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Influence of oxygen conditions on bacterial interactions within biofilms related with cystic fibrosis

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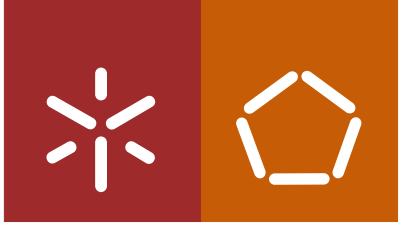

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Influence of oxygen conditions on bacterial interactions within biofilms related with cystic fibrosis

Master Dissertation
Master in Bioengineering

Work Supervised by:
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And Co-supervised by:
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*"Para ser um autêntico arqueiro, o domínio técnico é insuficiente. É necessário transcende-lo, de tal maneira que ele se converte numa **arte sem arte,** emanada do inconsciente."*

Eugen Herrigel, em A Arte Cavalheiresca do Arqueiro Zen.

ABSTRACT

Cystic Fibrosis (CF) is a genetic disorder associated with multispecies infections where interactions between classical and newly identified bacteria might be crucial for a better understanding of their persistent colonization in CF lungs. Nonetheless, little is known about the contributions of these microbes in the development of chronic biofilms, particularly under variable oxygen environments that are known to occur *in vivo* in the airways of CF patients. As such, this work aimed at giving insights into the physiology, phenotype and ecology of polymicrobial communities involving traditional (*Staphylococcus aureus* and *Pseudomonas aeruginosa)* and emergent bacteria (*Achromobacter baumannii*, Dolosigranulum *pigrum*, *Inquilinus limosus*, *Klebsiella pneumoniae* and Stenotrophomonas *maltophilia)* associated to CF.

At a first stage of this work, the ability of abovementioned bacteria to growth planktonically and to develop biofilms under *in vitro* atmospheres with different oxygen concentrations (aerobic and anaerobic) was examined. Results showed that all bacteria were able to growth and to develop biofilms under such conditions, demonstrating high number of cultivable cells even with a significant decreasing in the amount of biomass for low-oxygen atmospheres. Based on these initial findings, that revealed an easy adaptation of the emergent-species to the CF airways environments, it was considered crucial to investigate how they interact and contribute to the polymicrobial consortia when cultured with CF-common pathogens. As such, *S. aureus, I. limosus* and *S. maltophilia* were grown in dual-species populations with *P. aeruginosa* under variable oxygen atmospheres and these biofilms were thoroughly characterized for biomass, colony-forming units (CFU) and relative distribution of bacterial populations. Results demonstrated that dual-species biofilms, similarly to most single-species biofilms, produced more biomass under aerobic conditions. However, the presence of *S. aureus, I. limosus* and *S. maltophilia* in co-culture with *P. aeruginosa* significantly reduced the biofilm biomass formed comparatively with the mono-species *P. aeruginosa biofilm*, although the number of cultivable cells was not affected. Regarding microbial composition, the results obtained by CFU counting and PNA FISH under aerobic and anaerobic atmospheres, demonstrated that in all polymicrobial consortia, *P. aeruginosa* was still the dominant species.

The latest results have shown that populations encompassing CF-bacteria could easily adapt to planktonic and biofilm state under variable oxygen conditions resembling CF. But how these microorganisms contribute to disease progression and to antibiotic therapy was still to be unveiled. As such, those bacterial populations were grown under variable oxygen conditions and their antibiotic resistance profiles using ciprofloxacin were assessed. Results indicate that biofilms were notoriously more difficult to eradicate than their planktonic counterparts, for all oxygen atmospheres. Regarding polymicrobial populations, biofilm eradication was not achieved by using monotherapy, showing even an increased overall cell density when compared with mono-species *P. aeruginosa* biofilm*,* in all oxygen conditions. In general, biofilm compositions changed as a result of antibiotic treatment, with alterations being dependent on the antibiotic concentration and oxygen conditions implemented. For consortia formed between *P. aeruginosa* and *S. aureus*, the latter species predominated in the consortia for both oxygen conditions. Contrariwise, the consortia encompassing *P. aeruginosa – I. limosus* and *P. aeruginosa – S. maltophilia* were dominated by the CF-key pathogen *P. aeruginosa*. As such, the endurance of *P. aeruginosa* within the consortia, before and after antibiotic treatment, could be the basis for a higher contribution of this species to the antibiotic resistance presented by dual-species biofilms. However, the increasing survival of *S. maltophilia* and *I. limosus* (slight increase for *I. limosus*) in dual-species consortia with *P. aeruginosa* after antibiotic exposure, for all oxygen atmospheres, indicates that these species may have also a preponderant role in increasing the whole resistance within the consortia. The PNA FISH method was employed to directly localize and discriminate the bacterial populations within the consortia, corroborating the dominance of *P. aeruginosa* within the mixed-species consortia (determined by CFU counting), and allowed to observe a decreasing in the overall cell density for all consortia under low-oxygen atmospheres.

In summary, the results demonstrated that emergent- and traditional-species are able to live in association with key-CF pathogen *P. aeruginosa* commonly found in CF airways under variable oxygen atmospheres, developing highly resilient consortia towards antibiotic treatment. The interactions established between emergent-species and other major pathogens might be crucial to understanding the persistent microbial infection in CF airways and bring information about the pathogenic character of such emergent species.

RESUMO

A Fibrose Cística (FC) é uma doença genética associada a infecções multiespécie em que as interações, entre bactérias clássicas e recentemente identificadas, podem ser cruciais para melhor compreender a sua persistente colonização nos pulmões de pacientes com FC. No entanto, a contribuição destes microrganismos no desenvolvimento de biofilmes crônicos, particularmente em ambientes com diferentes concentrações de oxigênio como ocorrem *in vivo* nas vias respiratórias de pacientes com FC, é pouco compreendida e estudada. Neste cenário, este trabalho teve como objectivo contribuir para o conhecimento da fisiologia, fenótipo e ecologia de comunidades polimicrobianas envolvendo bactérias tradicionais (*Staphylococcus aureus* e *Pseudomonas aeruginosa*) e emergentes (*Achromobacter baumannii, Dolosigranulum pigrum, Inquilinus limosus, Klebsiella pneumoniae* e *Stenotrophomonas maltophilia*) associadas à FC.

Na primeira etapa do trabalho foi investigada a capacidade das bactérias acima referidas crescerem planctónicamente e desenvolver biofilmes *in vitro* em atmosferas com diferentes concentrações de oxigênio (aerobiose e anaerobiose). Os resultados mostraram que todas as bactérias exibiram capacidade para crescer e desenvolver biofilmes em tais condições, atingindo um elevado número de células cultiváveis mesmo com uma diminuição significativa na quantidade de biomassa formada para ambientes com deficiência em oxigénio. Com base nestes resultados preliminares, que revelaram uma fácil adaptação das espécies emergentes a ambientes similares aos das vias respiratórias de pacientes com FC, considerou-se crucial investigar como estas bactérias interagem entre si e contribuem para a resposta global dos consórcios polimicrobianas quando cultivadas com patogéneos comumente associados à FC. Desta forma, desenvolveram-se biofilmes duplos envolvendo *S. aureus* ou *I. limosus* ou *S. maltophilia* com *P. aeruginosa* em concentrações de oxigénio variáreis, tendo sido, posteriormente, caracterizados relativamente à quantidade de biomassa, células cultiváveis e distribuição relativa de populações bacterianas. Os resultados demonstraram que os biofilmes duplos, tal como os simples, produziram maior quantidade de biomassa em condições aeróbias. No entanto, a presença de *S. aureus, I. limosus e S. maltophilia* em co-cultura com *P. aeruginosa* reduziu significativamente a quantidade de biomassa dos biofilmes duplos formados, comparativamente com o biofilmes simples de *P. aeruginosa*, embora o número de células cultiváveis não fosse afectado. Em termos de composição microbiológica, os resultados obtidos por contagem de células cultiváveis e PNA FISH sob atmosferas aeróbias e anaeróbias, demonstrou que em todos os consórcios polimicrobianos *P. aeruginosa* foi a espécie dominante.

Como demostrado, bactérias associadas à FC parecem adaptar-se facilmente a ambientes com diferentes gradientes de oxigénio, crescendo planctónicamente e em biofilme. No entanto, não há ainda muita informação acerca de como esses microrganismos contribuem para a progressão da doença e reagem à antibioterapia, nas várias condições de oxigénio existentes nas vias respiratórias da FC. Sendo assim, os seus perfis de resistência à ciprofloxacina sob condições variáveis de oxigênio foram avaliados. Os resultados indicam que os biofilmes foram notoriamente mais difícil de erradicar do que as células planctónicas, para todas as atmosferas de oxigênio. Relativamente às populações polimicrobianas, a erradicação de biofilmes não foi conseguida através da monoterapia (ciprofloxacina), havendo mesmo um aumento da densidade celular quando comparado com o biofilme de *P. aeruginosa*, em todas as condições de oxigênio. De um modo geral, a composição de biofilmes polimicrobianos dependente da concentração do antibiótico e das condições de oxigénio implementadas. No caso dos biofilmes duplos formados entre *P. aeruginosa* e *S. aureus*, a última espécie predominou o consórcio para ambas as condições de oxigénio. Pelo contrário, os biofilmes duplos que englobam *P. aeruginosa - I. limosus* e *P. aeruginosa - S. maltophilia* foram dominados pela *P. aeruginosa*. Estes dados parecem indicar que a resistência dos consórcios polimicrobianos, antes e após o tratamento com ciprofloxacina, poderá estar na base de uma maior contribuição da *P. aeruginosa.* No entanto, o aumento da proporção de *S. maltophilia e I. limosus* (ligeiro aumento no caso de *I. limosus*) nos biofilmes duplos após a exposição à ciprofloxacina, em condições de aerobiose e anaerobiose, parece indicar que essas espécies têm um papel preponderante no aumento da resistência global dos consórcios. O método PNA FISH foi utilizado para localizar e distinguir as populações bactérias nos consórcios polimicrobianos, corroborando com a dominância de *P. aeruginosa* nos consórcios polimicrobianos, e com a diminuição na densidade celular dos consórcios para atmosferas com baixa concentração de oxigênio.

Conclui-se assim, que ambas as espécies (tradicionais e emergentes) são capazes de viver em associação com *P. aeruginosa* adaptando-se facilmente a condições de oxigénio semelhantes às encontradas nas vias respiratórias dos pacientes com FC, originando consórcios polimicrobianos com resistência a antibióticos aumentada. Assim, avaliar as interações estabelecidas entre espécies emergentes e tradicionais associadas à FC pode ser crucial para compreender as infecções microbianas persistentes que ocorrem nas vias aéreas de pacientes com FC, e trazer informações sobre a verdadeiro papel patogênico das espécies emergentes.

This study aimed to address the behaviour of traditional and emergent CF-bacteria, as well as to appraise the antibiotic susceptibility and characterize polymicrobial communities formed between these bacteria under variable oxygen conditions, similar to those locally found in CF airways *in vivo.*

This thesis is organized into five chapters. Chapter 1 briefly reviews relevant clinical aspects of CF, emphasising the composition of microbial communities in CF airways. The bacterial biofilms, including relevant particularities and importance for CF are also summarized in this chapter. In Chapter 2, the microorganisms, culture conditions, materials and techniques used in the work presented herein are described. Chapter 3 reports preliminary studies on planktonic and biofilm mono- and dual-species encompassing emergent and traditional CF-associated bacteria, developed under variable oxygen conditions (aerobic and anaerobic), resembling those occurring in CF airways *in vivo.* Chapter 4 focuses on resistance profiles of mono- and dual-species populations, formed by CF-associated unusual and traditional bacteria, and microbial compositions before and after antibiotic exposure, performed under aerobic and anaerobic conditions. Chapter 5 finalizes the thesis by presenting the main conclusions of the work presented and proposes future research lines.

TABLE OF CONTENTS

ºC: Celsius degrees
%: Percent
ΔF508: mutation in phenylalanine residue at position 508
μL: microliter
μm: micrometer

AI: Auto inducer
ANOVA: Analysis of variance
ASL: Airway surfaces liquid
ATCC: American Type Culture Collection
BCC: *Burkholderia cepacia* complex
BCSA: *Burkholderia cepacia* selective agar
BDSF: cis-2-dodecenoic acid
bp: base pair
CAMHB: Cation-adjusted Mueller-Hinton Broth
CF: Cystic fibrosis
CFTR: Cystic fibrosis transmembrane conductance regulator
CFU: Colony-forming units
CIP: Collection of Institute Pasteur or Ciprofloxacin
CLSM: Confocal laser scanning microscopy
cm: centimeter
CO_2: Carbon dioxide
CV: Crystal violet
DAPI: 4`, 6-diamidino-2-phenylindole
DNA: Deoxyribonucleic acid
DSF: Diffusible signal molecules
e.g.: (*exempli gratia*) for example
et al.: (*et alli*) and others
F: Fosfomycin
Fig.: Figure
FISH: Fluorescence *in situ* hybridization
FTI: Fosfomycin/Tobramycin
g: Relative centrifugal force
h: hour
HQNO: 2-heptyl-4-hydroxyquinolone-N-oxide
IL: Interleukin
kb: Kilobase
kHz: kilo-hertz
L: liter
log_{10}: logarithm with base 10
MBC: Minimum inhibitory concentration
MBEC: Minimum biofilm eradication concentration
MBIC: Minimum biofilm inhibitory concentration
mg: milligram

MIC: Minimum inhibitory concentration
min: minute
mL: milliliter
mM: millimolar
MMP: Matrix Metalloproteases
MRSA: Methicillin-resistant *Staphylococcus aureus*
NaCl: Sodium chloride
Ndk: Nucleoside diphosphate kinase
OD: Optical density
$OD_{570\ nm}$: Optical density at 570 nm
$OD_{640\ nm}$: Optical density at 640 nm
OF: Oropharyngeal flora
P: probability
PCL: Periciliary liquid layer
pH: potential hydrogen
PNA: Peptide nucleic acid
PQS: Pseudomonas quinolone signal
PS: Polystyrene
Psl: Polysaccharide synthesis locus
Q_2: Rate of epithelial oxygen consumption
QS: Quorum-sensing
rpm: revolutions per minute
rRNA: ribosomal ribonucleic acid
s: Second
SCVs: *Staphylococcus aureus* small variant
SD: Standard deviation
T: Tobramycin
TIP: Tobramycin inhalation powder
TSA: Tryptic soy agar
TSB: Tryptic soy broth
UP: ultrapure
vol: volume
w/w: weight-to-weight
wt: weight

LIST OF TABLES

Papers in peer reviewed journals

Magalhães, A.P., Azevedo, N., Pereira, M.O., Lopes, S.P. (2015) The cystic fibrosis microbiome in an ecological perspective**.** *Appl Microbiol and Biotechnol*. Submited

Abstracts in conferences

Magalhães, A.P., Azevedo, N., Pereira, M.O., Lopes, S.P. (2015) Cystic fibrosis emerging bacteria under variable oxygen tensions: biofilm formation ability and resilience to acute antibiotherapy**.** 4rd European Congress on Microbial Biofilms – Basic and Clinical aspects (EUROBIOFILMS 2015), Brno, Czech Republic, 23-26 June.

Chapter 1

GENERAL INTRODUCTION

This chapter provides a general outline of major aspects about cystic fibrosis (CF) lung disease, carefully emphasizing the heterogeneous CF airways environment, the composition of microbial communities, including the key pathogens and the emergent microorganisms. Special emphasis was also given to microbial biofilms and its impact for CF severity.

Considering the collective microbiome as a potential pathogenic entity in itself, it was intended to make a general overview, focusing on the social behavior within CF communities (microbe-microbe and microbe-host relationships), and analyzing whether a particular community causes or worsens disease, in a manner analogous to individual pathogens. It is believed that the relationship between a microbial community and disease is better understood from an ecological perspective and can improve clinical understanding, ultimately providing guidelines for an effective treatment and chronic infection suppression.

1.1 RELEVANT ASPECTS OF CF - PATHOGENESIS, REDUCED-OXYGEN ENVIRONMENT AND MICROBIAL COLONIZATION

Cystic fibrosis is a human genetic disorder that results from mutations in the CF transmembrane conductance regulator (CFTR) gene. The most prevalent of those mutations (ΔF508) is the deletion of three nucleotides at the position 508 of the CFTR protein sequence [1]. The CFTR protein acts as a channel for the chloride and sodium ions transport across the cell membranes. Therefore, a dysfunctional CFTR protein leads to the absence or a decreased chloride secretion, resulting in an intracellular accumulation of those ions and ultimately to the depletion of chloride, sodium and water from the airway lumen. This causes abnormal thick and viscous secretions and impairs mucociliary clearance in CF airways (figure 1-1) [2–4].

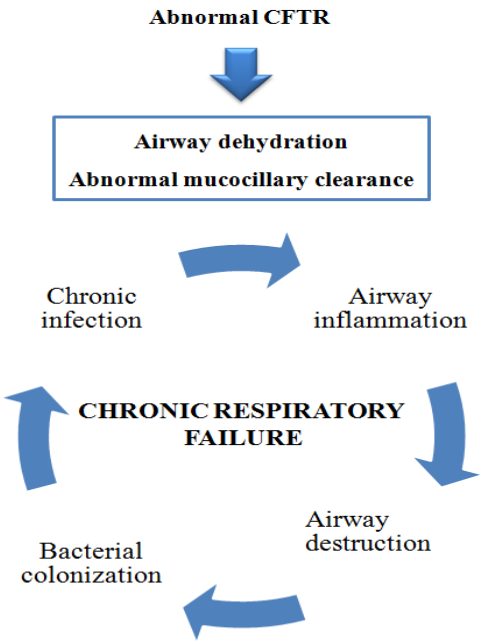

Figure 1-1 Mechanism of the CF pulmonary disease. In the lungs, the defective chloride ion transport results in the decrease of the volume of the periciliary fluid, compromising the mucociliary clearance and triggering the overproduction of dehydrated and viscous mucus. This leads to the persistent colonization of bacteria in the lungs, and the physiologic consequences are persistent inflammatory responses, obstructive lung physiology, respiratory insufficiency, which ultimately results in death from chronic respiratory failure. Adapted from Kirby et al. [5].

The clinical manifestations of CF are quite variable, affecting individuals throughout their entire life, that typically has a shortest timespan or around thirty-forty years [6]. It is well established

that the greatest contributor for the morbidity and/or mortality is the failure in lung function that generally occurs in the older patients, caused by the build-up of mucus that clogs the airways and leads to persistent colonization by different microorganisms (frequently bacterial species). Hence, recurrent cycles of infections and inflammations lead to a progressive airway and lung damage, respiratory failure and eventually death [7–9].

The existence of steep oxygen gradients within the CF airway mucus is well-known, with zones ranging from aerobic (in the top layers) to completely anaerobic (deeper mucus layers) (Figure 1-2 steps 1-3) [10]. Typically, the airway epithelial cells have a thin and hydrated mucus layer, located on top of the periciliary liquid layer (PCL), that enables an efficient mucociliary clearance [10–12]. A normal rate of epithelial O_2 consumption (Qo_2) produces no O_2 gradients within the thin airway surfaces liquid (ASL). In CF, the airway epithelium absorbs the sodium (Na^+) and chloride (Cl^-) ions and water from the lumen, depletes the PCL and slow down or even stop the mucus transport. The increased O_2 consumption associated with accelerated CF ion transport does not generate gradients in the thin biofilm of ASL, but the persistent mucus hyper secretion leads to the production of luminal mucus plugs, hence increasing the mucus layer on the epithelial cells and generating steep oxygen gradients, with zones ranging from aerobic (generally located at the top) and microaerobic and/or even completely anaerobic (located in the deeper layers) [10–12].

Patients suffering from CF are prone to develop severe biofilm-related infections, and are thought to contribute greatly to the emergence and dissemination of antibiotic resistance [13]. The biofilm formation represents a protective mode of growth that allows microorganisms to survive in hostile environments and disperse by seeding cells to colonize new niches under desirable conditions [14]. *P. aeruginosa* persists in the CF airways due to its ability to form biofilms, being considered the key CF pathogen [15]. *P. aeruginosa* presents a notorious ability to develop resilient biofilms in the form of "bacterial aggregates" within the CF mucus (Figure 1-2, steps 4-6) [10–12,14]. The persistence of chronic *P. aeruginosa* lung infections in CF patients is due to biofilm-growing mucoid strains, protected by alginate overproduction [16]. The persistence of these biofilms into the CF airway mucus often leads to a high tolerance to many antibiotics [17]. Conventional resistance mechanisms, such as the presence of a chromosomal β-lactamase, upregulated efflux pumps, and mutations of antibiotic target molecules in the bacteria, have also contributed to the adaptation of *P. aeruginosa* biofilms to the CF environment [16]. Although *P. aeruginosa* prefers oxygen respiration as the highest-energy-yielding process for growth, it can survive in the mucus anaerobic zones [11].

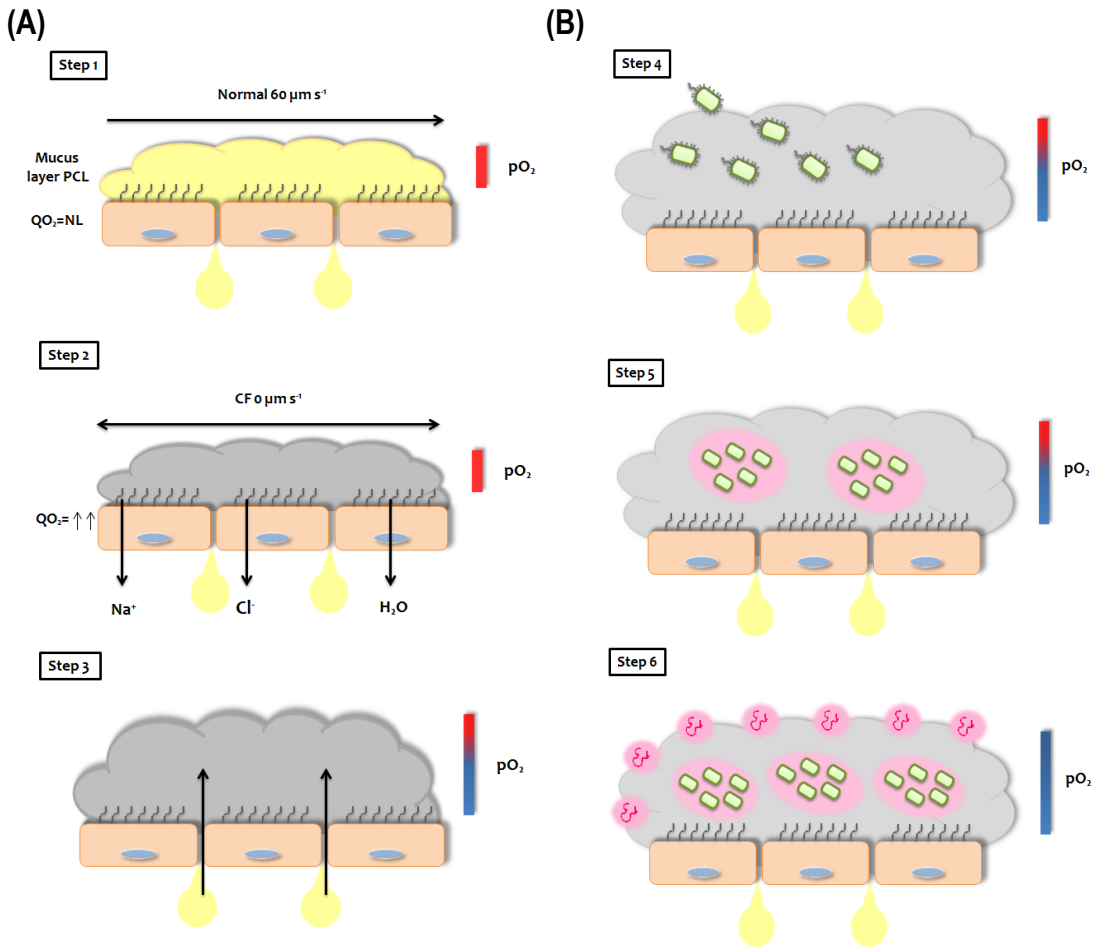

Figure 1-2 (A) Alterations in mucus of normal epithelial airway cells (Step 1 to 3): **(Step 1)** On a normal airway epithelia, a thin mucus layer (yellow) resides on top of the PCL (clear). The presence of the low-viscosity PCL facilitates efficient mucociliary clearance (denoted by black arrow). A normal rate of epithelial O_2 consumption (Qo_2; left) produces no O_2 gradients within this thin ASL (denoted by the red bar). **(Step 2)** Excessive CF volume depletion (denoted by vertical arrows) removes the PCL, mucus becomes adherent to epithelial surfaces, and mucus transport slows/stops (bidirectional black arrow). The raised O_2 consumption (left) associated with accelerated CF ion transport does not generate gradients in thin films of ASL. **(Step 3)** Persistent mucus hypersecretion (denoted as mucus secretory gland; gray) with time increases the height of luminal mucus masses/plugs. The raised CF epithelial Qo_2 generates steep hypoxic gradients (blue color in bar) in thickened mucus masses. **(B)** Schematic model for *P. aeruginosa* biofilm in the CF mucus (Step 4 to 6): **(Step 4)** *P. aeruginosa* are deposited on the thickened mucus surfaces and can penetrate the mucus actively (e.g. by inhalation, flagellum- or pili-dependent motitily) and/or passively (due to mucus turbulence) into the CF mucus. **(Step 5)** Afterwards, *P. aeruginosa* start to develop bacterial aggregates (the biofilms), which are protected by an alginate capsule. In this step, the consumption of O_2 is drastically increased by the bacterial cells and hypoxic and/or anaerobic pockets are formed. **(Step 6)** In the final stages, where O2 is almost depleted, the bacterial aggregates become highly resistant to the neutrophils and antibiotics, setting the stage for persistent chronic infection [10–12] .

The ability of this bacterium to adapt to the oxygen-limited environments is associated with a drastic physiological change in *P. aeruginosa* (e.g. increased alginate production; alterations in the outer membrane; biofilm development), which contributes to an increased antibiotic tolerance [18]. The alginate produced by the biofilm bacteria in CF lung infections also provides a physical barrier to host defense systems [10–12].

1.2 THE COMPLEX CF MICROBIOME

As stated above, the infections in the CF airway are frequently polymicrobial [19–21]. The CF airways offer a favorable environment for the colonization and proliferation of a large variety of organisms, including as bacteria, fungi and viruses, with bacterial species being the ones that are more frequently isolated [22].

Traditionally, the detection and identification of microbial species has relied on culture-based studies, using sputum or bronchial alveolar lavage samples for microbial detection and identification [23]. These techniques allow identifying several key microbial species that contribute to CF lung infection and disease progression, beginning early in life with *S. aureus* and *H. influenzae* and culminating in chronic infections caused by *P. aeruginosa* or *B. cepacia complex* species (Table 1-1) [23,24].

Table 1-1 Bacterial species most commonly associated with CF airway disease. Adapted from Huang et al. [25].

Species	Clinical Significance	References
Pseudomonas aeruginosa	Arguably the most important pathogen; Presents a prevalence of 80% at ages ≥ 18 years; Ability to develop biofilms that protect from host responses and numerous antibiotics;	[26–28]
Haemophilus influenza	Most frequently isolated during infancy and/or early childhood; Ability to form biofilms;	[29,30]
Staphylococcus aureus	Infects young patients, but can also be cultured from adolescents and adult patients; Ability to cause chronic infection;	[31–33]
Burkholderia cepacia complex	Important opportunistic pathogens Ability to cause a progressive, invasive and fatal pulmonary disease known as “cepacia syndrome”.	[34,35]

S. aureus, one of the first pathogens isolated from CF samples, is the most prevalent pathogen in children and adolescents, however 40% of adult patients still remain colonized [33]. *S. aureus* has the ability to cause chronic infection [31–33]. *H. influenzae* is also involved in chronic lung infections in CF pediatric patients, forming structures consistent with biofilms even before the onset of clinical signs or symptoms of lung disease [30]. *B. cepacia complex* is a group of 18 *Burkholderia* species infecting 2 to 8% of patients with CF, with some of them (*B. cenocepacia, B. multivorans, B cepacia and B. dolosa*) being highly transmissible, presenting pathogenic potential and very high resistance to antibiotic therapy [34–36].

Approximately 50% of CF patients are colonized with *P. aeruginosa* [37], which remains the most common pathogen isolated from CF sputum, being more prevalent in adults [38]. The presence of *P. aeruginosa* in CF airways is highly associated with poor lung function, morbidity and mortality of patients. After colonization with *P. aeruginosa*, consecutive episodes of re-colonization frequently occur, resulting in a chronic infection that can persist for years or even never being eradicated in CF patients lungs [39].

However, recent molecular methodologies have documented complex microbial ecosystems in CF samples, with a wide array of uncommon microorganisms co-existing with traditional pathogens (Table 1-2), acting collectively to facilitate disease progression [40]. These microorganisms include bacteria (e.g. *I. limosus*, *Dolosigranulum pigrum*, *Stenotrophomonas maltophilia*), fungi (e.g. *Aspergillus fumigatus*, *Candida albicans*) and viruses (e.g. rhinovirus, adenovirus, influenza). In addition, other anaerobic species are frequently isolated. Worlitzsch et al. [41] identified, in a cross-sectional study, 15 genera of obligate anaerobes in 91% of patients suffering from CF. Tunney et al. [42] also identified anaerobic species within the genera *Prevotella*, *Veillonella*, *Propionibacterium*, and *Actinomyces*, which were isolated in high numbers (>64%) in sputum samples from patients in adulthood. The high numbers of anaerobic bacteria detected in the CF airways may be a result of oxygen consumption by aerobic pathogens (such as *P. aeruginosa*) that often colonize the airways, creating a favorable niche for the proliferation of anaerobes [10,43]. Using molecular methods, Guss et al. [22] have identified more than 60 bacterial genera, including facultative and obligate anaerobes, oral bacteria and opportunistic pathogens, many of which have never before been found in the CF lung. These results indicate that the traditional culture-methods are insufficient to describe the polymicrobial populations actually present in the CF lung. A recent review, by Lopes et al. [1], provides a comprehensive understanding of the great complexity of the microbiome existing in CF, detected and/or identified employing recent molecular methodologies.

Table 1-2 Examples of microorganisms identified in patients with CF.

Species		References
Bacteria		
Acinetobacter baumanii	**Aerobic**	[44]
Achromobacter xylosoxidans		[45,46]
Brevundimonas diminuta		[44,47]
Chromobacterium violaceum		[20]
Escherichia coli		[22,45,46]
Inquilinus limosus		[44]
Klebsiella pneumoniae		[45]
Moraxella osloensis		[46]
Pseudomonas huttiensis		[20]
Pseudomonas stutzeri		[20]
Stenotrophomonas maltophilia		[44]
Dolosigranulum pigrum		[45]
Mycobacterium abscessus		[45,46]
Staphylococcus epidermidis		[42]
Actinomyces odontolyticus	**Anaerobic**	[22,41,42]
Capnocytophaga leadbetteri		[22,41,46]
Dialister pneumosintes		[22,41,45]
Fusobacterium necrophorum		[22,41,42,46]
Gemella morbillorum		[22,41,45]
Staphylococcus saccarolyticus		[41,42]
Streptococcus pneumoniae		[41,42,45,48]
Fungi		
Aspergillus fumigatus		[49]
Candida albicans		[49–52]
Geosmithia argillacea		[53,54]
Penicillium emersonii		[55]
Trichosporon mycotoxinivorans		[56]
Viruses		
Adenovirus		[57–59]
Human metapneumovirus		[57–59]
Influenza (A and *B) viruses*		[57–59]
Rhinovirus		[57–59]

1.3 ECOLOGICAL PERSPECTIVE OF THE CF MICROBIOME

Microbial interactions might exist within CF polymicrobial communities, so it is not surprising that these infections are increasingly viewed as complex communities of interacting organisms, with dynamic processes key to their pathogenicity. Similarly to the relative contribution to clinical status, disease progression, and the efficacy of antibiotic therapy by newly identified members of a polymicrobial community, which remain to be fully explored, also the know-how on the consequences of the interplay among potential pathogens and/or between them and their eukaryotic host are good supports for understanding and treat CF. These interactions can be mediated by a large number of mechanisms, which encompasses interspecies signaling, metabolite exchange, cell-cell contact and are often implicated in the modulation of microbial behavior, ultimately contributing to disease progress and clinical outcome. In addition, many types of infections are caused by biofilm-associated microorganisms [60], which are harder to eradicate compared with planktonic exponentially growing cells, due to several factors operating concurrently (e.g. changed structure and reduced diffusion rates of the compounds in the biofilm matrix, changed gene expression pattern and low growth rates of the biofilm-encased cells) [39]. This protective effect may be further enhanced if multiple species are present within the biofilm, where the dynamics between the resident species may potentially evolve and change the volume and function of the whole biofilm both qualitatively and quantitatively [61]. In these consortia, microorganisms frequently communicate via quorum-sensing (QS) complex systems, which play an important role in the social behaviour, regulation of microbial population density and expression of virulence factors (e.g. resistance genes and proteins) among members of a microbial community [62].

Although particular microbial communities may be associated with certain clinical outcomes, the heterogeneous nature of the airway environment (nutrients, as well as physiochemical characteristics, such as oxygen tension, temperature and pH) will influence the mix of microbes able to occupy it, through exerting selective pressures. In addition, it is increasingly recognized that the microbes can alter the characteristics of the niche in which they grow, by influencing the behavior of other colonizing species (such as pathogenicity [63]), or by directly interacting with the eukaryotic host (e.g., by damaging airway epithelia [64] or triggering inflammation [65]), as well as the impact of changes in antibiotic treatment that follow clinical worsening, such as the type and intensity of antibiotic exposure [66]. For instance, Tunney et al. [67] have reported that substantial shifts in bacterial abundance within the microbial community can be

detected following antibiotic therapy. However, Stressmann et al. [68] showed that antibiotic therapy can temporarily perturb these communities, which tend to return to their pretreatment configuration following cessation of antibiotics.

The pressures affecting microbiome composition are dynamic, and the comprehensively understanding of the drives of microbial community stability is fundamental for predicting the way in which a microbiome will respond to perturbation. Microbial activity will influence the processes that select for subsequent members of the microbiome, therefore the infection by one species can indirectly dictate microbiome composition [66].

Hence, it becomes imperative to understand the molecular basis and the biological effect of those interplay processes within multispecies communities to help improving clinical understanding and the in-use treatment regimens, devising new targets and disease control strategies.

An extensive research in recent literature has identified studies reporting interactions among microbes and between microbes and their host in the context of CF, which is summarized in Table 1-3. The interactions described within Table 1-3 are divided into two different categories, synergism and antagonism. Contrariwise to synergistic interactions, which represent mutual benefit to all species present, antagonistic interactions result in a negative effect for at least one species. As it is possible to observe, microorganisms can use simultaneously different mechanisms to interact with other species, which may be associated with the niche characteristics and selective pressures exerted that shaped the behavior and the way in which the species interact.

Table 1-3 Host-microbe and microbe-microbe interactions occurring in the context of CF and their predictive ecological effects.

	Microbes	Interaction	Mechanism	Predicted Ecological Interaction		References
				Within Microbes[a]	Effect in Host[b]	
Microbial – Microbial interplay	*B. cenocepacia* – *C. albicans*	*C. albicans* filamentation is inhibited by *B. cenocepacia*	*B. cenocepacia* QS signal, BDSF (cis-2-dodecenoic acid) inhibited the filament formation by *C. albicans.*	Antagonism	+	[69]
	P. aeruginosa – *A. fumigatus*	*A. fumigatus* biofilm formation is inhibited by direct contact with *P. aeruginosa.*	Exposure to the *P. aeruginosa* metabolites resulted in the inhibition of hyphal growth in *A. fumigatus*, decreasing biomass about 19%. Antagonistic relationships existed between *A. fumigatus* and *P. aeruginosa*, which were influenced by the release of small diffusible extracellular molecules.	Antagonism	+	[70]
		A. fumigatus and *P. aeruginosa* co-culture lead to an worst pulmonary function.	Unknown	Unknown	-	[71]
		A. fumigatus convert *P. aeruginosa* metabolites.	*P. aeruginosa* phenazine metabolites were converted by *A. fumigatus* into other chemical entities with alternative properties, that include fungal inhibitory activity.	Antagonism	Unknown	[72]

Table 1-3 *Continued*

P. aeruginosa – B. cenocepacia	The alginate produced by *P. aeruginosa* facilitates *B. cenocepacia* infection by interfering with host innate defence mechanisms.	The presence of alginate impaired *B. cenocepacia* phagocitosis both *in vivo* and *in vitro*. Alginate also reduced the proinflammatory responses of CF epithelial cells and alveolar macrophages against *B. cenocepacia* infection.	Synergism	-	[73]
	B. cenocepacia stimulates *P. aeruginosa* biofilm development; Co-infection in a mouse model by *P. aeruginosa* and *B. cenocepacia* lead to an increased host inflammatory response.	*B. cenocepacia* influenced biofilm formation by *P. aeruginosa,* leading to altered biofilm architecture and increased biomass. Co-infection of by both species increased the production of virulence factors (cytokines IL-1β and chemokines CCL2/JE and CXCL1/KC).	Synergism	-	[74]
P. aeruginosa – B. cepacia	*P. aeruginosa* increases the virulence by *B. cepacia.*	*P. aeruginosa* PAO1 exoproducts (autoinducers) increased the production of three virulence factors (siderophore, lipase and protease), by *B. cepacia.*	Synergism	-	[75]
P. aeruginosa – B. cepacia - S. aureus	*P. aeruginosa* dominates over *B. cepacia* and *S. aureus* in mixed culture under a variety of growth conditions.	*P. aeruginosa* dominated by more efficient substrate consumption in regard to obtain high cell concentrations. Conversely, the other bacterial species strongly reduced their viability in mixed culture.	Antagonism (*P. aeruginosa* to *B. cepacia*; (*P. aeruginosa* to *S. aureus*)	Unknown	[76]
P. aeruginosa – C. albicans	*C. albicans* morphology is significantly influenced by the presence of *P. aeruginosa*	3OC12HSL (3-oxo-C12 homoserine lactone), a cell-cell signalling molecule produced by *P. aeruginosa,* was sufficient to inhibit *C. albicans* filamentation without affecting fungal growth rates	Antagonism	+	[77]

Table 1-3 *Continued*

	In co-cultures, the presence of farnesol, a sesquiterpene produced by *C. albicans*, decreases the production of PQS (*Pseudomonas* quinolone signal) signalling by *P. aeruginosa*	Farnesol inhibited the production of PQS by inhibition of transcriptions on the pqs operon.	Antagonism	+	[78]
	Bacterial supernatant from four *P. aeruginosa* strains strongly reduces the ability of *C. albicans* to form biofilm on silicone.	Up-regulation of YWP1 gene by C. albicans, which encodes a protein known to inhibit biofilm formation, in response to bacterial supernatants of *P. aeruginosa.*	Antagonism	+	[79]
P. aeruginosa – I. limosus; P. aeruginosa – D. pigrum	The emergent CF species *I. limosus and D. pigrum* can grow together with *P. aeruginosa*, increasing tolerance of the overall consortia to a wide range of antibiotics.	A possible alteration in the overall biofilm structure and extracellular matrix by both emerging species comparing with *P. aeruginosa* biofilms alone is suggested.	Synergism	-	[80]
P. aeruginosa – Oropharyngeal flora (OF)	The presence of OF in the lung of a rat model enhances lung damage caused by *P. aeruginosa.*	Auto-inducer-2 (AI-2), a QS mediator used by OF bacteria use to interact with *P. aeruginosa*, modulated *P. aeruginosa* gene expression (up-regulation), increasing its pathogenicity.	Synergism	-	[81]

Table 1-3 *Continued*

P. aeruginosa-Phage (14/1, ϕKZ, PNM and PT) and Protist (*Tetrahymena termophila* and *Acanthamoebae polyphaga*)	*P. aeruginosa* in the presence of phage and protist decreases its potential for virulence.	Bacteria decreased the protease expression within the host, leading to a reduced virulence potential. The long-term adaptation to the host conditions of the environmental pathogens was associated with reduced defense against natural phages and protists.	Antagonism	+	[82]
P. aeruginosa – *S. aureus*	*P. aeruginosa* isolates trigger a wide range of biofilm-stimulatory activities when co-cultured with *S. aureus*.	The ability to stimulate *S. aureus* biofilm formation was strongly associated to the production of HQNO (2-heptyl-4-hydroxy quinolone N-oxide) and PQS (*Pseudomonas* Quinolone Signal) by *P. aeruginosa* isolates.	Unknown	-	[83]
	In a murine model of acute lung co-infection, early CF clinical isolate of *P. aeruginosa* could inhibit *S. aureus*. While late CF clinical isolate did not outcompete *S. aureus*	*P. aeruginosa* early CF clinical isolates presented high virulence in an acute infection.	Unknown	- / Unknown (For late CF clinical isolate)	[84]
	Wild type *P. aeruginosa* PAO1 facilitates *S. aureus* microcolony formation.	P. aeruginosa type IV pili mediated interactions between *P. aeruginosa* and *S. aureus* in co-culture biofilms and the level of P. aeruginosa piliation has an important impact on microcolony formation.	Synergism	Unknown	[85]

Table 1-3 *Continued*

	P. aeruginosa – *S. aureus small colony variants (SCVs)*	*P. aeruginosa* simultaneously suppresses *S. aureus* respiration and protects it from aminoglycoside antibiotics.	HQNO (2-hydroxy-2-heptyquinoline-N-oxide) produced by *P. aeruginosa* protected *S. aureus* from killing by aminoglycosides, by inhibiting electron transport that is required for aminoglycoside uptake. Furthermore, HQNO had the ability to inhibit *S. aureus* cytochrome activity.	Synergism	-	[86]
	P. aeruginosa – *S. maltophilia* *P. aeruginosa* – *B. cenocepacia*	The presence of diffusible signal molecules of DSF family from sputum of patients with CF, produced by *S. maltophilia* and *B. cenocepacia*, led to altered biofilm formation and increased resistance to antibiotics by *P. aeruginosa.*	The sensing of DSF by P. aeruginosa leads to alterations in expression of genes encoding a wide range of functions to include biofilm and increased tolerance to polymyxins.	Synergism	-	[87]
Microbial – Host interplay	*B. cenocepacia* - Host	The establishment of a *B. cenocepacia* infection delays the wound repair and also elicited a potent proinflammatory response.	An upregulation of metalloproteases (MMP) genes by 16HBE14o-cells and CFBE41o- cell lines, with increased matrix activity, was observed in response to *B. cenocepacia* infection.	Not determined	-	[88]
		B. cenocepacia infection induces proinflammatory response by the host	*B. cenocepacia* O antigen contributed to macrophage activation due the secretion of proinflammatory cytokine IL-1β.	Not determined	-	[89]

Table 1-3 *Continued*

P. aeruginosa – Host	Early *P. aeruginosa* CF isolates were lethal, while late isolates exhibit reduced or abolished acute virulence in the CF lungs.	The lesions caused by early *P. aeruginosa* strains were due the high leukocytes recruitment and bacterial load in the lungs of mice.	Not determined	- (early) / + (late)	[90]
	P. aeruginosa infection causes an excessive stimulated immune inflammatory response.	The expression of IL-8 was up-regulated by translocated Nucleoside diphosphate kinase (Ndk) into host cells. The massive influx of neutrophils into *P. aeruginosa*-infected sites was stimulated by an excessive inflammatory response caused by the production and release of IL-8.	Not determined	-	[91]
	The loss of bacterial motility enables non-motile P. *aeruginosa* to evade to association and ingestion by phagocytes both *in vitro* and *in vivo*.	The loss of bacterial motility resulted in reduced inflammatory activation and anti-bacterial IL-1β host response. These mechanisms enabled pathogens to evade the innate immune system.	Not determined	-	[92]
Rhinovirus – Host and influenza - Host	The presence o *rhinovirus* and *influenza* stimulate inflammatory responses by the host.	Rhinovirus had a pronounced effect on chemokine expression, being associated with greater than two-fold induction of five genes. Influenza induced a more potent response consisting of inflammation, being associated with overexpression of 20 genes, including those encoding the cytokines tumor necrosis factor and IL-12.	Not determined	-	[93]

[a] The terms antagonism refers, in this case, to the result of a negative relationship between the microbes; while the terms synergism is related with a positive or addictive relationship.

[b] Predictive ecological effect in host that results from interaction between the microbes. (The symbol (+) refers to a positive predictive effect in host; the symbol (-) refers to a negative predictive effect in host).

The majority of the studies found in the literature are carried out under *in vitro* conditions so that the effect of interaction in the host is only predictive. Although the predictive effects for most microbe-host interactions (most of them carried out *in vivo*) are considered negative, some mechanisms involve interactions that can have a predictable positive effect on the host and thereby be used as a therapeutic approach. Similarly, molecules that block key signal sensing or transduction steps in pathogens could represent lead compounds for new drugs.

In any polymicrobial infection, the combined effect of two or more microbes on the disease progression can be more dramatic that any of the individuals alone, and can display enhanced pathogen persistence in the infection site, increased disease severity, and increased antimicrobial resistance in a phenomenon known as polymicrobial synergism [40,94,95]. Synergistic interactions between different bacterial species allow to reap benefits that would be unattainable to them as individual cells, such as increased antibiotic tolerance, biofilm development, defense against competitors, adaptation to changing environments, increased tissue damage and declined pulmonary infection [81,94,96]. As examples of synergistic interactions occurring in the CF context, several authors [74,88] have demonstrated that a higher number of cells in the biofilm can be produced, which may have a great impact in antibiotic tolerance.

However, in some cases the antagonistic interactions between organisms within a community are unavoidable due to competition for finite resources, with effects on the growth or viability of competitors [97]. In CF, these interactions were found, for example, between *P. aeruginosa* and the fungal species *A. fumigatus* and *C. albicans*, with the small diffusive molecules secreted by *P. aeruginosa* inhibiting the biofilm formation of those fungal populations [70,77,78], and between *B. cenocepacia* and *C. albicans* with QS signal produced *by B. cenocepacia* inhibiting the filament formation by *C. albicans* [69]. Bacteria produce many types of diffusible molecules that can interact with other bacteria during disease. The various chemical cell-to-cell signaling mechanisms that are used by bacteria are collectively known as QS systems [98], a bacterial cell-to-cell communication process that involves the production, detection, and response to extracellular signaling molecules called autoinducers (AIs) [62]. Some signal molecules such as auto-inducer-2 (AI-2), *Pseudomonas* Quinolone Signal (PQS), 2-heptyl-4-hydroxy quinoline N-oxide (HQNO) and signal molecules of the diffusible signal factor (DSF) have been found to be produced during the infection and to influence other bacteria. For example, the ability to stimulate *S. aureus* biofilm formation was strongly associated to the production of HQNO and PQS by *P. aeruginosa* isolates [83].

While some studies have revealed the interplay among typical CF bacteria [86], only few have reported the role of emergent species for lung disease chronicity [1,99] or their interactions with eminent pathogens [80], which become more complex when microbes are encased in biofilms. Recently, Lopes et. al [80] showed that the CF atypical bacteria *I. limosus* and *D. pigrum* could interact synergistically with *P. aeruginosa,* developing dual-species consortia with increased tolerance to several antibiotics. This suggests that previously thought clinically insignificant species may influence the behaviour of individual species or even the whole microbial community. Based on these ecological interactions, it is strongly suggested a focus shift from an individual species to a polymicrobial community management and that modeling such multispecies interactions will help to predict the effects of new therapeutic interventions, dismissing much of the current antibiotic therapy empiricism and increasing its effectiveness.

In addition to microbial-microbial interactions, microbial-host interactions also exist in CF and the most significant features is the ability of the pathogens to deceive or modulate the multifaceted host response following colonization. The airway epithelium recognizes and responds to pathogens through the interaction between host pathogen recognition receptors and pathogen-associated membrane proteins [100]. The airway epithelium is one of several sources of chemokine interleukin-8 (IL-8) [101] that acts as the first line of host defense against pathogens. In CF patients, the ΔF508-CFTR mutation results in increased levels of IL-8 and neutrophils, responsible for the development of chronic obstructive and inflammatory lung diseases [102]. Furthermore, neutrophils resulting in DNA release and increased mucous viscosity, worsen the problem of bacterial attachment [100]. Recent studies have demonstrated that the conventional pathogens *P. aeruginosa* and *B. cenocepacia* can trigger an excessive inflammatory response in the host [88–91]. Deregulation of matrix metalloproteases (MMPs) in CF is another contributor to CF lung disease and to bacterial colonization [88]. So while many of the modifications and adaptations serve to promote inflammation and to benefit the colonization, other strategies are used to avoid and minimize the host response [92]. Additionally, colonization by multiple pathogens may trigger unknown repercussions in the host, although it is suspected that for most cases adverse effects can occur with greater impact in antibiotic therapy.

The majority of studies about interactions in the polymicrobial CF community focuses on the traditionally pathogen *P. aeruginosa*, due to its prevalence in CF lung, its ability to form biofilms that protect the organism to the host responses and to numerous antibiotics, and its potential do develop chronic infection. Therefore, more research is needed to provide a better

mechanistic insight into the complex interplay between potential pathogenic agents, commensal organisms, and the host response in the polymicrobial infections.

1.4 UNDERSTANDING POLYMICROBIAL INTERACTIONS TO BETTER TREAT CF

The resistance to antimicrobial agents is currently one of the major problems in the healthcare setting worldwide [103]. Antimicrobial resistance is potentiated in CF patients due to the extensive use of antimicrobial agents from a young age, both for the prophylaxis and treatment of respiratory infection [104].

When the chronic infection is established, pathogens such as *P. aeruginosa* growing as biofilms in the CF lung can exhibit increased resistance to antibiotics [28,105,106]. In fact, bacteria in the form of biofilms show increased resistance to several antibiotics when compared to planktonic or free living counterparts [107]. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of antibiotics to biofilm-growing bacteria may be up to 100–1000-fold higher compared with planktonic bacteria [108,109].

Apart from the conventional resistance mechanisms presented by bacteria (e.g. chromosomal beta-lactamase, up-regulated efflux pumps and mutations in antibiotic target molecules), biofilms also present an extracellular polymeric matrix. The reduced diffusion of antibiotics through the exopolysaccharide matrix (alginate, in the case of *P. aeruginosa* biofilm), retards the movement of antimicrobial agents [110–114] and contributes for the resistance and/or tolerance of biofilms to the antimicrobial agents [13,115].

When CF was first described in 1938 [116], the predicted survival age of patients was only 6 months [117]. For patients born in the 1990's median survival is now predicted to exceed 40 years, due to the introduction of multiple therapies that treat the symptoms of CF [118].

Antibiotic therapy for CF patients is directed at preventing, eradicating or controlling respiratory infections. The therapy generally starts with oral and inhaled therapies in an outpatient setting, and the use of intravenous route for patients with severe exacerbations [115,119].

The fluoroquinolones (e.g. ciprofloxacin), are the most commonly used oral agents to treat acute exacerbations caused by *P. aeruginosa* infection [115]. Other agents that have long been used by inhalation in CF patient for the treatment of *P. aeruginosa* lung infection are

tobramycin, aztreonam or colistin [115]. Current standard care guidelines for antibiotic recommend in CF patients for most commonly bacterial species are described in table 1-4.

Table 1-4 Antibiotic therapy used for bacterial species most commonly associated with CF airway disease [120].

Species	Infection phase	Antibiotic therapy
P. aeruginosa	1st isolated from patients	Oral ciprofloxacin or Inhaled colistin or tobramycin or aztreonam.
	Chronic infection	Two inhaled antibiotics among the following: colistin, tobramycin, aztreonam.
H. influenzae	-	Oral or intravenous amoxicillin + clavulanic acid depending on the severity.
S. aureus	1st isolated from patients	Oral flucloxacillin or Oral flucloxacillin + oral or intravenous rifampicin or fusidic acid.
	Chronic infection	Oral lucloxacillin.
MRSA: Methicillin-resistant Staphylococcus aureus	1st isolated from patients Chronic infection	Oral rifampicin + fusidic acid. Intravenous vancomycin or teicoplanin or linezolid.
B. cepacia	-	At least two intravenous antibiotics: Intravenous ticarcillin+ clavulanic acid or piperacillin+ tozabactam

Recently, new antibiotic combinations have been developed [119,121–123]. One example is the combination of fosfomycin/tobramycin (FTI), an inhaled antibiotic with broad-spectrum antibacterial activity for treatment of bacterial respiratory infections. FTI consists of fosfomycin (F) and tobramycin (T) in a 4:1 weight-to-weight ratio (w/w); this combination has promising activity against MRSA and *P. aeruginosa* with greater activity under aerobic and physiologically relevant anaerobic conditions, compared to F or T alone [119,121–123]. Lam et al. [124] reported that tobramycin inhalation powder (TIP) represents the first dry powder inhaled antibiotic available for use in CF. TIP was approved in the US in 2013 [125]. Inhaled antibiotics have been probably the safest and most effective therapy for *P. aeruginosa* chronic lung infection in CF patients [126]. The use of inhaled antibiotics allows delivered directly to the target area, with a lower dose than more conventional oral or intravenous delivery methods, with reduced systemic absorption and consequently reduced risk of toxic effects [127,128].

But with the increased antibiotic resistance in CF patients, the need for new strategies in the lifelong treatment of pulmonary infection has to be validated [129]. Because CF infection is no

longer viewed as being caused by a single pathogen, antibiotics used to target a small group of species recognized as key CF pathogens are generally ineffective when other atypical species are present [80,99] or fail in many cases [130]. This problem is compounded by the huge polymicrobial CF community and the bacterial interactions occurring in lung. Due the complex interactions that result between traditional and emergent CF pathogens – for instance, a study by Lopes et al. [80] demonstrated that the association among atypical and conventional CF bacterial could result in the impact of the antibiotic resistance - a new approach where antibiotic therapy is personalized to each patient, based on comprehensive microbiological analyses, could be development for treating lung infections [131].

Further, antivirulence drugs are a new type of therapeutic drug that target virulence factors, without killing or inhibiting bacterial growth. Many anti-virulence strategies are being explored, including inhibiting bacterial adhesion to the host cell (inhibiting biofilm formation), inhibiting cell-to-cell signaling (known as quorum quenchers by inhibiting QS systems), and interfering with gene regulation of virulence traits [62,132,133]. Other innovative therapeutic approach is the development of CFTR modulating drug as potential treatment for cystic fibrosis. Ivacaftor is the first licensed CFTR modulator drug and although only targets ~5% of CF patients, may indeed be one of many therapeutic agents that point to the emergence of a new era of personalized medicine [134]. These drugs will allow treatment of the basic defect in CF disease and open the door for therapy according to gene sequencing - true personalized medicine [118]. Moreover, every person with CF is unique and requires personalized diagnosis and therapy.

In addition to recognize the polymicrobial nature of CF community, understanding the molecular mechanisms and biological effects from the microbe-microbe and host-microbe interactions is also crucial to improve therapy regimens and also define new antimicrobial agents, new targets and strategies for CF disease control. We are facing a post-antibiotic era with limited capability to combat polymicrobial infections.

1.5 REFERENCES

1. Lopes SP, Azevedo NF, Pereira MO: **Microbiome in cystic fibrosis: Shaping polymicrobial interactions for advances in antibiotic therapy.** *Crit. Rev. Microbiol.* 2014, doi:10.3109/1040841X.2013.847898.

2. Davis PB: **Cystic fibrosis since 1938.** *Am. J. Respir. Crit. Care Med.* 2006, **173**:475–82.

3. Rowe SM, Miller S, Sorscher EJ: **Cystic fibrosis.** *N. Engl. J. Med.* 2005, **352**:1992–2001.

4. Farrell PM, Rosenstein BJ, White TB, Accurso FJ, Castellani C, Cutting GR, Durie PR, Legrys VA, Massie J, Parad RB, et al.: **Guidelines for diagnosis of cystic fibrosis in newborns through older adults: Cystic Fibrosis Foundation consensus report.** *J. Pediatr.* 2008, **153**:S4–S14.

5. Kirkby S, Novak K, McCoy K: **Aztreonam (for inhalation solution) for the treatment of chronic lung infections in patients with cystic fibrosis: an evidence-based review.** *Core Evid.* 2011, **6**:59–66.

6. Castellani C, Cuppens H, Macek M, Cassiman JJ, Kerem E, Durie P, Tullis E, Assael BM, Bombieri C, Brown A, et al.: **Consensus on the use and interpretation of cystic fibrosis mutation analysis in clinical practice.** *J. Cyst. Fibros.* 2008, **7**:179–96.

7. Nixon GM, Armstrong DS, Carzino R, Carlin JB, Olinsky A, Robertson CF, Grimwood K: **Clinical outcome after early Pseudomonas aeruginosa infection in cystic fibrosis.** *J. Pediatr.* 2001, **138**:699–704.

8. Boucher RC: **Evidence for airway surface dehydration as the initiating event in CF airway disease.** *J. Intern. Med.* 2007, **261**:5–16.

9. De Boeck K, Wilschanski M, Castellani C, Taylor C, Cuppens H, Dodge J, Sinaasappel M: **Cystic fibrosis: terminology and diagnostic algorithms.** *Thorax* 2006, **61**:627–35.

10. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, Birrer P, Bellon G, Berger J, Weiss T, et al.: **Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients.** *J. Clin. Invest.* 2002, **109**:317–25.

11. Hassett DJ, Cuppoletti J, Trapnell B, Lymar S V, Rowe JJ, Yoon SS, Hilliard GM, Parvatiyar K, Kamani MC, Wozniak DJ, et al.: **Anaerobic metabolism and quorum sensing by Pseudomonas aeruginosa biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets.** *Adv. Drug Deliv. Rev.* 2002, **54**:1425–43.

12. Boucher RC: **New concepts of the pathogenesis of cystic fibrosis lung disease.** *Eur. Respir. J.* 2004, **23**:146–58.

13. Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O: **Antibiotic resistance of bacterial biofilms.** *Int. J. Antimicrob. Agents* 2010, **35**:322–32.

14. Wei Q, Ma LZ: **Biofilm matrix and its regulation in Pseudomonas aeruginosa.** *Int. J. Mol. Sci.* 2013, **14**:20983–1005.

15. Hassett DJ, Korfhagen TR, Irvin RT, Schurr MJ, Sauer K, Lau GW, Sutton MD, Yu H, Hoiby N: **Pseudomonas aeruginosa biofilm infections in cystic fibrosis: insights into pathogenic processes and treatment strategies.** *Expert Opin. Ther. Targets* 2010, **14**:117–30.

16. Høiby N, Ciofu O, Bjarnsholt T: **Pseudomonas aeruginosa biofilms in cystic fibrosis.** *Future Microbiol.* 2010, **5**:1663–74.

17. Borriello G, Werner E, Roe F, Kim AM, Ehrlich GD, Stewart PS: **Oxygen limitation contributes to antibiotic tolerance of Pseudomonas aeruginosa in biofilms.** *Antimicrob. Agents Chemother.* 2004, **48**:2659–64.

18. Schobert M, Jahn D: **Anaerobic physiology of Pseudomonas aeruginosa in the cystic fibrosis lung**. *Int. J. Med. Microbiol.* 2010, **300**:549–556.

19. Sibley CD, Rabin H, Surette MG: **Cystic fibrosis: a polymicrobial infectious disease.** *Future Microbiol.* 2006, **1**:53–61.

20. Rogers GB, Carroll MP, Serisier DJ, Hockey PM, Jones G, Bruce KD: **characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling.** *J. Clin. Microbiol.* 2004, **42**:5176–83.

21. Bittar F, Rolain J-M: **Detection and accurate identification of new or emerging bacteria in cystic fibrosis patients.** *Clin. Microbiol. Infect.* 2010, **16**:809–20.

22. Guss AM, Roeselers G, Newton ILG, Young CR, Klepac-Ceraj V, Lory S, Cavanaugh CM: **Phylogenetic and metabolic diversity of bacteria associated with cystic fibrosis.** *ISME J.* 2011, **5**:20–9.

23. Price KE, Hampton TH, Gifford AH, Dolben EL, Hogan DA, Morrison HG, Sogin ML, O'Toole GA: **Unique microbial communities persist in individual cystic fibrosis patients throughout a clinical exacerbation.** *Microbiome* 2013, **1**:27.

24. Razvi S, Quittell L, Sewall A, Quinton H, Marshall B, Saiman L: **Respiratory microbiology of patients with cystic fibrosis in the United States, 1995 to 2005.** *Chest* 2009, **136**:1554–60.

25. Huang YJ, Lynch S V: **The emerging relationship between the airway microbiota and chronic respiratory disease: clinical implications.** *Expert Rev. Respir. Med.* 2011, **5**:809–21.

26. Treggiari MM, Rosenfeld M, Retsch-Bogart G, Gibson R, Ramsey B: **Approach to eradication of initial Pseudomonas aeruginosa infection in children with cystic fibrosis.** *Pediatr. Pulmonol.* 2007, **42**:751–6.

27. Lambert PA: **Mechanisms of antibiotic resistance in Pseudomonas aeruginosa.** *J. R. Soc. Med.* 2002, **95 Suppl 4**:22–6.

28. Høiby N, Ciofu O, Johansen HK, Song Z, Moser C, Jensen PØ, Molin S, Givskov M, Tolker-Nielsen T, Bjarnsholt T: **The clinical impact of bacterial biofilms.** *Int. J. Oral Sci.* 2011, **3**:55–65.

29. Lyczak JB, Cannon CL, Pier GB: **Lung infections associated with cystic fibrosis.** *Clin. Microbiol. Rev.* 2002, **15**:194–222.

30. Starner TD, Zhang N, Kim G, Apicella MA, McCray PB: **Haemophilus influenzae forms biofilms on airway epithelia: implications in cystic fibrosis.** *Am. J. Respir. Crit. Care Med.* 2006, **174**:213–20.

31. Hauser AR, Jain M, Bar-Meir M, McColley SA: **Clinical significance of microbial infection and adaptation in cystic fibrosis.** *Clin. Microbiol. Rev.* 2011, **24**:29–70.

32. Alexander EH, Hudson MC: **Factors influencing the internalization of Staphylococcus aureus and impacts on the course of infections in humans.** *Appl. Microbiol. Biotechnol.* 2001, **56**:361–6.

33. Kahl BC: **Impact of Staphylococcus aureus on the pathogenesis of chronic cystic fibrosis lung disease.** *Int. J. Med. Microbiol.* 2010, **300**:514–9.

34. Yang JH, Spilker T, LiPuma JJ: **Simultaneous coinfection by multiple strains during Burkholderia cepacia complex infection in cystic fibrosis.** *Diagn. Microbiol. Infect. Dis.* 2006, **54**:95–8.

35. Drevinek P, Mahenthiralingam E: **Burkholderia cenocepacia in cystic fibrosis: epidemiology and molecular mechanisms of virulence**. *Clin. Microbiol. Infect.* 2010, **16**:821–830.

36. Lynch JP: **Burkholderia cepacia complex: impact on the cystic fibrosis lung lesion.** *Semin. Respir. Crit. Care Med.* 2009, **30**:596–610.

37. Government US a: **Annual Data Report**. 2013, [no volume].

38. Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Høiby N, Molin S: **Adaptation of Pseudomonas aeruginosa to the cystic fibrosis airway: an evolutionary perspective.** *Nat. Rev. Microbiol.* 2012, **10**:841–51.

39. Sousa AM, Pereira MO: **Pseudomonas aeruginosa Diversification during Infection Development in Cystic Fibrosis Lungs-A Review.** *Pathog. (Basel, Switzerland)* 2014, **3**:680–703.

40. Peters BM, Jabra-Rizk MA, O'May G a., Costerton JW, Shirtliff ME, William Costerton J, Shirtliff ME: **Polymicrobial interactions: Impact on pathogenesis and human disease**. *Clin. Microbiol. Rev.* 2012, **25**:193–213.

41. Worlitzsch D, Rintelen C, Böhm K, Wollschläger B, Merkel N, Borneff-Lipp M, Döring G: **Antibiotic-resistant obligate anaerobes during exacerbations of cystic fibrosis patients.** *Clin. Microbiol. Infect.* 2009, **15**:454–60.

42. Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, Wolfgang MC, Boucher R, Gilpin DF, McDowell A, et al.: **Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis.** *Am. J. Respir. Crit. Care Med.* 2008, **177**:995–1001.

43. Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, Kamani MC, Allen HL, DeKievit TR, Gardner PR, Schwab U, et al.: **Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis.** *Dev. Cell* 2002, **3**:593–603.

44. Coenye T, Goris J, Spilker T, Vandamme P, LiPuma JJ: **Characterization of unusual bacteria isolated from respiratory secretions of cystic fibrosis patients and description of Inquilinus limosus gen. nov., sp. nov.** *J. Clin. Microbiol.* 2002, **40**:2062–9.

45. Bittar F, Richet H, Dubus J-C, Reynaud-Gaubert M, Stremler N, Sarles J, Raoult D, Rolain J-M: **Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients.** *PLoS One* 2008, **3**:e2908.

46. Harris JK, De Groote MA, Sagel SD, Zemanick ET, Kapsner R, Penvari C, Kaess H, Deterding RR, Accurso FJ, Pace NR: **Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis.** *Proc. Natl. Acad. Sci. U. S. A.* 2007, **104**:20529–33.

47. Menuet M, Bittar F, Stremler N, Dubus J-C, Sarles J, Raoult D, Rolain J-M: **First isolation of two colistin-resistant emerging pathogens, Brevundimonas diminuta and Ochrobactrum anthropi, in a woman with cystic fibrosis: a case report.** *J. Med. Case Rep.* 2008, **2**:373.

48. Khanbabaee G, Akbarizadeh M, Sayyari A, Ashayeri-Panah M, Abdollahgorji F, Sheibani K, Rezaeig N: **A survey on pulmonary pathogens and their antibiotic susceptibility among cystic fibrosis patients.** *Braz. J. Infect. Dis.* **16**:122–8.

49. Bakare N, Rickerts V, Bargon J, Just-Nübling G: **Prevalence of Aspergillus fumigatus and other fungal species in the sputum of adult patients with cystic fibrosis.** *Mycoses* 2003, **46**:19–23.

50. Bouchara J-P, Hsieh HY, Croquefer S, Barton R, Marchais V, Pihet M, Chang TC: **Development of an oligonucleotide array for direct detection of fungi in sputum samples from patients with cystic fibrosis.** *J. Clin. Microbiol.* 2009, **47**:142–52.

51. Chotirmall SH, O'Donoghue E, Bennett K, Gunaratnam C, O'Neill SJ, McElvaney NG: **Sputum Candida albicans presages FEV_1 decline and hospital-treated exacerbations in cystic fibrosis.** *Chest* 2010, **138**:1186–95.

52. Delhaes L, Monchy S, Fréalle E, Hubans C, Salleron J, Leroy S, Prevotat A, Wallet F, Wallaert B, Dei-Cas E, et al.: **The airway microbiota in cystic fibrosis: a complex fungal and bacterial community--implications for therapeutic management.** *PLoS One* 2012, **7**:e36313.

53. Barton RC, Borman AM, Johnson EM, Houbraken J, Hobson RP, Denton M, Conway SP, Brownlee KG, Peckham D, Lee TWR: **Isolation of the fungus Geosmithia argillacea in sputum of people with cystic fibrosis.** *J. Clin. Microbiol.* 2010, **48**:2615–7.

54. Giraud S, Pihet M, Razafimandimby B, Carrère J, Degand N, Mely L, Favennec L, Dannaoui E, Bouchara J-P, Calenda A: **Geosmithia argillacea: an emerging pathogen in patients with cystic fibrosis.** *J. Clin. Microbiol.* 2010, **48**:2381–6.

55. Cimon B, Carrere J, Chazalette JP, Vinatier JF, Chabasse D, Bouchara JP: **Chronic airway colonization by Penicillium emersonii in a patient with cystic fibrosis.** *Med. Mycol.* 1999, **37**:291–3.

56. Hickey PW, Sutton DA, Fothergill AW, Rinaldi MG, Wickes BL, Schmidt HJ, Walsh TJ: **Trichosporon mycotoxinivorans, a novel respiratory pathogen in patients with cystic fibrosis.** *J. Clin. Microbiol.* 2009, **47**:3091–7.

57. Smyth AR, Smyth RL, Tong CY, Hart CA, Heaf DP: **Effect of respiratory virus infections including rhinovirus on clinical status in cystic fibrosis.** *Arch. Dis. Child.* 1995, **73**:117–20.

58. Punch G, Syrmis MW, Rose BR, Harbour C, Bye PTP, Nissen MD, Elkins MR, Sloots TP: **Method for detection of respiratory viruses in the sputa of patients with cystic fibrosis.** *Eur. J. Clin. Microbiol. Infect. Dis.* 2005, **24**:54–7.

59. Olesen HV, Nielsen LP, Schiotz PO: **Viral and atypical bacterial infections in the outpatient pediatric cystic fibrosis clinic.** *Pediatr. Pulmonol.* 2006, **41**:1197–204.

60. Burmølle M, Thomsen TR, Fazli M, Dige I, Christensen L, Homøe P, Tvede M, Nyvad B, Tolker-Nielsen T, Givskov M, et al.: **Biofilms in chronic infections - a matter of opportunity - monospecies biofilms in multispecies infections.** *FEMS Immunol. Med. Microbiol.* 2010, **59**:324–36.

61. Burmølle M, Ren D, Bjarnsholt T, Sørensen SJ: **Interactions in multispecies biofilms: do they actually matter?** *Trends Microbiol.* 2014, **22**:84–91.

62. Rutherford ST, Bassler BL: **Bacterial quorum sensing: its role in virulence and possibilities for its control.** *Cold Spring Harb. Perspect. Med.* 2012, **2**.

63. Sibley CD, Duan K, Fischer C, Parkins MD, Storey DG, Rabin HR, Surette MG: **Discerning the complexity of community interactions using a Drosophila model of polymicrobial infections.** *PLoS Pathog.* 2008, **4**:e1000184.

64. King P: **Pathogenesis of bronchiectasis.** *Paediatr. Respir. Rev.* 2011, **12**:104–10.

65. Essilfie A-T, Simpson JL, Dunkley ML, Morgan LC, Oliver BG, Gibson PG, Foster PS, Hansbro PM: **Combined Haemophilus influenzae respiratory infection and allergic airways disease drives chronic infection and features of neutrophilic asthma.** *Thorax* 2012, **67**:588–99.

66. Rogers GB, Hoffman LR, Carroll MP, Bruce KD: **Interpreting infective microbiota: the importance of an ecological perspective.** *Trends Microbiol.* 2013, **21**:271–6.

67. Tunney MM, Klem ER, Fodor AA, Gilpin DF, Moriarty TF, Mcgrath SJ, Muhlebach MS, Boucher RC, Cardwell C, Doering G, et al.: **Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis.** *Thorax* 2011, **66**:579–84.

68. Stressmann FA, Rogers GB, van der Gast CJ, Marsh P, Vermeer LS, Carroll MP, Hoffman L, Daniels TW V, Patel N, Forbes B, et al.: **Long-term cultivation-independent microbial diversity analysis demonstrates that bacterial communities infecting the adult cystic fibrosis lung show stability and resilience.** *Thorax* 2012, **67**:867–73.

69. Boon C, Deng Y, Wang L-H, He Y, Xu J-L, Fan Y, Pan SQ, Zhang L-H: **A novel DSF-like signal from Burkholderia cenocepacia interferes with Candida albicans morphological transition.** *ISME J.* 2008, **2**:27–36.

70. Mowat E, Rajendran R, Williams C, McCulloch E, Jones B, Lang S, Ramage G: **Pseudomonas aeruginosa and their small diffusible extracellular molecules inhibit Aspergillus fumigatus biofilm formation**. *FEMS Microbiol. Lett.* 2010, **313**:96–102.

71. Amin R, Dupuis A, Aaron SD, Ratjen F: **The effect of chronic infection with Aspergillus fumigatus on lung function and hospitalization in patients with cystic fibrosis**. *Chest* 2010, **137**:171–176.

72. Moree WJ, Phelan V V., Wu C-H, Bandeira N, Cornett DS, Duggan BM, Dorrestein PC: **Interkingdom metabolic transformations captured by microbial imaging mass spectrometry**. *Proc. Natl. Acad. Sci.* 2012, **109**:13811–13816.

73. Chattoraj SS, Murthy R, Ganesan S, Goldberg JB, Zhao Y, Hershenson MB, Sajjan US: **Pseudomonas aeruginosa alginate promotes Burkholderia cenocepacia persistence in cystic fibrosis transmembrane conductance regulator knockout mice**. *Infect. Immun.* 2010, **78**:984–993.

74. Bragonzi A, Farulla I, Paroni M, Twomey KB, Pirone L, Lorè NI, Bianconi I, Dalmastri C, Ryan RP, Bevivino A: **Modelling Co-Infection of the Cystic Fibrosis Lung by Pseudomonas aeruginosa and Burkholderia cenocepacia Reveals Influences on Biofilm Formation and Host Response**. *PLoS One* 2012, **7**.

75. Kenney DMC, Brown KE, Allison DG: **Influence of Pseudomonas aeruginosa exoproducts on virulence factor production in Burkholderia cepacia : evidence of interspecies communication . Influence of Pseudomonas aeruginosa Exoproducts on Virulence Factor Production in Burkholderia cepacia : Evid**. 1995, **177**:6989–6992.

76. Rüger M, Ackermann M, Reichl U: **Species-specific viability analysis of Pseudomonas aeruginosa, Burkholderia cepacia and Staphylococcus aureus in mixed culture by flow cytometry.** *BMC Microbiol.* 2014, **14**:56.

77. Hogan D a., Vik Å°, Kolter R: **A Pseudomonas aeruginosa quorum-sensing molecule influences Candida albicans morphology**. *Mol. Microbiol.* 2004, **54**:1212–1223.

78. Cugini C, Calfee MW, Farrow JM, Morales DK, Pesci EC, Hogan D a.: **Farnesol, a common sesquiterpene, inhibits PQS production in Pseudomonas aeruginosa**. *Mol. Microbiol.* 2007, **65**:896–906.

79. Holcombe LJ, McAlester G, Munro C a., Enjalbert B, Brown AJP, Gow N a R, Ding C, Butler G, O'Gara F, Morrissey JP: **Pseudomonas aeruginosa secreted factors impair biofilm development in Candida albicans**. *Microbiology* 2010, **156**:1476–1485.

80. Lopes SP, Ceri H, Azevedo NF, Pereira MO: **Antibiotic resistance of mixed biofilms in cystic fibrosis: impact of emerging microorganisms on treatment of infection.** *Int. J. Antimicrob. Agents* 2012, **40**:260–3.

81. Duan K, Dammel C, Stein J, Rabin H, Surette MG: **Modulation of Pseudomonas aeruginosa gene expression by host microflora through interspecies communication**. *Mol. Microbiol.* 2003, **50**:1477–1491.

82. Friman VP, Ghoul M, Molin S, Johansen HK, Buckling A: **Pseudomonas aeruginosa Adaptation to Lungs of Cystic Fibrosis Patients Leads to Lowered Resistance to Phage and Protist Enemies**. *PLoS One* 2013, **8**:1–9.

83. Fugère A, Séguin DL, Mitchell G, Déziel E, Dekimpe V, Cantin AM, Frost E, Malouin F: **Interspecific small molecule interactions between clinical isolates of Pseudomonas aeruginosa and Staphylococcus aureus from adult cystic fibrosis patients**. *PLoS One* 2014, **9**.

84. Baldan R, Cigana C, Testa F, Bianconi I, De Simone M, Pellin D, Di Serio C, Bragonzi A, Cirillo DM: **Adaptation of Pseudomonas aeruginosa in cystic fibrosis airways influences virulence of Staphylococcus aureus in vitro and murine models of co-infection**. *PLoS One* 2014, **9**.

85. Yang L, Liu Y, Markussen T, Høiby N, Tolker-Nielsen T, Molin S: **Pattern differentiation in co-culture biofilms formed by Staphylococcus aureus and Pseudomonas aeruginosa**. *FEMS Immunol. Med. Microbiol.* 2011, **62**:339–347.

86. Hoffman LR, Déziel E, D'Argenio D a, Lépine F, Emerson J, McNamara S, Gibson RL, Ramsey BW, Miller SI: **Selection for Staphylococcus aureus small-colony variants due to growth in the presence of Pseudomonas aeruginosa.** *Proc. Natl. Acad. Sci. U. S. A.* 2006, **103**:19890–19895.

87. Twomey KB, O'Connell OJ, McCarthy Y, Dow JM, O'Toole G a, Plant BJ, Ryan RP: **Bacterial cis-2-unsaturated fatty acids found in the cystic fibrosis airway modulate virulence and persistence of Pseudomonas aeruginosa**. *ISME J.* 2012, **6**:939–950.

88. Pilkington R, Callaghan M, McClean S: **Activation of MMP-9 by Human Lung Epithelial Cells in Response to the Cystic Fibrosis-Associated Pathogen Burkholderia cenocepacia Reduced Wound Healing in**. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* 2011, **301**:L575–586.

89. Kotrange S, Kopp B, Akhter A, Abdelaziz D, Abu Khweek A, Caution K, Abdulrahman B, Wewers MD, McCoy K, Marsh C, et al.: **Burkholderia cenocepacia O polysaccharide chain contributes to caspase-1-dependent IL-1beta production in macrophages.** *J. Leukoc. Biol.* 2011, **89**:481–488.

90. Lorè NI, Cigana C, De Fino I, Riva C, Juhas M, Schwager S, Eberl L, Bragonzi A: **Cystic Fibrosis-Niche Adaptation of Pseudomonas aeruginosa Reduces Virulence in Multiple Infection Hosts**. *PLoS One* 2012, **7**:e35648.

91. Kim YJ, Paek SH, Jin S, Park BS, Ha UH: **A novel Pseudomonas aeruginosa-derived effector cooperates with flagella to mediate the upregulation of interleukin 8 in human epithelial cells**. *Microb. Pathog.* 2014, **66**:24–28.

92. Patankar YR, Lovewell RR, Poynter ME, Jyot J, Kazmierczak BI, Berwin B: **Flagellar motility is a key determinant of the magnitude of the inflammasome response to Pseudomonas aeruginosa**. *Infect. Immun.* 2013, **81**:2043–2052.

93. Ramirez I a., Caverly LL, Kalikin LM, Goldsmith AM, Lewis TC, Burke DT, LiPuma JJ, Sajjan US, Hershenson MB: **Differential responses to rhinovirus- and influenza-associated pulmonary exacerbations in patients with cystic fibrosis**. *Ann. Am. Thorac. Soc.* 2014, **11**:554–561.

94. Dalton T, Dowd SE, Wolcott RD, Sun Y, Watters C, Griswold JA, Rumbaugh KP: **An In Vivo Polymicrobial Biofilm Wound Infection Model to Study Interspecies Interactions**. *PLoS One* 2011, **6**:e27317.

95. Murray JL, Connell JL, Stacy A, Turner KH, Whiteley M: **Mechanisms of synergy in polymicrobial infections.** *J. Microbiol.* 2014, **52**:188–99.

96. Jacques I, Derelle J, Weber M, Vidailhet M: **Pulmonary evolution of cystic fibrosis patients colonized by Pseudomonas aeruginosa and/or Burkholderia cepacia.** *Eur. J. Pediatr.* 1998, **157**:427–31.

97. Harrison F: **Microbial ecology of the cystic fibrosis lung.** *Microbiology* 2007, **153**:917–23.

98. Fuqua WC, Winans SC, Greenberg EP: **Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators.** *J. Bacteriol.* 1994, **176**:269–75.

99. Lopes SP, Azevedo NF, Pereira MO: **Emergent bacteria in cystic fibrosis: in vitro biofilm formation and resilience under variable oxygen conditions.** *Biomed Res. Int.* 2014, **2014**:678301.

100. Callaghan M, McClean S: **Bacterial host interactions in cystic fibrosis.** *Curr. Opin. Microbiol.* 2012, **15**:71–7.

101. Standiford TJ, Kunkel SL, Basha MA, Chensue SW, Lynch JP, Toews GB, Westwick J, Strieter RM: **Interleukin-8 gene expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung.** *J. Clin. Invest.* 1990, **86**:1945–53.

102. Bodas M, Vij N: **The NF-kappaB signaling in cystic fibrosis lung disease: pathophysiology and therapeutic potential.** *Discov. Med.* 2010, **9**:346–56.

103. French GL: **The continuing crisis in antibiotic resistance.** *Int. J. Antimicrob. Agents* 2010, **36 Suppl 3**:S3–7.

104. Oliver A: **Mutators in cystic fibrosis chronic lung infection: Prevalence, mechanisms, and consequences for antimicrobial therapy.** *Int. J. Med. Microbiol.* 2010, **300**:563–72.

105. Bjarnsholt T, Jensen PØ, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, Pressler T, Givskov M, Høiby N: **Pseudomonas aeruginosa biofilms in the respiratory tract of cystic fibrosis patients.** *Pediatr. Pulmonol.* 2009, **44**:547–58.

106. Hassett DJ, Sutton MD, Schurr MJ, Herr AB, Caldwell CC, Matu JO: **Pseudomonas aeruginosa hypoxic or anaerobic biofilm infections within cystic fibrosis airways.** *Trends Microbiol.* 2009, **17**:130–8.

107. Sriramulu DD, Lünsdorf H, Lam JS, Römling U: **Microcolony formation: a novel biofilm model of Pseudomonas aeruginosa for the cystic fibrosis lung.** *J. Med. Microbiol.* 2005, **54**:667–76.

108. Moskowitz SM, Foster JM, Emerson J, Burns JL: **Clinically feasible biofilm susceptibility assay for isolates of Pseudomonas aeruginosa from patients with cystic fibrosis.** *J. Clin. Microbiol.* 2004, **42**:1915–22.

109. Anwar H, Dasgupta MK, Costerton JW: **Testing the susceptibility of bacteria in biofilms to antibacterial agents.** *Antimicrob. Agents Chemother.* 1990, **34**:2043–6.

110. Costerton JW: **Cystic fibrosis pathogenesis and the role of biofilms in persistent infection.** *Trends Microbiol.* 2001, **9**:50–2.

111. Anderson GG, O'Toole GA: **Innate and induced resistance mechanisms of bacterial biofilms.** *Curr. Top. Microbiol. Immunol.* 2008, **322**:85–105.

112. Bagge N, Schuster M, Hentzer M, Ciofu O, Givskov M, Greenberg EP, Høiby N: **Pseudomonas aeruginosa biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production.** *Antimicrob. Agents Chemother.* 2004, **48**:1175–87.

113. Vettoretti L, Plésiat P, Muller C, El Garch F, Phan G, Attrée I, Ducruix A, Llanes C: **Efflux unbalance in Pseudomonas aeruginosa isolates from cystic fibrosis patients.** *Antimicrob. Agents Chemother.* 2009, **53**:1987–97.

114. Chan C, Burrows LL, Deber CM: **Alginate as an auxiliary bacterial membrane: binding of membrane-active peptides by polysaccharides.** *J. Pept. Res.* 2005, **65**:343–51.

115. Sriramulu D: **Evolution and impact of bacterial drug resistance in the context of cystic fibrosis disease and nosocomial settings.** *Microbiol. insights* 2013, **6**:29–36.

116. ANDERSEN DH: **CYSTIC FIBROSIS OF THE PANCREAS AND ITS RELATION TO CELIAC DISEASE**. *Am. J. Dis. Child.* 1938, **56**:344.

117. Cohen-Cymberknoh M, Shoseyov D, Kerem E: **Managing cystic fibrosis: strategies that increase life expectancy and improve quality of life.** *Am. J. Respir. Crit. Care Med.* 2011, **183**:1463–71.

118. Wilschanski M: **Novel therapeutic approaches for cystic fibrosis.** *Discov. Med.* 2013, **15**:127–33.

119. McCaughey G, McKevitt M, Elborn JS, Tunney MM: **Antimicrobial activity of fosfomycin and tobramycin in combination against cystic fibrosis pathogens under aerobic and anaerobic conditions.** *J. Cyst. Fibros.* 2012, **11**:163–72.

120. Döring G, Flume P, Heijerman H, Elborn JS: **Treatment of lung infection in patients with cystic fibrosis: current and future strategies.** *J. Cyst. Fibros.* 2012, **11**:461–79.

121. MacLeod DL, Barker LM, Sutherland JL, Moss SC, Gurgel JL, Kenney TF, Burns JL, Baker WR: **Antibacterial activities of a fosfomycin/tobramycin combination: a novel inhaled antibiotic for bronchiectasis.** *J. Antimicrob. Chemother.* 2009, **64**:829–36.

122. Anderson GG, Kenney TF, Macleod DL, Henig NR, O'Toole GA: **Eradication of Pseudomonas aeruginosa biofilms on cultured airway cells by a fosfomycin/tobramycin antibiotic combination.** *Pathog. Dis.* 2013, **67**:39–45.

123. McCaughey G, Diamond P, Elborn JS, McKevitt M, Tunney MM: **Resistance development of cystic fibrosis respiratory pathogens when exposed to fosfomycin and tobramycin alone and in combination under aerobic and anaerobic conditions.** *PLoS One* 2013, **8**:e69763.

124. Lam J, Vaughan S, Parkins MD: **Tobramycin Inhalation Powder (TIP): An Efficient Treatment Strategy for the Management of Chronic Pseudomonas Aeruginosa Infection in Cystic Fibrosis.** *Clin. Med. Insights. Circ. Respir. Pulm. Med.* 2013, **7**:61–77.

125. Fiel SB: **Aerosolized antibiotics in cystic fibrosis: an update.** *Expert Rev. Respir. Med.* 2014, **8**:305–14.

126. Máiz L, Girón RM, Olveira C, Quintana E, Lamas A, Pastor D, Cantón R, Mensa J: **Inhaled antibiotics for the treatment of chronic bronchopulmonary Pseudomonas aeruginosa infection in cystic**

fibrosis: systematic review of randomised controlled trials. *Expert Opin. Pharmacother.* 2013, **14**:1135–49.

127. Traini D, Young PM: **Delivery of antibiotics to the respiratory tract: an update.** *Expert Opin. Drug Deliv.* 2009, **6**:897–905.

128. Hoppentocht M, Hagedoorn P, Frijlink HW, de Boer AH: **Developments and strategies for inhaled antibiotic drugs in tuberculosis therapy: a critical evaluation.** *Eur. J. Pharm. Biopharm.* 2014, **86**:23–30.

129. Van Westreenen M, Tiddens HAWM: **New antimicrobial strategies in cystic fibrosis.** *Paediatr. Drugs* 2010, **12**:343–52.

130. Leekha S, Terrell CL, Edson RS: **General principles of antimicrobial therapy.** *Mayo Clin. Proc.* 2011, **86**:156–67.

131. Short FL, Murdoch SL, Ryan RP: **Polybacterial human disease: the ills of social networking.** *Trends Microbiol.* 2014, **22**:508–16.

132. Rasko DA, Sperandio V: **Anti-virulence strategies to combat bacteria-mediated disease.** *Nat. Rev. Drug Discov.* 2010, **9**:117–28.

133. Allen RC, Popat R, Diggle SP, Brown SP: **Targeting virulence: can we make evolution-proof drugs?** *Nat. Rev. Microbiol.* 2014, **12**:300–8.

134. Ramsey BW, Davies J, McElvaney NG, Tullis E, Bell SC, Dřevínek P, Griese M, McKone EF, Wainwright CE, Konstan MW, et al.: **A CFTR potentiator in patients with cystic fibrosis and the G551D mutation.** *N. Engl. J. Med.* 2011, **365**:1663–72.

METHODOLOGY

This chapter describes the microorganisms, culture conditions and the methodologies used throughout this work, together with the rationale beyond their employment.

2.1 MICROORGANISMS AND CULTURE CONDITIONS

2.1.1 Microorganisms

Seven bacterial species were used throughout this work (Table 2-1).

Table 2-1 Bacterial species used throughout this work and their clinical significance associated with CF disease.

Species	Clinical Significance	References
Pseudomonas aeruginosa, clinical isolate UCBPP-PA14	Arguably the most important pathogen; Presents a prevalence of 80% at ages ≥ 18 years; Ability to develop biofilms that protect from host responses and numerous antibiotics;	[1–3]
Staphylococcus aureus ATCC 25923	Infects young patients, but can also be cultured from adolescents and adult patients; Ability to cause chronic infection;	[4–6]
Achromobacter baumannii, clinical isolate form sputum	Is being increasingly identified in the CF population. Increasing multidrug-resistance; It is not clear what role this bacterium plays in the airway inflammatory response.	[7–9]
Dolosigranulum pigrum, type strain CIP 104051 T	First described in 1993; Little is know about the implication for CF as well as their contributions for the disease progression.	[10,11]
Inquilinus limosus, strain M53, isolated from CF sputum	First isolated in 1999 from a lung transplant patient with CF; Ability to persist in the respiratory tract; Resistant to many antimicrobial drugs; Pathogenic potential remains unknown.	[12–15]
Klebsiella pneumonia, clinical isolate form sputum	Pulmonary infections are often characterized by a rapid progressive clinical course; Many clinical strains are multi-drug resistant.	[16,17]
Stenotrophomonas maltophilia, clinical isolated from CF sputum	Data from several CF centers worldwide indicate that the prevalence has increased in recent years; Ability to cause an increasing advance in disease and lung function decline; Intrinsic resistance to broad-spectrum antimicrobial agents.	[18–20]

2.1.2 Bacteria preservation

All strains were stored at – 70 ± 2ºC in 20 % (vol/vol) glycerol. Prior to each assay, bacteria were subcultured from frozen stock preparations onto Tryptic Soy Broth (TSB; Liofilchem®, Roseto degli Abruzzi, Italy) supplemented with 1.2 % (wt/vol) agar (Liofilchem®) plates and incubated aerobically at 37ºC for 24-48 h.

2.1.3 Culture media and buffers

Pure liquid cultures of all bacteria were grown in TSB medium. Tryptic Soy Agar (TSB supplemented with 1.2 % wt/vol agar) was used as non-selective culture medium, whereas *Pseudomonas* Isolation Agar (PIA; Sigma, St. Louis, MO, USA), Mannitol Salt Agar (MSA; Liofilchem®, Roseto degli Abruzzi, Italy) and *Burkholderia cepacia* Selective Agar (BCSA; Oxoid Limited, Hampshire, UK) supplemented with 30 mg/L polymyxin B (Biochrom, Berlin, Germany) and 100 mg/L ticarcillin (Sigma) were used as selective growth media for specific isolation of *P. aeruginosa, S. aureus* and *I. limosus*, respectively. Unless otherwise stated, all rinse steps were performed using ultrapure (UP) sterile water.

2.1.4 Preparation of bacterial suspensions

To prepare the bacterial suspension, several colonies from a fresh subculture of each organism were suspended in TSB (autoclaved at 121 ºC for 20 min) and grown overnight at 37 ºC and 120 rpm. Subsequently, bacteria were washed twice with UP sterile water. Standardized cell suspensions were prepared in TSB at a cell density of 1×10^7 CFU/mL, unless otherwise stated. The standard concentration was obtained from the calibration curve, previously determined, that relates CFU/mL versus OD_{640nm} for each bacterium. For dual-species cultures, the suspended inoculum of each species was combined in a 1:1 ratio.

2.1.5 Antibiotic agents

Ciprofloxacin (Sigma), a broad-spectrum antimicrobial from the fluroquinolone drug class, was used throughout this work. This antibiotic is frequently used to control pulmonary infections caused by *P. aeruginosa* in CF patients [21,22].

Stock solutions were prepared at 5120 mg/L and stored according to the manufacturer instructions. For susceptibility testing, antibiotic was serially two-fold diluted in cation adjusted Mueller-Hinton broth (CAMHB; Sigma). The antibiotic concentrations ranged from 0.125 to 512 mg/L.

2.2 PLANKTONIC ASSAYS

2.2.1 Single- and mixed-species planktonic growth

2.2.1.1 Planktonic inoculum

Cell suspension cultures prepared in broth medium (TSB) at ~1×10^7 CFU/mL were used as inoculum for planktonic growth.

2.2.1.2 Planktonic growth curves

For bacterial growth, the wells of 96-well round-bottom polystyrene microtiter plates (Orange) were filled with 200 μL (per well) of cell suspensions (1×10^7 CFU/mL) and plates were incubated under aerobic and anaerobic conditions at 37 ºC on a horizontal shaker (120 rpm). Bacterial growth was monitored each 2 h, until reach 24 h, by optical density measurement at 640 nm ($OD_{640\ nm}$). For dual-species cultures, the suspended inoculum of each species was combined in a 1:1 ratio.

For aerobic assays, plates were placed in an incubator without oxygen restriction (n-biotek, Model NB-205Q, Korea). The anaerobic atmosphere was created by sealing the plates containing the cell suspensions in plastic boxes with AnaeroGen sachets (Oxoid), which reduces oxygen concentration to below 1 % (vol/vol).

2.2.1.3 Planktonic susceptibility (MIC and MBC determination)

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by broth microdilution according to the EUCAST guidelines [23].

Serial two-fold antibiotic dilutions were prepared on CAMHB, with antibiotic concentration ranged from 0.125 to 512 mg/L. Briefly, 96-well round-bottom polystyrene microtiter plates (Orange) with a total well capacity of 300 μL were used. In each well, 100 μL of increasing concentrations of ciprofloxacin were added to 100 μL of each bacterial suspension (1×10^6 CFU/mL) to obtain the specific final concentration of 5×10^5 CFU/mL. The plates were incubated for 24 h aerobically at 37 ºC under static conditions.

The MIC of the planktonic cell was obtained by reading the optical density at 640 nm (OD_{640nm}), where clear wells ($OD_{640\ nm} < 0.1$) were evidence of bacterial growth inhibition. MBC values were determined by transferring 10 μL of culture from each well into TSA plates. The MBC corresponded to the lowest concentration of an antibiotic that had resulted on 99.9 % killing of planktonic cells, in other words, no colony growth after 24-48 h at 37 ºC.

2.2.1.4 Determination of planktonic time-kill curves

Microtiter plates (96-well) containing 100 μL of TSB supplemented with ciprofloxacin in a range of concentrations selected for each bacteria (¼ MIC, ½ MIC, MIC, 2 MIC and 4 MIC), were incubated under aerobic and anaerobic conditions at 37 ºC (120 rpm) with 100 μL of each bacterial suspension (1×10^6 CFU/mL) to obtain the specific final concentration of 5×10^5 CFU/mL. Each plate include positive control wells comprising 100 μL of TSB and 100 μL of each bacterial species. Negative controls were also performed comprising 100 μL of ciprofloxacin solution at each concentration tested, and 100 μL of TSB.

After 2 h of growth, the content of each well (planktonic suspension) was transferred to a new plate and the optical density at 640 nm ($OD_{640\ nm}$) was recorded using an automated plate reader (Tecan Sunrise®). The bacterial growth was measured every 2 h until reach 24 h. For dual-species cultures, the suspended inoculum of each species was combined in a 1:1 ratio, and the range of concentrations was the selected for *P. aeruginosa.*

2.3 BIOFILM ASSAYS

2.3.1 Single- and mixed-species biofilm formation

2.3.1.1 Biofilm inoculum

Cell suspension cultures prepared in broth medium (TSB) at 1×10^7 CFU/mL were used as inoculum for biofilm formation.

2.3.1.2 Biofilm development

The methodology used to grow the bacterial was based on the microtiter plate test developed by Stepanovic and colleagues [24]. For this, the wells of microtiter plates were filled with 200 μL (per well) of cell suspensions (1×10^7 CFU/mL) and plates were incubated for 24 h under aerobic and anaerobic conditions at 37ºC on a horizontal shaker (120 rpm). For dual-species cultures, the suspended inoculum of each species was combined in a 1:1 ratio.

After the desired period of time, the planktonic cell suspensions were aspirated and biofilms were washed twice with UP sterile water (200 μL per well), in order to remove weakly and non-adherent bacteria.

2.3.1.3 Biofilm growth curves

The single- and dual-species biofilms that were grown aerobically and anaerobically on microtiter plate wells were evaluated for specific time points. Hence, at each 2 h until reach 24 h, planktonic cell fractions were aspirated from microtiter plate wells. Then, biofilms formed were rinsed once with UP sterile water (200 μL per well), being then detached by ultrasonic bath (Sonicor model SC-52, UK), operating at 50 kHz, during 10 min. The disrupted biofilms were serially diluted in UP sterile water and plated onto TSA plates, which were incubated aerobically at 37 ºC for 24-48 h for cultivable cell counting.

2.3.1.4 Determination of biofilm time-kill curves

Microtiter plates 96-well containing 100 μL of TSB supplemented with ciprofloxacin in a range of concentrations selected for each bacteria (¼ MIC, ½ MIC, MIC, 2 MIC and 4 MIC), were incubated under aerobic and anaerobic conditions at 37 ºC (120 rpm) with 100 μL of each bacterial suspension (1×10^6 CFU/mL) to obtain the specific final concentration of 5×10^5

CFU/mL.

Biofilm time-kill curves were determined by strictly following the planktonic time-kill curve procedure, but adapting the protocol to the biofilm mode of growth. Briefly, after 2 h of growth the content of each well (planktonic suspension) was aspirated and the attached biofilms were detached by ultrasonic bath (Sonicor model SC-52, UK), operating at 50 kHz, during 10 min. The following procedure was identical to the one described above.

After 2 h of growth, the content of each well (planktonic suspension) was aspirated. Then, biofilms formed were rinsed once with UP sterile water (200 μL per well), being then detached by ultrasonic bath (Sonicor model SC-52, UK), operating at 50 kHz, during 10 min. The disrupted biofilms were serially diluted in UP sterile water and plated onto TSA plates, which were incubated aerobically at 37 ºC for 24-48 h for cultivable cell counting.

2.3.1.5 Relative distribution of dual-species biofilms

Total cultivable cell numbers were enumerated by plating serially diluted biofilm-detached samples onto TSA plates. For *P. aeruginosa, S. aureus* and *I. limosus* enumeration, spots were plated onto PIA, MSA and onto supplemented BCSA, respectively (see above). Plates were then incubated for 24-48 h before cell counts.

Relative distribution in dual-species biofilms was reported as the percentage of each single-species (A) in the mixed consortia (single-species A + single-species B) calculated before and after antibiotic exposure, as follows:

$$\mathrm{Re}\,lative\ distribution\ (\%) = \frac{\log_{10} CFU/cm^2(\text{single - species A})}{\log_{10} CFU/cm^2_{Total}(\text{single - species A + single - specie B})} \times 100$$

2.3.2 Methodologies for biofilms analysis

2.3.2.1 Biofilm mass

The mass of biofilms was determined by using the crystal violet (CV) staining method initially described by Stepanovic *et al.* [24]. CV is a basic dye, which binds to negatively charged molecules from the surface and to polysaccharides from the extracelular matrix [25].

Briefly, after biofilm formation, the planktonic fraction in the wells was aspirated and the attached biofilms were left to air dry (10 min). Afterwards, 200 μL of pure methanol (Valente e Ribeiro Lda., Belas, Portugal) were transferred to each well in order to fix the remaining attached bacteria (15 min). The plates were emptied and left to air dry again. Biofilms were then stained with 200 μL of 1 % (vol/vol) CV (Pro-Labs Diagnostics Inc.) for 5 min and were washed twice with UP sterile water. After the staining step, the plates were washed with running tap water and air dried for approximately 20 min and, at last, 200 μL of 33 % (v/v) of acetic acid (Fisher Scientific, UK) were added to each well in order to solubilize the CV bound to the adherent bacteria. The quantitative analysis of biofilm production was performed through the measurement of optical density at 570 nm ($OD_{570\ nm}$) using an automated plate reader (Tecan Sunrise®). Control experiments to avoid false results were also performed in order to determine whether the tested media and the plate material could absorb CV and interfere with biomass quantification. When the optical density was higher 1.0 the sample was diluted with 33 % (v/v) of acetic acid.

2.3.2.2 Cultivable biofilm-encased cells

The number of cultivable cells within biofilms formed on microtiter plate wells was determined as previously described for biofilm growth curves. The disrupted biofilms were then serially diluted UP sterile water and plated onto non-selective agar (TSA) plates. Selective agar media for *P. aeruginosa* (PIA), *S. aureus* (MSA) and *I. limosus* (supplemented BCSA) CFU determination was also used. Agar plates were incubated aerobically at 37 ºC for 24-48 h for cultivable cell counting. Values of cultivable sessile cells were expressed as $\log_{10}$ CFU per area (cm^2).

2.3.2.3 PNA FISH

The technique of fluorescence *in* situ hybridization (FISH) is based on the same principle as any DNA hybridization method that uses the ability of single-stranded DNA to anneal to complementary DNA [26]. Traditionally, FISH uses labelled DNA probes for the *in situ* identification of microorganisms by hybridization to ribosomal RNA [27]. For certain applications, particularly in clinical diagnostics and microbial ecology, FISH limitations (cell permeability, hybridization affinity and target site accessibility [28,29]) could be overcome by the use of peptide nucleic acid (PNA) probes [30–33]. PNA is a synthetic DNA analogue that presents a quicker and stronger binding to DNA/RNA attributed to the lack of charge repulsion between the neutral PNA strand and the complementary RNA strand [34]. The hydrophobic nature of the PNA molecule allows an easy penetration in the cell, and theoretically a better diffusion thought the biofilm matrix [31].

Multiplex PNA FISH allows detecting and discriminating between bacterial populations within biofilms, by using several fluorescent labelled probes in simultaneous [35]. In CF, FISH has been directly applied to sputum samples for the rapid identification of bacteria within sputum [36–38], particularly at looking for the spatial distribution of bacteria [39]. In combination with confocal laser scanning microscopy (CLSM), FISH has allowed defining the three-dimensional distribution of microbial populations in mixed-species biofilms [40,41].

2.3.2.3.1 Biofilm formation on polystyrene (PS) coupons

In order to further apply PNA FISH method to biofilms, these were developed in PS coupons (1 × 1 cm) placed in the bottom of the wells of 24-well plates (Orange). Before testing, PS surfaces were submerged 3 min in a commercial detergent (Sonasol, Henkel Ibérica Portugal, Bobadela, Portugal), washed three times in UP sterile water and allowed to air dry for 3 h. To promote biofilm formation on PS surfaces, biofilm inoculum was prepared as described in sub-chapter 2.3.1.1 and dispensed in the wells containing the coupons. After biofilm formation, coupons were washed twice with 1 mL of UP sterile water and air dried (~60 ºC) for 15 min. Biofilm was fixed with methanol (100 % vol/vol) for 20 min. The initial step of fixing the biofilm with methanol is crucial to avoid the detachment of bacterial cells during the hybridization procedure. Fixed biofilms were stored at 4 ºC for a maximum of 48 h before the multiplex PNA FISH procedure.

2.3.2.3.2 PNA FISH applied to biofilms

Multiplex assay was assessed on multi-species biofilms encompassing P. *aeruginosa* with *S. aureus* or *I. limosus* or *S. maltophilia* (Fig 2-1).

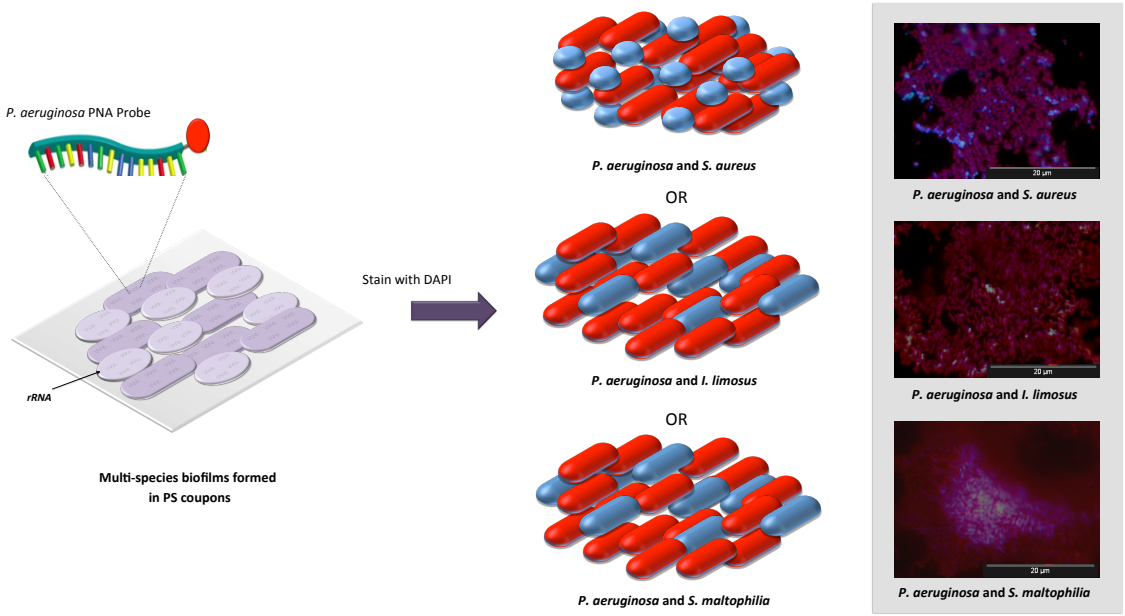

Fig 2-1 Basic steps in multiplex PNA FISH for identification and discrimination of *P. aeruginosa* with *S. aureus* or *I. limosus* or *S. maltophilia* within polymicrobial communities.

P. aeruginosa was identified using the specifically probe Paer565 (5'- GCTGAACCACCTACG - 3') coupled to the fluorochrome Alexa 594. This probe was previously designed and optimized by Lopes et al. [42]. Other than P. aeruginosa organisms were identified by counterstaining the samples with 4`, 6-diamidino-2-phenylindole (DAPI; Sigma) at the end of the hybridization procedure. After biofilms formation on PET coupons, 30 μl of each solution of 4 % (wt/vol) paraformaldehyde followed by 50 % (vol/vol) ethanol was dispensed in the PET coupons for 10 min each and allowed to air dry. This step enables the fixation of the cells and increase the permeabilization of the cell membrane to the subsequent hybridization allowing the labeled oligonucleotide probes to diffuse to their intracellular rRNA target molecules [28]. Afterwards, 20 μl of hybridization solution containing the probe at 200 nM were dispensed on the coupons, which were finally covered with coverslips and incubated in the dark for 1 h at 65 ºC. After hybridization, coupons were carefully removed and were submersed for 30 min in 24-well plates containing 1 mL per well of a prewarmed (65 ºC) washing solution composed of 5 mM Tris Base, 15 mM NaCl and 1 % (vol/vol) Triton X-100 (all from Sigma). Finally, the coupons

were removed from the plates and allowed to air dry in the dark before counterstaining with DAPI. For this, each coupon was covered with 20 μL of DAPI (40 μg/mL) for 5 min at room temperature in the dark before immediate observation in the fluorescence microscope. Negative controls were performed for each experiment, with no probes added to the hybridization solution. For microscopic visualization, a fluorescence microscope (Olympus BX51, Perafita, Portugal) equipped with the filters sensitive to DAPI (BP 365-370, FT 400, LP 421) and to the signaling molecule of the PNA probes (BP 530-550, FT 570, LP 591, for Alexa 594) was used.

2.4 STATISCTICAL ANALYSIS

Data were analyzed using the Prism software package (GraphPad Software version 6.0 for Macintosh). Otherwise stated, data were expressed as means ± standard deviation (SD). Data were compared by one-way analysis of variance (ANOVA) and subsequent comparisons were performed using Turkey multiple-comparisons test. Differences were considered statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

2.5 REFERENCES

1. Treggiari MM, Rosenfeld M, Retsch-Bogart G, Gibson R, Ramsey B: **Approach to eradication of initial Pseudomonas aeruginosa infection in children with cystic fibrosis.** *Pediatr. Pulmonol.* 2007, **42**:751–6.

2. Lambert PA: **Mechanisms of antibiotic resistance in Pseudomonas aeruginosa.** *J. R. Soc. Med.* 2002, **95 Suppl 4**:22–6.

3. Høiby N, Ciofu O, Johansen HK, Song Z, Moser C, Jensen PØ, Molin S, Givskov M, Tolker-Nielsen T, Bjarnsholt T: **The clinical impact of bacterial biofilms.** *Int. J. Oral Sci.* 2011, **3**:55–65.

4. Hauser AR, Jain M, Bar-Meir M, McColley SA: **Clinical significance of microbial infection and adaptation in cystic fibrosis.** *Clin. Microbiol. Rev.* 2011, **24**:29–70.

5. Alexander EH, Hudson MC: **Factors influencing the internalization of Staphylococcus aureus and impacts on the course of infections in humans.** *Appl. Microbiol. Biotechnol.* 2001, **56**:361–6.

6. Kahl BC: **Impact of Staphylococcus aureus on the pathogenesis of chronic cystic fibrosis lung disease.** *Int. J. Med. Microbiol.* 2010, **300**:514–9.

7. Peleg AY, Seifert H, Paterson DL: **Acinetobacter baumannii: emergence of a successful pathogen.** *Clin. Microbiol. Rev.* 2008, **21**:538–82.

8. Forster DH, Daschner FD: **Acinetobacter species as nosocomial pathogens.** *Eur. J. Clin. Microbiol. Infect. Dis.* 1998, **17**:73–7.

9. Falagas ME, Kasiakou SK, Michalopoulos A: **Treatment of multidrug-resistant Pseudomonas aeruginosa and Acinetobacter baumannii pneumonia.** *J. Cyst. Fibros.* 2005, **4**:149–50.

10. Aguirre M, Morrison D, Cookson BD, Gay FW, Collins MD: **Phenotypic and phylogenetic characterization of some Gemella-like organisms from human infections: description of Dolosigranulum pigrum gen. nov., sp. nov.** *J. Appl. Bacteriol.* 1993, **75**:608–12.

11. Bittar F, Richet H, Dubus J-C, Reynaud-Gaubert M, Stremler N, Sarles J, Raoult D, Rolain J-M: **Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients.** *PLoS One* 2008, **3**:e2908.

12. Pitulle C, Citron DM, Bochner B, Barbers R, Appleman MD: **Novel bacterium isolated from a lung transplant patient with cystic fibrosis.** *J. Clin. Microbiol.* 1999, **37**:3851–5.

13. Salvador-García C, Yagüe-Guirao G, Pastor-Vivero MD, Sáez-Nieto JA: **Chronic colonization of Inquilinus limosus in a patient with cystic fibrosis: first report in Spain.** *Enferm. Infecc. Microbiol. Clin.* **31**:414–5.

14. Bittar F, Leydier A, Bosdure E, Toro A, Reynaud-Gaubert M, Boniface S, Stremler N, Dubus J-C, Sarles J, Raoult D, et al.: **Inquilinus limosus and cystic fibrosis.** *Emerg. Infect. Dis.* 2008, **14**:993–5.

15. Chiron R, Marchandin H, Counil F, Jumas-Bilak E, Freydière A-M, Bellon G, Husson M-O, Turck D, Brémont F, Chabanon G, et al.: **Clinical and microbiological features of Inquilinus sp. isolates from five patients with cystic fibrosis.** *J. Clin. Microbiol.* 2005, **43**:3938–43.

16. Rice LB: **The clinical consequences of antimicrobial resistance.** *Curr. Opin. Microbiol.* 2009, **12**:476–81.

17. Cortés G, Alvarez D, Saus C, Albertí S: **Role of lung epithelial cells in defense against Klebsiella pneumoniae pneumonia.** *Infect. Immun.* 2002, **70**:1075–80.

18. De Vrankrijker AMM, Wolfs TFW, van der Ent CK: **Challenging and emerging pathogens in cystic fibrosis.** *Paediatr. Respir. Rev.* 2010, **11**:246–54.

19. Stanojevic S, Ratjen F, Stephens D, Lu A, Yau Y, Tullis E, Waters V: **Factors influencing the acquisition of Stenotrophomonas maltophilia infection in cystic fibrosis patients.** *J. Cyst. Fibros.* 2013, **12**:575–83.

20. Goss CH, Mayer-Hamblett N, Aitken ML, Rubenfeld GD, Ramsey BW: **Association between Stenotrophomonas maltophilia and lung function in cystic fibrosis.** *Thorax* 2004, **59**:955–9.

21. Döring G, Flume P, Heijerman H, Elborn JS: **Treatment of lung infection in patients with cystic fibrosis: current and future strategies.** *J. Cyst. Fibros.* 2012, **11**:461–79.

22. Flume PA, O'Sullivan BP, Robinson KA, Goss CH, Mogayzel PJ, Willey-Courand DB, Bujan J, Finder J, Lester M, Quittell L, et al.: **Cystic fibrosis pulmonary guidelines: chronic medications for maintenance of lung health.** *Am. J. Respir. Crit. Care Med.* 2007, **176**:957–69.

23. EUCAST: **Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution**. *Clin. Microbiol. Infect.* 2003, **9**:ix–xv.

24. Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M: **A modified microtiter-plate test for quantification of staphylococcal biofilm formation.** *J. Microbiol. Methods* 2000, **40**:175–9.

25. Li X, Yan Z, Xu J: **Quantitative variation of biofilms among strains in natural populations of Candida albicans.** *Microbiology* 2003, **149**:353–62.

26. Anderson R: **Multiplex fluorescence in situ hybridization (M-FISH).** *Methods Mol. Biol.* 2010, **659**:83–97.

27. Almeida C, Azevedo NF, Santos S, Keevil CW, Vieira MJ: **Discriminating multi-species populations in biofilms with peptide nucleic acid fluorescence in situ hybridization (PNA FISH)**. *PLoS One* 2011, **6**.

28. Amann R, Fuchs BM: **Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques.** *Nat. Rev. Microbiol.* 2008, **6**:339–48.

29. Daims H, Wagner M: **Quantification of uncultured microorganisms by fluorescence microscopy and digital image analysis.** *Appl. Microbiol. Biotechnol.* 2007, **75**:237–48.

30. Stender H: **PNA FISH: an intelligent stain for rapid diagnosis of infectious diseases.** *Expert Rev. Mol. Diagn.* 2003, **3**:649–55.

31. Stender H, Fiandaca M, Hyldig-Nielsen JJ, Coull J: **PNA for rapid microbiology.** *J. Microbiol. Methods* 2002, **48**:1–17.

32. Pavlekovic M, Schmid MC, Schmider-Poignee N, Spring S, Pilhofer M, Gaul T, Fiandaca M, Löffler FE, Jetten M, Schleifer K-H, et al.: **Optimization of three FISH procedures for in situ detection of anaerobic ammonium oxidizing bacteria in biological wastewater treatment.** *J. Microbiol. Methods* 2009, **78**:119–26.

33. Almeida C, Azevedo NF, Iversen C, Fanning S, Keevil CW, Vieira MJ: **Development and application of a novel peptide nucleic acid probe for the specific detection of Cronobacter genomospecies (Enterobacter sakazakii) in powdered infant formula.** *Appl. Environ. Microbiol.* 2009, **75**:2925–30.

34. Cerqueira L, Azevedo NF, Almeida C, Jardim T, Keevil CW, Vieira MJ: **DNA mimics for the rapid identification of microorganisms by fluorescence in situ hybridization (FISH).** *Int. J. Mol. Sci.* 2008, **9**:1944–60.

35. Mothershed EA, Whitney AM: **Nucleic acid-based methods for the detection of bacterial pathogens: present and future considerations for the clinical laboratory.** *Clin. Chim. Acta.* 2006, **363**:206–20.

36. Wellinghausen N, Wirths B, Poppert S: **Fluorescence in situ hybridization for rapid identification of Achromobacter xylosoxidans and Alcaligenes faecalis recovered from cystic fibrosis patients.** *J. Clin. Microbiol.* 2006, **44**:3415–7.

37. Brown AR, Govan JRW: **Assessment of fluorescent in situ hybridization and PCR-based methods for rapid identification of Burkholderia cepacia complex organisms directly from sputum samples.** *J. Clin. Microbiol.* 2007, **45**:1920–6.

38. Rudkjøbing VB, Thomsen TR, Alhede M, Kragh KN, Nielsen PH, Johansen UR, Givskov M, Høiby N, Bjarnsholt T: **The microorganisms in chronically infected end-stage and non-end-stage cystic fibrosis patients.** *FEMS Immunol. Med. Microbiol.* 2012, **65**:236–44.

39. Yang L, Haagensen JAJ, Jelsbak L, Johansen HK, Sternberg C, Høiby N, Molin S: **In situ growth rates and biofilm development of Pseudomonas aeruginosa populations in chronic lung infections.** *J. Bacteriol.* 2008, **190**:2767–76.

40. Lopez C, Pons MN, Morgenroth E: **Evaluation of microscopic techniques (epifluorescence microscopy, CLSM, TPE-LSM) as a basis for the quantitative image analysis of activated sludge.** *Water Res.* **39**:456–68.

41. Sunde PT, Olsen I, Göbel UB, Theegarten D, Winter S, Debelian GJ, Tronstad L, Moter A: **Fluorescence in situ hybridization (FISH) for direct visualization of bacteria in periapical lesions of asymptomatic root-filled teeth.** *Microbiology* 2003, **149**:1095–102.

42. Lopes SP: **Insights into the ecology of polymicrobial biofilms involved in cystic fibrosis**. 2013, [no volume].

COMPARISON OF PLANKTONIC AND BIOFILM GROWTH BY CF BACTERIA UNDER VARIABLE OXYGEN CONDITIONS

Cystic Fibrosis (CF) is a genetic disorder associated with multispecies infections where interactions between classical and newly identified bacteria might be crucial to understanding the persistent colonization in CF lungs. Nonetheless, little is known about the contributions of these microbes in the development of chronic biofilms, particularly under variable oxygen environments that are known to occur *in vivo* in the mucus of CF patients.

As such, five CF-emergent bacterial species, *Achromobacter baumannii*, Dolosigranulum *pigrum*, *Inquilinus limosus*, *Klebsiella pneumoniae* and *Stenotrophomonas maltophilia,* and the conventional pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa* were firstly studied in terms of planktonic growth and biofilm development under *in vitro* atmospheres with different oxygen concentrations.

All single-species were able to growth planktonically and develop biofilms under aerobic and anaerobic environments, demonstrating high number of cultivable cells even with a significant decreasing in the amount of biomass for low-oxygen atmospheres. The results obtained for dual-species populations, under the different oxygen-available environments, showed that *I. limosus, S. aureus* and *S. maltophilia* in co-culture with *P. aeruginosa* tend to produced significantly less biomass than *P. aeruginosa* mono-specie, although the number of cultivable cells was not affected.

Moreover, this study highlighted that emergent species are able to establish polymicrobial consortia with common pathogens, modulating different social activities into such communities, which may have impact on CF therapies.

3.1 INTRODUCTION

Cystic Fibrosis (CF) is a common lethal disease affecting nearly 70 000 people around the world. It is characterized by the build-up of stick mucus overlying lung epithelial cells, wherein persistent cycles of chronic infection and inflammation occur [1,2]. The CF airways offer a favorable environment, of heterogeneous availabilities of oxygen, pH, nutrient, and antibiotics, which contributes largely for the proliferation of a phylogenetically diverse ecosystem and influencing the mix of microbes able to occupy it [3]. The existence of steep oxygen gradients within the CF airway mucus is well-known, with zones ranging from aerobic to completely anaerobic [4].

A complex and infective microbiome have been described in the context of CF (e.g. [5]). These polymicrobial infections are developed throughout a complex process involving several pathogens, which encompasses species that are believed to be clinically significant and species of which no direct evidence exists to support their impact in the disease. *Pseudomonas aeruginosa* stands out in CF infections, worsening CF pulmonary status due to chronic infections and being reliable for higher fatality rates [6]. But novel molecular technologies have recently detected and identified a diverse microbial community inhabiting CF lungs involving emergent species of unexplored relevance in CF disease. Although *P. aeruginosa* has been extensively studied in CF environments [7–11] no reports were found to evaluate the performance of other bacteria studied while associated to biofilms in the oxygen conditions found in *in vivo* CF airways.

Thus, evaluating the behavior of mono- and polymicrobial communities forming between traditional and emergent bacteria in *in vitro* conditions resembling CF airways may give major insights into their contributions for CF pathophysiology, which may be a starting point to determine their potential for pathogenicity.

3.2 MATERIAL AND METHODS

Bacterial strains and culture conditions

The seven CF-related bacterial species, *P. aeruginosa* (strain UCBPP-PA14), *S. aureus (*ATCC 25923), *A. baumannii (*clinical isolate form sputum), *I. limosus (*isolated strain M53), *D. pigrum* (CIP 104051^T), *K. pneumonia (*clinical isolate form sputum) and *S. maltophilia, (*clinical isolated from CF sputum) were used throughout this work. Dual species-biofilms were formed between *S. aureus* or *S. maltophilia* or *I. limosus* with the key-pathogen *P. aeruginosa*. Bacteria were preserved and cultured as described in sub-chapter 2.1.

Planktonic growth (planktonic growth curves)

Planktonic suspension of single- and dual-species were developed under environments with different oxygen concentrations (aerobic and anaerobic), as described in sub-chapter 2.2.1. Bacterial growth was monitored by optical density measurement at 640 nm (sub-chapter 2.2.1.2). Three-independent assays were performed in triplicate for each time point/species/condition.

Biofilm growth (biofilm growth curves)

Biofilms of single- and dual-species were developed under aerobic and anaerobic conditions in microtiter plate wells, as described in sub-chapter 2.3.1. The adhesion of bacteria to microtiter plate wells was monitored by growth curves (log_{10} CFU over time), as outlined in the sub-chapter 2.3.1.3. Three-independent assays were performed in triplicate for each time point/species/condition.

Analysis of biofilms – biomass and cultivability

After incubation under aerobic and anaerobic environments, biofilms were then analysed in terms of biomass, through the crystal violet (CV) staining method (sub-chapter 2.3.2.1). The number of total cultivable cells for single and dual-species biofilms was determined by cell counting onto non-selective agar medium (TSA) (see sub-chapter 2.3.2.2). At least three independent assays were performed, with six replicates were run and for both assays.

Quantification of biofilm attachment

Microtiter plates containing 24-h-old biofilms developed under environments with different oxygen

concentrations (aerobic and anaerobic), were washed with UP sterile water (200 μL per well), and after discarding the planktonic fraction, the attached bacteria were analysed for biomass, The total CFU number within biofilms was determined with TSA as described in sub-chapter 2.3.2.2. Selective agar media was used for *P. aeruginosa, S. aureus* and *I. limosus* quantification in the dual-species biofilms. The CFU number corresponding to *S. maltophilia* was calculated by the difference between the total CFUs and the CFUs obtained by selective media for other bacteria in the consortia. All tests were run in triplicate in three-independent assays.

Localization and distribution of biofilm populations by PNA FISH

In order to further apply PNA FISH to biofilms, single-species biofilms and dual-species consortia encompassing *P. aeruginosa*, S. aureus *I. limosus* and *S. maltophilia* were also formed on polystyrene (PET) coupons under the variable oxygen conditions mentioned before (sub-chapter 2.3.2.3.1). Biofilms were then fixed and hybridized with Paer565 peptide nucleic acid (PNA) probes previously described in sub-chapter 2.3.2.3.2. The PNA FISH procedure is described in sub-chapter 2.3.2.3.2. All tests were run in duplicate in three-independent assays.

Statistical analysis

Data were analyzed using the Prism software package (GraphPad Software version 6.0 for Macintosh). Otherwise stated, data were expressed as means ± standard deviation (SD). Data were compared by one-way analysis of variance (ANOVA) and subsequent comparisons were performed using Turkey multiple-comparisons test. Differences were considered statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

3.3 RESULTS

3.1.1 Effect of variable oxygen conditions in planktonic and biofilm growth

Single- and dual-species planktonic growth

This study aimed to appraise the adaptation to planktonic mode of growth of traditional and emerging CF related bacteria, and also to address the behaviour of polymicrobial (dual-species) consortia involving the key pathogen *P. aeruginosa* together with *S. aureus,* and with two emergent bacteria, *I. limosus and S. maltophilia,* under variable-oxygen atmospheres resembling CF airways. The selected single- and dual-species cultures were developed in polystyrene (PS) microtiter plate wells and were then incubated under aerobic and anaerobic environments and the planktonic growth was monitored each 2 h, by reading the OD at 640 nm of the planktonic fraction, up until 24 h (Fig. 3-1).

Despite slight differences in the growth rates for single-species under the different oxygen conditions (higher for aerobic than for anaerobic environments), the results revealed that all bacteria are able to adapt and survive in variable atmospheres at which the experiments were carried out (Fig. 3-1), showing high specific growth rates (in orders or magnitude ranging between 10^6 and 10^8 cells/mL/h calculated for the first 8 h, supplementary material, Table S3-1). *S. aureus* and *K. pneumoniae* presented the best initial growth rates in aerobic (5×10^8 cells/mL/h, $r^2 = 0.92$ and 2×10^8 cells/mL/h, $r^2 = 0.69$, respectively) and anaerobic atmospheres (2×10^8 cells/mL/h, $r^2 = 0.97$ and 1×10^8 cells/mL/h, $r^2 = 0.92$, respectively). The poorest growth rates were observed for *I. limosus (*2×10^2 cells/mL/h, for both atmospheres), which seems to be the less adapted species to the aforementioned environments or, suggesting that more extended times are necessary for its adaptation and growth.

In a general point of view, planktonic studies showed that both bacterial species, the traditional and emergent, were able to proliferate under the distinct environments tested, which may denote a possible potential prospect on the impact of nonconventional organisms on CF lung infection, as occurs for most traditional pathogens.

Similarly, dual-species growth pattern was equivalent under aerobic and anaerobic environments, weakly decreasing for low-oxygen conditions for all populations. As can be observed, dual-species formed between *P. aeruginosa* and each of the traditional- and emergent-species reached a similar growth pattern of *P. aeruginosa* alone (Fig. 3-1 (F)). These suggest that the

relationship established between all dual-species, in planktonic mixed cultures, do not affect the growth of *P. aeruginosa.*

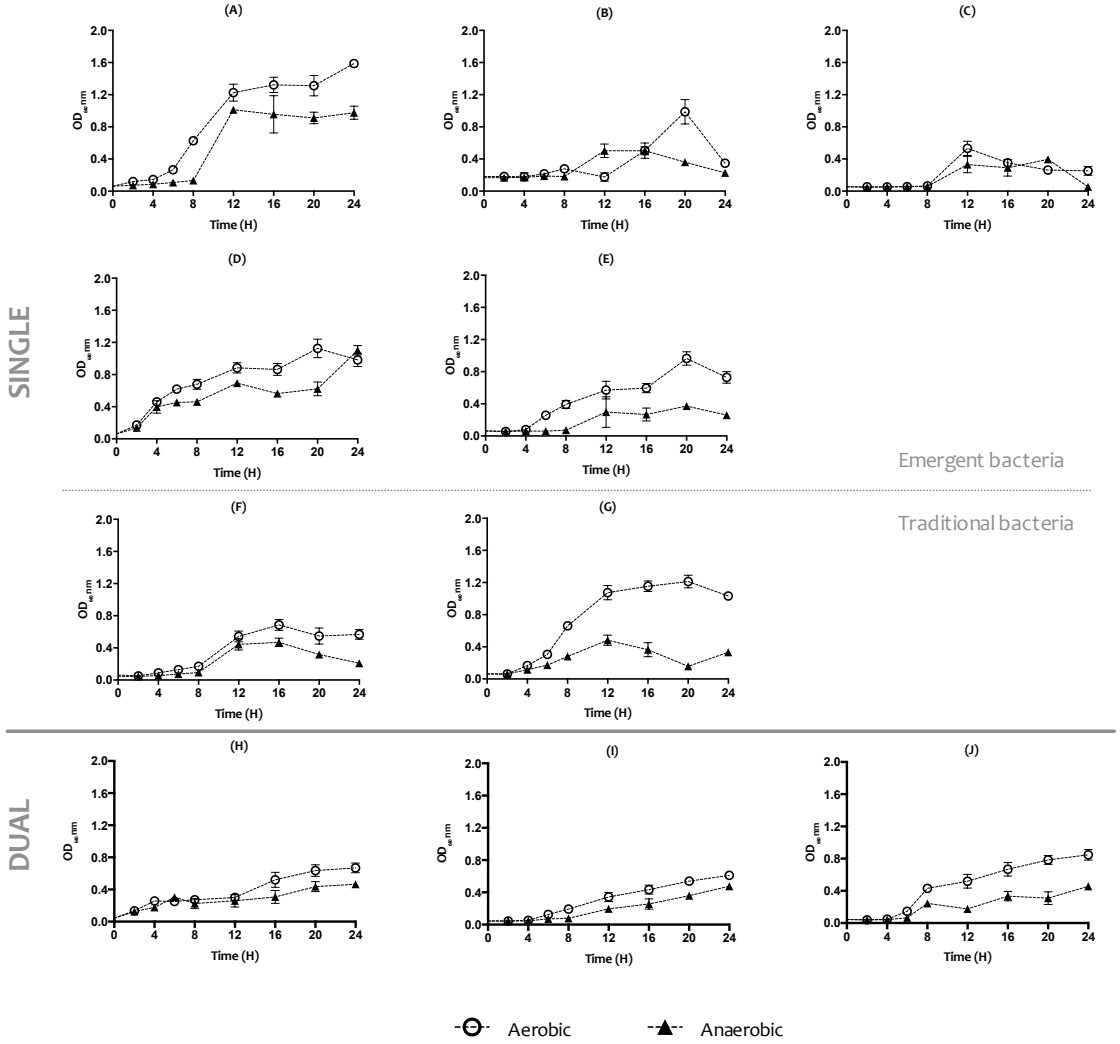

Fig. 3-1 Planktonic growth curves obtained for single-species *A. baumannii* **(A)**, *D. pigrum* **(B)**, *I. limosus* **(C)**, *K. pneumoniae* **(D)**, *S. maltophilia* **(E)**, *P. aeruginosa* **(F)**, *S. aureus* **(G)** and for dual-species involving *P. aeruginosa* and *S. aureus* **(H)**, *P. aeruginosa* and *I. limosus* **(I)** and *P. aeruginosa* and *S. maltophilia* **(J)** growing under aerobic and anaerobic environments. Bars represent means ± standard deviations (SDs) for three independent assays.

Single- and dual-species biofilm growth

Following the previous practical procedure, after analyzing the planktonic fraction, the biofilm growth was also monitored, by determining biofilm CFU number per area after detaching by sonication the cells adhered to the microtiter plates wells. As for planktonic growth, single- and dual-species biofilm populations were followed up until 24 h. Fig. 3-2 shows the biofilm growth curves (log_{10} CFU per cm^2 per time) for each single population (*A. baumannii*, *D. pigrum*, *I. limosus*, *K. pneumoniae*, *S. maltophilia*, *P. aeruginosa* and *S. aureus*) and for dual-species

cultures (*P. aeruginosa* and *S. aureus* or *P. aeruginosa* and *I. limosus, P. aeruginosa* and *S. maltophilia*) obtained under aerobic and anaerobic environments.

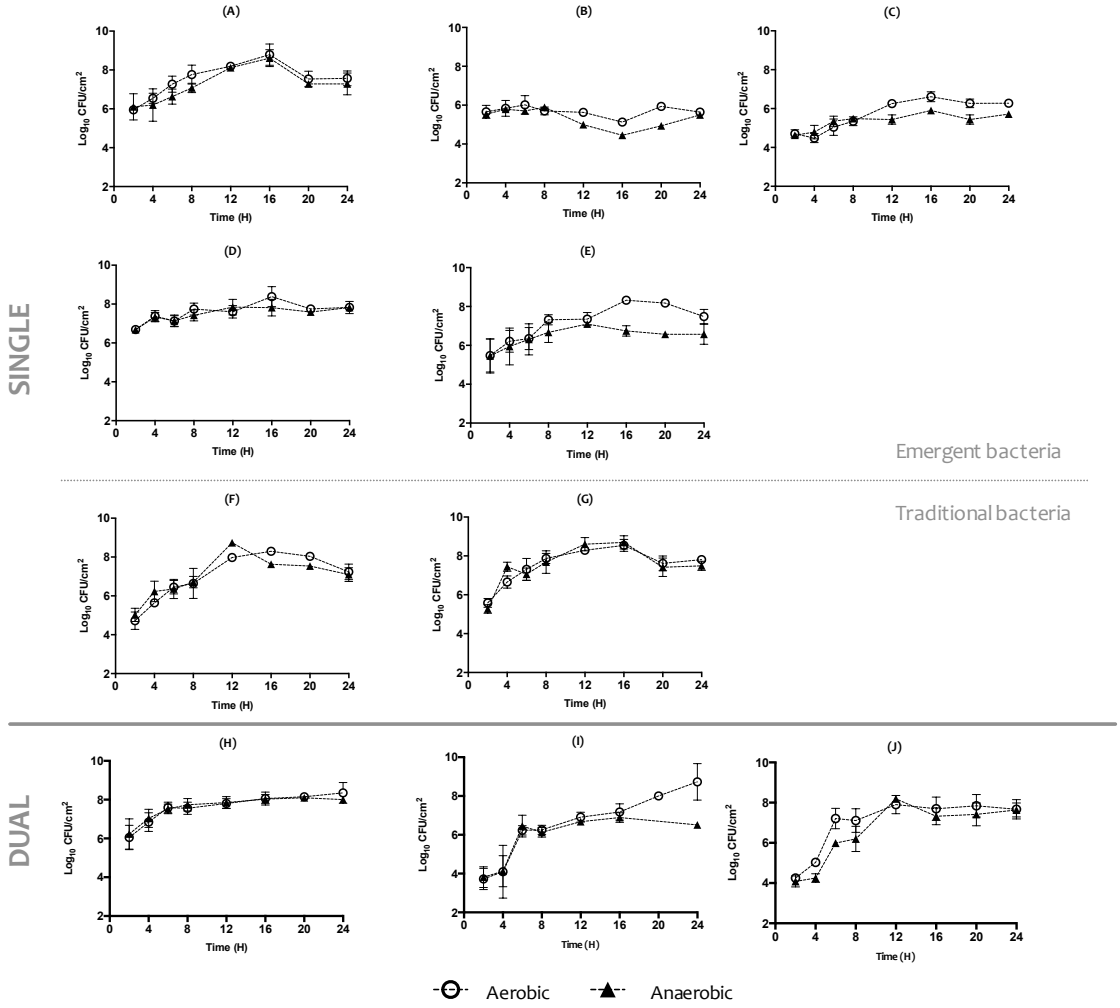

Fig. 3-2 Biofilm growth curves obtained for single-species *A. baumannii* **(A)**, *D. pigrum* **(B)**, *I. limosus* **(C)**, *K. pneumoniae* **(D)**, *S. maltophilia* **(E)**, *P. aeruginosa* **(F)**, *S. aureus* **(G)** and for dual-species involving *P. aeruginosa* and *S. aureus* **(H)**, *P. aeruginosa* and *I. limosus* **(I)**, *P. aeruginosa* and *S. maltophilia* **(J)** growing under aerobic and anaerobic environments. Bars represent means ± standard deviations (SDs) for three independent assays.

Results revealed that, as for traditional species, the emergent bacteria were able to adapt in aerobic and anaerobic atmospheres growing as biofilms, showing high specific adhesion rates (calculated for the first 8 h, supplementary material, Table S3-2). The poorest initial adhesion rates were observed for single-species *I. limosus* and *D. pigrum* (~10^4 cells/cm^2/h), which may be due to the slow growth under these conditions. Despite slight differences in the adhesion rates under the different atmospheric conditions, all single-species populations presented increasing CFUs over time under all environments, achieving abundant cell densities ranging from 10^5 to 10^8 adhered cells per cm^2 upon 24 h.

Dual-species biofilms showed high initial adhesion rates (supplementary material, Table S3-3) and seems that the growth of *P. aeruginosa,* in mixed cultures, was not disturbed by the presence of *S. aureus, I. limosus* and *S. maltophilia.* Despite the observed delay in the early growth stage of biofilms encompassing *P. aeruginosa* with *I. limosus* and with *S. maltophilia*, all populations could present initial adhesion rates greater than *P. aeruginosa* alone. For dual-species biofilms formed by *P. aeruginosa* and *I. limosus*, a reduction (< 1 log) in cell number upon 24 h was observed, when compared with *P. aeruginosa* alone, but this was considered not significant.

In general, as occurs for dual-species planktonic growth, biofilm studies suggest that the oxygen availability within the milieu has not a preponderant effect on the planktonic and biofilm growth, with most species being able to thrive significant cell numbers under aerobic and anaerobic conditions. Additionally, the emergent species may have a significant effect in CF infections by strengthen the overall biofilm when co-cultured with traditional pathogens.

3.1.2 Single and dual-species biofilms analysis: biomass, cultivable cells and biofilm populations discrimination

Biofilm mass and cultivable cells

After growing for 24 h under aerobic and anaerobic environments in PS microtiter plate wells, single- and dual-species biofilms were analyzed in terms of biomass and cultivable cells (Fig. 3-3).

As demonstrated by Fig. 3-3 (A), all single-bacteria produced significantly ($P < 0.0001$) more biomass in aerobic environments. The only exception was observed for *K. pneumoniae*, which presented greater amount of biomass in low-oxygen conditions ($P < 0.01$). Although there were large deviations on the biomass produced by each bacterial species under aerobic and anaerobic environments, most bacteria showed high but similar CFU counts for both (Figure 3-3 (B)). It was also noticed that *I. limosus* biofilms exhibited the lowest amount of biomass and number of cells under both conditions, which may be consequence from a slow growth.

Similarly to most single-species biofilms, dual-species consortia also produced more biomass and obtained more cells under aerobic conditions. It could be observed a great decrease in the biomass produced by the biofilms involving *P. aeruginosa* with *S. aureus* and with *I. limosus*, for example when compared with *P. aeruginosa* single biofilms. Contrariwise, the number of biofilm-encased cells of *P. aeruginosa* was not affected by the presence of other species, reaching high cell numbers (~ 10^7 to10^8 CFU/cm^2) for both aerobic and anaerobic conditions. These results (less biomass, with no significant changes in the number of cells compared with *P. aeruginosa* alone)

could be potentially associated with a decrease in the amount of extracellular matrix produced by the biofilm cells. The only exception was found for biofilms involving *P. aeruginosa* with *S. maltophilia,* with the amount of biomass increased significantly ($P < 0.0001$) under aerobic conditions, although the number of cultivable cells was not affected. These result that in co-culture these two species may reap benefit, once the improved in the amount of biomass could protect the dual-consortia.

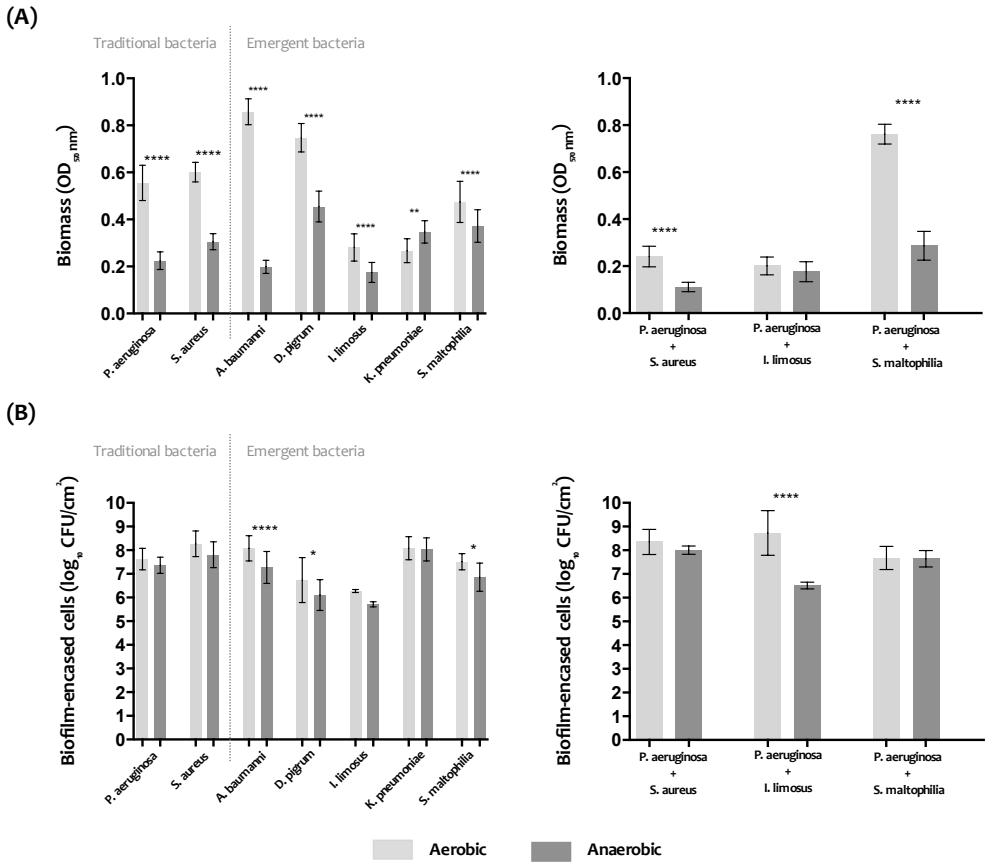

Fig. 3-3 Quantification in terms of biomass **(A)** and cultivable biofilm-encased cells **(B)** obtained for single-species biofilms formed by *P. aeruginosa*, *S. aureus, A. baumannii*, *D. pigrum*, *I. limosus*, *K. pneumoniae* and *S. maltophilia*, and for dual-species biofilms encompassing *P. aeruginosa*, *S. aureus, I. limosus* and *S. maltophilia* developed under aerobic and anaerobic environments. Bars represent means ± standard deviations (SDs) for at least three independent assays.

In order to investigate which species were prevailing in the polymicrobial (dual-species) consortia, under variable oxygen conditions, the CFU number was discriminated for each species included in the consortia, by using selective agar media, and the microbial composition of the polymicrobial biofilms was evaluated (Fig. 3-4).

Cell counts within 24-h-old biofilms were estimated by plating dilutions of biofilm samples onto non-selective and selective agar media, in order to obtain the total number of CFUs present in

biofilms, as well as to evaluate the amount of cells of each bacteria in the overall population (Fig. 3-4 A). The estimation of each species within the biofilms was made counting CFUs on selective agar media (PIA, MSA and supplemented BCSA for isolation of *P. aeruginosa, S. aureus* and *I. limosus*, respectively). Due to the complexity of selective media described in the literature for *S. maltophilia*, this species was estimated by the difference between the average total cell number (in TSA) and the average of *P. aeruginosa* (in PIA) present in the consortia.

(A)

Biofilm	Atmosphere	All species	*P. aeruginosa*	*S. aureus*	*I. limosus*	*S. maltophilia*
P. aeruginosa* + *S. aureus	AEROBIC	8.49 ± 0.53	7.54 ± 0.07	8.46 ± 0.40	-	-
	ANAEROBIC	8.00 ± 0.20	7.43 ± 0.77	8.11 ± 0.36		
P. aeruginosa* + *I. limosus	AEROBIC	9.03 ± 0.94	6.86 ± 0.30	-	4.08 ± 0.53	-
	ANAEROBIC	6.81 ± 0.14	6,54 ± 0.88		4.06 ± 0.18	
P. aeruginosa* + *S. maltophilia	AEROBIC	7.45 ± 0.49	7.04 ± 0.54	-	-	2,76 ± 0.18
	ANAEROBIC	7.42 ± 0.35	7.37 ± 0.28			1,99 ± 0.09

(B)

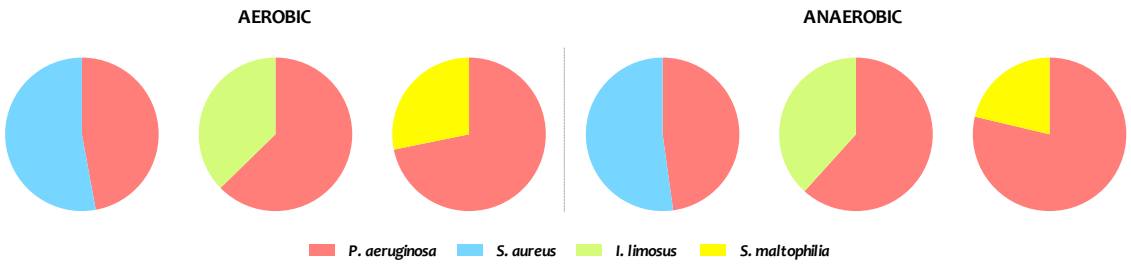

Fig. 3-4 Quantification of dual-species biofilms formed by *P. aeruginosa* (PA), *S. aureus* (SA), *I. limosus* (IL) and *S. maltophilia* (SM) in terms of cultivable cells (log_{10} CFU/cm^2) **(A)** and their relative distributions **(B)** within polymicrobial consortia under aerobic and anaerobic environments. For cultivable cells, log_{10} CFU/cm^2 are represented. TSA was used to determine total cell numbers, whereas PIA was used as selective agar medium for PA, BCSA (supplemented with polymixin B and ticarcillin) and MSA was used for IL and SA countings, respectively. SM was estimated by the difference between the average total cell number (in TSA) and the average of PA (in PIA). The means ± SDs for three independent assays are represented all cases.

After 24 h of incubation under aerobic and anaerobic conditions, dual-species biofilms of *P. aeruginosa* and *S. aureus* were more or less equally composed by both populations under all environments, although with *S. aureus* covering about 53% (in average) of the whole consortia. When *I. l*imosus was grown together with *P. aeruginosa*, the consortia was dominating by the key-pathogen, for aerobic and anaerobic conditions. Similarly, for both atmospheres, dual-species biofilms of *P. aeruginosa* and S. *maltophilia* consortia were almost entirely dominated by *P. aeruginosa.* In general, *P. aeruginosa* seems be the predominate species in the polymicrobial consortia.

Biofilm populations discrimination by PNA FISH

In order to confirm the results obtained by biofilm CFU counts under variable environments, and also to visualize directly the distribution and the location of each bacterial population within the polymicrobial communities, biofilms were also observed under a fluorescence microscope after applying a multiplex PNA FISH methodology counterstained with 4`, 6-diamidino-2-phenylindole (DAPI).

In order to observe the morphology of each individual population and also for comparison purposes, PNA FISH was also applied to mono-species biofilms (Fig. 3-5). According the cell counts determined by culture techniques, PNA FISH allowed to observe that *P. aeruginosa, S. aureus* and *S. maltophilia* are present in high numbers, with I. *limosus* producing less cells (Fig. 3-5, top row). Additionally, all populations were more perceptible under aerobic atmospheres than for anaerobiosis. In terms of cell arrangement, all bacteria formed a more pronounced aggregated-like structures under low-oxygen conditions, which could be a result of a biofilm protection against oxygen stringency or even an adaptive response in this environment.

In Fig. 3-6, it can be seen that all microorganisms could be easily distinguished with distinct cell morphologies in the multiplex experiment. The strength of each fluorescent signal allowed to distinguish *P. aeruginosa* (red rod-shaped cells) of *S. aureus* (blue cocci cells), *S. maltophilia* (blue bacillus cells) and I. *limosus* (blue rod cells) within the polymicrobial consortia. In agreement with cell counts determined by culture techniques, when *S. aureus* is present in the consortia, it still occupies a significant extent within the overall biofilms, together with *P. aeruginosa* populations (column 1). In aerobic atmospheres the dual-species biofilm formed between *P. aeruginosa and S. aureus* appearing in close proximity, with *P. aeruginosa* population seeming to cover *S. aureus* cells in protective manner.

Dual-species biofilms involving *P. aeruginosa* and *I. limosus* were composed by both species, although with I. limosus low-size populations being sporadically distributed within the consortia, and with *P. aeruginosa* population being significantly more perceptible under the different atmospheres (column 2).

Contrarily to the relative distribution determined by culture techniques*, S. maltophilia* occupy a significant portion together with *P. aeruginosa* in the overall biofilm (column 3), with *P. aeruginosa* more present under aerobic atmospheres and *S. maltophilia* dominating in low-oxygen conditions.

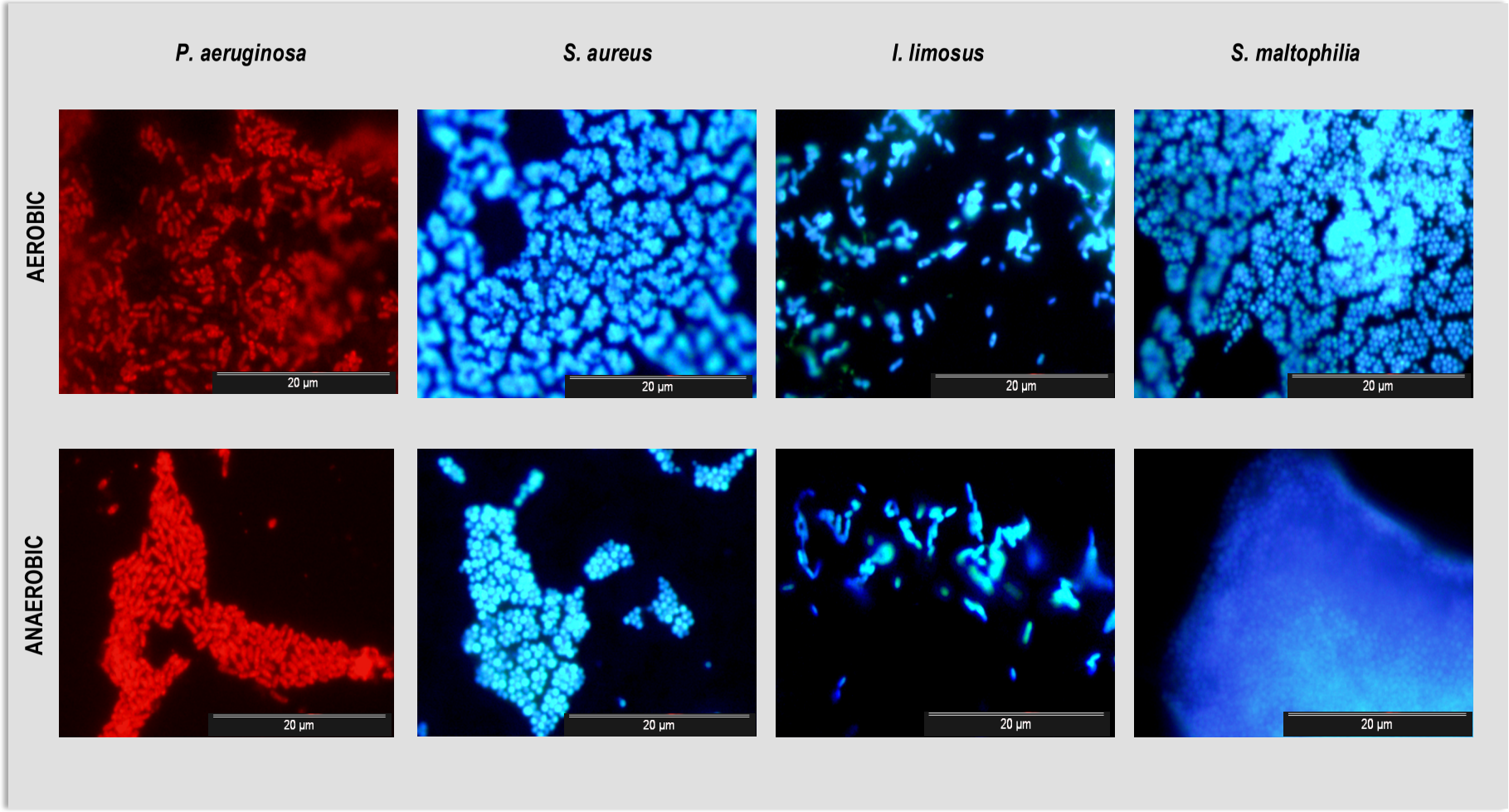

Fig. 3-5 PNA-FISH applied to single-species biofilms of *P. aeruginosa* (red rod cells), *S. aureus* (blue clustered cocci cells), *I. limosus* (blue rod cells) and *S. maltophilia* (blue clustered bacillus cells) grown for 24 h at aerobic and anaerobic conditions. Images resulted from two channels used to visualize the fluorochromes used (Alexa fluor 594, red: *P. aeruginosa*; DAPI, blue: *S. aureus, I. limosus and S. maltohilia*).

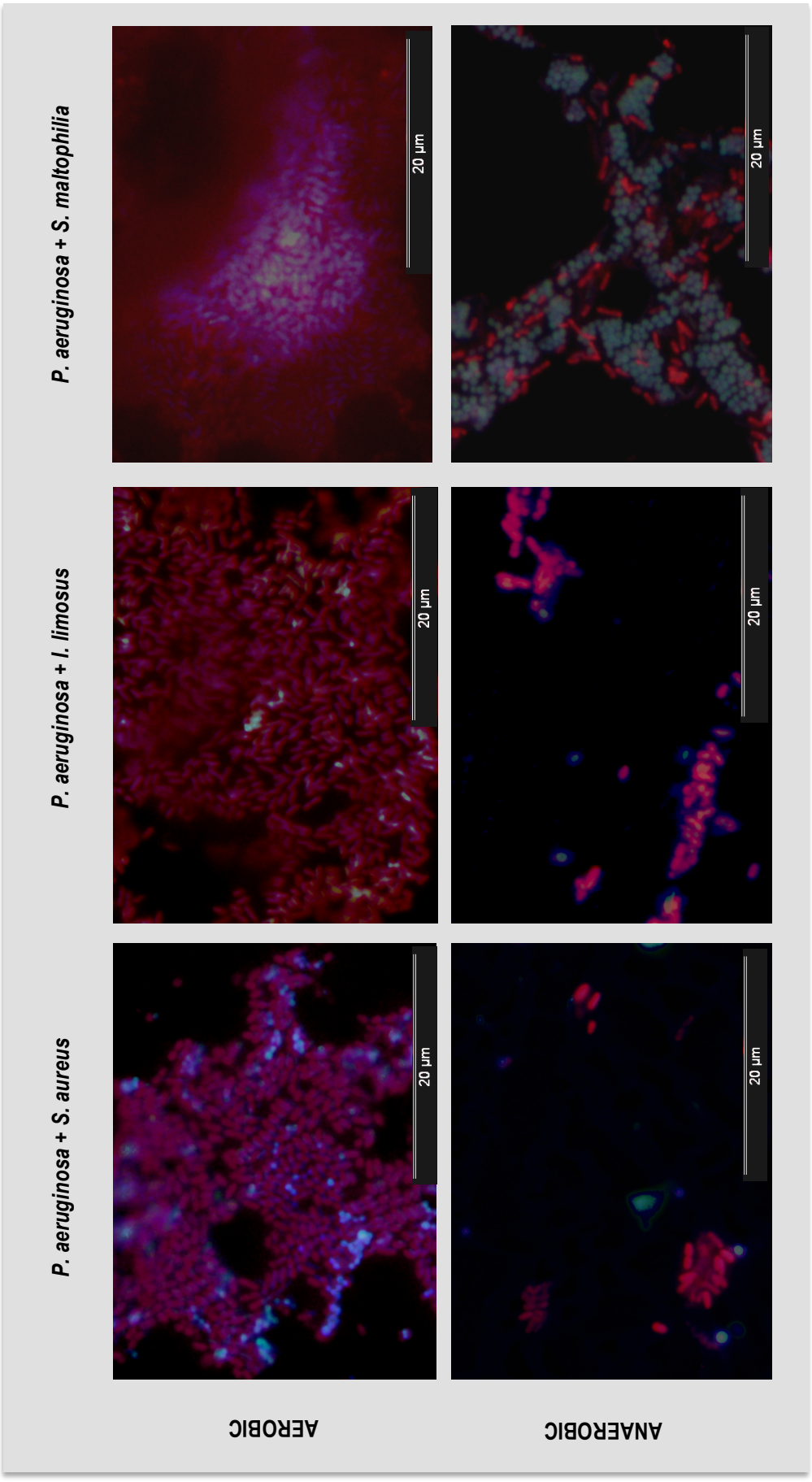

Fig. 3-6 Multiplex PNA-FISH applied to dual-species biofilms involving *P. aeruginosa* (red rod cells), *S. aureus* (blue cocci cells), *I. limosus* (blue rod cells) and *S. maltophilia* blue bacillus cells) grown for 24 h at aerobic and anaerobic conditions. Images resulted from the bands superposition of the two channels used to visualize the fluorochromes used (Alexa fluor 594, red: *P. aeruginosa*; DAPI, blue: *S. aureus, I. limosus and S. maltohilia*).

3.4 DISCUSSION

Although it was initially believed that only a limited number of organisms could cause symptomatic infection and lung damage in CF, it has now been shown that the microbial ecology of the CF lung is far more complex (e.g. [5]), with uncommon species co-existing and establishing dynamic interactions with traditional pathogens.

The sputum matrix in the CF lung has been extensively studied and is reported to contain aerobic and anaerobic regions that can promote and sustain microbial colonization. An oxygen gradient is present, where the highest levels of oxygen are found at the top of the sputum layer and an anaerobic environment is present near the epithelial surface [4]. Although *P. aeruginosa* is considered an aerobe, it is widely recognized that *P. aeruginosa* is capable of growth under anaerobic conditions. Furthermore, Yoon et al. [12] showed by confocal laser scanning microscopy that *P. aeruginosa* formed robust biofilms under anaerobic conditions.

The main goal of this study was to address the behavior of traditional and emergent CF-bacteria under oxygen-atmospheres resembling CF airways. Unlike *P. aeruginosa*, which has been extensively studied in such environments [7–11] no reports were found to evaluate the performance of other bacteria studied while associated to biofilms in the oxygen conditions found in *in vivo* CF airways, failing to consider the role of biofilms, oxygen availability and the interplay among microorganisms within polymicrobial infections in CF context.

Results revealed that most bacteria, including both traditional and emergent, were able to adapt and survive under variable oxygen atmospheres (with no significant variations between both conditions), achieving high cell densities and showing high specific growth rates and initial adhesion rates, for planktonic and biofilm growth, respectively. The fact of uncommon organisms showing their great capacity to easily adapt under atmospheres with restricted oxygen conditions, that are similar to those occurring in CF airways, may highlight their potential impact in the disease progression and contributing for CF infection.

Most studies involving mixed-species in CF have only included classical pathogens such as *P. aeruginosa*, *B. cenopacia* and *S. aureus* [13–17]. Co-infections of traditional pathogens with emergent species in CF lungs remain largely unexplored, limiting the understanding of the importance of interspecies interactions and a possible pathogenic potential associated to unusual species. The fitness of dual-species populations was evaluated by comparison with the fitness of P. aeruginosa single-specie. *P. aeruginosa* was used as a reference because this bacterium is typically the prevailing pathogen in the airways of CF patients. In this study, it was demonstrated

that the presence of other species, namely *S. aureus and I. limosus* in the same consortia as *P. aeruginosa* led to a reduced biomass, but have no altered the number of cells within the overall biofilms. These results, obtained for aerobic and anaerobic conditions, indicate that these species could interfere eventually with the exopolysaccharyde matrix of these biofilms but not with the cells. This result may have impact in the clinical context, for example, by decreasing the resistance of these biofilms against antibiotic action. The polymeric matrix of biofilms, formed for a mixture of polysaccharides, extracellular DNA, and proteins, acts as both a structural scaffold and a protective barrier to harsh environments [18]. At least three polysaccharides (polysaccharide synthesis locus (Psl), Pel polysaccharide (Pel) and alginate) have been identified in *P. aeruginosa* that play important roles in structure maintenance and antibiotic resistance of biofilm [19–22].

The establishment of bacterial biofilms under variable oxygen conditions similar to those locally found in CF airway *in vivo* is becoming increasingly recognized [4,23] enabling the well adaptation of pathogens, such as *P. aeruginosa*. Although there are significantly differences in the ability to produce biomass, for low oxygen conditions, all single- and dual-species bacteria showed high CFU counts for all environments. It is important to note that *I. limosus,* from all bacteria, exhibited the lowest amount of biomass and number of produced cells by their single biofilm. Accordingly to Chiron et al. [24] the presence of strains with nonmucoid and mucoid phenotypes suggesting that *I. limosus* might be able to undergo a switch to a mucoid phenotype. In this study, the CF clinical isolate *I. limosus* M53 presented a mucoid physiology, which likely contributed to the slow growth and consequently to the limited biomass and number of cells.

In order to exhaustively characterize these polymicrobial communities formed by traditional and emergent bacteria associated to CF under variable oxygen conditions, several culture techniques were employed in this study. Additionally to conventional culture methods, a multiplex approach of the PNA FISH method was applied. This technique provides information not only about the specific detection and discrimination between the biofilm-forming populations as also it is possible to observe the *in situ* spatial distribution and arrangement of the bacterial cells within the consortia [25], leading to a better understanding of the real bacterial interactions occurring in CF polymicrobial consortia. Basically, consisted in the application of a PNA oligonucleotide probe (designed by the group to specifically detect and localize the CF-classical opportunist *P. aeruginosa)* in dual-species biofilms formed by the abovementioned bacteria, developed on PS coupons. The identification of the *S. aureus*, *I. limosus* and *S. maltophilia* organisms within the dual-species biofilms populations was possible by counterstaining the samples with the non-

specific dye DAPI at the end of the hybridization process. PNA oligonucleotide probes are synthetic DNA analogue molecules, with the main difference residing in the backbone of the molecule. Whilst DNA possesses a negatively charged sugar-phosphate backbone, the PNA molecule consists of repeating *N*-(2-aminoethyl)glycine units, resulting in an achiral and neutral backbone [26,27]. This enables PNA molecules to better penetrate through the cell wall and cell membrane of the target microorganism and specifically hybridize with the complementary rRNA sequence [28]. The versatility and the fast procedure, taking no longer that 3 h, make FISH a valuable tool for the rapid diagnosis of bacteria in CF samples. The multiplex PNA FISH assay, counterstained with DAPI, was successfully applied directly dual-species biofilms, enabling an accurate identification of the bacteria involved in the consortia.

In this study, all single-species populations were more perceptible under aerobic atmospheres than for anaerobiosis. Moreover, all bacteria formed a more pronounced aggregated-like structures under low-oxygen conditions. In agreement with this results, Yoon and colleagues [29] demonstrated that during anaerobic respiration was observed a highly cohesive autoaggregates in the *P. aeruginosa.* This alteration in the biofilm structure could be a result of a biofilm protection against oxygen stringency or even an adaptive response in this environment.

For polymicrobial biofilm communities, the results obtained with PNA FISH allow to see that *P. aeruginosa* was the predominate species in the dual-species consortia, corroborating those results obtained by culture techniques, using selective agar media. The cocci cells of *S. aureus*, visualized by DAPI, were present in a great extension and in close proximity with *P. aeruginosa*, suggesting a synergistic interaction between both bacteria. This result is consistent with recent studies that shown a positive relationship between these two pathogens, which may be result from synergistic interactions [15,17]. The synergism among *P. aeruginosa* and *S. maltophilia* was pronounced by increased biomass, although the global cultivable cell number remains constant. The results obtained with PNA FISH allowed complementing these findings, with both species similarly distributed in consortia, which was not visible on cultivability data, and established a close relationship. The discrepancies found between culture techniques and PNA FISH methodology may have related with the loss of cultivability by *S. maltophilia* when it is cultured with *P. aeruginosa*. Many factors can be pointed out to justify the nonculturability of bacteria, this even be related with a survival strategy used for bacteria that are introduced into a new environments. Roszak and Colwell [30] created the term “viable but nonculturable” for characterize those bacteria.

The competitive or even antagonistic interaction among *P. aeruginosa* and *I. limosus,* in low-oxygen atmospheres, was related with the reduction of the cell density of the dual consortia comparatively with both monospecies. The few blue cells identified by epifluorescence microscopy after 24 h of incubation, and the reduction in the number of *P. aeruginosa* cells, lead to believe that *I. limosus* was outcompeted by *P. aeruginosa.* This result is also observed when conventional culture techniques were used, with *P. aeruginosa* presenting a high relative cell proportion comparatively with *I. limosus*. The negative interactions among these species could eventually result from competition for space and for finite resources or even for other growth parameters, unavoidable within any community [31]. In CF, interactions between classic pathogens residing in the airways are well documented [13–17,32–34] but only a limited number of studies have focused on interactions involving emergent species [35–37], leading to the development of the well-organized biofilms community structure, for the adaptation of CF airways niche and for disease progression.

Although the most common CF pathogen is *P. aeruginosa*, this study has evidenced the pronounced ability of *S. aureus* and the emergent species *A. baumannii, D. pigrum, I. limosus, K. pneumoniae* and *S. maltophilia* to grow and develop biofilms under variable oxygen atmospheres, similar to those that occur in CF airways. In effect, the presence of *S. aureus, I. limosus* and *S. maltophilia* in the same consortia as *P. aeruginosa,* can became more drastic in the clinical context, because they tend to reduce the biofilm matrix and decreasing the restriction of such consortia against antibiotherapy. Nonetheless, these experiments was performed in vitro which is a limitation in that the bacteria were allowed to adhere to polystyrene microtiter plate wells rather than airway epithelium, where a number of host-derived and bacterial-specific factors, which may include increased levels of DNA and actin that contribute colonization sites and/or biofilm matrix components, mucus production, specific receptors on the epithelial surface, contribute for bacterial adherence and invasion [51].

It is clear that the complex interactions among bacteria play an important role in the complex pathology of the disease and may be often responsible for the increase in antibiotic tolerance.

3.5 REFERENCES

1. Gibson RL, Burns JL, Ramsey BW: **Pathophysiology and management of pulmonary infections in cystic fibrosis.** *Am. J. Respir. Crit. Care Med.* 2003, **168**:918–51.

2. Goss CH, Burns JL: **Exacerbations in cystic fibrosis. 1: Epidemiology and pathogenesis.** *Thorax* 2007, **62**:360–7.

3. Yang L, Jelsbak L, Molin S: **Microbial ecology and adaptation in cystic fibrosis airways.** *Environ. Microbiol.* 2011, **13**:1682–9.

4. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, Birrer P, Bellon G, Berger J, Weiss T, et al.: **Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients.** *J. Clin. Invest.* 2002, **109**:317–25.

5. Lopes SP, Azevedo NF, Pereira MO: **Microbiome in cystic fibrosis: Shaping polymicrobial interactions for advances in antibiotic therapy.** *Crit. Rev. Microbiol.* 2014, doi:10.3109/1040841X.2013.847898.

6. Winstanley C, Fothergill JL: **The role of quorum sensing in chronic cystic fibrosis Pseudomonas aeruginosa infections.** *FEMS Microbiol. Lett.* 2009, **290**:1–9.

7. Schobert M, Jahn D: **Anaerobic physiology of Pseudomonas aeruginosa in the cystic fibrosis lung**. *Int. J. Med. Microbiol.* 2010, **300**:549–556.

8. Schertzer JW, Brown SA, Whiteley M: **Oxygen levels rapidly modulate Pseudomonas aeruginosa social behaviours via substrate limitation of PqsH.** *Mol. Microbiol.* 2010, **77**:1527–38.

9. Hogardt M, Heesemann J: **Adaptation of Pseudomonas aeruginosa during persistence in the cystic fibrosis lung.** *Int. J. Med. Microbiol.* 2010, **300**:557–62.

10. Field TR, White A, Elborn JS, Tunney MM: **Effect of oxygen limitation on the in vitro antimicrobial susceptibility of clinical isolates of Pseudomonas aeruginosa grown planktonically and as biofilms.** *Eur. J. Clin. Microbiol. Infect. Dis.* 2005, **24**:677–87.

11. King P, Citron DM, Griffith DC, Lomovskaya O, Dudley MN: **Effect of oxygen limitation on the in vitro activity of levofloxacin and other antibiotics administered by the aerosol route against Pseudomonas aeruginosa from cystic fibrosis patients.** *Diagn. Microbiol. Infect. Dis.* 2010, **66**:181–6.

12. Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, Kamani MC, Allen HL, DeKievit TR, Gardner PR, Schwab U, et al.: **Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis.** *Dev. Cell* 2002, **3**:593–603.

13. Chattoraj SS, Murthy R, Ganesan S, Goldberg JB, Zhao Y, Hershenson MB, Sajjan US: **Pseudomonas aeruginosa alginate promotes Burkholderia cenocepacia persistence in cystic fibrosis transmembrane conductance regulator knockout mice**. *Infect. Immun.* 2010, **78**:984–993.

14. Bragonzi A, Farulla I, Paroni M, Twomey KB, Pirone L, Lorè NI, Bianconi I, Dalmastri C, Ryan RP, Bevivino A: **Modelling Co-Infection of the Cystic Fibrosis Lung by Pseudomonas aeruginosa and Burkholderia cenocepacia Reveals Influences on Biofilm Formation and Host Response**. *PLoS One* 2012, **7**.

15. Fugère A, Séguin DL, Mitchell G, Déziel E, Dekimpe V, Cantin AM, Frost E, Malouin F: **Interspecific small molecule interactions between clinical isolates of Pseudomonas aeruginosa and Staphylococcus aureus from adult cystic fibrosis patients**. *PLoS One* 2014, **9**.

16. Baldan R, Cigana C, Testa F, Bianconi I, De Simone M, Pellin D, Di Serio C, Bragonzi A, Cirillo DM: **Adaptation of Pseudomonas aeruginosa in cystic fibrosis airways influences virulence of Staphylococcus aureus in vitro and murine models of co-infection**. *PLoS One* 2014, **9**.

17. Yang L, Liu Y, Markussen T, Høiby N, Tolker-Nielsen T, Molin S: **Pattern differentiation in co-culture biofilms formed by Staphylococcus aureus and Pseudomonas aeruginosa**. *FEMS Immunol. Med. Microbiol.* 2011, **62**:339–347.

18. Wei Q, Ma LZ: **Biofilm matrix and its regulation in Pseudomonas aeruginosa.** *Int. J. Mol. Sci.* 2013, **14**:20983–1005.

19. Ryder C, Byrd M, Wozniak DJ: **Role of polysaccharides in Pseudomonas aeruginosa biofilm development.** *Curr. Opin. Microbiol.* 2007, **10**:644–8.

20. Friedman L, Kolter R: **Two Genetic Loci Produce Distinct Carbohydrate-Rich Structural Components of the Pseudomonas aeruginosa Biofilm Matrix**. *J. Bacteriol.* 2004, **186**:4457–4465.

21. Jackson KD, Starkey M, Kremer S, Parsek MR, Wozniak DJ: **Identification of psl, a locus encoding a potential exopolysaccharide that is essential for Pseudomonas aeruginosa PAO1 biofilm formation.** *J. Bacteriol.* 2004, **186**:4466–75.

22. Colvin KM, Gordon VD, Murakami K, Borlee BR, Wozniak DJ, Wong GCL, Parsek MR: **The pel polysaccharide can serve a structural and protective role in the biofilm matrix of Pseudomonas aeruginosa.** *PLoS Pathog.* 2011, **7**:e1001264.

23. Bjarnsholt T, Jensen PØ, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, Pressler T, Givskov M, Høiby N: **Pseudomonas aeruginosa biofilms in the respiratory tract of cystic fibrosis patients.** *Pediatr. Pulmonol.* 2009, **44**:547–58.

24. Chiron R, Marchandin H, Counil F, Jumas-Bilak E, Freydière A-M, Bellon G, Husson M-O, Turck D, Brémont F, Chabanon G, et al.: **Clinical and microbiological features of Inquilinus sp. isolates from five patients with cystic fibrosis.** *J. Clin. Microbiol.* 2005, **43**:3938–43.

25. Almeida C, Azevedo NF, Santos S, Keevil CW, Vieira MJ: **Discriminating multi-species populations in biofilms with peptide nucleic acid fluorescence in situ hybridization (PNA FISH)**. *PLoS One* 2011, **6**.

26. Nielsen PE, Egholm M, Berg RH, Buchardt O: **Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide.** *Science* 1991, **254**:1497–500.

27. Nielsen PE: **Peptide nucleic acid: a versatile tool in genetic diagnostics and molecular biology.** *Curr. Opin. Biotechnol.* 2001, **12**:16–20.

28. Stender H: **PNA FISH: an intelligent stain for rapid diagnosis of infectious diseases.** *Expert Rev. Mol. Diagn.* 2003, **3**:649–55.

29. Yoon MY, Lee K-M, Park Y, Yoon SS: **Contribution of cell elongation to the biofilm formation of Pseudomonas aeruginosa during anaerobic respiration.** *PLoS One* 2011, **6**:e16105.

30. Roszak DB, Colwell RR: **Metabolic activity of bacterial cells enumerated by direct viable count.** *Appl. Environ. Microbiol.* 1987, **53**:2889–93.

31. Hibbing ME, Fuqua C, Parsek MR, Peterson SB: **Bacterial competition: surviving and thriving in the microbial jungle.** *Nat. Rev. Microbiol.* 2010, **8**:15–25.

32. Kenney DMC, Brown KE, Allison DG: **Influence of Pseudomonas aeruginosa exoproducts on virulence factor production in Burkholderia cepacia : evidence of interspecies communication . Influence of**

Pseudomonas aeruginosa Exoproducts on Virulence Factor Production in Burkholderia cepacia : Evid. 1995, **177**:6989–6992.

33. Rüger M, Ackermann M, Reichl U: **Species-specific viability analysis of Pseudomonas aeruginosa, Burkholderia cepacia and Staphylococcus aureus in mixed culture by flow cytometry.** *BMC Microbiol.* 2014, **14**:56.

34. Hoffman LR, Déziel E, D'Argenio D a, Lépine F, Emerson J, McNamara S, Gibson RL, Ramsey BW, Miller SI: **Selection for Staphylococcus aureus small-colony variants due to growth in the presence of Pseudomonas aeruginosa.** *Proc. Natl. Acad. Sci. U. S. A.* 2006, **103**:19890–19895.

35. Lopes SP, Ceri H, Azevedo NF, Pereira MO: **Antibiotic resistance of mixed biofilms in cystic fibrosis: impact of emerging microorganisms on treatment of infection.** *Int. J. Antimicrob. Agents* 2012, **40**:260–3.

36. Twomey KB, O'Connell OJ, McCarthy Y, Dow JM, O'Toole G a, Plant BJ, Ryan RP: **Bacterial cis-2-unsaturated fatty acids found in the cystic fibrosis airway modulate virulence and persistence of Pseudomonas aeruginosa**. *ISME J.* 2012, **6**:939–950.

37. Mowat E, Rajendran R, Williams C, McCulloch E, Jones B, Lang S, Ramage G: **Pseudomonas aeruginosa and their small diffusible extracellular molecules inhibit Aspergillus fumigatus biofilm formation**. *FEMS Microbiol. Lett.* 2010, **313**:96–102.

ANTIMICROBIAL SUSCEPTIBILITY OF CYSTIC FIBROSIS PLANKTONIC AND BIOFILM POPULATIONS UNDER VARIABLE OXYGEN CONDITIONS

Previous findings showed that populations encompassing cystic fibrosis (CF) bacteria could easily adapt as biofilms under variable oxygen conditions resembling CF, but how these pathogens may contribute to disease progression and to antibiotic therapy is still to be disclosed. As such, those bacterial populations were grown under variable oxygen conditions and their antibiotic resistance profiles were assessed. For dual-species consortia, a more deep characterization was performed, in terms of antibiotic resistance profiles and relative distributions of bacterial populations within those biofilms.

Results indicate that, for all oxygen atmospheres, biofilms were notoriously more difficult to eradicate than their planktonic counterparts. Regarding polymicrobial populations, biofilm eradication was not achieved by using monotherapy (ciprofloxacin), showing even an increased overall cell density when compared with monospecies *P. aeruginosa*. In general, biofilm compositions changed as a result of antibiotic treatment, with alterations being dependent on the antibiotic concentration and oxygen conditions implemented. For consortia formed between *P. aeruginosa* and *S. aureus*, *S. aureus* predominated in the consortia for both oxygen conditions. Contrariwise, the consortia encompassing *P. aeruginosa* – *I. limosus* and *P. aeruginosa* – *S. maltophilia* were dominated by the CF-key pathogen *P. aeruginosa*. As such, the endurance of *P. aeruginosa* within the consortia, before and after antibiotic treatment, could be the basis for a higher contribution of this species to the antibiotic resistance presented by dual-species biofilms. However, the increasing survival of *S. maltophilia* and *I. limosus* (slight increase for *I. limosus*) in dual-species consortia with *P. aeruginosa* after antibiotic exposure, for all oxygen atmospheres, indicates that these species may have also a preponderant role in increasing the whole resistance within the consortia.

Data highlighted that emergent species are able to establish polymicrobial consortia with common pathogens, and that multispecies biofilms gain fitness advantages compared with mono-species, modulating and impacting the CF therapeutics.

4.1 INTRODUCTION

The Cystic fibrosis (CF) airways offer a favorable environment for the colonization and proliferation of a large variety of organisms, including bacteria, fungi and viruses, with bacterial species being the ones that are more frequently isolated [1]. However, CF infections were originally characterized as being monomicrobial, likely due to the extensive use of culture-dependent isolation techniques. These techniques allow identifying several key microbial species that contribute to disease progression, beginning early in life with *S. aureus* and *H. influenzae* and culminating in chronic infections caused by *P. aeruginosa* or *B. cepacia complex* species [2,3]. Recent molecular methodologies have documented complex microbial ecosystems in CF samples, with a wide array of uncommon microorganisms co-existing with traditional pathogens, acting collectively to facilitate disease progression [4].

Microbial interactions might exist within CF polymicrobial communities, so it is not surprising that these infections are increasingly viewed as complex communities of interacting organisms, with dynamic processes key to their pathogenicity and contributing to clinical status, disease progression, and resistance to antibiotic therapy.

This study aimed at investigating the susceptibility profiles of single-species and even in dual-species populations encompassing *S. aureus*, *I. limosus* and *S. maltophilia* when associated with the CF-conventional pathogen *P. aeruginosa* growing planktonically and as biofilms under atmospheric conditions resembling CF airways. The polymicrobial assemblages were thoroughly characterized in terms of antibiotic resistance profiles as well as microbial compositions within biofilms and regarding biofilm-cell reductions. Moreover, PNA FISH was employed to better discriminate populations within the consortia, inferring about the population arrangement and prevalence in polymicrobial consortia. Lastly, it was determined if these polymicrobial communities offered enhanced fitness compared to biofilms from the individual species.

4.2 MATERIAL AND METHODS

Bacterial strains and culture conditions

The seven CF-related bacterial species, *P. aeruginosa* (strain UCBPP-PA14), *S. aureus (*ATCC 25923), *A. baumannii (*clinical isolate form sputum), *I. limosus (*isolated strain M53), *D. pigrum* (CIP 104051^T), *K. pneumonia (*clinical isolate form sputum) and *S. maltophilia, (*clinical isolated from CF sputum) were used throughout this work. Dual species-biofilms were between *S. aureus*, *S. maltophilia* and *I. limosus* with the key-pathogen *P. aeruginosa*. Bacteria were preserved and cultured as described in sub-chapter 2.1.

Antibiotics

Ciprofloxacin was used throughout this work for antibiotic susceptibility testing. Stock solutions were prepared at 5120 mg/L and stored according with the manufacturers 'instructions, as described in sub-chapter 2.1.5.

Antibiotic susceptibility testing

Planktonic cells were exposed for 24 h to ciprofloxacin with concentrations ranging from 0.125 to 512 mg/L, as fully described in sub-chapter 2.2.1.3. The minimum inhibitory concentration (MIC) was considered the minimum concentration to which no growth was observed (measured by $OD_{640\ nm} < 0.1$). The values of minimum bactericidal concentration (MBC) were determined by enumerating spot plates for bacterial growth, and corresponded to the minimum antibiotic concentration required to eliminate at least 99.9 % of planktonic bacteria. At least three independent assays were performed in triplicate.

Planktonic time-kill curves

Planktonic cells of single- and dual-species, developed under aerobic and anaerobic environments, were checked by bacterial growth in the presence of increasing concentrations of ciprofloxacin as described in sub-chapter 2.2.1.4. After each 2 h of growth the optical density of the planktonic suspension was recorded at 640 nm, according with the procedure outlined in the same sub-chapter. All tests were run in triplicate in three-independent assays.

Biofilm time-kill curves

*S*ingle- and dual-species biofilm*s* were developed in the presence of increasing concentrations of ciprofloxacin, under aerobic and anaerobic environments as described in sub-chapter 2.3.1.4. The adhesion of bacteria to microtiter plate wells in the presence of ciprofloxacin was determined by cell counting onto TSA, according with the procedure outlined in the same sub-chapter. All tests were run in triplicate in three-independent assays.

Relative distribution of dual-species biofilms

Relative distribution in dual-species biofilms was performed before and after antibiotic exposure under aerobic and anaerobic environments as outlined in sub-chapter 2.3.1.5. The total CFU number within biofilms was determined with TSA. Selective agar media was used for *P. aeruginosa, S. aureus* and *I. limosus* quantification in the dual-species biofilms. The CFU number corresponding to *S. maltophilia* was calculated by the difference between the total CFUs and the CFUs obtained by selective media for other bacteria in the consortia. All tests were run in triplicate in three-independent assays.

Localization and distribution of biofilm populations by PNA FISH

In order to further apply PNA FISH to biofilms, dual-species consortia encompassing *P. aeruginosa* and S. aureus or *I. limosus* or *S. maltophilia* were also formed on polystyrene (PS) coupons under aerobic and anaerobic environments (sub-chapter 2.3.2.3.1). Dual-species biofilms were also performed in the presence of ciprofloxacin concentration corresponding to MIC of P. aeruginosa single-specie. Biofilms were then fixed and hybridized with Paer565 peptide nucleic acid (PNA) probes previously described in sub-chapter 2.3.2.3.2. The PNA FISH procedure is described in sub-chapter 2.3.2.3.2. All tests were run in three-independent assays.

Statistical analysis

Data were analyzed using the Prism software package (GraphPad Software version 6.0 for Macintosh). Otherwise stated, data were expressed as means ± standard deviation (SD). Data were compared by one-way analysis of variance (ANOVA) and subsequent comparisons were performed using Turkey multiple-comparisons test. Differences were considered statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

4.3 RESULTS

4.3.1 Effect of variable oxygen conditions on planktonic and biofilm antimicrobial susceptibilities

Antibiotic susceptibility of planktonic populations

The concentrations of antibiotic able to inhibit planktonic bacteria (Minimum inhibitory concentration, MIC) and those required to kill at least 99,9 % (Minimum bactericidal concentration, MBC) of standard inoculum planktonic cells, are summarised in Table 4-1. Minimum inhibitory concentrations were determined by broth microdilution according to the EUCAST guidelines [5]. The MIC value of the antibiotic against the quality control strain *Pseudomonas aeruginosa* ATCC 27853 was within the accuracy range described by EUCAST through the study [5].

Table 4-1 *In vitro* susceptibility patterns of single-species planktonic cultures for *P. aeruginosa*, *S. aureus, A. baumannii*, *D. pigrum*, *I. limosus*, *K. pneumoniae* and *S. maltophilia*, against ciprofloxacin.

	Ciprofloxacin		
	MIC[a]	**MBC**	**Intrinsic antibiotic resistance[c]**
P. aeruginosa	≤ 0.125	1	S
S. aureus	0.5	0.5	S
A. baumannii	0.25	2	S
D. pigrum	512	4	n/r
I. limosus	≤ 0.125	> 512	n/r
K. pneumoniae	16	> 512	R
S. maltophilia	0.5	1	n/r
P. aeruginosa ATCC 27853[b]	0.25	0.25	S

[a]MIC and MBC values are expressed in mg/L

[b]Quality control strain

[c]S, susceptible; R, resistant and n/r, not reported, acording EUCAST guidelines (www.eucast.org/)

In general, ciprofloxacin was effective in inhibiting planktonic growth of single species at low concentrations. In contrast, the ability of ciprofloxacin to inhibit *D. pigrum* was reduced, requiring abnormal doses (MIC=512 mg/L) to inhibit its planktonic growth. For most bacteria, MBC results revealed that the bactericidal efficacy of ciprofloxacin was achieved with values at least equal than the corresponding MIC values. However, with exception for *D. pigrum*, a great increase in the MBC values was observed in particular for the emergent species *A. baumannii*, *I. limosus*, *K. pneumoniae* and *S. maltophilia*, with values 2 times higher than their respective MICs.

Bacteria are normally categorised as susceptible or resistant to antibiotics though comparison of the MIC to breakpoint concentrations of the antibiotic [6]. Regarding the intrinsic antibiotic resistance profiles, while traditional species are susceptible to ciprofloxacin it seems to be a lack in the clinical breakpoints for majority of the emergent species presented, once EUCAST provides clinical breakpoints only for *A. baumannii* and *K. pneumoniae.* It is important to highlight that for *D. pigrum* and *K. pneumonia*e, MIC values (512 and 16 mg/L, respectively) exceed the maximum serum concentration for ciprofloxacin that is, accordingly with Food and Drug Administration, 4.3 mg/L following a 750 mg dose administration.

Planktonic and biofilm time-kill curves of single-species populations

In the present study, time-kill experiments were performed to assess the activity of ciprofloxacin on planktonic and biofilm growth by CF bacteria, under variable-oxygen conditions. The advantage lies in that killing curves provide a dynamic picture of antimicrobial action against those populations. For this, single-species cultures were grown in the presence of increasing concentrations of the antimicrobial agent (¼ MIC, ½ MIC, MIC, 2 MIC and 4 MIC), incubated under aerobic and anaerobic environments and the growth was followed up until 24 h (Fig. 4-1).
The results revealed that time-kill curves exhibited a dose- and time-dependent pattern, both for planktonic and biofilm populations. In general, there were no significant differences in the susceptibility profiles of all bacteria in variable oxygen conditions; with time-kill curves demonstrating that growth rate (planktonic) and rate of adhesion (biofilm) were equivalent, calculated for the first 8 h, under aerobic and anaerobic atmospheres (supplementary material, Table S4-1 and S4-2). Nonetheless, the results of time-kill curve for planktonic bacteria showed that for both aerobic and anaerobic environments, ciprofloxacin had a rapid and pronounced inhibitory effect (OD_{640} nm < 0.1) for *D. pigrum, I. limosus, S. maltophilia* and *P. aeruginosa,* which was even found with the employment of ¼ MIC of ciprofloxacin. Emergent species *A. baumannii* and *K. pneumoniae* showed increased resistance to ciprofloxacin, with a increasing in OD values over time. To reach the maximum inhibitory effect for this species was necessary abnormal concentrations of ciprofloxacin (with a concentration of ciprofloxacin ≥ 2 MIC), with specific time points of treatment. However, the inhibitory effect was incomplete allowing bacterial regrowth after 24 h incubation, at levels lower that those observed for untreated control. Although ciprofloxacin affected notably *S. aureus* growth for both oxygen conditions, the susceptibility pattern seems notably affected for low oxygen conditions, with only ½ MIC of ciprofloxacin.

Traditionally, the MIC and the MBC as a single parameter *in* vitro are used for the selection of antimicrobial agents to treat planktonic bacterial infections. Therefore, the clinical target of antibiotic treatment for biofilm infections is different from that for infections caused by planktonic cells. As demonstrated by Fig. 4-1, ciprofloxacin was ineffective in eradicating most biofilms, with cultivable cells being slightly disturbed along time for all both oxygen conditions. The results of biofilm time-kill curve analysis showed that *K. pneumoniae* was considerably the most resistance specie to ciprofloxacin, the maximum concentration used was not sufficient to disturb the grow of *K. pneumoniae* causing less that 1-log reduction in total cultivable cells after 24 h, for all environments. In parallel, for both oxygen conditions, ciprofloxacin showed only a slight inhibitory effect for *A. baumannii* and *I. limosus* causing no more that 2-log reduction in total cultivable cells after 24 h. Contrariwise, ciprofloxacin exerted a pronounced antimicrobial activity against *S. aureus*, reducing the number of total cultivable cells in 3-log with a concentration of ciprofloxacin ≥ MIC under aerobic and anaerobic atmospheres.

The dissimilarities in susceptibility patterns of ciprofloxacin related with variable-oxygen atmospheres were found for *S. maltophilia*. In aerobic environments there is a reduction of 4-log in total cultivable cells with ≥ 2 MIC of ciprofloxacin after 24 h. While low oxygen conditions protect this specie from ciprofloxacin action, causing no more that 2-log reduction in total cultivable cells after 24 h of treatment with ≥ 2 MIC. Interestingly, total eradication biofilm cultivable cells it was achieved for *D. pigrum* with at least 24 h of treatment (with a concentration of ciprofloxacin ≥ MIC) or 16 h of treatment with a concentration of ciprofloxacin equal to 4 MIC. Conversely to *S. maltophilia,* low oxygen conditions seems protect *D. pigrum* and to reach the maximum inhibitory effect of ciprofloxacin, treatment was required for 16 h and at least 4 MIC of ciprofloxacin concentration.

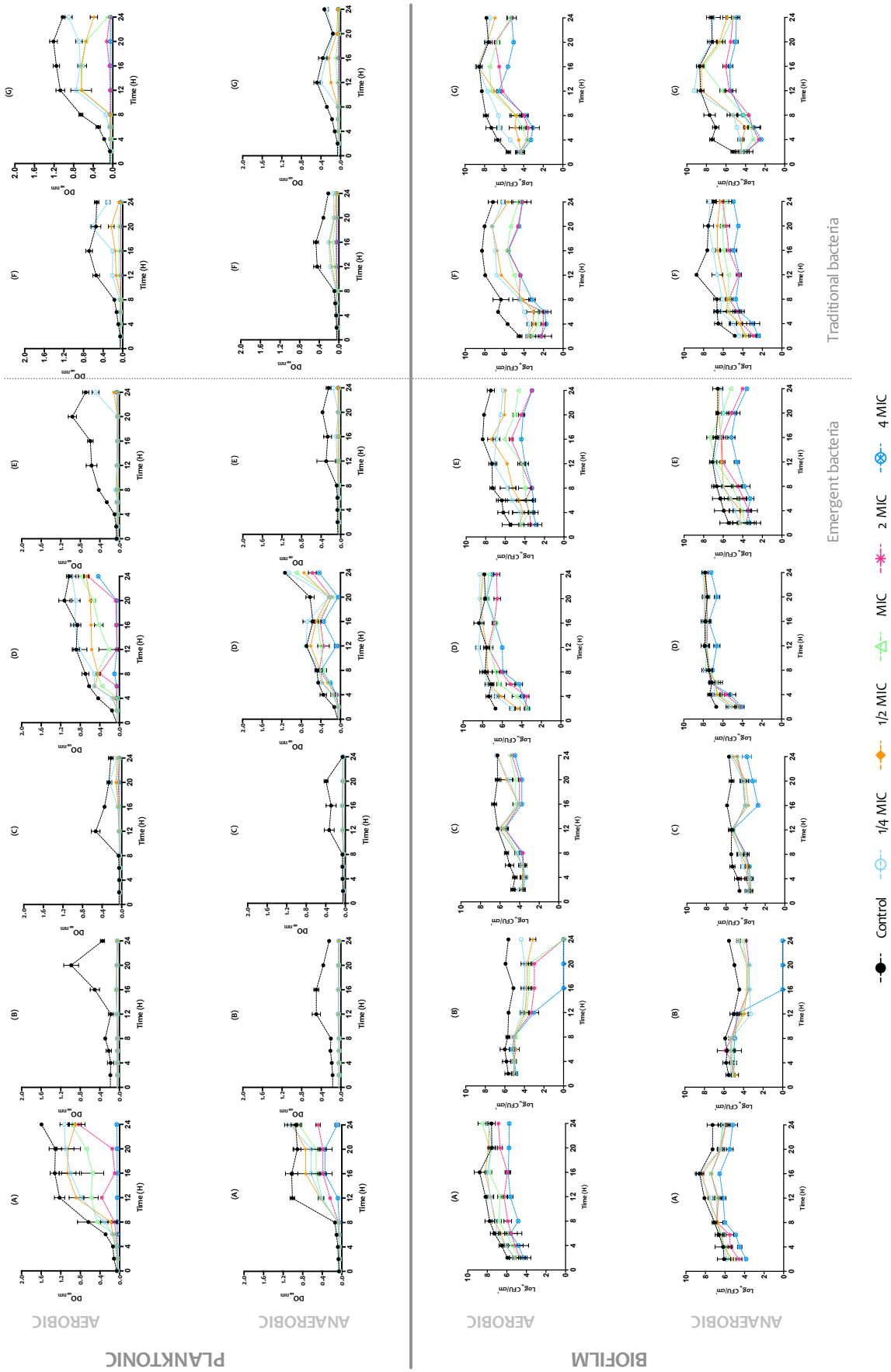

Fig. 4-1 Time-kill curves of planktonic and biofilm single-species obtained for *A. baumannii* (**A**), *D. pigrum* (**B**), *I. limosus* (**C**), *K. pneumoniae* (**D**), *S. maltophilia* (**E**), *P. aeruginosa* (**F**) and *S. aureus* (**G**) growing under aerobic and anaerobic environments. Bars represent means ± standard deviations (SDs) for three independent assays.

4.3.2 Impact of variable oxygen conditions in CF polymicrobial populations

Similarly to the aforementioned experiments, dual-species populations involving *P. aeruginosa* and *S. aureus, I. limosus*, *or S. maltophilia* were grown in the presence of ciprofloxacin concentrations ranging between ¼ MIC and 4 MIC (it was considered the MIC = 0.125 mg/L, previously achieved for P. *aeruginosa* PA14*)*. Fig. 4-2 shows the planktonic time-kill curves (OD_{640} nm per time) for those populations obtained under aerobic and anaerobic environments.

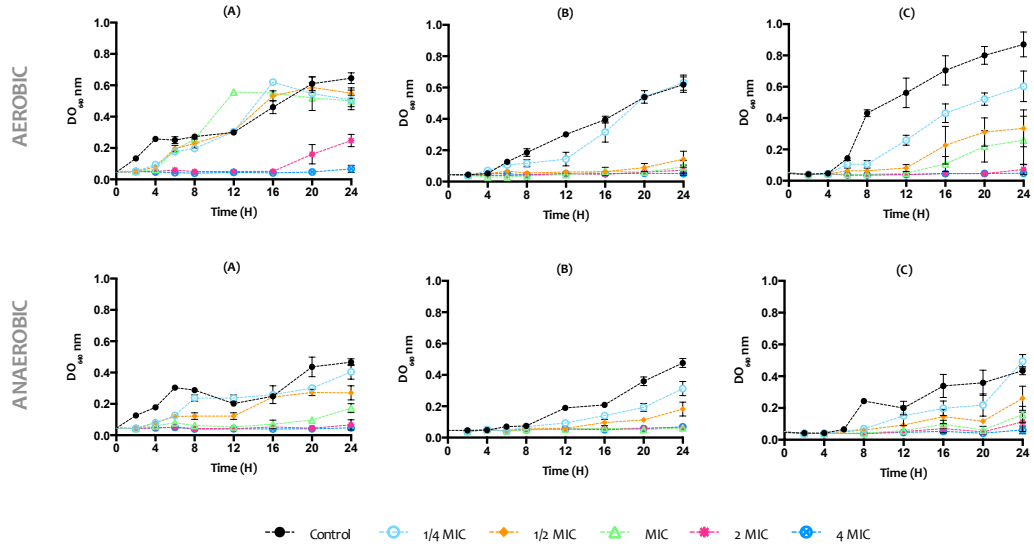

Fig. 4-2 Time-kill curves of planktonic dual-species obtained for *P. aeruginosa* and *S. aureus* **(A)**, *P. aeruginosa* and *I. limosus* **(B)**, *P. aeruginosa* and *S. maltophilia* **(C)** growing under aerobic and anaerobic environments. Bars represent means ± standard deviations (SDs) for three independent assays.

The results revealed that the planktonic populations exhibited a dose- and time-dependent growth pattern. As can be observed, the growth pattern was decreasing in low oxygen conditions for all population. However, the susceptibility profiles were equivalent under both oxygen conditions when compared with the untreated control, with a similar pattern of reduction in OD values. Nonetheless, for dual-species populations formed between *P. aeruginosa – I. limosus* time-kill curve analysis showed that ciprofloxacin was a rapid inhibitory effect (OD_{640} nm < 0.1), being found at the beginning of treatment with only ½ MIC of ciprofloxacin for aerobic conditions. Although ciprofloxacin affected *P. aeruginosa – S. aureus* and *P. aeruginosa – S. maltophilia* growth for both oxygen conditions, the susceptibility pattern showed that was necessary high concentrations of ciprofloxacin (≥ 2 MIC) to notably affect the growth of these consortia (OD_{640} nm < 0.1).

As can be observed, dual-species formed between *P. aeruginosa* and each of the traditional- and emergent-species reached a better susceptibility pattern than *P. aeruginosa* alone. (Fig. 4-1, (F)).

This may suggest that the relationship established between all dual-species, in mixed cultures, results in a synergistic response by *P. aeruginosa.*

After evaluating the susceptibility pattern of ciprofloxacin in planktonic populations, the next step was to evaluate how this antibiotic could impact the biofilm growth of those polymicrobial consortia under different oxygen-available environments (Fig. 4-3).

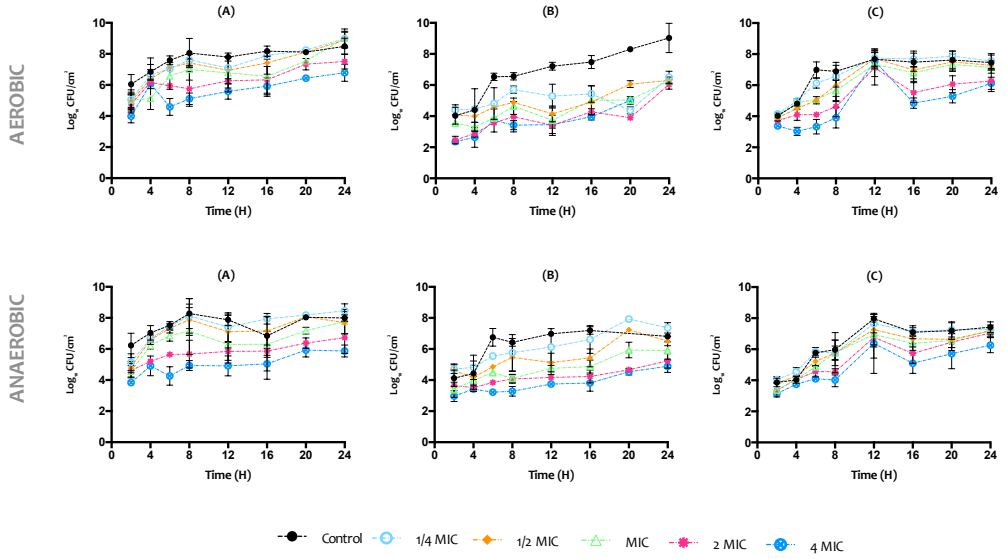

Fig. 4-3 Time-kill curves of biofilm dual-species obtained for *P. aeruginosa* and *S. aureus* **(A)**, *P. aeruginosa* and *I. limosus* **(B)**, *P. aeruginosa* and *S. maltophilia* **(C)** growing under aerobic and anaerobic environments. Bars represent means ± standard deviations (SDs) for three independent assays.

As demonstrated by Fig. 4-3, most biofilm populations follow the same growth tendency even in aerobic and anaerobic environments. As observed for single-species populations, no significant changes were noticed for biofilms growing in the presence of increasing concentrations of antibiotic under aerobic and anaerobic atmospheres (supplementary material, Table S4-3). As expected, the highest antibiotic concentrations display the greatest reductions in biofilm cultivable cells. However, 4 MIC (or 0.5 mg/L) of ciprofloxacin were not enough to obtain a total reduction of biofilm cultivable cells. The best reductions (3 log) were observed for *P. aeruginosa - I. limosus* dual-species consortia (Figure 4-3, B, top row). But comparing these results with *P. aeruginosa* biofilm alone (Fig.4-1, (F)), it can be noticed an increase in the overall cell density (> 1 log) of polymicrobial consortia, which indicates that the presence of other bacteria in the consortia did not alter the susceptibility of the whole consortia. For *P. aeruginosa* single specie the best reduction in total number of cultivable cells, obtained with a concentration of ciprofloxacin equal to 4 MIC, was 3-log for aerobic conditions and 2-log under low oxygen atmosphere.

The results of biofilm time-kill curve analysis showed that *P. aeruginosa – S. aureus* and *P. aeruginosa – S. maltophilia* were the most resistant consortia, the maximum concentration of

ciprofloxacin used (0.5 mg/L) was not sufficient to disturb their grow causing at most 2-log reduction in cultivable cells after 24 h, for both oxygen conditions.

Discrimination of bacterial populations within biofilms with culture techniques and PNA FISH

In order to investigate which species were prevailing in the biofilm consortia during antibiotic exposure, the microbial composition of the polymicrobial biofilms was evaluated by determining CFU number for each bacterial population (using selective agar media and the strategy described in Chapter 2 for *S. maltophilia*) at each time point (Fig. 4-4).

Before antibiotic treatment the consortia were equally distributed by both bacterial populations, with exception of dual-species biofilms formed by *P. aeruginosa* and *S. maltophilia* (Fig. 4-4C) where the latest species represented the smallest representative population. It is of great interest to observe a gradual increasing in the overall proportion of *P. aeruginosa* over time. In general, biofilm compositions changed in result of antibiotic treatment, with alterations being dependent on the antibiotic concentration and oxygen conditions implemented. Results showed that for *P. aeruginosa* and *S. aureus* consortia (Fig. 4-4A), the latter specie predominated the consortia under aerobic and anaerobic conditions. Moreover, in initial time points of treatment, under high oxygen conditions, the results indicated a complete eradication of *P. aeruginosa* cultivable cells, however after 6 h of growth *P. aeruginosa* achieved *S. aureus* cell density. The absence of *P. aeruginosa* cultivable cells may have related with the inhibition ability of ciprofloxacin, once these antibiotic is targeted to the treatment of *P. aeruginosa*, allowing that *S. aureus* cultivable cells take advantage dominating the whole consortia. For biofilms encompassing *P. aeruginosa* and *I. limosus* (Fig. 4-4B), before ciprofloxacin exposure the consortium was dominated for the CF-key pathogen *P. aeruginosa*. However, after antibiotic treatment *I. limosus* was able to survive towards antibiotics and inhabiting nearly half portion of the overall consortia, in conjunction with *P. aeruginosa* for both oxygen conditions. Regarding the microbial composition for the dual-species biofilms formed between *P. aeruginosa* and *S. maltophilia* (Fig. 4-4C), a clear dominance of the consortia was attained for *P. aeruginosa*, with an increasing in *S. maltophilia* proportion after antibiotic exposure. Furthermore, after antibiotic exposure the proportion of *S. maltophilia* is higher under aerobic conditions than anaerobiosis.

In addition, both traditional and emergent populations within these biofilms were resistant to ciprofloxacin, suggesting that antibiotics often used to treat *P. aeruginosa* infections in CF fail to eradicate these bacteria in the whole consortia.

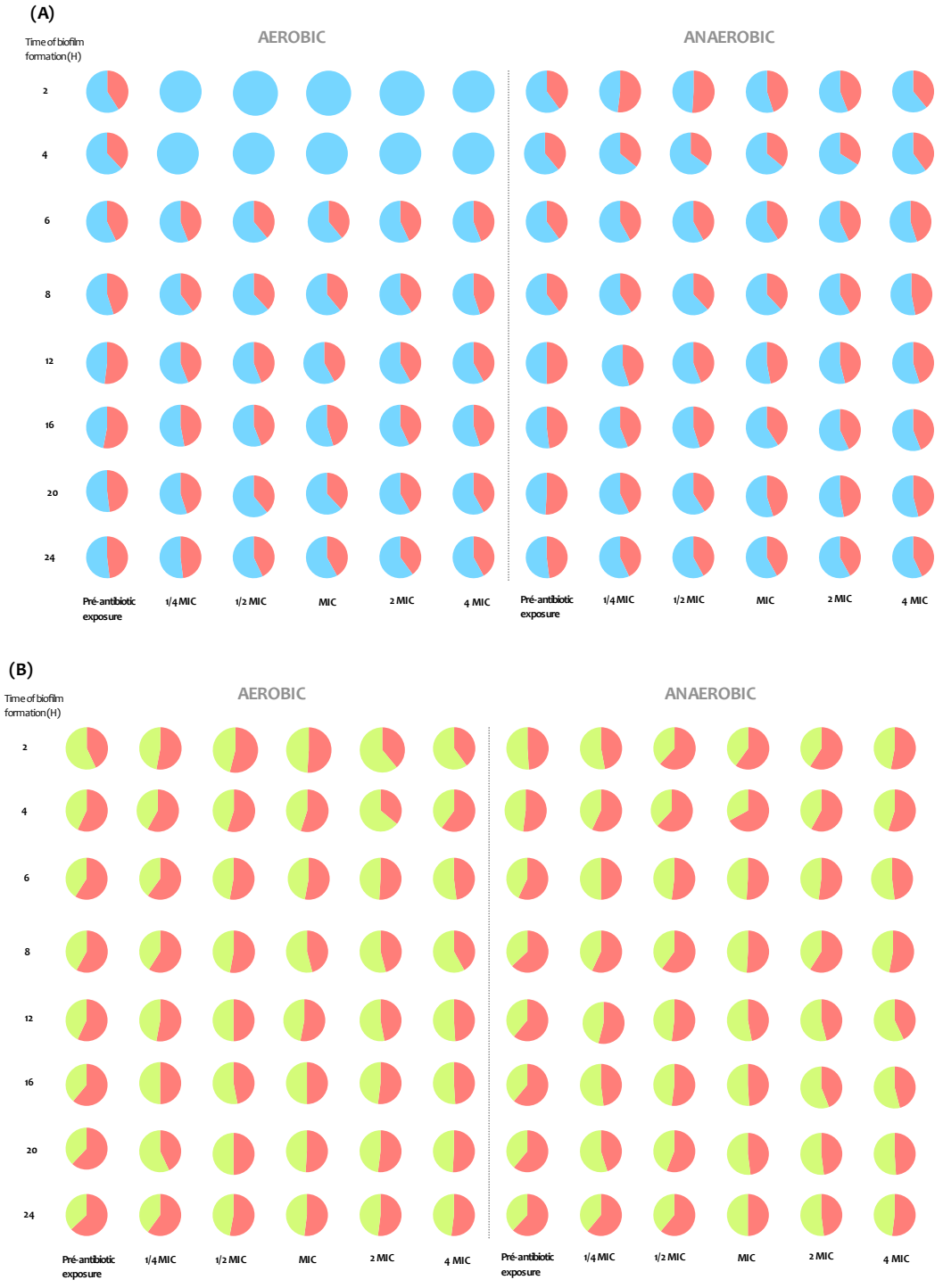

Fig. 4-4 Relative distributions of *P. aeruginosa* (salmon), *S. aureus* (blue), *I. limosus* (green) and *S. maltophilia* (yellow) within polymicrobial consortia involving *P. aeruginosa* and *S. aureus* **(A)**, *P. aeruginosa* and *I. limosus* **(B)** and *P. aeruginosa* and *S. maltophilia* **(C)** before and after exposure to antibiotics at ¼ MIC, ½ MIC, MIC, 2 MIC and 4 MIC (according with *P. aeruginosa* susceptibility), under aerobic and anaerobic environments.

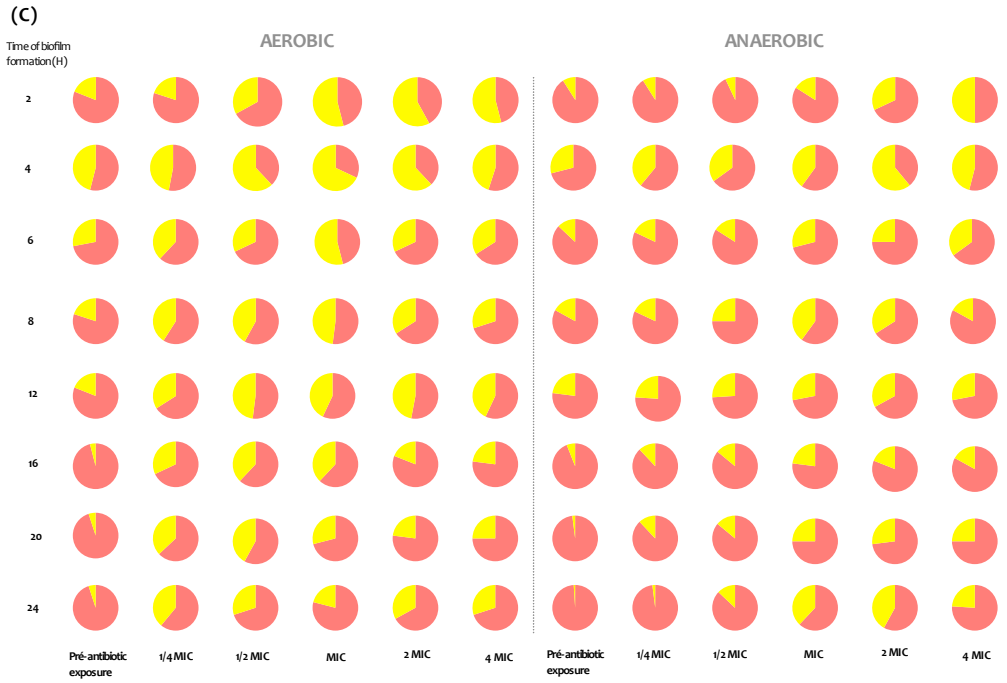

Fig. 4-4 Continued.

The aforementioned dual-species biofilms were then analyzed by PNA FISH methodology counterstained with DAPI to directly visualize the location and distribution of bacterial populations before (Fig.4-5) and after antibiotic treatment (Fig. 4-6). All bacterial species could be easily distinguished with distinct cell morphologies by the PNA FISH multiplex experiment. The strength of each fluorescent signal allowed distinguishing *P. aeruginosa* (red rod cells) of *S. aureus* (blue cocci cells), *S. maltophilia* (blue bacillus cells) and *I. limosus* (blue rod cells) within the polymicrobial consortia.

When *S. aureus* is present in the consortia, it occupies a significant extent within the overall biofilms together with *P. aeruginosa* populations (Fig. 4-5, column 1). This was visible even after treatment with ciprofloxacin (at 0.125 mg/L), with both populations equally distributed (Fig. 4-6, column 1). Dual-species biofilms involving *P. aeruginosa* and *I. limosus* were composed by both species, although with *P. aeruginosa* populations being significantly more perceptible under the different atmospheres (Fig 4-5, column 2). In agreement with cell counts determined by culture techniques, after treatment with ciprofloxacin the dual consortia, seems to be composed by similar proportions of both populations (Fig 4-6, column 2), although under low-oxygen atmospheres a reduction in total cell numbers could be detected. Dual-species biofilms formed between *P. aeruginosa* and *S. maltophilia* seems to be distributed by both species (Fig. 4-6, column 3). The abundance of *P. aeruginosa* compared with *S. maltophilia* demonstrated by CFU counting was not fully perceptible by PNA FISH.

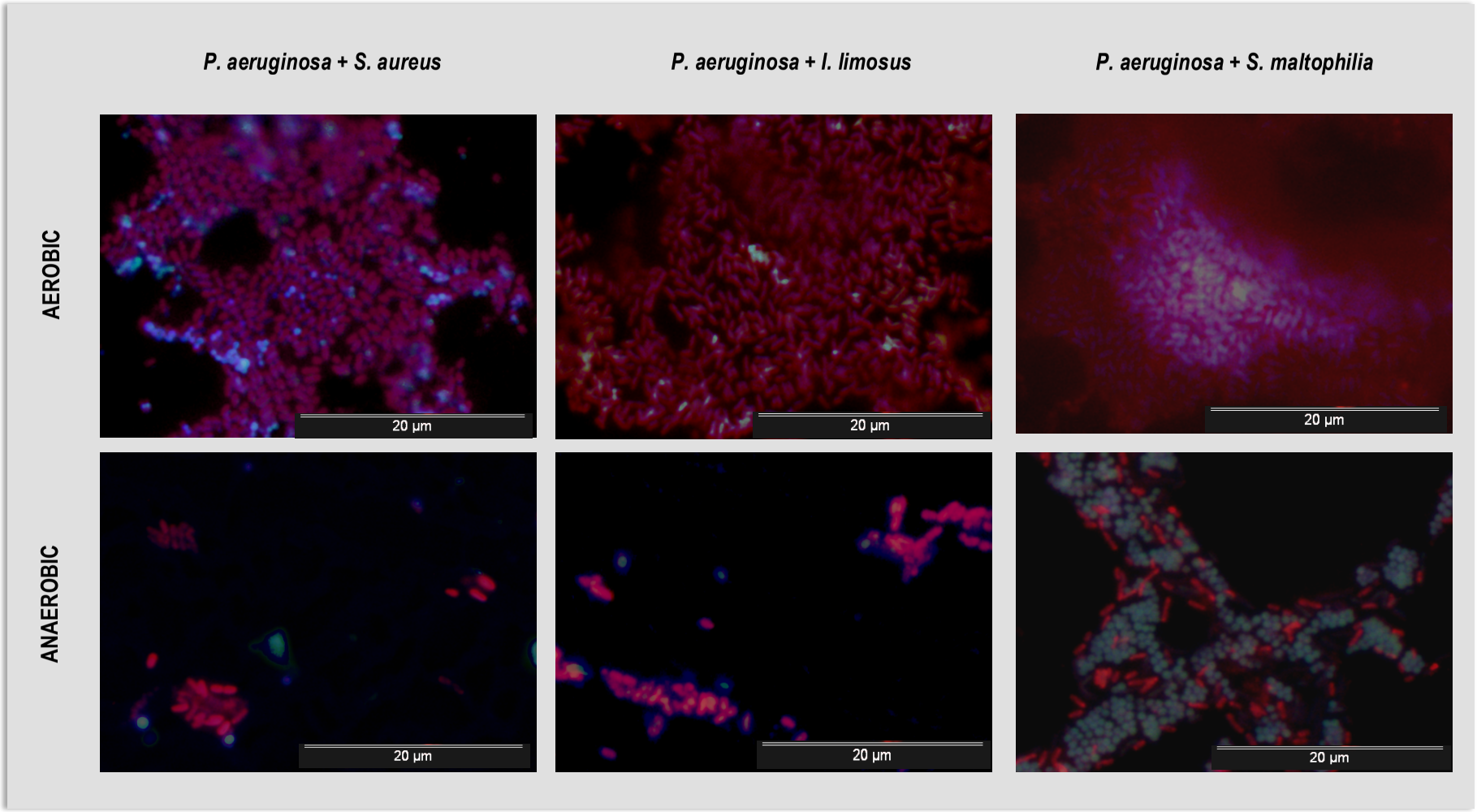

Fig. 4-5 Multiplex PNA-FISH applied to dual-species biofilms involving *P. aeruginosa* (red rod cells), *S. aureus* (blue cocci cells), *I. limosus* (blue rod cells) and *S. maltophilia* blue bacillus cells) grown for 24 h at aerobic and anaerobic conditions. Images resulted from the bands superposition of the two channels used to visualize the fluorochromes used (Alexa fluor 594, red: *P. aeruginosa*; DAPI, blue: *S. aureus, I. limosus and S. maltohilia*).

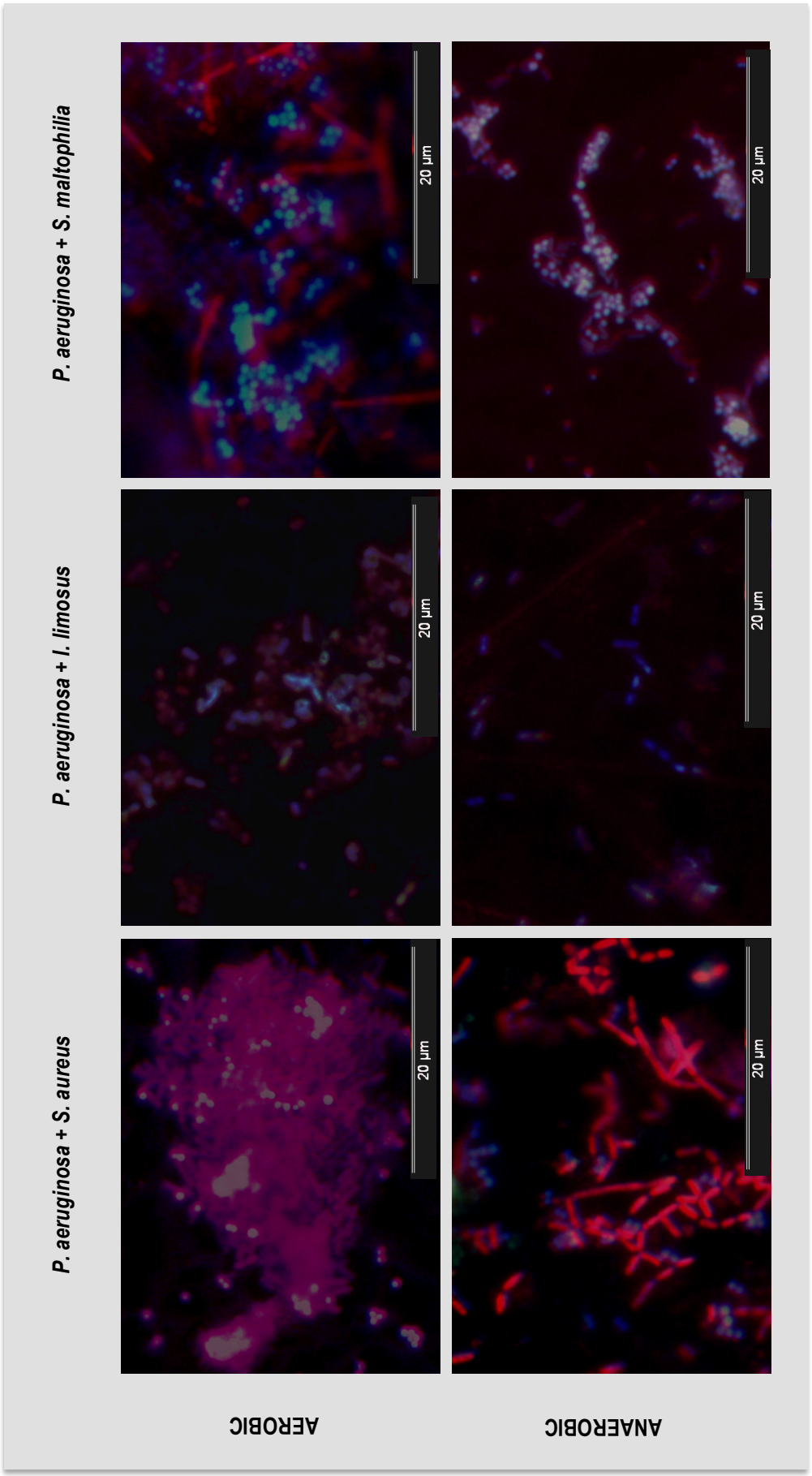

Fig. 4-6 Multiplex PNA-FISH applied to dual-species biofilms involving *P. aeruginosa* (red rod cells), *S. aureus* (blue cocci cells), *I. limosus* (blue rod cells) and *S. maltophilia* blue bacillus cells) grown for 24 h at aerobic and anaerobic conditions, in the present of ciprofloxacin (0,125 mg/L, MIC according with *P. aeruginosa* susceptibility) . Images resulted from the bands superposition of the two channels used to visualize the fluorochromes used (Alexa fluor 594, red: *P. aeruginosa*; DAPI, blue: *S. aureus, I. limosus and S. maltohilia*).

4.4 DISCUSSION

This study aimed to appraise the antibiotic susceptibility of traditional and emergent bacteria associated to CF and characterize polymicrobial communities formed between these bacteria under variable oxygen conditions, similar to those locally found in CF airways *in vivo*.

There is currently a lack of detail within the literature regarding the antimicrobial susceptibility of emergent bacteria in aerobic and anaerobic environments. The impact of these potential emerging pathogens on morbidity and mortality remains under study, demonstrated only their resistance patterns under standard aerobic environments [7–11], failing to consider the role of anaerobiosis and polymicrobial infections in CF.

Antibiotic therapy in CF patients generally targets only a limited number of microorganisms, in particular the major pathogen *P. aeruginosa* [12–14], disregarding the impact of emergent species that are actually present. Aminoglycosides (*e.g.* tobramycin), fluorquinolones (*e.g.* ciprofloxacin), monobactams (*e.g* aztreonam) and polymixins (*e.g.* colistin) are often used to control pulmonary infections caused by *P. aeruginosa* in CF patients [15,16]. In this study, the susceptibilities of *P. aeruginosa, S. aureus, A. baumannii*, *D. pigrum*, *I. limosus*, *K. pneumoniae* and *S. maltophilia* against ciprofloxacin, were addressed and compared. In general, planktonic bacteria could be inhibited at low concentrations of ciprofloxacin, with the exception of *D. pigrum* and *K. pneumonia*, which were only disturbed by abnormal doses (512 and 16 mg/L, respectively). Additionally, only with high concentrations of ciprofloxacin was possible to inhibit bacterial growth in solid agar medium, which MBCs being greater than MICs. This result suggests a resistance propensity for most species towards this antibiotic. However, this situation was not observed for *D. pigrum*, which presented a MBC lower than its MIC. This unusual result may be explained by the reduced cultivability of this bacterium on solid medium, already demonstrated before by Lopes et al. [17].

According with the recommended EUCAST standard guidelines, antibiotics are applied to a planktonic culture under conditions that do not mirror well what occurs in *in vivo* CF infections. These methods are generally straightforward to complete but they do not accurately simulate the polymicrobial and chronically infected lung environment of CF where communities of bacteria might grow as a biofilm [18]. The biofilm mode of growth is the main reason for the failure of antibiotic treatment to eradicate airway infection, allowing the bacteria to persist for decades in the CF lung [19]. Bacteria growing in biofilms are embedded in a matrix of exopolysacaccharide, DNA, and protein and have been shown to be much more resistant to antibiotics than organisms growing planktonically [20]. Wu and colleagues [21] showed that when *S. maltophilia* was grown

as a biofilm, significantly fewer isolates were susceptible to several antibiotics including β-lactams and fluoroquinolones than when they were grown planktonically. Another biofilm-specific resistance mechanism was discovered by Zhang and Mah [22] who reported that the specific genes in *P. aeruginosa* encode a multi-drug efflux pumps that is expressed only during the biofilm mode of growth, and mediates tolerance to many antibiotics, including to ciprofloxacin. Therefore, the traditional antibiotic regimens based on the MIC and MBC are problematic for treatment of biofilm infections [23,24], suggesting that the strategies of antimicrobial therapy should be based on the parameters of biofilm susceptibility assay. Comparing to the parameters of MIC and MBC, minimal biofilm inhibitory concentration (MBIC) and minimal biofilm eradication concentration (MBEC) are more appropriate parameters to follow in treating biofilm infections, as shown *in vitro* and *in vivo* animal studies [25–27]. However, MBEC of antibiotics are difficult to reach *in vivo* due to the side effects and toxicity of the antibiotics [27]. Furthermore, these values only provide a still photo of the effect of an antibiotic at a single concentration value, which is regarded as an "all or nothing" threshold value. Contrariwise, time-kill curves can follow microbial killing and growth as a function of both time and antibiotic concentration, elucidating the dynamic relationship between bacteria and antibiotic [28].

In the present study, time-kill experiments were used to assess the activity of ciprofloxacin on the planktonic and biofilm mode of growth of traditional and emergent single-species bacteria, under variable-oxygen atmospheres. As expected, the results showed that, independently of the atmosphere implemented, bacteria growing in biofilms were notoriously more difficult to eradicate that when growing planktonically. In fact, biofilm tolerance is thought to be multifactorial, resulting by (i) decreased growth rates, resultant by the oxygen and nutrient microscale heterogeneities within the biofilm; (ii) the protective barrier provided by the exopolysaccaride matrix (EPM), retarding or inactivating the penetration of antibiotics into the biofilm; (iii) the number and spatial distribution of bacterial cells within biofilms; (iv) the expression of biofilm-specific resistance genes; (vi) the presence of "persisters", *i.e.* a subpopulation of microorganisms that differentiate into a dormant and protected state, like a spore-bacterial form [29,30].

In the airways of patients with CF, antibiotic treatment is an important selective pressure that influences the adaptation and evolution of *P. aeruginosa.* As a result of this adaptation, the bacteria persist for long periods of time, undergoing up to 200 000 generations in the CF lung [31]. Long-term persistence of bacteria in the presence of antibiotics might not only result from expression or acquisition of genetic mechanisms of resistance but also be affected by the microenvironment that exists and develops in cystic fibrosis airways [18]. The anaerobic

conditions inside the thick CF mucus might also decrease the efficacy of fluoroquinolones, β-lactams and aminoglycosides antibiotics than for aerobically grown isolates [29,32]. In here, it was shown that for *S. maltophilia* biofilms there was a decrease in the efficacy of ciprofloxacin under anaerobic conditions. These results corroborate with previous studies, where anaerobic conditions led cells residing in the biofilms (the "persisters") to rapidly decrease their metabolic activity, leading to an increase in antibiotic tolerance of the whole consortia [23,33,34]. In general, all species growing in a biofilm demonstrated sharply increased resistance to ciprofloxacin, which strongly suggest that these organisms and eventually other unusual species might have a great importance in the outcome and treatment of infection in CF.

Dynamic compositional changes within microbial populations which are dependent from the environmental heterogeneity conditions found in CF [35], as well as social interactions between microorganisms within polymicrobial communities should not be dismissed. This ecological perspective is believed to have important impact for CF therapeutics, offering the prospect of novel approaches to antibiotic treatment. As such, there is a need to fundamentally address this microbe-microbe interplay within a given ecosystem, which may ultimately determine the properties and behaviors of the overall consortia. In light of these circumstances, previous data obtained under aerobic and anaerobic conditions (provided in Chapter 3) had already supported the hypotheses about a crucial role of emergent species in CF infections; with emergent species interacting positively with *P. aeruginosa*.

In this study it was demonstrated that ciprofloxacin presented a poor activity against dual-species biofilms of *P. aeruginosa* with an emergent species, with the implemented doses not altering the behaviour and even inhibiting of the growth of these overall consortia. This result was observed in both aerobic and anaerobic conditions. The results obtained by selective media, showing the endurance of *P. aeruginosa* within the consortia, before and after antibiotic treatment, could be the basis for a higher contribution of *P. aeruginosa* to the antibiotic resistances presented by dual-species biofilms. Additionally, for *P. aeruginosa* - *S. maltophilia* and *P. aeruginosa* – *I. limosus* dual-species consortia the increased survival of *S. maltophilia* and *I. limosus* (although slightly to I. limosus) populations within the biofilm after application of increasing doses of antibiotics indicates that theses species are potentially the organism conferring resistance to the whole biofilm under all oxygen environments, once the sensitive to ciprofloxacin in mono-species seems to be the same. In chapter 3 was shown that the reduced biofilm biomass formed in dual-species biofilms could decrease the restriction of such consortia against antibiotherapy. The arrangement or even the high number of biofilm-encased cells in the overall biofilms could be eventually

enough to imply an increased resistance on the whole consortia [36,37] once the performance of dual consortia was equal or better when compared with *P. aeruginosa* single specie. The results obtained with PNA FISH allowed complementing those obtained by the culture techniques. It was noticed that both *P. aeruginosa* - *S. aureus* and *P. aeruginosa* - *I. limosus* consortia were equally distributed in terms of microbial populations before and even after antibiotic exposure. This result, together which corroborates with a large number of earlier studies that have shown the abundance of this species in the clinical samples recovered from CF patients [38–40]. In fact, in dual-species biofilms all species were present in a great extension and in close proximity with *P. aeruginosa* after antibiotic exposure. An exception was observed for *P. aeruginosa* - *S. maltophilia* biofilm, where culture techniques have been shown preponderance of *P. aeruginosa* in comparison with *S. maltophilia*. This result indicates eventually the lack of sensitivity of these routine techniques in identifying bacteria difficult to grow on solid medium.

In order to evaluate whether living in polymicrobial consortia may reap benefits to the bacteria, the fitness of polymicrobial biofilms were compared to mono-species biofilms by each individual species. In effect, *P. aeruginosa* growing associated with *S. aureus, I. limosus* or *S. maltophilia*, had similar or increased resistance to ciprofloxacin. Thus, the CF-related bacteria - *S. aureus* and the emergent species *I. limosus* and *S. maltophilia* - are able to exert synergistic relationships with *P. aeruginosa*, leading to polymicrobial biofilms with increased performances than *P. aeruginosa* biofilms growing alone. Synergistic interactions between bacteria may confer a fitness advantage to bacterial communities from residing in multispecies biofilms, throughout an increased virulence and pathogenicity associated to these consortia [41–44]. Previous studies have reported synergistic interactions between these bacterial species, which support our thinking. For example, Lopes et al. [17] shown that the emergent CF species *I. limosus* can grow together with *P. aeruginosa*, increasing the tolerance of the overall consortia to a wide range of antibiotics. Twomey and colleagues [44] presented several lines of evidence supporting the contention that diffusible signal factor (DSF) family contributes to *P. aeruginosa* persistence and antibiotic resistance in CF lung infection. DSF is found at physiologically relevant levels in CF sputum, where this presence is correlated with *S. maltophilia* colonization. This study provides evidence that interspecies DSF-mediated bacterial interactions occur in the CF lung and may influence the efficacy of antibiotic treatment. In addition, some studies [45,46] underline that the interactions between the CF typically bacteria *P. aeruginosa* and *S. aureus* allows to reap benefits in ability to *S. aureus* biofilm formation, which may benefit the overall consortia. Although it is believed that the bacterial interplay in those biofilms is made via quorum-sensing (QS) [47,48] ,a bacterial cell-

to-cell communication process that involves the production, detection, and response to extracellular signaling molecules called autoinducers (AIs) [49], more intensive work is indispensable to understand the full mechanisms behind the microbial interplay.

All together the results demonstrated that emergent- and traditional-species are able to live in association with key-CF pathogen *P. aeruginosa* commonly found in CF airways under variable oxygen atmospheres, developing highly resilient consortia towards antibiotic treatment. Additionally, these microbial-microbial interactions with CF related species might have great implications by changing the clinical course of the disease. As such, disregard the interactions between all bacteria may lead to ineffective antibiotic therapeutic strategies that could select for antibiotic-resistant pathogens. Additionally, these experiments was performed *in vitro* which is a limitation in that the bacteria were allowed to adhere to polystyrene microtiter plate wells rather than airway epithelium, where a number of host-derived and bacterial-specific factors, which may include increased levels of DNA and actin that contribute colonization sites and/or biofilm matrix components, mucus production, specific receptors on the epithelial surface, contribute for bacterial adherence and invasion [50].

Our results highlight the importance for an adjustment to the actual therapeutic strategies, which are majority focused on conventional pathogens, is necessary in face of the complex bacterial multiplicity and the high resistant patterns associated to other than conventional organisms found in CF airways.

4.5 REFERENCES

1. Guss AM, Roeselers G, Newton ILG, Young CR, Klepac-Ceraj V, Lory S, Cavanaugh CM: **Phylogenetic and metabolic diversity of bacteria associated with cystic fibrosis.** *ISME J.* 2011, **5**:20–9.

2. Price KE, Hampton TH, Gifford AH, Dolben EL, Hogan DA, Morrison HG, Sogin ML, O'Toole GA: **Unique microbial communities persist in individual cystic fibrosis patients throughout a clinical exacerbation.** *Microbiome* 2013, **1**:27.

3. Razvi S, Quittell L, Sewall A, Quinton H, Marshall B, Saiman L: **Respiratory microbiology of patients with cystic fibrosis in the United States, 1995 to 2005.** *Chest* 2009, **136**:1554–60.

4. Peters BM, Jabra-Rizk MA, O'May G a., Costerton JW, Shirtliff ME, William Costerton J, Shirtliff ME: **Polymicrobial interactions: Impact on pathogenesis and human disease**. *Clin. Microbiol. Rev.* 2012, **25**:193–213.

5. EUCAST: **Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution**. *Clin. Microbiol. Infect.* 2003, **9**:ix–xv.

6. MacGowan AP, Wise R: **Establishing MIC breakpoints and the interpretation of in vitro susceptibility tests.** *J. Antimicrob. Chemother.* 2001, **48 Suppl 1**:17–28.

7. Chiron R, Marchandin H, Counil F, Jumas-Bilak E, Freydière A-M, Bellon G, Husson M-O, Turck D, Brémont F, Chabanon G, et al.: **Clinical and microbiological features of Inquilinus sp. isolates from five patients with cystic fibrosis.** *J. Clin. Microbiol.* 2005, **43**:3938–43.

8. Laclaire L, Facklam R: **Antimicrobial susceptibility and clinical sources of Dolosigranulum pigrum cultures.** *Antimicrob. Agents Chemother.* 2000, **44**:2001–3.

9. Pompilio A, Crocetta V, Scocchi M, Pomponio S, Di Vincenzo V, Mardirossian M, Gherardi G, Fiscarelli E, Dicuonzo G, Gennaro R, et al.: **Potential novel therapeutic strategies in cystic fibrosis: antimicrobial and anti-biofilm activity of natural and designed α-helical peptides against Staphylococcus aureus, Pseudomonas aeruginosa, and Stenotrophomonas maltophilia.** *BMC Microbiol.* 2012, **12**:145.

10. Vidaillac C, Benichou L, Duval RE: **In vitro synergy of colistin combinations against colistin-resistant Acinetobacter baumannii, Pseudomonas aeruginosa, and Klebsiella pneumoniae isolates.** *Antimicrob. Agents Chemother.* 2012, **56**:4856–61.

11. Saiman L, Chen Y, Gabriel PS, Knirsch C: **Synergistic activities of macrolide antibiotics against Pseudomonas aeruginosa, Burkholderia cepacia, Stenotrophomonas maltophilia, and Alcaligenes xylosoxidans isolated from patients with cystic fibrosis.** *Antimicrob. Agents Chemother.* 2002, **46**:1105–7.

12. Döring G, Flume P, Heijerman H, Elborn JS: **Treatment of lung infection in patients with cystic fibrosis: current and future strategies.** *J. Cyst. Fibros.* 2012, **11**:461–79.

13. Döring G, Hoiby N: **Early intervention and prevention of lung disease in cystic fibrosis: a European consensus.** *J. Cyst. Fibros.* 2004, **3**:67–91.

14. Flume PA, O'Sullivan BP, Robinson KA, Goss CH, Mogayzel PJ, Willey-Courand DB, Bujan J, Finder J, Lester M, Quittell L, et al.: **Cystic fibrosis pulmonary guidelines: chronic medications for maintenance of lung health.** *Am. J. Respir. Crit. Care Med.* 2007, **176**:957–69.

15. Ciofu O, Mandsberg LF, Wang H, Høiby N: **Phenotypes selected during chronic lung infection in cystic fibrosis patients: implications for the treatment of Pseudomonas aeruginosa biofilm infections.** *FEMS Immunol. Med. Microbiol.* 2012, **65**:215–25.

16. Rogers GB, Hoffman LR, Whiteley M, Daniels TW V, Carroll MP, Bruce KD: **Revealing the dynamics of polymicrobial infections: implications for antibiotic therapy.** *Trends Microbiol.* 2010, **18**:357–64.

17. Lopes SP, Azevedo NF, Pereira MO: **Emergent bacteria in cystic fibrosis: in vitro biofilm formation and resilience under variable oxygen conditions.** *Biomed Res. Int.* 2014, **2014**:678301.

18. Sherrard LJ, Tunney MM, Elborn JS: **Antimicrobial resistance in the respiratory microbiota of people with cystic fibrosis.** *Lancet* 2014, **384**:703–13.

19. Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Høiby N, Molin S: **Adaptation of Pseudomonas aeruginosa to the cystic fibrosis airway: an evolutionary perspective.** *Nat. Rev. Microbiol.* 2012, **10**:841–51.

20. Moskowitz SM, Foster JM, Emerson J, Burns JL: **Clinically feasible biofilm susceptibility assay for isolates of Pseudomonas aeruginosa from patients with cystic fibrosis.** *J. Clin. Microbiol.* 2004, **42**:1915–22.

21. Wu K, Yau YCW, Matukas L, Waters V: **Biofilm compared to conventional antimicrobial susceptibility of Stenotrophomonas maltophilia Isolates from cystic fibrosis patients.** *Antimicrob. Agents Chemother.* 2013, **57**:1546–8.

22. Zhang L, Mah T-F: **Involvement of a novel efflux system in biofilm-specific resistance to antibiotics.** *J. Bacteriol.* 2008, **190**:4447–52.

23. Werner E, Roe F, Bugnicourt A, Franklin MJ, Heydorn A, Molin S, Pitts B, Stewart PS: **Stratified growth in Pseudomonas aeruginosa biofilms.** *Appl. Environ. Microbiol.* 2004, **70**:6188–96.

24. Høiby N, Krogh Johansen H, Moser C, Song Z, Ciofu O, Kharazmi A: **Pseudomonas aeruginosa and the in vitro and in vivo biofilm mode of growth.** *Microbes Infect.* 2001, **3**:23–35.

25. Hengzhuang W, Ciofu O, Yang L, Wu H, Song Z, Oliver A, Høiby N: **High β-lactamase levels change the pharmacodynamics of β-lactam antibiotics in Pseudomonas aeruginosa biofilms.** *Antimicrob. Agents Chemother.* 2013, **57**:196–204.

26. Hengzhuang W, Wu H, Ciofu O, Song Z, Høiby N: **In vivo pharmacokinetics/pharmacodynamics of colistin and imipenem in Pseudomonas aeruginosa biofilm infection.** *Antimicrob. Agents Chemother.* 2012, **56**:2683–90.

27. Hengzhuang W, Wu H, Ciofu O, Song Z, Høiby N: **Pharmacokinetics/pharmacodynamics of colistin and imipenem on mucoid and nonmucoid Pseudomonas aeruginosa biofilms.** *Antimicrob. Agents Chemother.* 2011, **55**:4469–74.

28. Mueller M, de la Peña A, Derendorf H: **Issues in pharmacokinetics and pharmacodynamics of anti-infective agents: kill curves versus MIC.** *Antimicrob. Agents Chemother.* 2004, **48**:369–77.

29. Hill D, Rose B, Pajkos A, Robinson M, Bye P, Bell S, Elkins M, Thompson B, Macleod C, Aaron SD, et al.: **Antibiotic susceptabilities of Pseudomonas aeruginosa isolates derived from patients with cystic fibrosis under aerobic, anaerobic, and biofilm conditions.** *J. Clin. Microbiol.* 2005, **43**:5085–90.

30. Hassett DJ, Korfhagen TR, Irvin RT, Schurr MJ, Sauer K, Lau GW, Sutton MD, Yu H, Hoiby N: **Pseudomonas aeruginosa biofilm infections in cystic fibrosis: insights into pathogenic processes and treatment strategies.** *Expert Opin. Ther. Targets* 2010, **14**:117–30.

31. Yang L, Jelsbak L, Marvig RL, Damkiær S, Workman CT, Rau MH, Hansen SK, Folkesson A, Johansen HK, Ciofu O, et al.: **Evolutionary dynamics of bacteria in a human host environment.** *Proc. Natl. Acad. Sci. U. S. A.* 2011, **108**:7481–6.

32. Kohanski MA, Dwyer DJ, Collins JJ: **How antibiotics kill bacteria: from targets to networks.** *Nat. Rev. Microbiol.* 2010, **8**:423–35.

33. Yang L, Haagensen JAJ, Jelsbak L, Johansen HK, Sternberg C, Høiby N, Molin S: **In situ growth rates and biofilm development of Pseudomonas aeruginosa populations in chronic lung infections.** *J. Bacteriol.* 2008, **190**:2767–76.

34. Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K: **Persister cells and tolerance to antimicrobials.** *FEMS Microbiol. Lett.* 2004, **230**:13–8.

35. Hauser AR, Jain M, Bar-Meir M, McColley SA: **Clinical significance of microbial infection and adaptation in cystic fibrosis.** *Clin. Microbiol. Rev.* 2011, **24**:29–70.

36. Lopes SP, Ceri H, Azevedo NF, Pereira MO: **Antibiotic resistance of mixed biofilms in cystic fibrosis: impact of emerging microorganisms on treatment of infection.** *Int. J. Antimicrob. Agents* 2012, **40**:260–3.

37. Mah TF, O'Toole GA: **Mechanisms of biofilm resistance to antimicrobial agents.** *Trends Microbiol.* 2001, **9**:34–9.

38. Bjarnsholt T, Jensen PØ, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, Pressler T, Givskov M, Høiby N: **Pseudomonas aeruginosa biofilms in the respiratory tract of cystic fibrosis patients.** *Pediatr. Pulmonol.* 2009, **44**:547–58.

39. Valenza G, Tappe D, Turnwald D, Frosch M, König C, Hebestreit H, Abele-Horn M: **Prevalence and antimicrobial susceptibility of microorganisms isolated from sputa of patients with cystic fibrosis.** *J. Cyst. Fibros.* 2008, **7**:123–7.

40. Rudkjøbing VB, Thomsen TR, Alhede M, Kragh KN, Nielsen PH, Johansen UR, Givskov M, Høiby N, Bjarnsholt T: **True microbiota involved in chronic lung infection of cystic fibrosis patients found by culturing and 16S rRNA gene analysis.** *J. Clin. Microbiol.* 2011, **49**:4352–5.

41. Dalton T, Dowd SE, Wolcott RD, Sun Y, Watters C, Griswold JA, Rumbaugh KP: **An In Vivo Polymicrobial Biofilm Wound Infection Model to Study Interspecies Interactions**. *PLoS One* 2011, **6**:e27317.

42. Bragonzi A, Farulla I, Paroni M, Twomey KB, Pirone L, Lorè NI, Bianconi I, Dalmastri C, Ryan RP, Bevivino A: **Modelling Co-Infection of the Cystic Fibrosis Lung by Pseudomonas aeruginosa and Burkholderia cenocepacia Reveals Influences on Biofilm Formation and Host Response**. *PLoS One* 2012, **7**.

43. Yang L, Liu Y, Markussen T, Høiby N, Tolker-Nielsen T, Molin S: **Pattern differentiation in co-culture biofilms formed by Staphylococcus aureus and Pseudomonas aeruginosa**. *FEMS Immunol. Med. Microbiol.* 2011, **62**:339–347.

44. Twomey KB, O'Connell OJ, McCarthy Y, Dow JM, O'Toole G a, Plant BJ, Ryan RP: **Bacterial cis-2-unsaturated fatty acids found in the cystic fibrosis airway modulate virulence and persistence of Pseudomonas aeruginosa**. *ISME J.* 2012, **6**:939–950.

45. Fugère A, Séguin DL, Mitchell G, Déziel E, Dekimpe V, Cantin AM, Frost E, Malouin F: **Interspecific small molecule interactions between clinical isolates of Pseudomonas aeruginosa and Staphylococcus aureus from adult cystic fibrosis patients**. *PLoS One* 2014, **9**.

46. Baldan R, Cigana C, Testa F, Bianconi I, De Simone M, Pellin D, Di Serio C, Bragonzi A, Cirillo DM: **Adaptation of Pseudomonas aeruginosa in cystic fibrosis airways influences virulence of Staphylococcus aureus in vitro and murine models of co-infection**. *PLoS One* 2014, **9**.

47. Rutherford ST, Bassler BL: **Bacterial quorum sensing: its role in virulence and possibilities for its control.** *Cold Spring Harb. Perspect. Med.* 2012, **2**.

48. Köhler T, Guanella R, Carlet J, van Delden C: **Quorum sensing-dependent virulence during Pseudomonas aeruginosa colonisation and pneumonia in mechanically ventilated patients.** *Thorax* 2010, **65**:703–10.

49. Lazar V: **Quorum sensing in biofilms--how to destroy the bacterial citadels or their cohesion/power?** *Anaerobe* 2011, **17**:280–5.

50. Moreau-Marquis S, Stanton BA, O'Toole GA: **Pseudomonas aeruginosa biofilm formation in the cystic fibrosis airway.** *Pulm. Pharmacol. Ther.* 2008, **21**:595–9.

Chapter 5

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In this Chapter, the major conclusions of the present thesis are addressed. Suggestions for future work are also proposed.

5.1 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The multispecies microbiome composition in CF is constantly shaped by selective pressures exerted by the niche characteristics at sites of infection, such as heterogeneous availabilities of oxygen present in the mucus of CF patients. It is increasingly recognized that the properties of such communities may be distinct from those of their individual members. Given the polymicrobial nature of the CF lung, it is not surprising that CF pathogens, traditional and emergent, can establish interactions that have the potential to alter the course of airway disease and also pose significant challenges for the management of these patients. The full extent and implications of many of these interactions remains largely unknown and more investigation is needed to fill this gap.

As shown in Chapter 3, the conventional pathogens *P. aeruginosa* and *S. aureus,* and the CF-emergent bacterial species *A. baumannii, D. pigrum, I. limosus, K. pneumoniae* and *S. maltophilia* had pronounced ability to grow planktonically and to develop biofilms under variable oxygen atmospheres, resembling the *in vivo* CF airways. All species achieved high number of cultivable cells for both oxygen conditions, with a significant decreasing in the amount of biomass under low-oxygen atmospheres. The fact that uncommon organisms showed great ability to atmospheres with restricted oxygen conditions similar to those occurring in CF airways, may highlight their potential impact in the disease progression and their eventual contributions for the infection progression.

Given these preliminary findings and knowing the polymicrobial nature of the CF airways, *P. aeruginosa* was grown in co-culture with two CF-emergent bacteria, *I. limosus* and *S. maltophilia,* and the conventional pathogen *S. aureus* in order to evaluate how the bacteria behave and interact with each other when in the polymicrobial consortia. Results demonstrated that dual-species biofilms, similarly to most single-species biofilms, produced more biomass under aerobic conditions. However, the presence of *S. aureus* and *I. limosus* and in co-culture with *P. aeruginosa* significantly reduced the biofilm biomass formed comparatively with *P. aeruginosa* biofilm alone, although the number of cultivable cells was not significantly affected. These results, obtained for aerobic and anaerobic conditions, indicate that these species could interfere eventually wit the exopolysaccharyde matrix of the whole biofilms but not with the cells, which may have impact in the clinical context, for example, by decreasing the resistance of these biofilms against antibiotic action. Regarding microbial composition, the results obtained by CFU counting and PNA FISH under aerobic and anaerobic atmospheres,

demonstrated that in all polymicrobial consortia *P. aeruginosa* was the dominant species. PNA FISH has been described as a robust method to confidently discriminate multispecies biofilms and thus infer about multi-species interplay. In this study, PNA FISH was employed for the qualitative study of biofilm populations, as well as to directly localize and discriminate those polymicrobial communities.

It is important to note that these infections usually involve more than one species, which are not distributed equally in the CF airways, with one species being firstly well-established when other species arrive. Because of this, further experiments are required to explore distinct scenarios of co-infection. In addition, given the diversity of microorganism within the CF microbiome, it would be interesting to study different polymicrobial populations, involving particularly bacteria that have been residing in the airway for different lengths of time or even study inter-kingdom consortia (e.g. bacteria-fungi). Moreover, studies using *in vivo* models that closely mimic features of human polymicrobial disease are key in bridging the gap from the lab to the clinic context.

Previous findings showed that populations encompassing CF-bacteria could easily adapt as planktonic and biofilm populations under variable oxygen conditions resembling CF, but how these pathogens contribute to disease progression and to antibiotic therapy was still to be disclosed. Results obtained in Chapter 4 indicated that biofilms were notoriously more difficult to eradicate than their planktonic counterparts using ciprofloxacin, for all oxygen atmospheres. Regarding polymicrobial populations, biofilm eradication was not achieved by using monotherapy, showing even an increased overall cell density when compared with *P. aeruginosa* mono-species, in all oxygen conditions. This work showed that the development of synergistic biofilms between these species (*P. aeruginosa* in co-culture with *S. aureus* or *I. limosus* or *S. maltophilia)* leads to the generation of greater performances than mono-culture of traditional pathogen *P. aeruginosa.* These results confirm that the fitness of dual-species biofilms may be not necessarily the sum of the characteristics of each single species. This is consistent with the emerging theme that some bacterial communities associated with chronic infection are gaining a fitness advantage from residing in multispecies biofilms. In general, biofilm compositions changed as a result of antibiotic treatment, with alterations being dependent on the antibiotic concentration and oxygen conditions implemented. For consortia formed between *P. aeruginosa* and *S. aureus*, the latter species predominated the consortia for both oxygen conditions. The consortia encompassing *P. aeruginosa – I. limosus* and *P. aeruginosa – S. maltophilia* were dominated by the CF-key pathogen P. aeruginosa. As such,

the endurance of *P. aeruginosa* within the consortia, before and after antibiotic treatment, could lead to the ciprofloxacin resistance presented by dual-species biofilms. However, the increasing survival of *S. maltophilia* and *I. limosus* in dual-species consortia after antibiotic exposure, for all oxygen atmospheres, could indicates that these species may have also a preponderant role in increasing the whole resistance of the consortia. The PNA FISH method employed corroborating the dominance of *P. aeruginosa* within the mixed-species consortia (determined by CFU counting), and allowed to observe a decreasing in the overall cell density for all consortia under low-oxygen atmospheres.

Altogether, the findings encountered in this study led to conclude that emergent- and traditional-species are able to live in association with key-CF pathogen *P. aeruginosa* under variable oxygen atmospheres, developing highly resilient consortia even after antibiotic treatment. However, several questions remain to be answered: i) *Which genes are differentially expressed in mono- versus multi-species biofilms, and what are the underlying molecular mechanisms and extracellular signals causing these changes?* ii) *Over which distances are bacterial populations capable of impacting each other in CF infections? iii) Is it possible to eradicate the pathogens without affecting the microbiota? iv) Is synergism highly prevalent in multispecies biofilms in nature?*

The challenge is now to explore multispecies biofilms in further detail, by examining their physiology, function and underlying mechanisms but specifically enhancing the focus for microbial-microbial and/or microbial-host interactions in these communities. Understanding the physical and chemical interactions between microorganisms in these polymicrobial communities will help to define potential new targets for disrupting biofilm-community development and, in CF, affect the ecology of biofilms in the airways of patients.

Supplementary Material

CHAPTER 3

Table S3-1. Initial specific growth rates for planktonic single-species for *P. aeruginosa*, *S. aureus, A. baumannii*, *D. pigrum*, *I. limosus*, *K. pneumonia* and *S. maltophilia* grown on microtiter plate wells at aerobic and anaerobic conditions. Growth rates were calculated for the first 8 h from planktonic growth curves as the slope of the linear regression of the cell-number-versus-time data.

		Specific growth rate (cells/mL/h)						
Atmosphere		**P. aeruginosa**	**S. aureus**	**A. baumannii**	**D. pigrum**	**I. limosus**	**K. pneumoniae**	**S. maltophilia**
AEROBIC	**Rate of growth**	2,00E+07	5,00E+08	8,00E+07	8,00E+07	6,00E+02	2,00E+08	6,00E+07
	R^2	0,99	0,92	0,82	0,82	0,69	0,94	0,93
ANAEROBIC	**Rate of growth**	8,00E+06	2,00E+08	1,00E+07	1,00E+07	5,00E+02	1,00E+08	2,00E+07
	R^2	0,98	0,97	0,98	0,98	0,9	0,92	0,75

Table S3-2. Initial adhesion rates for single-species biofilms for *P. aeruginosa*, *S. aureus, A. baumannii*, *D. pigrum*, *I. limosus*, *K. pneumonia* and *S. maltophilia* grown on microtiter plate wells at aerobic and anaerobic conditions. Adhesion rates were calculated for the first 8 h from growth curves as the slope of the linear regression of the cell-number-versus-time data.

		Rate of adhesion (cells/cm²/h)						
Atmosphere		**P. aeruginosa**	**S. aureus**	**A. baumannii**	**D. pigrum**	**I. limosus**	**K. pneumoniae**	**S. maltophilia**
AEROBIC	**Rate of adhesion**	5,40E+05	1,00E+07	9,00E+06	2,50E+04	2,90E+04	7,00E+06	4,00E+06
	R^2	0,47	0,81	0,84	0,06	0,79	0,68	0,71
ANAEROBIC	**Rate of adhesion**	7,00E+05	6,00E+06	4,00E+05	5,90E+04	4,70E+04	3,00E+06	4,90E+05
	R^2	0,79	0,62	0,07	0,69	0,93	0,64	0,74

Table S3-3. Initial adhesion rates for dual-species biofilms encompassing *P. aeruginosa* with *S. aureus* or *I. limosus* or *S. maltophilia* grown on microtiter plate wells at aerobic and anaerobic conditions. Adhesion rates were calculated for the first 8 h from growth curves as the slope of the linear regression of the cell-number-versus-time data.

		Rate of adhesion (cells/cm²/h)		
Atmosphere		**P. aeruginosa + S. aureus**	**P. aeruginosa + I. limosus**	**P. aeruginosa + S. maltophilia**
AEROBIC	**Rate of adhesion**	2,00E+07	7,00E+05	2,00E+06
	R^2	0,85	0,82	0,69
ANAEROBIC	**Rate of adhesion**	3,00E+07	7,00E+05	2,00E+05
	R^2	0,74	0,43	0,9

CHAPTER 4

Table S4-1. Initial specific growth rates for planktonic single-species for *P. aeruginosa*, *S. aureus, A. baumannii*, *D. pigrum*, *I. limosus*, *K. pneumonia* and *S. maltophilia* grown on microtiter plate wells in the presence of increasing concentrations of ciprofloxacin (¼ MIC, ½ MIC, MIC, 2 MIC and 4 MIC) at aerobic and anaerobic conditions. Growth rates were calculated for the first 8 h from planktonic growth curves as the slope of the linear regression of the cell-number-versus-time data.

			Specific growth rate (cells/mL/h)					
			CONTROLO	1/4 MIC	1/2 MIC	MIC	2 MIC	4 MIC
A. baumannii	AEROBIC	Rate of growth	8,00E+07	4,00E+07	2,00E+07	6,00E+07	9,00E+06	8,00E+06
		R^2	0,82	0,89	0,99	0,72	0,92	1
	ANAEROBIC	Rate of growth	1,00E+07	4,00E+06	4,00E+06	6,00E+06	4,00E+06	1,00E+06
		R^2	0,98	0,95	0,95	0,82	0,95	0,4
D. pigrum	AEROBIC	Rate of growth	2,00E+06	1,00E+06	7,00E+05	2,00E+06	3,00E+05	3,00E+04
		R^2	0,82	0,86	0,99	0,72	0,92	1
	ANAEROBIC	Rate of growth	3,00E+05	1,00E+05	1,00E+05	2,00E+05	1,00E+05	3,00E+04
		R^2	0,98	0,95	0,95	0,82	0,95	0,4
I. limosus	AEROBIC	Rate of growth	6,00E+02	5,00E+01	5,00E+01	5,00E+01	5,00E+01	5,00E+01
		R^2	0,69	0,02	0,02	0,02	0,02	0,02
	ANAEROBIC	Rate of growth	5,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02	1,00E+02
		R^2	0,90	0,04	0,04	0,04	0,04	0,04
K. pneumoniae	AEROBIC	Rate of growth	2,00E+08	2,00E+08	1,00E+08	3,00E+06	2,00E+06	6,00E+06
		R^2	0,94	0,91	0,98	0,77	0,28	0,81
	ANAEROBIC	Rate of growth	1,00E+08	1,00E+08	1,00E+08	2,00E+08	1,00E+07	1,00E+06
		R^2	0,92	0,93	0,95	0,96	0,08	0,08
S. maltophilia	AEROBIC	Rate of growth	2,00E+07	3,00E+05	2,00E+05	3,00E+05	2,00E+05	2,00E+05
		R^2	0,93	0,83	0,45	0,83	0,45	0,45
	ANAEROBIC	Rate of growth	1,00E+06	2,00E+05	2,00E+05	2,00E+05	2,00E+05	2,00E+05
		R^2	0,75	0,45	0,45	0,45	0,45	0,45
P. aeruginosa	AEROBIC	Rate of growth	1,00E+08	1,00E+07	4,00E+06	4,00E+06	4,00E+06	4,00E+06
		R^2	0,99	0,85	0,60	0,60	0,60	0,60
	ANAEROBIC	Rate of growth	8,00E+06	2,00E+06	2,00E+06	3,00E+06	3,00E+06	3,00E+06
		R^2	0,98	0,98	0,83	0,69	0,69	0,69
S. aureus	AEROBIC	Rate of growth	1,00E+08	2,00E+07	5,00E+05	3,00E+06	7,00E+05	8,00E+04
		R^2	0,92	0,83	0,36	0,95	0,64	0,02
	ANAEROBIC	Rate of growth	4,00E+07	1,00E+06	2,00E+06	2,00E+06	1,00E+06	8,00E+04
		R^2	0,97	0,75	0,89	0,5	0,72	0,01

Table S4-2. Initial adhesion rates for single-species biofilms for *P. aeruginosa*, *S. aureus, A. baumannii*, *D. pigrum*, *I. limosus*, *K. pneumonia* and *S. maltophilia* grown on microtiter plate wells in the presence of increasing concentrations of ciprofloxacin (¼ MIC, ½ MIC, MIC, 2 MIC and 4 MIC) at aerobic and anaerobic conditions. Adhesion rates were calculated for the first 8 h from growth curves as the slope of the linear regression of the cell-number-versus-time data.

			Rate of adhesion (cells/cm2/h)					
			CONTROLO	1/4 MIC	1/2 MIC	MIC	2 MIC	4 MIC
A. baumannii	AEROBIC	Rate of adhesion	9,00E+06	3,00E+06	3,00E+06	2,00E+06	1,00E+05	3,00E+04
		R^2	0,84	0,92	0,78	0,71	0,92	0,12
	ANAEROBIC	Rate of adhesion	4,00E+05	2,00E+06	-2,00E+06	1,00E+06	9,50E+05	2,00E+05
		R^2	0,07	0,85	0,19	0,93	0,63	0,63
D. pigrum	AEROBIC	Rate of adhesion	2,50E+04	7,80E+03	-4,30E+03	-1,60E+03	2,90E+03	9,60E+03
		R^2	0,06	0,22	0,68	0,04	0,07	0,42
	ANAEROBIC	Rate of adhesion	5,90E+04	-5,10E+03	1,70E+04	2,30E+03	3,20E+04	-6,40E+03
		R^2	0,69	0,12	0,64	0,04	0,08	0,53
I. Limosus	AEROBIC	Rate of adhesion	2,90E+04	1,40E+03	3,10E+03	2,20E+03	-1,20E+02	3,80E+02
		R^2	0,79	0,24	0,53	0,65	0,32	0,51
	ANAEROBIC	Rate of adhesion	4,70E+04	5,20E+03	4,50E+03	2,10E+03	8,90E+02	8,60E+02
		R^2	0,93	0,84	0,71	0,85	0,4	0,82
K. Pneumoniae	AEROBIC	Rate of adhesion	7,00E+06	1,00E+07	6,00E+06	8,00E+05	2,00E+05	1,00E+05
		R^2	0,68	0,87	0,92	0,89	0,69	0,65
	ANAEROBIC	Rate of adhesion	3,00E+06	9,00E+06	5,00E+06	3,00E+06	5,00E+06	3,00E+06
		R^2	0,64	0,66	0,73	0,88	0,9	0,49
S. Maltophilia	AEROBIC	Rate of adhesion	4,00E+06	2,40E+04	-3,40E+03	-3,40E+03	2,50E+02	1,60E+02
		R^2	0,71	0,62	0,55	0,55	0,07	0,77
	ANAEROBIC	Rate of adhesion	4,90E+05	1,80E+05	1,50E+05	1,90E+04	4,40E+03	6,90E+02
		R^2	0,74	0,72	0,74	0,78	0,7	0,53
P. aeruginosa	AEROBIC	Rate of adhesion	5,40E+05	3,50E+03	1,20E+03	2,60E+03	5,00E+03	2,10E+02
		R^2	0,47	0,77	0,35	0,54	0,6	0,55
	ANAEROBIC	Rate of adhesion	7,00E+05	2,00E+05	7,60E+04	5,30E+04	3,90E+04	8,90E+02
		R^2	0,79	0,59	0,76	0,78	0,72	0,87
S. aureus	AEROBIC	Rate of adhesion	1,00E+07	7,13E+05	8,36E+03	8,27E+03	-2,47E+03	-1,45E+03
		R^2	0,81	0,89	0,72	0,59	0,38	0,28
	ANAEROBIC	Rate of adhesion	6,00E+06	2,52E+04	2,84E+04	3,63E+02	-3,59E+02	1,27E+03
		R^2	0,62	0,85	0,52	0,02	0,09	0,15

Table S3-3. Initial adhesion rates for dual-species biofilms encompassing *P. aeruginosa* with *S. aureus* or *I. limosus* or *S. maltophilia* grown on microtiter plate wells in the presence of increasing concentrations of ciprofloxacin (¼ MIC, ½ MIC, MIC, 2 MIC and 4 MIC) at aerobic and anaerobic conditions. Adhesion rates were calculated for the first 8 h from growth curves as the slope of the linear regression of the cell-number-versus-time data.

			Rate of adhesion (cells/cm²/h)					
			CONTROLO	1/4 MIC	1/2 MIC	MIC	2 MIC	4 MIC
P. aeruginosa + S. aureus	AEROBIC	Rate of adhesion	2,00E+07	7,00E+06	6,00E+06	2,00E+06	5,00E+04	-4,00E+04
		R^2	0,85	0,80	0,87	0,85	0,05	0,03
	ANAEROBIC	Rate of adhesion	3,00E+07	2,00E+07	1,00E+07	2,00E+06	8,00E+04	9,00E+03
		R^2	0,74	0,75	0,82	0,94	0,92	0,30
P. aeruginosa + I. Limosus	AEROBIC	Rate of adhesion	7,00E+05	7,00E+04	1,00E+04	6,00E+03	1,00E+03	8,00E+02
		R^2	0,82	0,66	0,85	0,68	0,88	0,63
	ANAEROBIC	Rate of adhesion	7,00E+05	5,00E+05	4,00E+04	3,00E+03	1,00E+03	9,00E+01
		R^2	0,43	0,67	0,68	0,33	0,78	0,13
P. aeruginosa + S. Maltophilia	AEROBIC	Rate of adhesion	2,00E+06	6,00E+05	1,00E+05	7,00E+04	5,00E+03	9,00E+02
		R^2	0,69	0,85	0,68	0,8	0,76	0,55
	ANAEROBIC	Rate of adhesion	2,00E+05	1,00E+05	9,00E+04	9,00E+04	6,00E+03	2,00E+03
		R^2	0,90	0,71	0,81	0,70	0,87	0,78