleaved cycle and period of time *P. aeruginosa* cells did not achieve any recovery. Thus, the number of cultivable *P. aeruginosa* cells remained null up to 120 h.

For the *C. albicans* strain, a high number of cultivable cells (~ 6 log CFU/cm^2) was also observed after 24 h biofilm development. However, the dual-species biofilms grown for 24 h under antimicrobial combinations was characterized by a total reduction in the number of cultivable *C. albicans* cells. After a recovery period of 24 h, where the growth occurred in the absence of antimicrobials combinations, *C. albicans* cells entrapped in dual-species biofilms had recovered the initials levels of cultivable cells. Biofilm recovery post ToB/PolyB (0.016 mg/L and 256 mg/L, respectively) combination (Figure 3.13 A) did not reach a number of cultivable *C. albicans* cells as high as the recorded immediately after 24 h biofilm development (~ 4 log CFU/cm^2).

Regarding the treatment with AmB/PolyB (0.016 mg/L and 256 mg/L, respectively) combination (Figure 3.13 B) the number of cultivable *C. albicans* cells led to biofilms with higher numbers of cultivable cells (~ 6 log CFU/cm^2). This trend was similar to that observed in the 24 h pre-established dual-species biofilms without any stress factor. The second cycle of biofilm growth under the pressure of antimicrobial combinations clearly reduced the number of cultivable *C. albicans* cells entrapped in biofilms, for both combinations. However, despite the total reduction of the number of cultivable cells for ToB/PolyB (0.016 mg/L and 256 mg/L, respectively) it was similar to that obtained after the first cycle of antimicrobial combination treatment (Figure 3.13 A). For AmB/ PolyB (0.016 mg/L and 256 mg/L, respectively) combination, these reductions were lower than those obtained after the first cycle of antimicrobial combination (~2 log CFU/cm^2) (Figure 3.13 B). Nevertheless, this reduction was not total, allowing biofilm re-growth during the second interleaved period. In fact, the resulting 120 h-old biofilms had recovered its levels of cultivable *C. albicans* cells, reaching values in the same order of magnitude as those determined in the 24 h pre-established dual-species biofilms without any stress factor.

In general, the post-antimicrobial effects observed after the second interleaved cycle of antimicrobial combinations treatment is similar to that observed after the first biofilm growth under the pressure of antimicrobial combinations. The exception was the second

interleaved cycle of AmB/ToB with concentrations at 0.016 mg/L and 256 mg/L, respectively. In fact, the number of cultivable cells of the 96 h-old biofilms was lower but not totally reduced as observed in the first cycle of antimicrobial combinations treatment.

CHAPTER IV

DISCUSSION

4.1. Single- and dual-species biofilms (24 and 48 h) phenotype

Numerous reports have recently demonstrated complex interactions occurring between the bacterial species *P. aeruginosa*, and the dimorphic fungal species *C. albicans*, two important microorganisms frequently involved in device-related NIs, such as the case of VAP[151][167][332][333][334][335][336][337][338][339]. However, the interactions occurring among these species are not fully understood[340]. Results obtained in this study did not show significant differences in single-species biofilms, between 24 h and 48 h of growing, which is in accordance with several previous studies (Figure 3.1)[340][341]. Unlike reported in literature[342][343][344][345], in our study the simultaneously presence of both bacterial and fungal species did not result in significant changes in the overall consortia (Figure 3.1). Such studies[342][343][344][345] have even suggested that these perturbations were caused by the interference of *P. aeruginosa* with *C. albicans,* affecting the whole biofilm. However, our findings are in accordance with the study conducted by El- Azizi *et al*.[334], suggesting that development of dual-species biofilm (involving both bacterial and fungal species) may be dependent on factors other than inter-species variations such as the composition of the culture medium used to grow the biofilms[346]. In fact, a few studies[151][334] have established that the possibility of *P. aeruginosa* to affect the growth of *C. albicans* is higher in nutrient-rich environments and under normoxic conditions.

According to the clinical laboratory standards, an optimal nutrient medium should provide good or at least an adequate growth of the microorganisms[347]. In this study, biofilms were developed in the RPMI 1640 culture medium, at pH 7.0. This is considered a poorer medium compared to glucose-rich culture media (e.g. yeast extract peptone dextrose (YPD)), since fungal species can grow slower[348]. This statement proves the low cell concentration found in RPMI 1640 (for 24 and 48 h biofilms of both strains) and corroborates the results show that the presence of *P. aeruginosa* having no effect on *C. albicans* when both strains are present in a biofilm.

SEM images (Figure 3.2) showed an increase of cell number for both strains between 24 h and 48 h. *C. albicans* presented, at both single- and dual-species biofilms, a combination of yeast, pseudohyphae and hyphae in a multilayered structure[349][350][351][352][353][354]. The increase in *C. albicans* filamentation observed in SEM could be explained by various environmental signals, namely media culture, the strain of the study and the time incubation[355].

C. albicans strain of this study (SC5314) is highly filamentous *in vitro* when compared with other strains of *C. albicans*[356]. The fungal genes associated with hyphal morphogenesis

likely contribute to differences virulence and differences severities of disease between strains of *C. albicans*[356][357]. On the other hand, the use of RPMI 1640 culture medium, and particularly its compounds (such as L-glutamine) are known as one of the factors that induce germ tube production and, consequently, the hyphae growth in *C. albicans* yeast cells[358][359][360][361][362][363]. The yeast-to-hyphae transition, associated to the composition of the culture media, has been considered one of the crucial factors of development stages and *C. albicans* surface attachment[354]. In addition, neutral pH promotes a denser biofilm structure composed of a basal layer of yeast cells followed by hyphae and contributes to better adhesion of *C. albicans* in RPMI 1640[361].

In addition, a further extension of the incubation time promotes a gradual increase of the amount of hyphae corroborating our findings, which showed an increase of *C. albicans* filamentation between 24 h and 48 h of biofilm development[364]. However, there was a decrease in the biomass, which can be explained by the detachment of biofilm-entrapped cells at 48 h leading to the crack that can be visualized in the SEM images (Figure 3.2). Sellam *et al*.[365] reported that cohesive (cell to cell) and relatively strong adhesive bonds are formed in an early stage of *C. albicans* biofilm when inoculated from the yeast form and grown in rich medium under flow. Through cells germination and hyphae growth by linear extension, the adhesive bonds are progressively weakened over an 8 h period. This loss of adhesion is accompanied by a structural reorganization of hyphae along the perimeter of the biofilm such that they become aligned in a direction perpendicular to the interfaces delineated by the biofilm-medium and biofilm-substratum boundaries. The most pronounced transition in both adhesion and structural reorganization occurs within the first 2 h of biofilm development and promotes the crack in the biofilm.

As expected, it is not possible to establish a correlation between quantitative and qualitative methods, since SEM images captured in a single plane, only allowing a qualitative analysis. Moreover, while CFUs enumeration shows the number of cultivable cells, the SEM analysis allows the observation of the presence or absence of viable, non-viable cells and matrix composition.

4.2. Effect of single antimicrobials

4.2.1. Susceptibility of planktonic populations

Several studies[366][367][368][369][370][371] have demonstrated that delaying the effective administration of antimicrobial therapy against VAP may lead to an increase of morbidity,

costs of care, and mortality, since it may adversely impact the ability of the antimicrobial agents to eradicate the infective pathogen.

The most recent recommendations from the American Thoracic Society (ATS) for the management of VAP by antimicrobial therapy rely on evidence-guidelines based on patient suffering from several pathologies and the MV duration[23]. For late onset VAP (occurring after 4 days of MV) or patients with comorbidities or risk factors for MDR pathogens, the ATS has recommended the use of broad spectrum antimicrobials. For critical ill without comorbidities or patients with early-onset VAP (occurring before 4 days of MV), ATS has suggested the treatment with limited spectrum antimicrobials[23].

From the analysis of the results obtained in this study (Table 3.1), and according to epidemiological cut-off values (ECOOFs) that represent epidemiological breakpoints set by EUCAST[372][373][374], *C. albicans* was susceptible to AmB and *P. aeruginosa* presented susceptibility to all antibiotics in study: ToB, CoL, PolyB, Merp and CIP.

However, the higher MIC values of AmB against *P. aeruginosa* and higher MIC values for all antibiotics for *C. albicans* obtained show that the antifungal agents have reduced activity on bacterial species and antibacterial agents do not promote effect on fungi. This observation could be explained by the comparison between the mechanisms of action of antifungals and antibiotics. The antimicrobial action is limited by several factors such as the structures of fungi and bacteria which diverge in very significant ways (such as the diploid nature of most fungi and the longer generation time of fungi in comparison to bacteria). Thus, the available antibacterial and antifungal agents target structures and functions that are most important to the microorganisms to be inhibited[375]. Many antibacterial agents inhibit steps essential on peptidoglycan formation, which is the vital constituent of the bacterial cell wall. In opposition, most antifungal compounds target either the formation or the function of ergosterol, an essential constituent of the fungal cell membrane[375].

Mixed planktonic cultures require stronger antimicrobial dosing treatments because the microorganisms involved in mixed cultures are more unmanageable (eventually by protection to each other) to antimicrobials in comparison to the same microorganisms in single cultures[376]. Li *et al*.[377], demonstrated that any antimicrobial agent used alone promoted obvious effects against mixed planktonic cultures, since it was observed that in mixed planktonic cultures concentrations equal to or even higher than those used to inhibit planktonic growth of single populations were required. According to Tato *et al*.[378], the MMC values were consistently equal or 1- to 2-fold higher than the equivalent MIC results (MMC/MIC ratios ≤ 4), showing that the antimicrobial behavior of all drugs was kept equal in spite of the increase or decrease of their activity subjected to different environments. Data

suggest that the MMC effect of the drugs implies the use of higher concentrations of antimicrobials than the concentrations used when only want to inhibit planktonic cells (MIC).

In this study, the 8-fold increase obtained for MMC of AmB and ToB in *C. albicans* and in *P. aeruginosa*, respectively, in mixed planktonic consortium is corroborated by Mohan *et al*.[379]). The increase of antimicrobial tolerance observed for both strains in mixed cultures can be associated to well-known mechanisms associated to microbial species: the difficulty of the interactions between the antimicrobial agents and target sites; the efflux of the antimicrobial agent from the bacterial cells before reaching target sites of attack, and/or the destruction or modification of the antimicrobial molecule; and also other resistance mechanisms provided by the mixed culture: protection by one of the species, cell rearrangement, interactions among the resident species that confer this antimicrobial resistance[380].

In the first mechanism, the antimicrobial resistance generally occur in situations where the metabolic activity of bacteria is lower in mixed cultures in comparison when their growth alone, which in turn leads to lower activity of the antimicrobial target sites[381]. Furthermore, the low metabolic activity may be the consequence for the need to conserve energy due to the competition for available nutrients in mixed cultures. In fact, bacteria growing in nutrient-limited conditions (e.g. RPMI 1640 medium) can initiate a mechanism known as stringent response, which leads to growth arrest and subsequent inactivity of the antimicrobial target sites (e.g., binding elements such as rRNA), thereby resulting in increased antimicrobial tolerance[381]. Regarding the second mechanism, the interspecies communication in bacteria is known to change gene expression patterns, which may cause efflux of antimicrobial molecules, leading to increased antimicrobial resistance. For example, the efflux pump genes in *P. aeruginosa* can be up-regulated in mixed planktonic cultures[382]. Antimicrobial resistance involving the destruction or modification of antimicrobial molecules may be different in single and mixed cultures due to differences in the production of molecules that can modify antimicrobials. For example, *P. aeruginosa* secretes various enzymes and small molecules, including PlcH, phenazines, the QS molecule 3OC12HSL and PYO which influence the biology and survival of *C. albicans*[160][161][162][163]. *C. albicans* also produces a QS molecule, FOH, which regulates its own morphology[170]. Like PYO, FOH is also associated with changes in *C. albicans* metabolic pathways, and these changes in fungal metabolism may indirectly effect other microorganisms such as *P. aeruginosa* in the consortium[159].

The production of small molecules by both microorganisms observed in mixed cultures can exert effects on microbial behavior with increased virulence, dissemination, survival and increased resistance to antimicrobial agents[159][383][384].

4.2.2. Effect on biofilms

In most natural environments, microorganisms exist mainly as biofilms rather than as planktonic cells and they are significantly less susceptible to antimicrobial agents, including antimicrobials, antiseptics and industrial biocides[183][385][386]. Resistance is reportedly up to 10-1000 fold greater in bacterial or fungi cells in biofilms than antibiotic or antifungal susceptibilities of planktonic cells, which could be the explanation of frequent therapeutic failure of antimicrobials against biofilm infections[183][273][274][275][276][277][278]. Cells in a biofilm (sessile cells) are phenotypical and physiologically different from non-adhered (planktonic cells) and one of the typical properties of cells in a mature biofilm concerns to the higher concentrations of antimicrobial drugs requirement to kill sessile cells compared to planktonic cells[188].

In this study, AmB did not promoted any effect in pre-established *C. albicans* biofilms (Figure 3.3). In a previous report (Uppuluri *et al*.[387]), it was verified that in single-biofilms of *C. albicans* the reductions in the number of cultivable cells have occurred after employment of AmB at 1 mg/L, whereas AmB at 0.25 mg/L resulted in only minimal effects on biofilm dispersion. In part, our results are in a concordance with this previous study[387], although the authors have evaluated the activity of AmB under flow conditions. Also, our results are in agreement with other studies[388][389][390], who have reported that *C. albicans* biofilms are relatively resistant to a wide spectrum of clinical antifungal agents, including to AmB. The increase of the structure complexity of *Candida* species biofilms could be the explanation for the antifungal resistance[391]. The development of complex architectures resulting in the production of hyphae or pseudohyphae in *C. albicans* cells in RPMI medium (as shown in Figure 3.2) limits the penetration of drugs through the ECM, also creating a barrier to the access of these antimicrobials to the biofilm-entrapped cells[274][386][392]. Moreover, several mechanisms detected in *C. albicans* biofilms may also justify these results, such as the overexpression of drug targets. Ramage *et al*.[393] demonstrated that *C. albicans* biofilms exposed to fluconazole induces an upregulation of genes involved in ergosterol biosynthesis, which caused the production of several sterols and, consequently, the antifungal resistance. Additionally, persister cells, described as being "highly tolerant to antibiotics", have been involved in the biofilms resistance as well[394]. Some studies[393][395] demonstrated the presence of these cells in *Candida* species biofilms including *C. albicans* biofilms when exposed to antifungal agents. The physiological stress that causes mutations in proteins affects biofilm formation and, consequently, the resistance to antifungal agents[393]. Another factor involved in the antifungal resistance of biofilms is the efflux pumps, which are differentially expressed

only in the early phase of biofilm development[388]. The decrease of metabolic activity and the high anti-oxidative capacities are other mechanisms of antifungal resistance that have been indicated for *Candida* species biofilms[396]. ECM has been referred as an important mechanism in the antifungal resistance of biofilms because it acts as a protective barrier of cells embedded in it, by hampering the diffusion of antimicrobials agents[388][393].

The effect of antibiotics such as ToB, CoL and PolyB, often used for treatment of *P. aeruginosa*[317], in single- and dual-species biofilms can be observed in Figure 3.3. The reduction of the number of cultivable cells entrapped in biofilms was more evident in concentrations 2x and 4x above the respective MIC value. According to the literature[273][397][398], a growing number of assays and techniques have been developed to enumerate the susceptibility of cells entrapped in biofilms, however without supplying a rational comparison with the standard MIC assay for planktonic cells. This occurs because EUCAST standards determinates the practice with a low inoculum of the planktonic cells (0.5 - 2.5 x 10^5 CFU/mL)[319]. Even if many clinical practices still depend on MIC determinations with microbial suspensions[399][400][401]; it is important to evaluate biofilm cells' susceptibility to antimicrobials rather than testing the susceptibility of planktonic cells, once they are not similar. So, for the application of the susceptibility assays to bacterial cells entrapped in a biofilm, it becomes advantageous to determine the minimum biofilm inhibitory concentration (MBIC) and the minimum biofilm eradication concentration (MBEC), instead to compare with the traditional susceptibility test based on planktonic cells[277].

In addition, many antimicrobials in clinical use present adverse effects (e.g., nephrotoxicity, ototoxicity, and neuromuscular blockade) caused by an extension of the antimicrobial´s normal pharmacology and complications of high serum levels[402][403][404]. Therefore, maximizing the effectiveness and minimizing the toxicity of different antimicrobials are critical settings for clinical implications. Thus, these parameters are of extremely importance to better screen the therapeutic decisions in the treatment of VAP infections[235][405]. In this way, the PK and PD profiles of an antimicrobial delivery agent is an important evidence to help establishing an efficient dosing regimen[278].

In general, mostly antimicrobials used in this study (ToB, CoL and PolyB) showed activity against single- and dual-species pre-established biofilms, in particular for *P. aeruginosa* to higher concentrations than the respective MIC (2x and/or 4x MIC value) (Figure 3.4). However, most of them only presented significant effect over time, being more noticed in dual-species biofilms. It is believed, therefore, that the antimicrobial dosages at certain defined time interval may even lead to a suitable therapeutic efficacy to treat polymicrobial biofilm-associated infections. Our findings have corroborated several authors

[234][235][236][278][405][406][407][408], showing that all antimicrobials (AmB, ToB, CoL and PolyB) presented concentration-dependent killing along with prolonged persistent effects. Cmax/MIC (AmB and ToB) or the AUC/MIC ratios (CoL and PolyB) are PK/PD indexes for antimicrobials in our study. The prolonged persistent effects protect against re-growth when active antimicrobials concentration decreases to below the MIC[234][235][236]. Moreover, in previous studies developed by Hengzhuang *et al*.[277], it was reported that to reach the clinical target for biofilm infections compared with planktonic cell infections, higher-dose and longer-term treatments are required in the presence of CoL. Such as for CoL, the initial killing of *P. aeruginosa* by PolyB was quicker and in higher dosage than the standard dose (2.5 mg/kg/day)[409]. Thus it may be necessary to suppress *P. aeruginosa* resistance in immunocompromised hosts[409]. In relation to ToB, the efficacy of high-dose extended-interval aminoglycoside therapy has been recommended by current guidelines of the Cystic Fibrosis Foundation (CFF). The reason behind is that is proven by the increase of pulmonary function assays and reduction of the incidence of side effects[410][411][412][413]. The killing effects of AmB on *C. albicans* biofilms were quicker in a linear concentration-dependent. This antifungal agent generally requires higher concentrations, even above its therapeutic margin, to start the therapeutic potential[405].

4.3. Effect of the combination antimicrobials'

4.3.1. Susceptibility of planktonic populations

ICU have been reporting, in the past decade, increased rates of *P. aeruginosa* strains resistant to monotherapy (eg: fluoroquinolones 46 %, piperacillin–tazobactam 40 %, and carbapenems 43 %) leading to infections where VAP management shows a demanding task itself[69][414][415][416]. In general, VAP treatment involving drug combination is recommended for patients with prolonged MV (> 3 – 5 days), risk factors for MDR pathogens or with a history of previous MDR infection, empiric broad-spectrum[50][417][418]. To ensure that the infective pathogens are susceptible to at least one of the antimicrobials, combination therapy should provide a greater spectrum of activity against these microorganisms, including in *P. aeruginosa* - *C. albicans* infections[50][418].

It is expected that using combination therapy will decrease the probability of wrong initial treatment, which has been associated with significantly increased mortality, and the use of reduced doses of each antimicrobial will cause a reduced toxicity[250][419].

Certain combinations of antimicrobials exhibit synergistic effect against several pathogens. It was defined as a significantly greater activity, the effect provided by two antimicrobials agents combined in comparison with the effect provided by the sum of each antimicrobial agent alone[420][421]. Among the synergy assays, the checkerboard assay is the most common technique that allows to obtain the best therapeutic clinical outcomes based in the evaluation of the potentially of two antimicrobials in patients with VAP mainly by *Pseudomonas* species[257][422]. The checkerboard method employs a methodology similar to that performed for determination of MIC, where the results of the checkerboard assay are interpreted by calculating the FIC index for the two antimicrobials agents[423][424].A FIC index of 0.5 or less shows a synergistic effect; between 0.5 and 1 is assumed to be an additive effect; between 1 and 4 shows indifference; and a FIC index greater than 4 symbolizes an antagonistic effect[317]. It is important to refer that most studies only report susceptibility assays regarding only one species rather than mixed cultures. The results obtained in this section (Table 3.2) revealed a similarity with the susceptibility of both strains (*P. aeruginosa* and *C. albicans)* to antimicrobials agents alone (Table 3.1). Thus, most of the antimicrobials tested against single planktonic cultures demonstrated to inhibit the growth of both strains (*P. aeruginosa* and *C. albicans*) at low concentrations. However concentrations equal to or even higher than those used to inhibit single planktonic cultures were necessary for mixed cultures. These results demonstrate that *P. aeruginosa* and *C. albicans* were susceptible to most antimicrobials combinations, particularly when these strains are in single planktonic cultures (Table 3.2).

Furthermore, it was found that most tested antimicrobial combinations demonstrated a synergistic effect against *P. aeruginosa* planktonic cultures and in mixed planktonic cultures, with the exception of the combination ToB/PolyB which had shown an antagonistic effect against mixed planktonic cultures. An antagonist effect was also observed for all tested antimicrobial combinations against *C. albicans* cultures (Table 3.2).

Bozkurt-Guzel *et al*.[425] had shown results similar to the obtained in this study, reporting a percentage of 38 % of synergistic effect between CoL and ToB against *P. aeruginosa* strains. Most studies concerning antimicrobial combinations generally use CoL rather than PolyB[263]. This is most likely due to the wider geographical use of CoL. In addition it is important to refer that these therapies are often applied for *P. aeruginosa* strains, being the microorganism most commonly presented in a wide number of studies[263].

The antagonistic effect observed for most antimicrobial combinations (AmB/CoL and AmB/PolyB) in *C. albicans* populations (Table 3.2) was not consistent with previously studies[426][427][428]. These authors[426][427][428] demonstrated that AmB/CoL FIC value (0.27) shows a synergistic effect against *C. albicans*. According to previous literature[426][429][430][431], not all

membrane-targeting drugs are synergistic with echinocandins, for instance, azoles and polyenes are not generally regarded as synergistic with echinocandins in *Candida* species, although some examples of synergy have been reported. However, the use of different antimicrobial combinations may be one possible explanation for the aforementioned contradictory results.

4.3.2. Effect on biofilms

According to the results obtained, the antimicrobial combinations did not promoted a strongly reduction in the number of cultivable cells in single- and dual-species pre-established biofilms (Figure 3.5 and 3.7, respectively). Exceptions were observed for AmB/PolyB and ToB/PolyB, particularly with PolyB at high concentration (256 mg/L). These results can be explained by mechanisms of resistance commonly reported in bacterial-fungal biofilms to antimicrobials: slow penetration of the antimicrobial agent through the ECM biofilm, changes in the chemical microenvironment within the biofilm (leading to zones of slow growth rate), adaptive stress responses such as oxygen gradients leading to protection against antimicrobial, changes in cell metabolism, presence of a small population of extremely tolerant 'persister' cells (i.e. they can tolerate certain antimicrobial agents and they are not killed) and efflux of the antimicrobial agent before reaching target sites of attack[188][307][310][432]. Other resistance mechanisms include horizontal gene transfer[433], being typically higher in biofilms than in planktonic cultures and the increased transfer of antimicrobial resistance determinants on mobile genetic elements in biofilms of various microorganisms[434].

It was also found that only the combinations involving PolyB at high concentration (at 256 mg/L), in particular AmB/PolyB and ToB/PolyB, could disturb *P. aeruginosa* biofilm cells and leading to the total inhibition of these cells in dual-species biofilms. Therefore, this antimicrobial resistance was lower when both strains were presented in dual-species biofilms in comparison with single-species biofilm for each strain. Thus it exhibited a greater reduction in the number of cultivable cells for both strains in dual-species biofilms. In the present study, these findings strongly suggest that both strains may even mediate by different fungal-bacterial interactions. The antagonistic interaction in fungal-bacterial interactions contributes to the pathogenicity and subsequent failure of antimicrobial treatment comparatively when they were alone in biofilms[159]. It can alter the overall community structure[435], fungal cellular morphology[165][436][437], bacterial motility[438] and the survival[151] of the interacting between *P. aeruginosa* and *C. albicans*. It is believed that the fungal- bacterial interactions interplaying in those biofilms are mediated by QS, a phenomenum by which microorganisms monitor and

regulate their population density through chemical signaling[160][161][162][163][439][440]. An example of the QS in *P. aeruginosa- C. albicans* biofilm is the production of enzymes and small molecules by both microorganisms (such as hemolytic PlcH, phenazines, PYO and 3OC12HSL by *P. aeruginosa* and FOH by *C. albicans*), facilitating host invasion[159][441]. Thus, their competitive mechanism observed leads to more susceptibility of the consortium, facilitating the subsequent eradication by antimicrobials which induces great implications by modifying the actually clinical therapy of the VAP[442][443][444]. This conclusion implies that antimicrobial strategies more effective can be developed and validated in order to better control the overall consortium[445]. Although, more exhaustive studies are required to understand the fully mechanism behind the microbial consortium

It should be noted, however, that the strong reduction in the number of cultivable cells, mainly in dual-species biofilms, may be linked only to the action of PolyB at 256 mg/L concentration. However this concentration is higher than permissible in clinical use. According to the literature[446] at this concentration of PolyB (256 mg/L) is revealed a strong toxicity for humans. Based on this, concentrations of PolyB below to permissible in clinical use (6.25 – 50 mg/L concentration range acceptable[446]) were tested in AmB/PolyB and ToB/PolyB combinations, in particular 8 and 16 mg/L of PolyB. It has been found that only combinations with 16 mg/L PolyB showed reduction in the number of cultivable cells in single-species biofilms of *P. aeruginosa* whereas combinations with 8 and 16 mg/L PolyB concentrations had reduction in the number of cultivable cells in dual-species biofilms.

These results are corroborated by those found by Furtado *et al*.[447], where reported that PolyB combinations did not provide additional benefit over PolyB monotherapy for pneumonia.

Although the potential benefits of PolyB combinations therapy over monotherapy, some studies[448][449] have demonstrated that PolyB combinations suggested are generally limited to retrospective analyses and small, low-powered, prospective studies using traditional dosage regimens that achieve low plasma concentrations. So, given the associated limitations with existing clinical data, well-designed clinical trials that include higher-dose PolyB regimens are urgently required to provide an optimization of PolyB combination therapies compared with monotherapy[263]. It is also important to take into account the ethical and logistical challenges of conducting such investigations[263].

The results mentioned above were evaluated by CFUs enumeration (Figure 3.5 and 3.7) and biomass quantification (Figure 3.7 and 3.8). Despite having different results obtained with both methods, it is essential to mention that the CFUs enumeration and CV staining are complementary techniques used to assess biofilm ability formation and effectively measure

distinct data. CFUs enumeration assesses the number of cultivable cells and CV method determines the total biomass values (viable, non-viable cells and the matrix present in biofilm)[450].

In order to confirm those results, PNA-FISH methodology counterstained with DAPI was performed to examine cell enumeration and distribution within the biofilms (Figure 3.9 and 3.10). Nonetheless, contradictory results between the quantitative (CFUs enumeration and biomass quantification) and qualitative methods (PNA-FISH analysis) regarding the antimicrobial effect in single- and dual-species biofilm were observed mainly in the AmB/PolyB and ToB/PolyB combinations.

In AmB/PolyB and ToB/PolyB combinations with PolyB at 256 mg/L, it was observed an overall reduction in the number of cultivable cells in dual-species biofilms for both strains by CFUs enumeration. In the evaluation by PNA FISH analysis showed that both strains (*P. aeruginosa* and *C. albicans*) are present in abundance, in particular for *P. aeruginosa*. Hence, bacterial cells are predominately located around the hyphae. Lopes *et al*.[451] reported the predominance of *P. aeruginosa* within the consortium as the higher contribution of *P. aeruginosa* to the antimicrobial resistances presented by dual-species biofilms. The dual-species biofilms alter the metabolic activity of the consortium and hence may alter the susceptibility patterns of the population. This can reflect itself as an alteration in the overall biofilm structure and ECM by both microorganisms impairing access of antimicrobials into the consortium, or by decreasing the antimicrobial uptake rate through the cell membrane[451].

Furthermore, an *in vitro* study[151] indicates that *P. aeruginosa* preferentially attaches to the filamentous form of the *C. albicans*. Subsequently, the bacterial cells are able to form a biofilm along with *C. albicans* filaments increasing nutrient acquisition from the fungi.

Applying the Live/Dead BacLight Bacterial Viability Kit, it was demonstrated that bacterial cells in dual-species biofilms, were still viable even after treatment with PolyB combinations at 256 mg/L (Figure 3.11). Therefore, these results allow us to conclude that those are viable but not cultivable (VBNC) cells on solid media. It is important to note that the kit used in this assay is specific for bacterial specie; hence the orange color of the hyphae could not match the reality of the yeast cells state. Nonetheless, the assay was addressed primarily to bacteria since this had shown the most contradictory results in previous assays.

The VBNC state is an unique survival strategy adopted by many bacteria, including *P. aeruginosa* in response to adverse environmental conditions such as antimicrobial pressure, high/low temperature, starvation, chlorination, change in the pH, and oxygen stress[452][453][454][455][456][457]. Yeasts are also capable to undergo a VBNC state by the same reasons mentioned for bacteria[458][459]. In the presence of VBNC cells, the total number of

viable cells in a sample will be underestimated by the CFUs enumeration methodology due to the inherent non-cultivability and subsequently non-detection of these cells[460]. This situation has been explained by the contradictory results obtained by different methods of evaluation (CFUs enumeration and PNA FISH analysis). Additionally, the ability of microorganism to enter the VBNC state may be advantageous for cells, but the underestimation or non-detection of viable cells in clinical samples induces a serious risk to human health. In this way, several human infections, such as VAP can be developed. The risks appear from the fact that pathogenic microorganism can be a virulent in the VBNC state or regain virulence after resuscitation in to cultivable cells under suitable conditions[460]. Moreover, the inherent characteristic of cells being VBNC cells may lead to latency and consequently, to the recurrence of disease in patients who were already submitted to treatment[461][462]. Hence, it is important to understand what species of human pathogens can enter the VBNC state and also apply reliable detection methodologies to quantify the accurate population of viable cells, including both cultivable and VBNC cells[463].

In order to know when this will actually total reduce and whether it will be reversible or not, it was performed the kinetic effect and post antimicrobial effect for certain concentrations of AmB/PolyB and ToB/PolyB to dual-species pre-established biofilms.

So, it was shown that the total reduction in the number of cultivable cells of *P. aeruginosa* entrapped in dual-species biofilms occur immediately after the contact with AmB/PolyB (0.016mg/L and 256mg/L, respectively) and Tob/PolyB (0.016mg/L and 256mg/L, respectively) combinations (Figure 3.12). Furthermore, in the presence of both combinations, *P. aeruginosa* cells entrapped in dual-species biofilms did not recover their ability for cultivability, noting a lack of the cultivable *P. aeruginosa* remaining inexistent until to 120 h (Figure 3.13). Whereas for *C. albicans* strain the total reduction in the number of cultivable cells occur after 20h of the presence of ToB/PolyB (0.016mg/L and 256mg/L, respectively) and Amb/PolyB (0.016mg/L and 256mg/L, respectively) combinations. The number of cultivable *C. albicans* cells entrapped in dual-species biofilms decreased dramatically, even leading to the total reduction after 12 h (Figure 3.12). However for both combinations, *C. albicans* strain recovered its ability for cultivability even after removal of the antimicrobial combination (stress factor) (Figure 3.13). In fact, it has been described by Weckwerth *et al*. [464], which demonstrated that in presence of stress factor (such as high temperatures in their study) *C. albicans* cells can enter the VBNC state. Furthermore, according to the literature[465][466][467], many species have the ability to rehabilitate from the VBNC state back to the cultivable state when the stress is removed.

Lastly, it was necessary to understand whether any antimicrobial combination could be

used in clinical treatment below its permissible concentration but also if it was able to promote better effects in single- and dual-species biofilms of both strains. So, it was decided to evaluate the effect of the PolyB at a 32 mg/L concentration when combined with AmB and ToB at a 0.016 mg/L concentration by quantitative methods (CFUs enumeration and biomass quantification) (Figure 3.5 and 3.6). However it was shown that AmB/PolyB (0.016mg/L and 32mg/L, respectively) and ToB/PolyB (0.016mg/L and 32mg/L, respectively) combinations present a more similar behavior to PolyB at 8mg/L and 16 mg/L concentrations when combined to AmB and ToB. As such, a non-significant reduction of cultivable cells for both strains in single- and dual-species biofilms was seen.

Thus, a prolonged administration of antimicrobial therapy involving AmB/PolyB (0.016mg/L and 256mg/L, respectively) combination is the best therapeutic which presents potential to treatment of both species in dual-species biofilms. However, further studies involving antimicrobial combinations with PolyB at high concentrations (256 mg/L) are needed to better clarify the concentrations for clinical usage for treatment of polymicrobial infections in VAP, such as bacterial-yeast infections, avoiding concentrations toxic for humans.

CHAPTER V

CONCLUSION AND WORK PERSPECTIVES

5.1. Conclusions

This work aimed to give new insights concerning polymicrobial infections in VAP. The interaction between *P. aeruginosa* and *C. albicans*, emphasizing their biofilm formation ability and their antimicrobial resistance profiles were features evaluated throughout this work. Although the particular interaction of *P. aeruginosa* and *C. albicans* in VAP infections is frequently exhibited and despite of their importance in the development of health disorders, the mechanism is not completely understood. Therefore, more research is needed in order to complete the comprehensive information regarding the role of this polymicrobial infection in VAP.

Considering the first results of this work it was possible to conclude that the presence of different incubation times (24 h and 48 h) did not result in significant changes to biofilm formation in the overall consortia by both pathogens.

Concerning to single antimicrobials, the planktonic populations involving *P. aeruginosa* and *C. albicans* are susceptible to most of the antimicrobials tested at low concentrations in single planktonic cultures and at higher concentrations in presence of mixed planktonic cultures. Given these preliminary findings and knowing the susceptibility to different single antimicrobials of both strains in planktonic cultures, *P. aeruginosa* and *C. albicans* were evaluated for biofilm-forming ability and for antimicrobial resistance profiles under different single antimicrobials. Thus, *P. aeruginosa,* both in single- and dual-species, was apparently the pathogen more sensitive to the most tested single antimicrobial agents and these reductions were concentration-dependent used and time factor mainly in dual-species biofilms.

Based on these previous results and since the effect of the single antimicrobials was not in accordance to the expected one, it was decided to study the behavior of *P. aeruginosa* and *C. albicans* under the effect of different antimicrobial combinations. Initially, it was shown that the synergistic effect of most tested antimicrobial combinations was found mostly for *P. aeruginosa* planktonic single cultures and in mixed planktonic cultures. Subsequently, it was applied the concentrations of the most interesting antimicrobial combinations to single- and dual-species biofilms of *P. aeruginosa* and *C. albicans*. Thus, only AmB/PolyB and ToB/PolyB combinations promoted a significant reduction in the number of cultivable cells in single- or even in dual-species biofilms of both strains, particularly in combinations with PolyB at high concentration (256 mg/L) which could strongly disturb in *P. aeruginosa* biofilm cells leading to the total inhibition of these cells in dual-species biofilms. So, a greater reduction in the number of cultivable cells was exhibited and consequently, a lower antimicrobial resistance in dual-species biofilms. These findings evidenced an interaction between both strains when

simultaneously present promoting a higher sensitivity to antimicrobial therapies. Therefore such antimicrobial therapies seem to be novel methodologies for VAP therapy.

However, the evaluation by PNA FISH showed that both strains are present in abundance, in particular *P. aeruginosa* strain. The application of the Live/Dead BacLight Bacterial Viability Kit demonstrated that bacterial cells in dual-species biofilms still viable even after treatment with PolyB combinations at 256 mg/L. Therefore, these results allow the conclusion that those are VBNC on solid media. The ability *P. aeruginosa* to enter the VBNC state may induce the underestimation or non-detection of viable cells in clinical samples promoting a serious risk to human health.

In conclusion, the different antimicrobials therapies used in this work did not display any effectiveness in the treatment of dual-species biofilms involving *P. aeruginosa* and *C. albicans*. The antimicrobial combinations with PolyB at 256 mg/L present a strong potential to treat both strains, mainly in dual-species biofilms. However, the aforementioned concentration shows to be higher than the permissible for clinical use with strong toxicity for humans.

In order to disclose the suitable concentrations for clinical usage, further studies with these antimicrobial combinations have to be performed. Thus, they can be used as novel methodologies for VAP therapy in polymicrobial infections, particularly in *P. aeruginosa- C. albicans* infections.

5.2. Work perspectives

The work presented in this thesis provides the first findings about several aspects of *P. aeruginosa* and *C. albicans* interactions, namely, the importance of their polymicrobial biofilms characteristics for worsening VAP infection.

Evidently, this work raised interesting new questions for further research in regards to optimization and brings new breakthroughs in this field. Some of the suggestions that should be considered for future investigation are given below:

- Apply the PNA-FISH methodology while quantitative method once in the present work the methodology was only used in qualitative terms (presence or absence of cells of both strains), and therefore it is not known whether the antimicrobial combinations actually used in biofilms had some effect with respect to reducing the number of cells of both strains;

- Apply the LIVE/DEAD staining for yeasts in order to evaluate the state of *C. albican*s, once this method was only applied for bacterial cells and so there was no information about the real state of *C. albicans;*

- Bacteria-yeast polymicrobial infections are often primarily induced by bacterial infection and further fungi infections are exhibited alongside due to the immunocompromised patient state. Therefore, we would aim to study the antimicrobial effect in the final consortia by applying a different methodology for the VAP treatment. Future studies would rely on the investigation of the antimicrobial agents effect on biofilm formation by induction of co-infection by both species (*P. aeruginosa* and *C. albicans*). The adopted methodology would concern a dual-phase infection whereas the development of bacterial biofilms would be promoted prior to induction of *C. albicans* infection;

- Since it has been suggested that the both strains involved in this work presented antimicrobial resistance profiles, it is important to study virulence genes involved in the interaction between both strains;

- In order to simulate the real environment found in VAP infections, it is suggested the use of different conditions, such as the culture medium, the use of artificial sputum medium (ASM) instead of RPMI 1640 medium; carry out the biofilms assays adhering the endotracheal tube segments instead PS microtiter plates; and use clinical isolates instead of references strains.

CHAPTER VI

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