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P. aeruginosa adaptation in cystic fibrosis environment challenged by the presence of other bacteria

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ABSTRACT

Cystic fibrosis (CF) is a recessive genetic disease caused by mutations in the cystic fibrosis transmembrane regulator resulting in thick mucus that difficults proper airflow. CF airways are infected by several opportunistic pathogens but *P. aeruginosa* is the major responsible for the high mortality rate due to the development of chronic infections. To long persist, *P. aeruginosa* uses sophisticated mechanisms to achieve full-adaptation mainly triggered by the harsh environmental conditions of CF lungs. CF lungs are colonized by several other bacterial species, as the early colonizers, with which *P. aeruginosa* have to co-exist. Some interactions between the typical early colonizers of CF lungs, such as *S. aureus*, *S. maltophilia*, and *P. aeruginosa,* have been reported. For instance, it has been proposed that *S. aureus* may somewhat help *P. aeruginosa* adaptation to CF lungs inducing the emergence of *P. aeruginosa* small colony variants (SCV). *P. aeruginosa* SCV are known to be excellent biofilm formers and typically exhibit resistance against multiple antibiotic classes. But, the current *in vitro* studies have using models that do not closely mimic the complex CF environment. Therefore, the main objective of this study was to determine the impact of *S. maltophilia* and *S. aureus* on *P. aeruginosa* pathogenicity using a new *in vitro* model that mimics CF lungs environment. To accomplish this feature, a clinical isolate of *S. maltophilia* and two *P. aeruginosa* strains, one antibiotic sensitive and one resistant, were grown in artificial CF sputum medium (ASM) and cultured at different timings to simulate a typical CF lung colonization. *S. maltophilia* was first cultured for 3 days, and then *P. aeruginosa* was co-cultured for more 4 days. In the last 4 days the mixed cultures were exposed to aggressive ciprofloxacin treatment. Samples were collected every 24 h to investigate bacterial growth kinetics, ciprofloxacin time killing and phenotypic diversity. The same procedure was carried out to assess the impact of *S. aureus* (an antibiotic sensitive strain and one resistant) on *P. aeruginosa*. To gain insights in the biological processes involved in *P. aeruginosa* adaptation in ASM and response to ciprofloxacin, the proteomic profile of *P. aeruginosa* strains was studied using a 2-D gel electrophoresis (2-DE).

Growth kinetics showed that apparently *P. aeruginosa* outcompeted *S. maltophilia* in ASM. Currently, there is no solid media able to effectively distinguish *P. aeruginosa* from *S. maltophilia* and without this differentiation it was not possible to infer if the outcompetition occurred indeed in ASM or in solid medium. Therefore, other methods, such as fluorescent differentiation or molecular methods, have to be used.

Data showed that *P. aeruginosa* growth kinetics and phenotypic diversity were not affected by the presence of *S. aureus*. Interestingly, the presence of *S. aureus* seemed to inhibit the emergence of *P. aeruginosa* SCV that have emerged when the resistant strain was cultured alone. These results do not substantiate the role of *S. aureus* on *P. aeruginosa* persistence pointed out by other studies where it was reported that *S. aureus* extracellular factors increased *P. aeruginosa* growth activity and resistance towards antibiotics by the emergence of SCV. It was hypothesized that these discrepancies among results could be caused by the effect of initial *P. aeruginosa* concentration or by the short period of time for *S. aureus* adapt to CF environment. The effect of *P. aeruginosa* concentration revealed no impact on *P. aeruginosa* adaptation. In contrast, CF-adapted *S. aureus* seemed to trigger the emergence of *P. aeruginosa* SCV, but the *P. aeruginosa* resistant strain was also able to produce SCV when grown alone in ASM. Therefore, both hypothesis were discarded which led to conclude that *S. aureus* seemed to have no role in *P. aeruginosa* pathogenicity in CF lungs. It was also concluded that the discrepancy of this study with literature may be explained by the different *in vitro* conditions used. The majority of the studies used standard laboratory medium that does not fully mimic the chemical and nutritional complex environment found in CF lungs. Moreover, this model used different inoculation timings of bacterial species to better mimic *in vivo* conditions, issue that was not took into account in the previous studies.

2-DE revealed some differences in protein expression by *P. aeruginosa* strains that could be involved in important biological processes, such as iron transport, carbon catabolism and lipids biosynthesis.

RESUMO

A fibrose quística (FQ) é uma doença genética causada por mutações no regulador transmembranar da fibrose quística (CFTR), originando um muco espesso que dificulta o correto fluxo de ar. Nos doentes com FQ as vias respiratórias são infetadas por um grupo característico de patógenos, onde se destacam as bactérias, por serem a principal causa de morte. A elevada taxa de mortalidade em doentes com fibrose cística deve-se essencialmente ao desenvolvimento de infeções crónicas de *P. aeruginosa*. De forma a persistir no ambiente de FQ, a *P. aeruginosa* recorre a mecanismos complexos de forma a atingir uma completa adaptação e sobreviver às condições adversas dos pulmões em FQ. Em FQ os pulmões são colonizados por muitas outras espécies bacterianas, sendo que a *P. aeruginosa* tem que co-existir com estas, nomeadamente com as que colonizam numa fase inicial da doença. Interações entre algumas espécies que colonizam os pulmões nomeadamente as que colonizam numa fase inicial e P. aeruginosa tem sido reportadas. Interações entre *S. aureus*, *S. maltophilia*, os colonizadores de uma fase inicial, com *P. aeruginosa* tem sido reportadas sendo, no entanto, um assunto ainda muito pouco compreendido. Assim, este estudo teve como principal objetivo investigar o impacto da S*. maltophilia* e *S. aureus* na adaptação de *P. aeruginosa* usando para isso um modelo *in vitro* que mimetiza as condições pulmonares de doentes com FQ. Para atingir este objetivo, um isolado clínico de *S. maltophilia* e duas estirpes de *P. aeruginosa*, uma resistente e outro sensível a antibióticos, foram crescidas em muco artificial de FQ (MAFQ) e em diferentes tempos de ensaio para simular uma situação real de colonização dos pulmões. *S. maltophilia* foi primeiramente incubada durante 3 dias após os quais *P. aeruginosa* foi então adicionada à cultura por mais 4 dias. Nos últimos 4 dias, a cultura mista foi exposta a agressivos tratamentos com ciprofloxacina de forma a avaliar o comportamento num ambiente de stresse. Foram recolhidas amostras a cada 24 h para proceder à análise da cinética de crescimento e da diversidade populacional através da observação da morfologia de colónia. O mesmo procedimento foi seguido para estudar o impacto de *S. aureus* na *P. aeruginosa*. Para estudar os processos biológicos subjacentes aos diferentes comportamentos observados entre a PAI e PAO1 em muco artificial de fibrose quística e em resposta a antibióticos, procedeu-se ao estudo proteómico destas duas estirpes recorrendo para tal à técnica de eletroforese bidimensional (2D).

Os resultados da cinética de crescimento bacteriano demonstraram que *P. aeruginosa* aparentemente elimina *S. maltophilia* em MAFQ. Contudo estes resultados têm que ser reforçados com o uso de um meio sólido diferencial de forma a garantir a qualidade dos resultados. Atualmente, não existem meios sólidos que consigam diferenciar colónias de *P. aeruginosa* das colónias de *S. maltophilia*. Assim, sem estes meios é impossível concluir se *S. maltophilia* é ou não eliminada em muco artificial de fibrose quística.

Relativamente aos resultados referentes à influência de *S. aureus* na patogenicidade da *P. aeruginosa*, verificou-se pela cinética de crescimento e a diversidade populacional da *P. aeruginosa* que estes aparentemente não foram afetados pela presença de *S. aureus*. Interessantemente, a presença de *S. aureus* pareceu inibir o surgimento de SCV de *P. aeruginosa* que surgiram aquando da cultura sozinha. Estes resultados não corroboram os resultados de outros estudos que reportaram que os fatores extracelulares de *S. aureus* induziam a formação de SCV em *P. aeruginosa* na presença de antibióticos. Estas discrepâncias de resultados podem ser causadas pela possível elevada concentração inicial de P. aeruginosa utilizada para a inoculação de MAFQ ou ainda pelo curto período de adaptação de *S. aureus* ao meio de cultura. Ambas as hipóteses foram testadas sendo que o efeito da concentração inicial de incubação não revelou nenhum impacto no comportamento da *P. aeruginosa*. Por outro lado, *S. aureus* adaptado induziu o surgimento de SCV de *P. aeruginosa* que apareciam aquando da cultura sozinha. Contudo, a emergência de SCV foi igualmente detetada em monocultura de *P. aeruginosa* em ASM. Assim sendo, as discrepâncias dos resultados obtidos com a literatura podem ser explicadas pelas condições *in vitro* usadas. A maioria dos estudos usa meio laboratorial *standard* que não mimetiza o ambiente nos pulmões em FQ.

O estudo do proteoma de ambas as estirpes de *P. aeruginosa* usadas neste estudo revelou que de facto estas estirpes diferem nas proteínas que expressam. Estas diferenças podem estar relacionadas com o transporte de ferro, com o catabolismo de carbono e biossíntese de lípidos.

SCOPE AND AIMS OF THE THESIS

1. AIMS OF THE THESIS

Cystic fibrosis is an autossomal recessive disease being the average life expectancy around 42 years. *P. aeruginosa* is the main pathogenic agent in CF disease causing chronic infections and high mortality rates. Several microbiological studies have been focused on determining the mechanisms used by *P. aeruginosa* to persist in CF lungs and to resist against antibiotics. These studies have provided relevant evidences about the mechanisms of *P. aeruginosa* adaptation to CF lungs environment, helping to understand, for instance, that prevention of *P. aeruginosa* lungs colonization is the best approach to avoid or, at least, to retard the development of chronic infections. However, this is not enough. CF patients still die because of *P. aeruginosa*-associated infections and thus clinical community demands more investigation to understand *P. aeruginosa* infections.

P. aeruginosa shares CF environment with other species, such as *S. aureus* and *S. maltophilia* and thus they have to interact, to communicate and to adapt to each other. These interactions could play a role in *P. aeruginosa* persistence and antibiotic resistance and, therefore, their understanding could provide valuable insights to design effective treatments or even to develop new antimicrobial molecules. The focus of clinical community must thus be directed to early interactions established by *P. aeruginosa* with the earlier colonizers of CF lungs as an attempt to avoid the establishment of *P. aeruginosa* chronic infection.

The main research question of this project was to determine which bacterial species augment the pathogenic potential of *P. aeruginosa*, contributing for its persistence and antibiotic resistance. Therefore, this project aimed to study the alterations undergone by *P. aeruginosa* caused by other bacterial species in CF environment. In addition, this project aimed to evaluate the impact of these alterations in response to antibiotic treatments. To accomplish these objectives, this project focused on two of the most abundant pathogens at CF early stage infection, *S. aureus* and *S. maltophilia.* Co-culture of these pathogens with *P. aeruginosa* was performed using a novel *in vitro* CF model that closely simulate the CF conditions. This *in vitro* model had special concerns to the culture medium, an artificial sputum medium, and different timings of bacterial growth to closely approximating to *in vivo* CF lungs. Moreover, advanced techniques, such as proteomics were used to disclose the mechanisms underlying *P. aeruginosa* adaptation.

2. OUTLINE OF THE THESIS

This thesis is organized in four chapters. Chapter 1 briefly reviews some major topics of the theme in study, namely cystic fibrosis disease and its microbiome, the main interactions established between *P. aeruginosa* and *S. aureus* and *S. maltophilia* and the major antimicrobial treatments currently administered to CF patients.

In chapter 2, all methods and materials used in this work, including bacterial strains, culture conditions, *in vitro* CF model, microbiological and proteomic techniques are described in detail.

Chapter 3 focuses on the presentation of the results obtained related to the study of co-culture of *P. aeruginosa* with *S. aureus*, and *P. aeruginosa* with *S. maltophilia* in CF artificial sputum medium regarding the phenotypic alterations undergone by *P. aeruginosa* in the presence of these species, and the impact of these alterations on persistence and antibiotic resistance. Moreover, the optimization of 2-D gel electrophoresis procedure and the *P. aeruginosa* proteomic profile is also presented and accurately discussed.

In chapter 4 the main conclusions and the future work perspectives are described finalizing this thesis.

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List of Abreviations and Acronyms

2-DE	Two dimensional gel electrophoresis
AI-2	Autoinduce 2
AprA	Alkaline protease
ASL	Airway surface liquid
ASM	Artificial sputum medium
BAM	Blood Agar medium
CF	Cystic Fibrosis
CFTR	Cystic fibrosis transmembrane regulator
CFU	Colony forming units
CFU/mL	Colony forming units per milliliter
CIP	Ciprofloxacin
DNA	Deoxyribonucleic acid
g	G-force
g	Gram
g/L	Gram *per* litre
h	Hour(s)
HQNO	4-hydroxyl-2-heptylquinoline N-oxide
Ig	Immunoglobulin
LB	Luria Bertani
LPS	Lipopolysaccharide
Mg	Milligram
mL	Millilitre
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin-sensitive *Staphylococcus aureus*
PBPs	Penicillin-binding proteins
PIA	Pseudomonas isolation agar
rpm	Rotations per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SCV	Small colony variants
Sec	Seconds

SpA	Staphylococcal protein A
TMP-SMX	Trimethoprim-sulfamethoxazole
Tris-HCl	Trizma hydrochloride
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TTC	Tiarcillin/clauvulanic acid
VIA	Vancomycin, imipenem, amphotericin
µg	Microgram

1. INTRODUCTION

1.1 Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive disease found predominantly in Caucasian populations caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) [1]. *CFTR* is a gene localized in the long arm of chromosome 7 that plays a direct role in multiple organs, such as the hypothalamus, kidney and bone [2]. Its malfunction contributes to a linear growth retardation, delayed pubertal onset, bone density modulation and also affects natural immunity or immune response [2–6]. CF is thus a disease that affects several organs with significant complications, but its effects on airway tract are the most problematic and responsible for the increased mortality rate [7]. The clinical features of this disease include worsening cough, increased sputum expectoration, chest pain, shortness of breath, fatigue, weight loss and decline in lung function [8].

CFTR gene encodes an important protein, also called CFTR, implicated on ion transport, cell signalling and inflammation pathways [9]. In the bronchial epithelial cell, CFTR normally regulates chloride and sodium ions flux across the cell membrane, and its defect or malfunction causes abnormalities in fluid secretion resulting in the secretion of viscous and thick mucus. This accumulated mucus is difficult to clear, leading to plugged and atrophic ducts (Figure 1.1) [10,11]. CFTR also conducts bicarbonate and its dysfunction changes the pH of airway surface liquid (ASL) [12–14]. Hyperabsorption of sodium ions through the epithelial sodium channel also occurs, creating osmotic gradient that favours ASL and mucous dehydration [15–17]. In CF patients, the decreased availability of extracellular water, an increase in actin molecules and an increase in anionic polyelectrolytes, including DNA derived from the invading bacteria and lyses inflammatory cells, seem to contribute to the formation of thick mucus [18]. The lack of CFTR function is also involved in increased inflammatory response and reduced activity of natural defence mechanisms that facilitates the development of microbial infection [3].

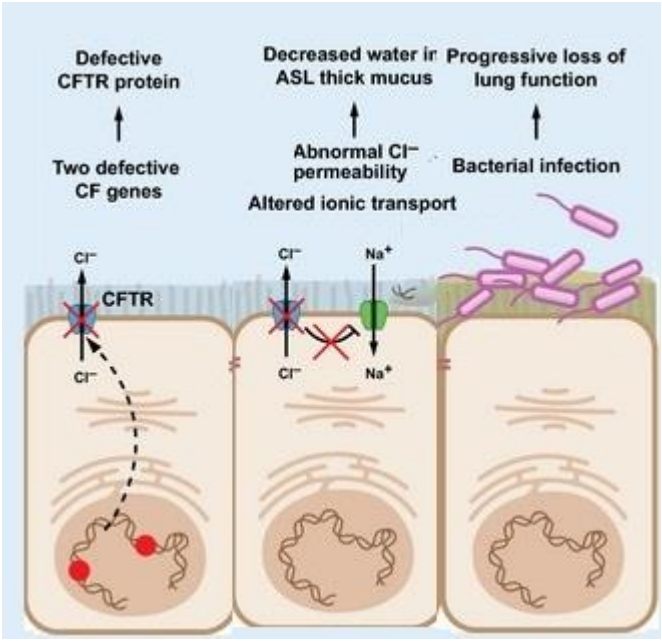


Figure 1.1 Pathogenic cascade of events occurring in the CF lungs. *CFTR,* cystic fibrosis transmembrane conductance regulator; ASL airway surface liquid. Adapted from: Amaral *et al*. (2015) [19].

The CF lung environment provides excellent conditions for microbial growth because it is rich in nutrients and presents optimal temperature and humidity. Consequently, the majority of CF patients is colonized in early childhood and continue to be infected along their life [20,21]. 80 to 95% of CF patients succumb to respiratory failure brought because of chronic infections and concomitant airway inflammation [3,22]. The life expectancy of CF patients was established as 42 years by the Cystic Fibrosis Foundation, which is considerably reduced in the 21st century mainly because of the microbial infections developed in the CF lungs [23]. For this reason, the clinical community has performed an intense investigation about these airway microbial infections in the attempt to augment the medium life expectancy of CF patients and to provide better quality of life. Therefore, in the next sections the main microorganisms responsible for these infections in CF lungs will be explored.

1.2 Cystic Fibrosis Microbiome

The CF airway represents a permissive environment for microbial colonization [24]. Typically, at birth CF airways appear not to be infected, but few years later CF airways are infected by a characteristic group of opportunistic pathogens that include viruses, fungus and bacteria [25]. Bacterial and fungal pathogens, such as *Aspergillus fumigatus, Candida albicans, Haemophilus influenzae*, *Staphyloccocus aureus*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans*, appear to have an appreciated role in infection development and persistence in CF lungs [20,26,27].

The role of fungal pathogens in the course of CF lung disease remains poorly understood, but it is thought to be associated to a more rapid decline in pulmonary function [27]. Viral pathogens, such as rhinoviruses are associated to pulmonary aggravations leading to severe complications in patients especially in children [28–30]. Pulmonary aggravation is caused by the increased susceptibility of CF epithelial cells [20,31]. Moreover, viruses facilitate the bacterial adhesion, which also greatly contributes to decline of lung function. Despite these evidences, bacterial species are the major cause of mortality in CF because they are able to evade immune response or harness host responses to their benefit and even to resist against the most aggressive antimicrobial therapies [24,32]. By this reason, the infecting bacterial species will be the focus of this study.

1.2.1 Bacterial pathogens during infection phases

The microbiome of CF lungs has suffered some alterations over the years. These alterations may be explained by the design of more effective treatments and the development of new antibiotics that may interfere with the prevalence of the infecting species. Nevertheless, bacterial infections in CF lungs seem to follow a profile that changes with patient age and infection progression (Figure 1.2). In the past years, several studies focusing on the bacterial identification led to realize the complexity of the polymicrobial communities resident in the CF airways, but also their dynamic overtime [33]. In infancy and early childhood, CF patients are often colonized with organisms, such as *S. aureus*, *H. influenzae* and *S. maltophilia* and the reason for this incidence remains unclear [24,34,35]. These early colonizing species have been associated to inflammation, damage of the airways and worse pulmonary function.

As patients get older and the disease progresses, early colonizing species decreased their incidence and patients become more susceptible to a range of Gram-negative bacteria, including *P. aeruginosa* (Figure 1.2) and entering thus in a later or chronic phase of the disease. The decreased prevalence of the early colonizing species may be explained by antibiotic treatments administered to patients or by competition among co-colonizing microorganisms [36]. However, some bacteria, such as *S. maltophilia* and methicillin-resistant *Staphylococcus aureus* (MRSA) can persist along the life of CF patients (Figure 1.2).

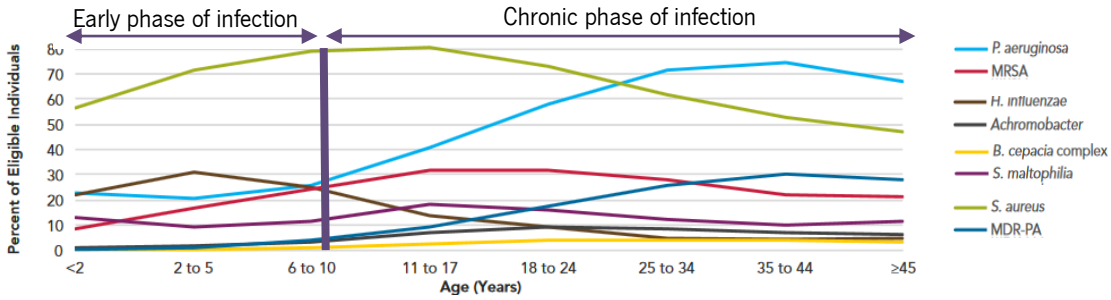


Figure 1.2. Prevalence of respiratory microorganisms by age cohort. Adapted from Cystic Fibrosis Foundation Patient Registry (CFFPR), 2014 [23].

Despite the impressive diversity of bacteria found in CF lungs at older ages, a limited number of organisms are associated to chronic lung infections and high mortality rates (Figure 1.2). Currently, *P. aeruginosa* is considered the most relevant pathogenic agent responsible for decreased quality of life, long hospitalisations, chronic infection development and ultimately the impressive number of deaths [37]. The source for the initial acquisition of *P. aeruginosa* seems to be relative to environmental reservoirs and cross-infection [38]. Once *P. aeruginosa* enters in CF lungs, bacteria cause chronic infections virtually impossible to eradicate even using aggressive and long antibiotic treatments [10]. Therefore, airways colonization by *P. aeruginosa* is considered a risk factor for CF patient and retard or avoid *P. aeruginosa* colonisation is the current gold standard of CF therapies.

P. aeruginosa colonization has even worsened prognosis, especially if associated with initial *S. aureus* colonization, because seriously compromise the growth rate of CF patient, as well as his social life [10]. Based on these evidences, it is hypothesized that early colonizers may positively impact on *P. aeruginosa* colonization, adaptation to CF environment and even on antibiotic resistance. In the next sections, the early colonizers *S. aureus* and *S. maltophilia* and also *P. aeruginosa* will be presented and the kind of interactions established will be reviewed in detail to understand their impact on CF disease.

1.3 Pseudomonas aeruginosa: the main pathogenic agent

P. aeruginosa is a gram-negative, rod-shaped, high versatile bacteria and the most problematic CF pathogen [39–41]. This bacterium is present in diverse environments, such as water, soil and plants, as well as in diverse abiotic surfaces such as medical equipment and indwelling devices [42].

This opportunist pathogen is capable of causing a wide range of infections, such as pneumonia, bloodstream infections, surgical site infections and urinary tract infections [43]. Regarding CF disease, *P. aeruginosa* is found in approximately 80 % of CF adult patients and it is the main responsible for

chronic infections that lead to a decline in pulmonary function, increased frequency of daily cough and worse chest radiological imaging scores [44–47]. Consequently, this pathogen is the major target of antibiotic treatment [48,49]. Although the long and aggressive antibiotic treatments administered to CF patients and the constant assault of the host immune system, *P. aeruginosa* infections are extremely difficult to eradicate, leading to morbidity and even death [50,51]. *P. aeruginosa* persistence is due to its impressive phenotypic plasticity to adapt to so complex environment as CF lungs, but more interestingly, *P. aeruginosa* is able to adapt to the different stages of CF disease development. These adaptations include the progressive transition toward a persistent and low virulence state, conversion to the mucoid phenotype [52] and to small colony variants (SCVs), antibiotic resistance, biofilm formation, alterations in lipopolysaccharide (LPS) and loss of motility [53–57]. *P. aeruginosa* undergoes some phenotypic and genetic adaptations in response to stressful conditions in a CF environment, such as osmotic stress, biological competition, the action of host immune molecules and antibiotic agents [56–59].

In early stages of infection, *P. aeruginosa* survival depends mainly on the expression of virulence factors, such as pyocyanin, proteases, phospholipase C, elastase, alkaline protease (AprA), exotoxin A, LPS, pili [15,47,60–64]. Pyocyanin has a particular role in the acute and chronic phase of infection because it enables anaerobic survival, serves as a redox-active antimicrobial compound and enables *P. aeruginosa* to successfully compete with other bacteria [65]. *P. aeruginosa* elastase cleaves many proteins, including collagen, fibrinogen and elastin, contributing to the invasion of bacteria into the lung parenchyma. AprA is thought to modulate the host response and prevent bacterial clearance [66,67]. The majority of *P. aeruginosa* virulence factors expression, including pyocyanin, elastase and alkaline protease are controlled by a cell-cell communication mechanism known as quorum sensing [68–70]. Quorum sensing is a cell-density-dependent regulatory system by which bacteria communicate with each other to control the expression of multiple genes [71]. This communication is performed by the secretion of autoinducers, which concentrations are detected by other bacteria.

P. aeruginosa CF isolates from chronic infections are generally distinct from those isolated at early stages of infection. *P. aeruginosa* phenotypic diversity in CF is one of the most interesting characteristic. As the infection progresses, *P aeruginosa* strains become less virulent, in order to acquire other adaptations, such as slow down its growth rate which is observed by phenotypic switching to SCV [72,73]. Chronic isolates exhibit frequently a mucoid phenotype and nowadays that feature is arguably one of the most clinically important features of chronic infections [50,74–76]. The mucoid phenotype

results from the overproduction of the exopolysaccharide alginate, a polymer of D-mannuronic acid and L-guluronic acid, which surrounds the cells and binds them together in aggregates and it is detected when bacteria are spread on solid media and colonies exhibit a mucoid appearance [52,77,78]. Alginate is one of three exopolysaccharides, along with Pel and PSI, that also play an important role in structural maintenance of biofilm matrix, enhancing thus biofilm formation that in turn prevents bacterial clearance by host phagocytes, opsonisation and antimicrobial therapy [79–82]. Alginate functions like a shield or barrier protecting bacteria that reside inside, reducing the penetration of the antimicrobial agents as well as the host molecules [51,78]. Mucoid phenotype presents a high resistance to antibiotic therapy, augmented by the enhanced production of biofilms due to the overproduction of alginate [51]. It has been shown that alginate can bind cationic antibiotics such as the aminoglycosides and restrict their diffusion [83].

SCV phenotype is considered a disease marker of the transition from early/intermediate infection stages to the chronic infection [84]. The isolation of SCVs is other phenotype detected in the sputum from CF patients with chronic infections [51]. SCVs are small size colonies (1-3 mm in diameter) after 24-48 h of growth on agar media [85]. They are typically hyperpiliated, slow growing isolates that show strong attachment to surfaces, autoaggregation, enhanced exopolysaccharide production and biofilm formation [86–88]. SCVs have an important role in *P. aeruginosa* pathogenesis, representing genetic adaptations to the hostile CF environment including resistance to phagocytosis, antimicrobial resistance due to slow growth [86,89,90]. The augmented resistance to antibiotics of SCVs is a great problem for the treatment of CF infections [91]. The appearance of antibiotic resistance lineages as SCV might be due to the prolonged time and repeated courses of antibiotics during which this pathogen inhabits the respiratory airways suffering adaptations [54]. *P. aeruginosa* antibiotic resistance has been also associated with modifications in the LPS, biofilm formation and QS mutations. The most prominent phenotype associated with SCV formation is the significantly enhanced production of one or more exopolysaccharides. The study of phenotypic diversity is thus very important in the determination of the CF infection stages once it gives information related to bacterial populations resident in CF lungs allowing to predict some information such as the resistance to antibiotics.

Biofilms may play a major role in the development of chronic lung infections in CF as they confer bacteria protection from host defences as well as tolerance to some antibiotics that target active and dividing cells [50]. Biofilm infections are generally difficult to eradicate even in hosts with intact immune system. Biofilm development *in vitro* was also found to be dependent on the quorum- sensing system

suggesting that the genes involved were critical for pathogenesis in the CF lung [43]. In CF, biofilms are particularly different due to the inexistence of an abiotic surface, forming biofilms in the mucus [92]. *P. aeruginosa* has the ability to form biofilms in CF lung which increases its resistance to antibiotic killing and phagocytosis [50]. These biofilms are multicellular aggregates within CF sputum where an oxygen gradient exists leading *P. aeruginosa* to undergo a switch from an aerobic to an anaerobic metabolism [93].

Samples from the airways of CF patients have revealed that *P. aeruginosa* may acquire mutations at an unusually high rate due to defects in the DNA repair system or proof reading system, namely in the *mutS*, *mutL*, and *uvrD* genes, being named hypermutable strains [94–96]. *P. aeruginosa* hypermutable strains become more frequent in later stages of the disease being their function a controversial theme. Hypermutable strains are likely to be mucoid or defective in quorum sensing, more resistant to antibiotics and in general more adaptable to the strict environment of the CF airways [57,97,98]. During antibiotic treatment of *P. aeruginosa* in CF patients, mutations in genes controlling the production of efflux pumps and β-lactamase frequently occur, originating resistant lineages that expand under the selective pressure of antimicrobial therapy, rising the risk of death or lung transplantation [57]. The effect of hypermutable strains in the clinical prognosis in CF is unclear, however theoretical considerations suggest that this phenotype is responsible for a more rapid clinical deterioration [51,99].

1.4 Early colonizers of cystic fibrosis lungs

1.4.1 Staphylococcus aureus

S. aureus is a human commensal commonly isolated from the upper respiratory tract and nasal passages of healthy individuals [100]. This gram-positive coccus, nonmotile, non sporeforming, unencapsulated bacterium that normally grows in aerobic conditions, but it can also be a facultative anaerobe [101,102].

Typically, *S. aureus* is the first pathogen to colonize the respiratory tract in CF [101]. *S. aureus* may inhabit the CF airways for years because it possesses an extensive group of virulent factors contributing for its significant pathogenesis, that are involved in attachment and persistence in human host [103,104]. *S. aureus* virulence factors, such as the biofilm formation and conversion to SCV are essential to ensure persistence in CF lungs [103]. Similarly to *P. aeruginosa, S. aureus* also produce SCVs to resist against antibiotics and they are also associated with chronic infections after patients

undergone prolonged antibiotic exposure [105–108]. SCV have been associated to a high ability to persist within eukaryotic cells without causing significant host-cell damage. By this way, bacteria are protected from host defences and antibiotic molecules, having the possibility to later revert to a more virulent wild-type resulting in recurrent infection [105,109–112]. SCV have been associated to persister cells because of the ability to survive to treatments and after treatment ending they are able to generate fast-growing offspring and when the conditions are right, can re-establish a major infection [113]. The slow-growing subpopulations can also be detected on solid media after 48 hours of growth.

The emergence of SCVs result from defects in electron transport, thymidine biosynthesis, carbon dioxide generation, or regulation of the stringent response, and it appears to be adaptive for intracellular survival [85,114,115]. It is known that SCVs contribute to infection and produce leukocidins that cause leukocyte destruction [116]. Once SCVs suffer a large range of microbiological changes, such as failure to metabolize mannitol, nonhemolytic colonies, lack of pigment, and the propensity to be overgrown by other bacteria, their identification could be problematic [117].

S. aureus hypermutable strains have also been detected. This phenotype seems to be related to mutations in both *mutS* and *mutL* genes [118,119]. Although it has not been made an association between *S. aureus* hypermutable phenotype and poorer clinical outcomes, this phenotype has been associated with SCVs and antibiotic resistance [118,119] .

The prevalence of MRSA has been rising, but MRSA has been detected in CF patients since 1980s. MRSA is a critical issue due to the lack of effective antimicrobial treatment. The resistance of *S. aureus* to methicillin, an β-lactam antimicrobial agent, is due to the production of β-lactamases that destroy these drugs and also due to the alteration in membrane-bound enzymes called penicillin-binding proteins (PBPs) [120,121].

Many contemporary strains of MRSA are resistant to multiple other classes of antimicrobial agents as tetracyclines, macrolides, lincosamides and streptogramins [122]. Several studies have reported that infection with MRSA is associated with worse clinical outcomes, but other some studies did not find an association between MRSA and decreasing of lung function and mortality [51,123]. The MRSA issue in CF lung, specifically the impact on quality of life and mortality rate, and the administration of prophylactic treatments or even the antimicrobial treatments to CF patients are quite controversial, therefore more studies must be addressed to expand the knowledge about the infection control strategies and about effective eradication of MRSA.

1.4.2 Stenotrophomonas maltophilia

S. maltophilia, previously known as both *Pseudomonas maltophilia* and *Xanthomonas maltophilia* is a gram-negative, non-fermentative, obligate aerobe, whose cells are straight or slightly curved [124–126] This bacterium is ubiquitously found in aqueous environments as single cells or in pairs [125,127]. This organism is commonly found in the lungs of CF patients and *S. maltophilia* colonization can occur in all ages, although *S. maltophilia* infections are more prevalent in older [23,128].

The role of *S. maltophilia* in the pathogenesis of CF disease remains unclear due to the difficulty to determine if it is only a colonizer or causative agent of infection [129,130] . Some studies report that *S. maltophilia* impairs lung function, while other studies did not find any difference in lung function [130–132].

The mechanisms involved in *S. maltophilia* pathogenesis are associated with adherence, biofilm formation, antibiotic resistance, production of hydrolytic enzymes and lipopolysaccharide. *S. maltophilia* colonization and, consequently development of disease, is favoured by the ability of pathogens to adhere to surfaces mediated by the fimbriae and flagella [133,134]. Flagella are highly immunogenic structures that mediates the adherence of *S. maltophilia* that together with production of fimbrial adhesion facilitate biofilm formation [135,136].

S. maltophilia has the ability to form biofilms resistant to antibiotics and to host immune response in CF, probably trigger by the selective adaptation imposed in CF airways [137,138]. Using confocal microscopy, *S. maltophilia* CF biofilms were observed and have revealed structures as microcolonies embedded in a matrix and also in a cell monolayer [137,139]. Biofilm formation by *S. maltophilia* seemed to be affected by environmental factors, such as phosphate and chloride concentrations, pH and temperature, the physicochemical properties of the bacterial cells and the surfaces to which the cells adhere [140,141]. In addition, alterations in LPS may alter biofilm formation and susceptibility of bacterial cells to antimicrobial compounds [142–145]. LPS is a major component of the outer membrane of most Gram-negative bacteria including *S. maltophilia* and comprises lipid A, core oligosaccharide, and O-antigen contributing for the development and maintenance of the bacterial cell adhesion to surfaces [146,147].

It has been reported that clinical CF isolates of *S. maltophilia* exhibit cytotoxic and hemolytic activity, and also demonstrate additional virulence factors expression, such as lecithinase activities [148].

However more studies are needed to understand the importance and the possible relationship between the infections caused by *S. maltophilia* and this cytotoxic activity.

Antibiotic resistance is a major problem in the eradication of *S. maltophilia* infections [141]. *In vitro* data of CF derived isolates suggest that tigecycline, trimethoprim-sulfamethoxazole, doxycycline, levofloxacin and ticarcillin-clavulanic acid have the highest *in vitro* rates of activity. Monitor the antibiotic susceptibility tests of isolates from patients are of upmost important to identify the sources of transmission of *S. maltophilia* and to reduce the prevalence of antibiotic resistance strains [141]. *S. maltophilia* multiresistance to antibiotics is due to several mechanisms, including plasmids, integrons, and transposon, reduction in outer membrane permeability and the activity of multidrug efflux pumps [149,150]. For instance, β-lactamase chromosomally-plasmid-encoded, multidrug efflux pumps, class 1 integrons and insertion element common region (ISCR) elements are associated with resistance to trimethoprim-sulfamethoxazole (TMP-SMX).

The presence of *S. maltophilia* is somewhat continuous, therefore it is very important the investigation of its evolutionary pathway and the mechanisms underlying antibiotic resistance during infection progression.

1.5 Antimicrobial treatments

As aforementioned, *P. aeruginosa* is the main pathogen in CF and an elevated number of patients succumb to chronic lung infection. Therefore, *P. aeruginosa* has become the main target of antimicrobial treatments, although the prevention of lungs colonization by *P. aeruginosa* is the main therapeutic goal. Despite this, *S. aureus* is also an important pathogen especially in an early stage of infection and some antibiotic treatments are focused on its eradication.

Early aggressive antibiotic treatment or prophylactic administration of antibiotics have been used over the years to delay or prevent chronic colonization by *P. aeruginosa* and *S. aureus* [151]. Despite the efforts to eradicate these pathogens, patient colonization occurs and consequently patient death.
Several antibiotics, combinations of agents and routes of administration have been used in antimicrobial treatment of *P. aeruginosa* infections. Treatments vary even across centres, countries and continent and, of course, according the stain isolated. Therefore, the most used antimicrobial treatments will be described in this section.

Ciprofloxacin has been used in CF therapy being administrated oral and intravenously combined with other antimicrobials, such as tobramycin, aztreonam or colistin [152,153]. This antibiotic is a broad-spectrum fluoroquinolone antibiotic effective against *P. aeruginosa* and *S. aureus*. As almost all antibiotics, the systemic administration of ciprofloxacin has side effects and thus it is only administrated to adults once it long term use in children can cause CF-associated musculoskeletal disorders [154].

Inhaled tobramycin and aztreonam have also been recommended for the treatment of *P. aeruginosa* infections mainly in children with at least 6 years [155]. Colistin, a polymyxin derivative colistimethate sodium, has also been used for the treatment of CF in children and adult with benefits demonstrated, but its long-term efficacy is poorly documented [156,157]. Gentamycin and tobramycin are especially administered to pediatric patients, however there is a cochleotoxis risk [158]. Piperacillin may also be administrated for CF treatment in conjunction with tazobactam, a potent inhibitor of β-lactamases causing some secondary effects such as urticaria and pruritic [151]. Ceftazidime is one of the most commonly used antimicrobials, causing the inhibition of cell wall synthesis [151].

Despite the use of several antibiotics for the treatment of CF, none of them is totally effective in the treatment of this disease given the existence of antibiotic resistance by pathogens and their interactions in the CF lung. Therefore, it is of upmost importance to take in account the mechanism used by bacteria to resist to antibiotic treatment and, more importantly, the interactions between pathogens when thinking about treatments for a specific disease once these interactions influence the resistance of pathogens to antibiotics.

In attempt to improve the efficacy results of the current antimicrobial treatments, several innovative treatments are being studied. For instance, administration of ciprofloxacin via dry powder inhaler has already been shown some efficacy [159]. The use of nanoparticles loaded with ciprofloxacin carriers is also a promising approach. These nanoparticles protect the drug from deactivation and increase mobility in mucous facilitating therefore the treatment of bacterial pathogens located inside the mucous [153,160–162]. These ciprofloxacin-loaded lipid-core nanocapsule are also advantageous in the prevention of *S. aureus* biofilm formation [153].

1.6 Interspecies Interactions

1.6.1 *S. aureus* – *P. aeruginosa* interactions

As referred previously, CF lungs are colonized by several bacterial species at the same time which "force" bacteria to interact with each other [163]. These interspecies interactions are thought to influence, or even determine, the disease progression [164]. In last years, clinical community has tried at elucidating how pathogens interact and, more importantly at determining the impact of these interactions on disease progression and the response to antimicrobial treatments. Unfortunately, few advances were made concerning bacterial interactions in CF lungs. In this section a review of the reported interactions established between early colonizers namely *S. maltophilia* and *S. aureus* and the most problematic pathogen, *P. aeruginosa*, will be described.

It is known that co-infecting species can interact both antagonistically and synergistically. Antagonist interactions refer to the inhibition of one population by the presence of the other, through competition of nutrients and/or space or, more directly, through an antibacterial effect of one species toward other. On the other hand, synergistic interactions are related to the benefit of one population by the presence of other bacterial species [165,166]. For instance, synergistic interactions have been proposed with respect to *S. aureus* "sensitizing" the lungs to subsequent infection by *P. aeruginosa* [167–169]. Nevertheless, antagonistic interactions between these two pathogens have also been described, which led to conclude that *S. aureus* and *P. aeruginosa* interactions are not well understood and more studies are needed to clarify this issue [170,171]. In Table 1 are presented some of the most important findings related to *S. aureus* and *P. aeruginosa* interactions.

Table 1.1. The main interactions reported in literature between *S. aureus and P. aeruginosa*.

Interaction	Reference
S. aureus production of peptidoglycan stimulates the production of virulence factors such as siderophores, exotoxins and proteases by *P. aeruginosa*;	[172]
S. aureus extracellular factors such as the SpA increase growth activity and suppress autolysis of *P. aeruginosa;*	[164]
S. aureus extracellular proteins, such as ClpP, induce selection for *P. aeruginosa* SCV under antibiotic pressure;	[164]
The presence of *P. aeruginosa* can inhibits the *S. aureus* growth by the production of hydrogen cyanide, quinoline N-oxides and phenazine pyocyanin;	[165,166,173]
The production of exoproducts by *P. aeruginosa,* including siderophes and HQNO can inhibit *S. aureus* respiration, induce resistance against antibiotic, virulence and biofilm formation;	[166,173,174]
P. aeruginosa can lyse *S. aureus* cells by the production of exoproducts such as HQNO;	[171]
P. aeruginosa coexists and then kills *S. aureus* in late-stage coculture.	[175]

It has been proposed that *S. aureus* could somehow prepare the respiratory tract epithelia of CF patients to promote the subsequent colonization of *P. aeruginosa* [43,176]. Armbruster *et al.* (2016) reported that *S. aureus* had impact in *P. aeruginosa* adhesion and phagocytosis resistance through the secretion of products, including the staphylococcal protein A (SpA), a virulence factor known to be expressed during *S. aureus* growth in CF [177]. SpA is known to mediate bacterial interactions in the specific case of *S. aureus* and *P. aeruginosa*, protecting *P. aeruginosa* against phagocytosis. On the other hand, SpA affects *P. aeruginosa* biofilm formation by binding to cell surface targets such as Psl polysaccharide and the PilA protein component of type IV pili, which are important in biofilm formation. Another study reported that the peptidoglycan shed by *S. aureus* stimulates the production of virulence factors, such as siderophores, exotoxins and proteases by *P. aeruginosa* [172]. *P. aeruginosa* uses peptidoglycan as a cue to stimulate production of antimicrobials molecules and multiple extracellular factors that possess lytic activity against prokaryotic and eukaryotic cells, enhancing host killing [172].

Michelsen *et al.* (2014) demonstrated that *S. aureus* extracellular proteins, such as ClpP, increase *P. aeruginosa* growth rate resulting in increased cell density, suppress autolysis and induce *P. aeruginosa* conversion to SCV. These interactions are dependent of the Agr quorum system which is responsible for the regulation of several *S. aureus* virulent factors [164].

On the other hand, *P. aeruginosa* produces several exoproducts inhibitor of *S. aureus* respiration, such as hydrogen cyanide, quinoline N-oxides and phenazine pyocyanine. The production of these exoproducts by *P. aeruginosa* was also associated to an increased resistance toward antibiotic killing and stimulation of pigment production, virulence potential and biofilm formation by *S. aureus* [165,166,173]. *P. aeruginosa* co-culture with *S. aureus* also results in *S. aureus* conversion to SCVs that appears to provide a mechanism for *S. aureus* persistence in co-culture with *P. aeruginosa*. The lysis of *S. aureus* by *P. aeruginosa* results in the release of iron that is an essential nutrient for *P. aeruginosa* especially in an acute infection where this nutrient is need in high dosage [171]. *S. aureus* switching to SCV phenotype seemed to partially protect from *P. aeruginosa* killing due to a mutation in the electron transport chain, despite this "degree of protection" from *P. aeruginosa* demonstrated to be quite variable [65,175].

Filkins *et al*. (2015) demonstrated that *P. aeruginosa* and *S. aureus* are able to coexist for a certain period of time being *S. aureus* then killed by *P. aeruginosa* in a late-stage of coculture [175]. The same study also demonstrated that the secretion of exoproducts by *P. aeruginosa* induces the shift of *S. aureus* from aerobic respiration to lactic acid fermentation. The produced products from *S. aureus* lactic acid fermentation, such as lactate, were consumed by *P. aeruginosa* for its growth. Moreover, exoproducts production and oxygen competition negatively impact on *S. aureus* growth and survival [175]. The production of these exoproducts, including siderophes and HQNO and oxygen utilization by *P. aeruginosa* inhibit the electron transport chain of *S. aureus* by sequestering iron that is a necessary cofactor of cytochromes increasing gene expression of fermentation pathways. The combination of poisoned electron transport chain, competition for nutrients and exposure to *P. aeruginosa* exoproducts leads to *S. aureus* death.

Despite these evidences, the studies about the interactions between *S. aureus* and *P. aeruginosa* in CF are scare [175]. The interspecies interactions established between these two most relevant pathogens detected in CF infections could be the key for deciphering the pathogenicity of CF disease and how CF-associated infections progress to chronic stages. Moreover, understanding these interspecies

interactions, new insights could rise helping to develop new therapeutic molecules for eradication of CF-associated infections.

1.6.2 *S. maltophilia – P. aeruginosa* interactions

S. maltophilia is often co-isolated with *P. aeruginosa* from CF samples and, thus, interactions between these two bacterial species are expected to occur [178]. *S. maltophilia* could represent an excellent assistant of *P. aeruginosa* pathogenesis (Table 1.2).

Table 1.2. Interactions between *P. aeruginosa* and *S. maltophilia*.

Interaction	***Reference***
S. maltophilia increases *P. aeruginosa* resistance to polymixin;	[179]
S. maltophilia may indirectly contribute to disease development by providing a favourable growth environment for *P. aeruginosa* and by modulating some virulence traits exhibited by *P. aeruginosa*;	[180]
P. aeruginosa outcompetes *S. maltophilia* during both exponential and stationary phases in CF biofilms once it exerts antibacterial *activity against S. maltophilia;*	[178]
The presence of *S. maltophilia* reduces the amount of EPS produced by *P. aeruginosa*, affecting its ability *to* adhere to substratum;	[178]
S. maltophilia ability to form biofilms is reduced in the presence of *P. aeruginosa;*	[178]
S. maltophilia may promote *P. aeruginosa* adaptation to the airways of CF patients by favoring phenotypic traits acquired by *P. aeruginosa*.	[178]

Pompilio *et al.* (2015) suggested that *S. maltophilia* could promote *P. aeruginosa* adaptation to the airways of CF patients by favouring the expression of advantageous phenotypic traits during chronic infections [178]. For instance, *S. maltophilia* favoured the up-regulation of both algD and aprA operon, which codifies for alkaline protease and alginate, respectively, and loss of LasR function. The increase expression of algD trigger by the presence of *S. maltophilia* favoured *P. aeruginosa* protection against oxygen radicals, having serious clinical implications in disease progression and response to antibiotic treatments [178,181].Alkaline protease, alginate and LasR have implications in antibiotic resistance because increase β-lactamases activity responsible for the tolerance of to β-lactam antibiotic, and thus promoting *P. aeruginosa* long-term survival in CF lungs. The presence of *S. maltophilia* seems also to increase *P. aeruginosa* resistance to polymyxin, imipenem or ceftazidime also caused by the

production of β-lactamases by *S. maltophilia*, that allow the growth of surrounding susceptible β-lactam bacteria as *P. aeruginosa* [179,180].

The presence of *S. maltophilia* significantly disturbs the amounts of EPS produced by *P. aeruginosa* affecting its adhesion [178,182]. However its capacity to form biofilms was unaffected by the presence of *S. maltophilia* [178].

S. maltophilia biofilm formation is significantly reduced in the presence of *P. aeruginosa* due to its antibacterial activity which seems to be not mediated by exoproducts, but apparently it requires direct contact between cells. *P. aeruginosa* may kill *S. maltophilia* by the injection of specific proteins via the type VI secretion system [178]. *P. aeruginosa* seemed dominate *S. maltophilia* during both exponential and stationary growth phases, probably due to the competition for nutrients and space, or even again to an antibacterial effect of *P. aeruginosa* over *S. maltophilia* by the same mechanism [178].

Much of the current knowledge about interactions between *S. maltophilia* and *P. aeruginosa* has resulted from the studies of mixed biofilms [178]. However, the *in vitro* conditions used in these studies are very different from the environmental conditions of CF lungs. Therefore, it is fundamental to start as soon as possible the investigation of *S. maltophilia* and *P. aeruginosa* interspecies interactions simulating the CF environment.

The understanding about the interactions between *P. aeruginosa* and other bacterial species at early and chronic infections stages is very limited, but they are quite valuable. Mapping the interactions patterns between species and how they affect the balance of lung colonization, the progression of disease and, more importantly, the response to antimicrobial treatments are essential to achieve new effective antimicrobial treatments [164]. Microbial interactions that favour *P. aeruginosa* adaptation to CF lungs could be the key to eradicate or, at least, to weaken this pathogen. An antibiotic could be used to target the bacterial species that assist *P. aeruginosa* adaptation, reducing thus the benefit to antibiotic resistance and chronic infection development. Being *P. aeruginosa* more susceptible in the absence of favouring interactions, eradication could be possible combining a second antibiotic agent targeting *P. aeruginosa*. In this logic, this thesis aimed to investigate the interactions established between *P. aeruginosa* and the typical early colonizers of CF lungs, including *S. aureus* and *S. maltophilia*. The novelty of this study is to investigate these interspecies interactions using *in vitro* conditions and an experimental setup quite similar to the conditions found in CF lungs. Therefore, the

outputs retrieved from this study will be more close to *in vivo* situation and *P. aeruginosa* adaptation and antibiotic resistance could be better understood.

2. MATERIALS AND METHODS

2.1 Bacterial strains and culture conditions

P. aeruginosa PAO1 and a clinical isolate from CF chronic phase of infection PAI, *S. aureus* ATCC 25293, a CF clinical isolate from a chronic phase of infection, SAI and a clinical CF isolate of *S. maltophilia* were used to perform this study. *P. aeruginosa* PAO1 and PAI were dry strains such as *S. aureus* strains. During this study, all bacterial strains were preserved in criovials (Nalgene) at -80 °C and they were grown in Tryptic Soy agar (TSA) (Liofilchem) for 24 h and after were cultured in Tryptic Soy broth (TSB) (Liofilchem) at 37 °C.

2.2 Phenotypic characterisation of bacterial strains

2.2.1 Antibiotic susceptibility profile

Before artificial sputum experiments, the antibiotic susceptibility of bacterial strains was studied given its importance for future comparisons in further studies of co-culture of *S. aureus* and *S. maltophilia* with *P. aeruginosa*.

The antibiotic susceptibility of *S. aureus* and *S. maltophilia* strains were determined using the disk diffusion method according CLSI guidelines [183]. Twelve antibiotics (Liofilchem) commonly used in the treatment of *S. aureus* infections belonging to 10 different classes were tested: ciprofloxacin 5 µg, erythromycin 15 µg, tetracycline 85 µg, rifampicin 6 µg, vancomycin 30 µg, ceftazidime 30 µg, gentamicin 10 µg, oxacillin 30 µg, linezolid 30 µg, clindamycin 2 µg, teicoplanin 30 µg and quindalfoprist 15 µg. Five antibiotics (Liofilchem) were used to assess *S. maltophilia* susceptibility: ciprofloxacin 5 µg, ceftazidime 30 µg, Tiarcillin/clavulanic acid 85 µg, gentamicin 10 µg and piperacillin 100 µg. After 18-21 h at 37 °C in aerobic conditions, the inhibition zones were measured and bacterial susceptibility categorized according CLSI guidelines [183]. The antibiotic susceptibility of *P. aeruginosa* was not performed because was previously determined [184].

2.2.2 Colony morphology observation

Phenotypic diversity is a bacterial mechanism of adaptation to CF environment and alterations in colony morphology could be a manifestation of this biological process [185]. Moreover, several phenotypic

alterations are clinically relevant, such as mucoid phenotype and SCV [166]. The assessment of the wild-colony morphology was performed as previously described [186].

Bacterial suspensions were serial diluted, plated on TSA and allowed to grow at 37 °C. In this colony observation study, selective solid media was not included because it was reported that colony morphology diversity is significantly reduced [186]. Colonies were observed at 24, 48 and 72 h of growth in order to determine the optimal growth time by which colonies maintain their morphological features.

2.3 Bacterial growth conditions in artificial sputum medium

Artificial sputum medium (ASM) was a medium used to mimic the sputum of CF patients. The ASM was prepared accordingly to Sriramulu, D. (2005) [187]. Briefly, ASM was prepared by mixing 5 g/L of mucin from pig stomach (Sigma-Aldrich), 4 g/L of DNA (Sigma-Aldrich), 5.9 mg/L of diethylene triamine pentaacetic acid (DTPA) (Sigma-Aldrich), 5 g/L of NaCl, 2.0 g/L of KCl and 5 g/L of casoaminoacids (AMESRO) adjusting the pH to 7.0 with Tris base. The mixture was then sterilized in an autoclave at 110 °C for 15 min and after cooled, 5 mL/L of egg yolk (Fluka) emulsion was added.

In a 24 well-plate, 2 mL of the ASM was transferred to each well and inoculated with *S. aureus* at a final concentration of 10^7 CFU/mL. The inocula were obtained by growth of *S. aureus* overnight in TSB at 37 °C and 120 rpm in air conditions. The resulting cell suspensions were washed in sterile water by centrifugation (9000 *g*, 7 min) and diluted to a concentration of 4 x 10^9 CFU/mL. 5 µL of these bacterial cell suspensions with of 4 x 10^9 CFU/mL were transferred to the top ASM of each well obtaining a final cellular concentration in each well of 10^7 CFU/mL. The inoculated ASM cultures were statically incubated at 37 °C for 3 days in air conditions. After 3 days, the time thought to be necessary to *S. aureus* adapt to the CF environment and thus mimicking the early phase of infection, the ASM containing *S. aureus* was inoculated with *P. aeruginosa* at a final concentration of 10^7 CFU/mL. The experiments were conducted over more 4 days (a total of 7 days) (Figure 2.1). The procedure to obtain the *P. aeruginosa* inocula was similar as described previously for *S. aureus*. The same method was used to coculture *P. aeruginosa* with *S. maltophilia*.

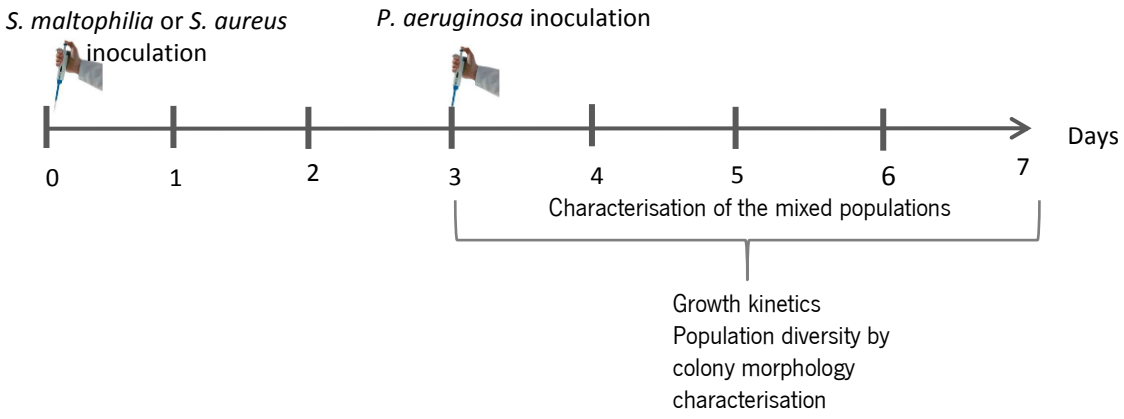


Figure 2.1. Time-line of the inoculation of each bacterial species for bacteria growth in ASM.

The mixed populations grown in ASM were monitored at scheduled intervals of time to investigate the interspecies interactions established (see next sections). The content of wells was collected aseptically during each day of bacterial growth and was vigorously shaken (using vortex) to detach cells from the small aggregates and those adhered to mucin. From these cell suspensions, the number of viable cells and the diversity of colony morphology was determined.

2.4 Kinetics growth of mixed populations

Every 24 h of bacterial growth in ASM, the number of viable cells was determined through colony forming unit (CFU) counting (Figure 2.1).

ASM cell suspensions were serial diluted in sterile water, plated on respective selective media, to distinguish each bacterial species, and incubated overnight at 37 °C. After that, the number of colonies were counted and verified after more 24 h of growth to detect bacteria with slow growth rate. *P. aeruginosa* was plated on *Pseudomonas* isolation agar (PIA) (Biotecnómica), *S. aureus* on mannitol salt agar (MSA) (Liofilchem) and *S. maltophilia* on Blood agar supplemented with 7 % sheep defibrinated blood (BAM) supplemented with vancomycin, amphotericin and imipenem (VIA) (HIMEDIA) as described elsewhere [188–190].

2.5 Time-killing curves

To investigate the influence of the earlier colonizers on *P. aeruginosa* response to antibiotic treatments, mixed populations grown in ASM were exposure to ciprofloxacin treatments. After 3 days of *P.*

aeruginosa co-culture with *S. aureus* in ASM, ciprofloxacin was added to the top of ASM cultures at a final concentration of 4 mg/L. New ciprofloxacin dosages were added every 24 h, over more 4 days. Ciprofloxacin time-killing curves were constructed based on the number of viable cells determined by CFU counting as described previously. The assays concerning the exposure of the mixed population relative to *S. maltophilia* were not performed given technical problems described in section 3.1.

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

In mixed cultures, the Competitive Index (CI) was calculated to determine if one species had competitive advantage relatively to other. CI was defined as the *S. aureus*/ *P. aeruginosa* output divided by the corresponding ratio in inoculation. Similar to CI, RIR was calculated based on monocultures growth results for each species [191]. For statistical analysis, the values of CI and RIR were subjected to a log transformation for normal distribution interpretation [178]. A CI value equal to 0 indicates equal competition of the two species; a positive CI value indicates a competitive advantage for *S. aureus*; a negative CI value indicates a competitive advantage for *P. aeruginosa*. CI and RIR were subject of a statistical analysis and significantly different values suggest a full competition between species.

2.7 Investigation of clonal diversification

Clonal diversification was inspected through observation of colony morphology of the mixed co-cultured populations in ASM. Bacterial populations were serial diluted, plated on TSA and allowed to grow at 37 °C during the optimal time determined previously in the phenotypic characterisation of the bacterial strains (section 2.2.2). Colonies were observed by directly placing petri plates on magnifying glass and photographed in a CCD camera. Colonies were evaluated according to their form, margin, type of surface, texture, size, sheath, elevation, opacity, consistency and colour as described by Sousa *et al.* (2015) [192].

2.8 *P. aeruginosa* proteome

To study the biological processes expressed by *P. aeruginosa* it was required the optimization of some major steps of the procedure, including the cell lysis (sonication time, and lysis buffer) and staining method of gels. Sample preparation is one of the most important steps in a 2-DE, since it determines

the quality of the sample and therefore the quality of the results. Bad quality samples could be responsible for the appearance of smears and sometimes for the nonappearance of specific spots.

2.8.1 Sample preparation

Proteins were extracted from plated colonies of running experiments on TSA, and were collected and suspended in ultrapure water being then centrifuged for 10 min at 6000 *g*. The pellet was collected and re-suspended in several lysis buffers (Table 2.1). Cells were then disrupted by 5 min of sonication (30 s on, 30 s off) on ice with amplitude of 30 %, being samples collected over time. Unbroken cells and cell debris were removed from the suspension by centrifugation at 9 000 *g* for 20 min at 4°C. The protein amount was measured using Pierce® BCA proteins assay kit (Thermofisher) according manufacturer's guide. Further, the supernatant was precipitated with TCA/Acetone method. This method consisted in the addition of 100 % TCA to obtain a final concentration of 20 % TCA to the volume of the protein sample being then incubated during 20 min and centrifuged at 7000 *g*, 4 °C during 10 min. After, the pellet was resuspended in acetone and the centrifugation procedure was repeated. The obtained pellet was re-suspended in rehydration buffer (Bio-Rad).

Table 2.1 Lysis buffers composition used in this study.

Lysis Buffer	Reagents
I	10 mM Tris-HCl; 1 mM PMSF; 1 mM EDTA
II	10 Mm Tris-HCl; 1 mM EDTA; 5 mM $MgCl_2$
III	7 M Urea; 2 M Thiourea; 40 mM DTT; 2% (w/v) CHAPS
IV	7 M Urea; 2 M Thiourea; 40 mM DTT; 2% (w/v) CHAPS; 10 Mm Tris-HCl; 1 mM EDTA

2.8.2 Protein separation by SDS-PAGE

Both *P. aeruginosa* strains proteins were analysed by an Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Eighty micrograms of proteins were added to sample solution (10 % (w/v) β-mercaptoethanol, 10 mM DTT, 20 % (v/v) glycerol, 0.2 M Tris-HCl pH 6.8, 0.05 % (w/v) Bromophenol Blue) and heated during 5 min at 100 °C. Further, proteins were separated using a 12 % (w/v) polyacrylamide running gel with a run at 150 V being then stained with silver nitrate.

2.8.3 Protein separation by two-dimensional gel electrophoresis

P. aeruginosa proteins were analyzed by two-dimensional gel electrophoresis (2-DE). One hundred micrograms of proteins were added to rehydration buffer (final volume, 125 μL). The first-dimension gel separation was carried out with 7 cm Ready Strip IPG gel (Bio-Rad) using Protean IEF apparatus (Bio-Rad). First the strips were rehydrated during 12-16 h at 50 V, 20 °C. Further, focusing protocol was executed according to table 2.2. The second dimension was obtained by SDS–PAGE using a 12% (w/v) polyacrylamide running gel with a run at 150 V. After migration, proteins were visualized by silver nitrate staining. The same procedure was followed for the 2-DE using 17 cm strips with exception of the focusing conditions (Table 2.3) and the running voltage used which in this case was 250 V.

Table 2.2. 2-DE focusing conditions for a gel size of 7 cm

	Voltage	**Set Time**	**Ramp**	**Temperature**
Step 1				
	250 V	15 min	Rapid	20 °C
	Voltage	**Set Time**	**Ramp**	**Temperature**
Step 2				
7 cm	8000	1h	Slow	20 °C
	Voltage	**Set Time**	**Ramp**	**Temperature**
Step 3				
7 cm	8.000	1h	Rapid	20 °C

Table 2.3 2-DE focusing conditions for a gel size of 17 cm

	Voltage	**Set Time**	**Ramp**	**Temperature**
Step 1				
	250 V	15 min	Rapid	20 °C
	Voltage	**Set Time**	**Ramp**	**Temperature**
Step 2				
7 cm	10000	2h	Slow	20 °C
	Voltage	**Set Time**	**Ramp**	**Temperature**
Step 3				
7 cm	10000	1h	Rapid	20 °C

2.9 Statistical Analysis

All statistical analysis was performed using Prism software package (GraphPad Software version 6.01) performing a one-way analysis of variance (ANOVA). Comparisons were performed using Turkey multiple-comparisons test with a 95 % confidence level and differences were considered statistically significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

P. aeruginosa is the main pathogen of CF-associated infections and, consequently, the major responsible of increase mortality rate because it is almost "impossible" to eradicate [84]. This pathogen cohabits with several other bacterial species in the CF lungs where interspecies interactions may occur determining the course of infection [193]. Although some studies have reported the existence of interactions between *S. aureus*, *S. maltophilia* and *P. aeruginosa*, these studies did not closely simulate the CF lung environment, which may greatly impact on the type of interactions established between pathogens [164,172,178]. Therefore, this study aimed to investigate the interspecies interactions established between the earlier colonizers of CF lungs, in particular, *S. aureus* and *S. maltophilia,* with *P. aeruginosa* [23,101]. More specifically, this study intended to determine the impact of these interactions on *P. aeruginosa* pathogenicity towards CF adaptation and antibiotic resistance. To perform that, a novel *in vitro* CF model was developed using ASM to closely mimic the CF lungs conditions experienced by bacteria in *in vivo*. Moreover, this model included different growth timings for each pathogen to simulate a typical CF lungs colonization profile composed by early infection stage (corresponding to early colonizer growth in ASM) and chronic stage (corresponding to P. aeruginosa growth in ASM).

3.1 Interspecies interactions between *S. maltophilia* and *P. aeruginosa* in cystic fibrosis environment

S. maltophilia is a common pathogen of CF lung and its isolation has been raising over the years [141]. This bacterium colonizes the lungs during all stages of infection, but its prevalence increases at early stage of the disease [194]. Interactions between *S. maltophilia* and *P. aeruginosa* have been described and it is believed that *S. maltophilia* may provide a favour environment for *P. aeruginosa* growth in CF lungs, contributing thus for disease development. For instance, *S. maltophilia* increases *P. aeruginosa* resistance to polymyxins and encourages *P. aeruginosa* growth in the presence of ceftazidime [179,180]. Despite these evidences, the role of *S. maltophilia* in CF disease and its impact on *P. aeruginosa* pathogenicity are still poorly understood. Therefore, more studies addressing the interactions between these two pathogens in a CF environment are essential mostly when clinical community is trying to find out new anti-pseudomonal treatments.

3.1.1 Phenotypic characterization of *S. maltophilia* isolate

To investigate the interactions established between *S. maltophilia* and *P. aeruginosa* in an early phase of infection, it was important to clearly characterise the "ruler" of this study, *S. maltophilia.* Therefore, some relevant phenotypic characteristics of *S. maltophilia* in CF context were studied, namely the antibiotic susceptibility and the colony morphology. Antibiotic susceptibility tests were performed in accordance with the CLSI guidelines [183]. Five antibiotics commonly used to treat *S. maltophilia* infection were tested and the *S. maltophilia* clinical isolate demonstrated to be resistant (R) to ciprofloxacin and piperacillin, intermediate resistant (I) to ceftazidime and susceptible (S) to gentamicin and ticarcillin-clavulanic acid (Table 3.1).

Table 3.1. Antibiotic susceptibility of *S. maltophilia* to the tested antibiotics according CLSI guidelines.

	CIP	**CN**	**CAZ**	**TTC**	**PRL**
S. maltophilia clinical isolate	R	S	I	S	R

CIP: Ciprofloxacin CAZ: ceftazidime CN: gentamicin PRL: piperacillin TTC: Ticarcillin /Clavulanic acid
S: susceptible; I: intermediate resistant; R: resistant

This antibiotic susceptibility profile was not surprising because it is known that *S. maltophilia* could be resistant to antibiotics of the β-lactam, aminoglycosides and quinolone classes similarly to the results obtained in this study [125]. Literature had also reported that ceftazidime has a reasonable activity against *S. maltophilia*, while ticarcillin/clavulanic acid is described as a good antibiotic against this pathogen which is corroborated by the obtained results [125]. On the other hand, *S. maltophilia* susceptibility to ciprofloxacin is quite variable according other studies and this *S. maltophilia* isolated showed to be resistance to ciprofloxacin [125,195,196].

The alteration of colony morphology by bacteria is one of the phenotypic manifestations of bacterial adaptation to new environment, such as CF lungs. In a CF environment, *S. maltophilia* is known to switch from the wild-morphotype to SCV [197]. Similarly, with other bacterial species, this *S. maltophilia* switching to SCV was detected after treatment with trimethoprim-sulfamethoxazole, suggesting that prolonged treatments select this *S. maltophilia* phenotype. *S. maltophilia* SCV has been associated with enhanced resistance to antibiotics [197].

To better detect alterations in colony morphology, in particular SCV, the characterization of colony wild-type morphology and the most adequate colony growth time is required. The study of the colony morphology was performed during 120 h in which the same colonies were observed and characterized at different growth times. To characterize the morphological traits exhibited by *S. maltophilia* colonies, a colony morphology system presented by *Sousa et al.* (2013) was used [186].

This *S. maltophilia* strain displayed only one colony morphology over the time of growth (Figure 3.1). This colony variant exhibited the same morphological characteristics over time with exception of size (Figure 3.1).

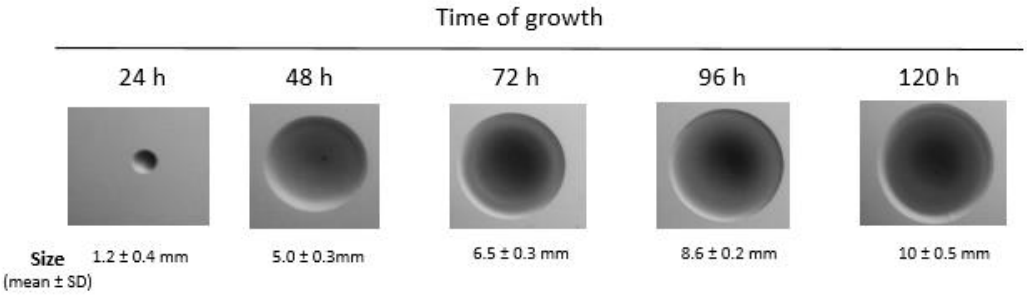


Figure 3.1. *S. maltophilia* colony morphology observed during 120 h of growth. *S. maltophilia* exhibited only one single colony morphotype over the 120 h.

The traits observed in *S. maltophilia* colonies were similar to those reported in other studies [125,141]. These colonies possess beige colour, entire margin and circular form (Supplemental material S1). Although there are some studies reporting the characteristics of *S. maltophilia* colonies, there are no studies addressing the optimal colony growth time to detect new variants, for instance SCV, or to accurately characterize their morphological traits. Therefore, any colony growth time (24, 48 and 72 h) could be used to inspect *S. maltophilia* colony morphology alterations. But, as this work aimed to study mixed cultures of *S. maltophilia* and *P. aeruginosa* that will be plated on the same agar medium, it would be convenient similar colony growth time for both species, facilitating then the analysis of mixed cultures. According previous results, *P. aeruginosa* colonies had a 48 h-optimal growth time [192]. Therefore, the time of growth of *S. maltophilia* colonies used in this study was also 48 h.

3.1.2 The impact of *S. maltophilia* on persistence of *P. aeruginosa* infections

To accomplish one of the main goals of this project and to clarify the impact of *S. maltophilia* on *P. aeruginosa* adaptation to CF environment, this study mimicked a progressive bacterial colonization and infection development in CF lungs. The early infection stage that corresponds to the childhood of CF

patients is typically characterized by the absence of *P. aeruginosa* and prevalence of other pathogens such as *S. maltophilia*. To investigate the influence of *S. maltophilia* on *P. aeruginosa* adaptation, *S. maltophilia* was previously cultured in ASM for 3 days to mimic an early phase of the disease. This period of time could be crucial, for instance, *S. maltophilia* adaptation to CF environment and exhibit identical phenotypic traits as those find in CF lungs. After *S. maltophilia* period of adaptation, *P. aeruginosa* was incubated and grown during 4 days with *S. maltophilia*. Samples were recovered every 24 h to investigate interspecies interactions.

The results obtained demonstrated that *P. aeruginosa* had outcompeted *S. maltophilia* (Figure 3.2) since no colony of *S. maltophilia* was visible in TSA plates. According Pompilio *et al.* (2015), *P. aeruginosa* also outcompeted *S. maltophilia* during stationary and growth phases being possibly caused by nutrients and space competition or caused by the antibacterial effect of one species toward the other [178,198,199].

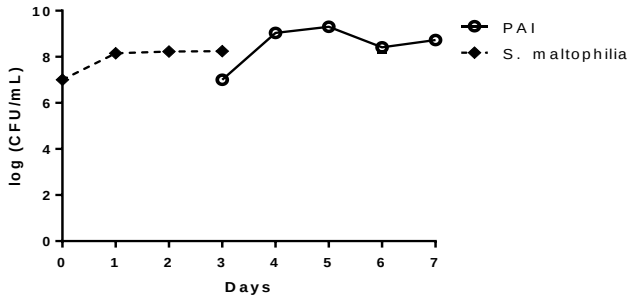


Figure 3.2. Co-culture of *P. aeruginosa* and *S. maltophilia* in ASM for 7 days. After the ASM inoculation with *P. aeruginosa*, *S. maltophilia* was not detected.

Based on the present results, *S. maltophilia* seemed to play no role in *P. aeruginosa* adaptation to CF environment as the growth of *P. aeruginosa* was quite similar to the one observed when cultured alone in ASM [184]. However, the growth kinetics observed was constructed based on colony counting on TSA plates for both species. The absence of *S. maltophilia* colonies on TSA could be caused by *P. aeruginosa* outcompetition of *S. maltophilia* not in ASM, but on TSA. To overcome this experimental limitation, selective media for each bacterial species should be used and thus the counting of colonies could be made without species competition or inhibition on agar medium. There are several selective media available for *P. aeruginosa* including PIA that clearly allow *P. aeruginosa* isolation. In what concerns *S. maltophilia*, there are no selective media for this species, but differential media, such as VIA agar medium, blood agar medium (BAM) supplemented with vancomycin, amphotericin and

imipenem [188,200]. BAM was used in this study to differentiate *S. maltophilia* from *P. aeruginosa* since the authors reported that *P. aeruginosa* could be distinguished [200].

Co-cultures from ASM were thus plated on BAM but *P. aeruginosa* was not different from *S. maltophilia*. Both species exhibited brown colonies with approximately the same size. To confirm the outcompetition of *S. maltophilia* in ASM more attempts to develop a selective medium of culture or an improved differential medium are needed to perform this experiment. Therefore, the differentiation of colonies belonging to *P. aeruginosa* and *S. maltophilia* was not possible maintaining the question if *S. maltophilia* is completely outcompeted by *P. aeruginosa*.

3.2 Interspecies interactions between *S. aureus* and *P. aeruginosa* in cystic fibrosis environment

S. aureus is the most prevalent pathogen at early phase of CF disease [101] and some studies have suggested that *S. aureus* have impact on *P. aeruginosa* augmenting its virulence and capacity to survive in stressful environments [164,175,201]. Despite these valuable insights about *S. aureus* and *P. aeruginosa* interactions, these studies did not closely simulate the CF lung environment and even the different lung colonization timings which may greatly impact on the type of interactions established between these two pathogens. This study aimed to clearly elucidate the CF community about this matter.

3.2.1 Phenotypic characterization of *Staphylococcus aureus* strains

The study of the interactions established between *S. aureus* and *P. aeruginosa* in an early phase of infection included two *S. aureus* strains with different genetic background, a reference strain – *S. aureus* ATCC 25293 – and a CF clinical isolate (SAI) and two *P. aeruginosa* strains – *P. aeruginosa* PAO1- and a clinical isolate (PAI). Using all these strains genetically unrelated, this work could strengthen its conclusions about the influence of *S. aureus* on *P. aeruginosa* adaptation to CF lungs and resistance to antibiotics.

Before the study of the interspecies interactions, it is of upmost importance to study first the phenotypic characteristics of *S. aureus* strains relevant for this work, namely susceptibility to antibiotics, colony morphology and kinetic growth in ASM. Phenotypic characterisation of *P. aeruginosa* strains was previously [184].

The antibiotic susceptibility profiles were determined using twelve antibiotic disks, according CLSI guidelines [183], revealing that both strains exhibited minor differences in response to antibiotics. Both strains were susceptible to all the antibiotic agents tested with the exception of erythromycin that SAI was resistant (Table 3.2). SAI is a clinical isolate from a chronic CF infection and thus some antibiotic resistance was expected.

Table 3.2 Antibiotic susceptibility profiles of the S. aureus ATCC 25293 and SAI strains according the CLSI guidelines

	CIP	**E**	**TE**	**RD**	**VA**	**CAZ**	**CN**	**OX**	**LNZ**	**CD**	**TEC**	**QDA**
***S. aureus* ATCC 25293**	S	S	S	S	S	S	S	S	S	S	S	S
SAI	S	R	S	S	S	S	S	S	S	S	S	S

CIP: Ciprofloxacin E: Erythromycin TE: Tethracyclin RD: Rifampicine VA: Vancomycine CAZ: ceftazidime CN: gentamicine OX: oxacillin LNZ: linezolid CD: clindamizine TEC: teicoplanin QDA: Quin-dalfoprist.
S: susceptible; R: resistant

In a CF environment, *S. aureus* often exhibits different colony morphologies being one of the most common the conversion to SCV. The conversion to SCV normally appears after antibiotic treatment providing to *S. aureus* enhanced resistance to the treatments and therefore a higher capability to survive in hostile environment [105–108]. Therefore, a particular attention was attributed to the detection of SCV during this study.

To better detect colony morphology alterations caused by the interspecies interactions between *S. aureus* and *P. aeruginosa*, a previous colony morphology study was performed. Therefore, it was needed to determine the wild-colony morphology for each *S. aureus* strains and the respective optimal colony time of growth that morphological traits do not modify (Figure 3.3). Unchangeable morphological traits are demanded when analysing colonies, being essential to avoid misinterpretations. Observations occurring before the stabilization of the morphology lead to inaccurate characterizations being sometimes even responsible for the non-visualization of colonies with reduce growth rate, such as SCV [186].

To characterize the morphological traits of *S. aureus* colonies again a colony morphology system presented by Sousa *et al.* (2013) was used [186]. As shown in Figure 3.3, each *S. aureus* strain displays only one variant of colony morphology over the time of growth differing only on the size (Table

S2 supplemental material). The observed colonies are similar to the ones observed in literature in what concerns to the form, margin, size and colour [202].

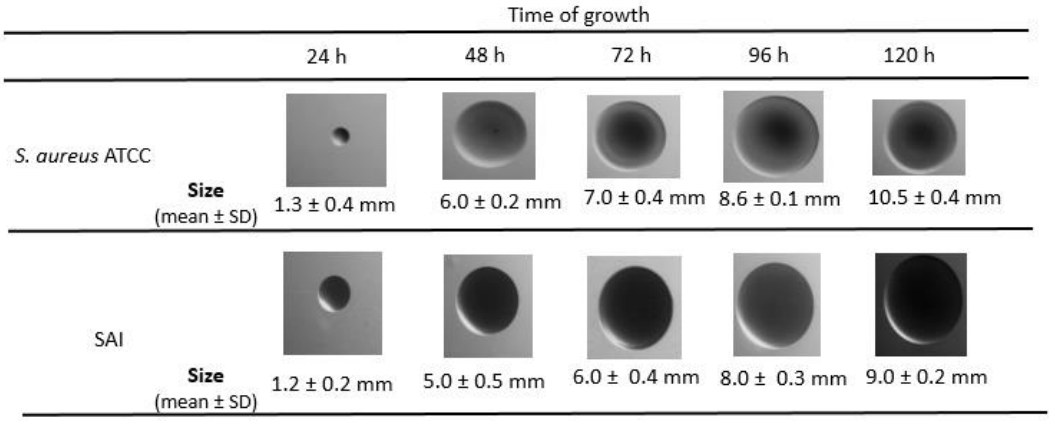


Figure 3.3. *S. aureus* ATCC 25293 and SAI colony morphological evolution during 120 h of growth in TSA. No alteration in the colonies was observed during the time of culture.

Despite the existence of literature describing the *S. aureus* colonies, no literature was found regarding the optimal time growth for an adequate observation of *S. aureus* colonies. According previous results obtained [192], *P. aeruginosa* colonies had a 48 h-optimal growth time. By the same reason described in section 3.1.1 for *S. maltophilia* colonies, the time of growth for *S. aureus* colonies was also 48 h facilitating the analysis of mixed culture.

3.2.2 Impact of *S. aureus* on *P. aeruginosa* adaptation to CF environment

To investigate the interactions between these pathogens and thus evaluate the role of *S. aureus* on *P. aeruginosa* pathogenicity, *S. aureus* was incubated 3 days in ASM before *P. aeruginosa*. This period of time aimed to mimic the initial phase (in infancy) of the disease where *P. aeruginosa* typically is absent, giving to *S. aureus* a period to adapt to CF environment. After *S. aureus* period of adaptation, *P. aeruginosa* was incubated at day 3 and samples were collected every 24 h. Four different co-cultures of *P. aeruginosa* and *S. aureus* were studied in order to strengthen the results inferences.

All *P. aeruginosa* strains had similar behaviour ($p \geq 0.05$), reaching a maximum of number of viable cells around 10^9 within 24h, following a "plateau" phase. In fact, these growth profiles were quite similar to those observed when these *P. aeruginosa* strains were grown alone in ASM [184] (Figure 3.4). Therefore, *S. aureus* seemed to have no effect on *P. aeruginosa* growth in ASM. Interestingly, the

analysis of *S. aureus* growth profiles suggested some negative impact of *P. aeruginosa* on *S. aureus*. *S. aureus* viable cells were slightly lower after *P. aeruginosa* inoculation.

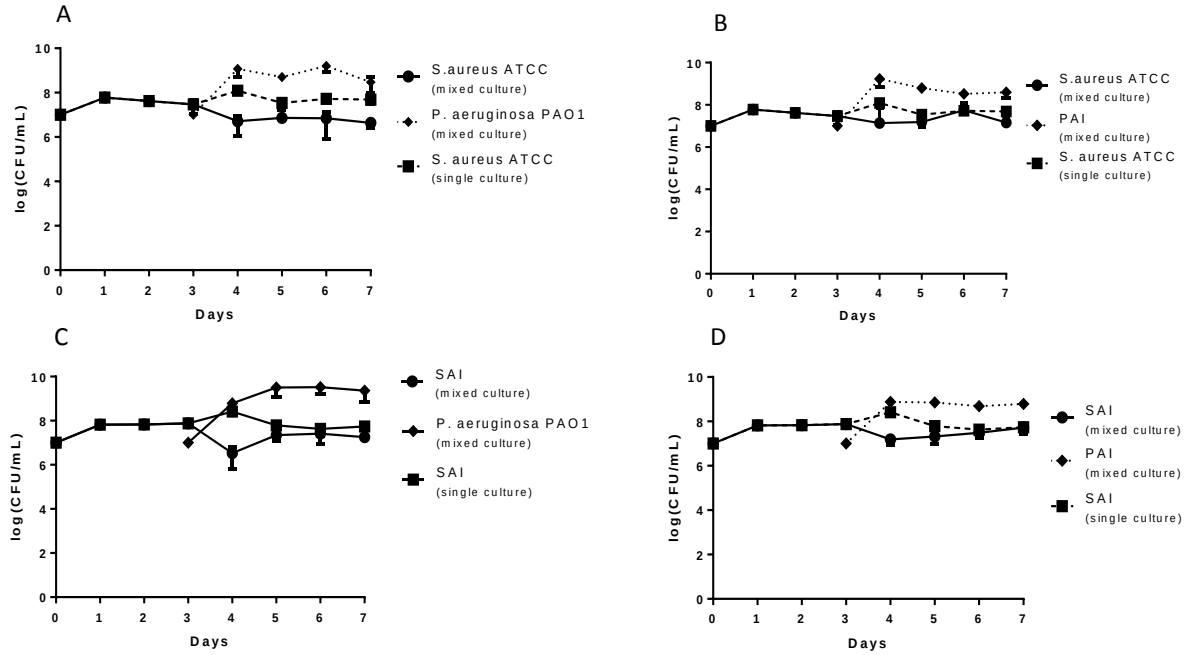


Figure 3.4. Viable cells counting of the consortia *S. aureus* ATCC 25293 and *P. aeruginosa* PAO1 (A), *S. aureus* ATCC 25293 and PAI (B) and the consortia SAI and *P. aeruginosa* PAO1 (C), SAI and PAI (D) in ASM during 7 days of culture. *P. aeruginosa* was added to culture on day 3.

For the four different ASM co-culture of *P. aeruginosa* and *S. aureus* the CI and the RIR were calculated in order to determine if one species had competitional advantage relatively to other. No significant differences between CI and RIR were observed ($p \geq 0.05$), thus, it seemed that none of the species was having full competitional advantage relatively to other, non-existing an antagonism interaction between the two species (Supplemental material II, Figure S 1). In literature, the type of interaction between *S. aureus* and *P. aeruginosa* is not consensual. There are some evidences supporting antagonism between *S. aureus* and *P. aeruginosa* in planktonic cultures, being *S. aureus* cells lysed by *P. aeruginosa* in order to use the released iron for its growth [171]. However, studies generally report the co-existence of these two species. For instance, Limoli *et al.* (2017) described *P. aeruginosa* co-existence with *S. aureus* in a medium that mimics CF sputum caused by the overproduction of alginate by *P. aeruginosa* [203]. Alginate overproduction causes a reduction in the generation of siderophores, HQNO, and rhamnolipids by *P. aeruginosa* being all of these three factors known to reduce *S. aureus* viability. Despite these evidences, studies generally report the co-existence of these two species only for

a period of growth of 24 h, however this time is very limited for the study of interactions in a CF context [175,166].

The impact *of S. aureus* should not be limited to the study of the number of viable cells. *S. aureus* could influence *P. aeruginosa* population to trigger the emergence of new phenotypes well adapted to CF environment. Analysing the morphological characteristics of the *P. aeruginosa* colonies obtained over the 4 days of growth, no population diversity was observed for both strains (Figure 3.5), being only detected the respective wild-morphotype (supplemental material Table S3).

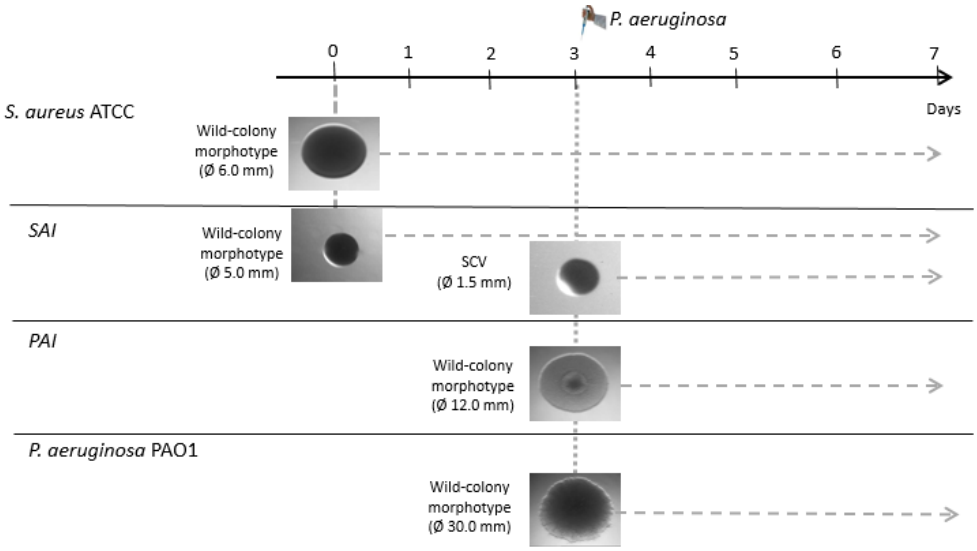


Figure 3.5. Population diversity of *S. aureus* ATCC 25293, SAI, PAI and *P. aeruginosa* PAO1 observed during 7 days of culture in ASM. The arrows represent the presence in all the subsequent days of culture.

The absence of population diversity is quite interesting in particular for PAI. Previous results demonstrated that PAI was able to generate several types of SCV when grown alone in ASM [184]. The absence of SCV in the present study led to suggest that *S. aureus* inhibited the emergence of SCV. *P. aeruginosa* could not have generated these variants because the surrounding environment provided by *S. aureus* was favourable. The secretion of several exoproducts by *S. aureus* such as autoinducer-2 (AI-2) and peptidoglycan are responsible for an upregulation of LasB, rhamnolipids and phenazines which are implicated in the virulence of *P. aeruginosa* [204]. For instance, LasB impairs uptake of *P. aeruginosa* by macrophages and its protease activity, leading to lung damage and a bigger dissemination into the bloodstream [68].

In contrast to the results obtained for *P. aeruginosa* population diversity, *S. aureus* strains showed a new type of colony, a SCV (Figure 3.6). The appearance of *S. aureus* SCV in both consortia happened

after the incubation of *P. aeruginosa* at day 3 which suggested the *P. aeruginosa* influences on *S. aureus*. The emergence of *S. aureus* SCV triggered by *P. aeruginosa* was also previously reported and it was suggested that *P. aeruginosa* exoproducts inhibit *S. aureus* respiration leading to a long term exposure to 4-hydroxy-2-heptylquinoline N-oxide (HQNO) that selected *S. aureus* SCV [164,205,206].

3.2.3 Impact of *S. aureus* on *P. aeruginosa* response to antibiotic treatments

Given the high mortality rates associated with *P. aeruginosa* infections, most of the treatments for CF disease are directed to the eradication of *P. aeruginosa* [37,46,47,151,8]. Even with the administration of aggressive antibiotic treatments, *P. aeruginosa* can persist for years or an entire life [50,51]. The previous results of this study suggested that CF lungs colonization by *S. aureus* prior to *P. aeruginosa* did not provide adaptive advantage to *P. aeruginosa* adaptation to CF environment. Therefore, this study aimed to investigate if CF lungs "pre-colonization" with *S. aureus* contribute for *P. aeruginosa* antibiotic resistance.

There are numerous antibiotic treatments varying agents, modes of administration and combination of agents [156,153]. There is no consensus about the antimicrobial treatments to administer to CF patients and thus this study simulated an antibiotic treatment using one of the most used antibiotic agents in CF therapy the ciprofloxacin [153,207,159].

To analyse the growth kinetics and population diversity of *P. aeruginosa* facing an aggressive antibiotic treatment, 4 mg/L of ciprofloxacin was added to ASM after the incubation of *P. aeruginosa* (Figure 3.6). *P. aeruginosa*, and also *S. aureus*, were exposure to ciprofloxacin only after ASM inoculation with *P. aeruginosa* to mimic a typical CF lung colonization timeline (first *S. aureus* and further *P. aeruginosa*), and more importantly, the begining of an anti-pseudomonal treatment only after *P. aeruginosa* detection. This experimental setup allowed approximating to the *in vitro* conditions.
The concentration of ciprofloxacin of 4 mg/L was used because previous results obtained by our research group demonstrated to be the most successful treatment in *P. aeruginosa* eradication in CF environment [184]. Testing with this treatment it would be easy to detect the possible *S. aureus* contribution for *P. aeruginosa* antibiotic resistance.

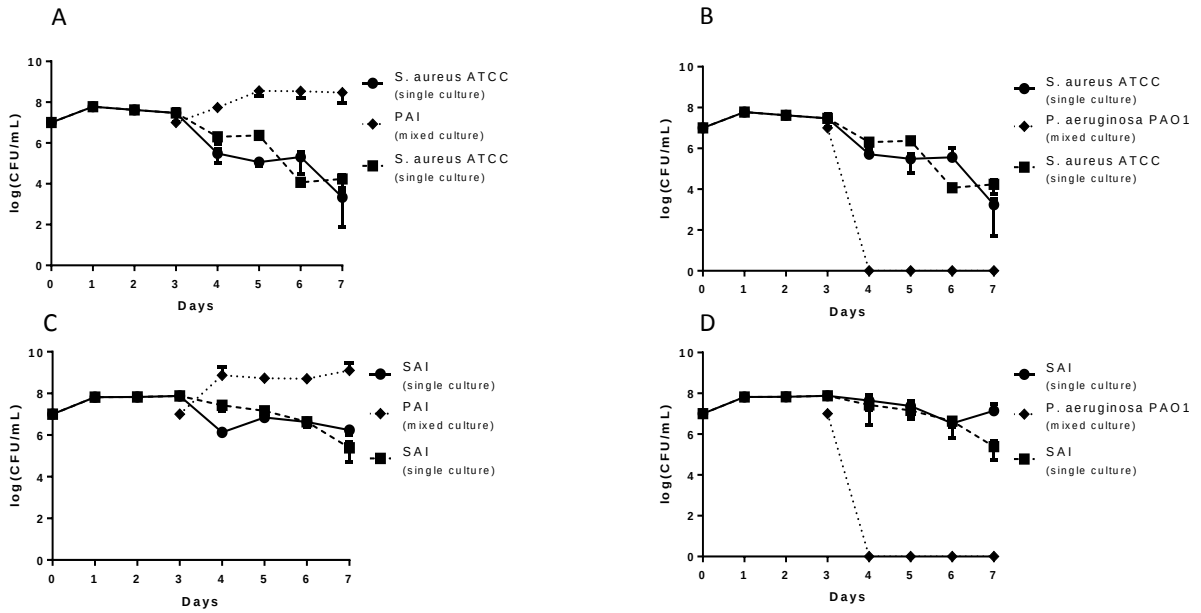


Figure 3.6. Growth kinetics of the consortia *S. aureus* ATCC 25293 and PAI (A), *S. aureus* ATCC 25293 and *P. aeruginosa* PAO1 (B) and the consortia SAI and PAI (C), SAI and *P. aeruginosa* PAO1 (D) in ASM during 7 days of culture. *P. aeruginosa* and 4 mg/L of ciprofloxacin were added to culture on day 3.

Ciprofloxacin treatment had different impact on the distinct consortia of *S. aureus* and *P. aeruginosa*. Ciprofloxacin was able to eradicate *P. aeruginosa* PAO1 within 24 h after exposure in the presence of both *S. aureus* strains (Figure 3.6). These results were similar to those obtained previously when PAO1 cultured alone in ASM and also exposed to 4 mg/L of CIP but the ciprofloxacin time-killing was reduced from 3 to 1 day [184]. In contrast to PA01 strain, *P. aeruginosa* PAI growth was not affected by the CIP treatment ($p \geq 0.05$). This PAI behaviour in the presence of ciprofloxacin was previously observed when PAI was cultured alone only in ASM [184]. Despite the different final results of the ciprofloxacin treatment over *P. aeruginosa* PAO1 and PAI, eradication and survival respectively, both results suggested that the presence of *S. aureus* had no impact in *P. aeruginosa* response against ciprofloxacin. Interestingly, ciprofloxacin seemed to have a considerable effect in *S. aureus* in the presence of *P. aeruginosa*, but it seemed to be strain-dependent.

In what concerns to the *P. aeruginosa* population diversity, again no population diversity was observed (Figure 3.5). There are evidences that *S. aureus* induces the selection for *P. aeruginosa* SCV under antibiotic pressure, but the results obtained suggested that *S. aureus* had no role in *P. aeruginosa* antibiotic resistance [164].

Intriguingly, PAI when cultured alone in ASM generated a different set of SCV, being some of those SCV ciprofloxacin resistant [184]. In that case, SCV were pointed out as a mechanism of PAI to resist against ciprofloxacin. When *P. aeruginosa* grown with *S. aureus*, the formation of *P. aeruginosa* SCV

seemed to be inhibit by the *S. aureus*. The differential behaviour of PAI in the presence of *S. aureus* led to question why PAI did not generate SCV and even so resist to ciprofloxacin. This issue must be addressed urgently because other mechanisms may be being used by *P. aeruginosa*. Later, a proteomic study was performed to investigate, among others, the mechanisms underlying ciprofloxacin resistance.

A new morphotype of SAI appeared when co-culture with *P. aeruginosa* PAO1 at day 7 (Figure S2 in Supplemental Material). The distinct feature from the wild-morphotype was the yellow colour that is associated to the production of staphyloxantin. Typically, some strains of *S. aureus* are capable of producing staphyloxantin which acts as a bacterial antioxidant against peroxide and hydroxyl radicals [208]. As so, *S. aureus* colonies regarding this feature are more likely to survive when exposed to human neutrophils as they are protected against oxidative stress allowing it to evade the reactive oxygen species which the host immune system uses to kill pathogens [208,209].

Generally, the results obtained until now seemed pointed out that *S. aureus* did not support *P. aeruginosa* infection persistence or resistance against antibiotics. This evidence did not corroborate other studies that reported positive influence of *S. aureus* towards *P. aeruginosa* pathogenicity like the phenotypic switching to SCV and the upregulation of some virulence factors [175,204].

The difference between the present results and those from literature may be caused by the *in vitro* conditions. In this study, ASM was used to closely approximate the culture conditions to the *in vivo* CF environment, while in the literature studies the interactions between *P. aeruginosa* and *S. aureus* are typically investigated using standard laboratory medium, such as TSB [164,166,177]. Laboratory rich media do not mimic the CF lung and thus the interactions established between these two pathogens could be different. Nutrients present in the media are known to be essential for bacterial functions like expression of virulence determinants, biofilm formation, secretion of exoproducts [170]. A study conducted by Palmer *et al.* (2005) concluded that CF sputum supports *P. aeruginosa* growth. The same study demonstrated that *P. aeruginosa* factors critical for lysis of *Staphylococcus aureus* were only induced in CF sputum increasing *P. aeruginosa* competitiveness during polymicrobial growth. Therefore it was being clear that CF experimental conditions significantly impacts polymicrobial interactions [170].

Despite ASM seemed to be main cause for the differences found between this study and the literature, other hypotheses were considered to explain the dissimilarities, such as the increased initial number of

viable cells of *P. aeruginosa* at the ASM inoculation and the limited adaptation time of *S. aureus* in ASM. These hypotheses were tested, and the results obtained are described and discussed in the next sections.

3.3 Impact of the experimental conditions on *S. aureus* and *P. aeruginosa* interactions

Increased initial concentration may impact on *P. aeruginosa* adaptation to a new environment and bacteria could not trigger their typical mechanisms of adaptation. *P. aeruginosa* incubation concentration of 10^7 CFU/mL could be high enough to facilitate *P. aeruginosa* adaptation and thus no need of SCV switching.

To verify if the initial concentration of *P. aeruginosa* used to inoculate ASM impact on *S. aureus* influence over *P. aeruginosa*, a reduction of 2 log of the initial concentration was tested. At day 3, ASM was inoculated with *P. aeruginosa* at final concentration of 10^5 CFU/mL and kinetic growth and population diversity was monitored.

The growth kinetics study revealed that *P. aeruginosa* and *S. aureus* had maintained similar growing profile (Figure 3.7) in comparison to the previous one using a concentration of 10^7 CFU/mL (Figure 3.4). It seemed thus that this experimental parameter had no influence on the interspecies interactions. To reinforce this evidence, *P. aeruginosa* population diversity was also studied, and the results demonstrated no *P. aeruginosa* SCV or other new colony variant was detected.

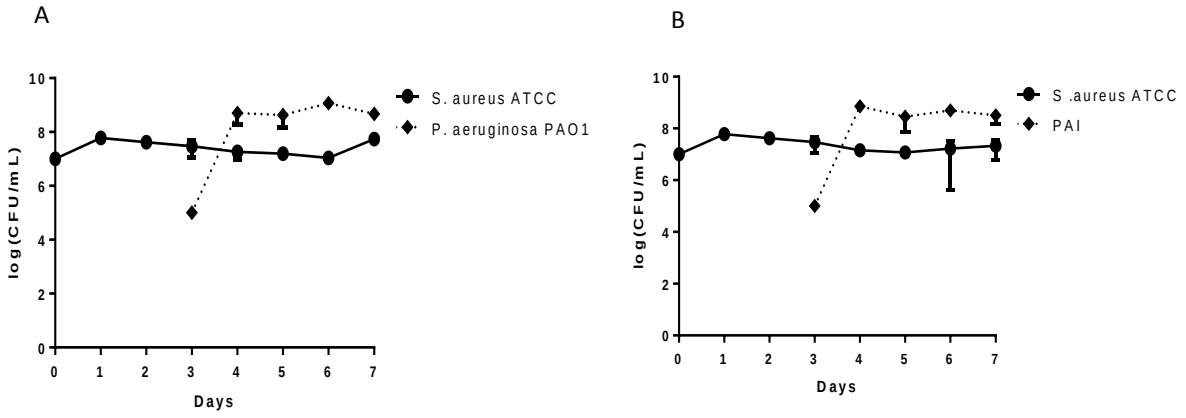


Figure 3.7. Growth kinetics of *S. aureus* and *P. aeruginosa* PAO1 (A) and *S. aureus* and PAI (B) grown in ASM over 7 days. In this study *P. aeruginosa* was incubated at a concentration of 1 x 10^5.

In overall, these results led to discard the hypothesis that the initial concentration of *P. aeruginosa* could be the parameter that causes different results of this study from the literature. Even with 2-log reduction of the initial concentration of *P. aeruginosa*, bacteria exhibit similar behaviour in the presence of *S. aureus* reinforcing that *S. aureus* did not contribute to *P. aeruginosa* adaptation and antibiotic resistance.

3.3.1 Effect of *S. aureus* adaptation timing on *P. aeruginosa* pathogenicity

The last hypothesis considered explaining the no influence of *S. aureus* on *P. aeruginosa* adaptation and antibiotic resistance was the reduced time of *S. aureus* growth in ASM before co-culture with *P. aeruginosa*. A previous study reported that *P. aeruginosa* needed 3 days in ASM to achieve adaptation to CF environment [184].

In the light of these evidences, this study allowed *S. aureus* to adapt to CF environment also during 3 days. However, this period of time could be not enough for *S. aureus* to achieve CF adaptation, expressing the proper phenotypic features, such as the production of peptidoglycan and autoinducer-2 (AI-2) impacting on *P. aeruginosa* adaptation [204]. This study aimed to clarify this issue allowing *S. aureus* grow in ASM during 15 days. Only after this longer period of *S. aureus* adaptation, *P. aeruginosa* was added to ASM and co-culture during more 5 days (a total of 20 days of experiment) to evaluate the impact of longer period of *S. aureus* adaptation on *P. aeruginosa* pathogenicity. Given the long duration of the experiment and based on previous studies that PAI is capable of forming SCV, this strain was chosen to perform these experiments [184].

Interestingly, the longer period of *S. aureus* adaptation to CF environment appeared to impact on *P. aeruginosa* adaptation (Figure 3.8).

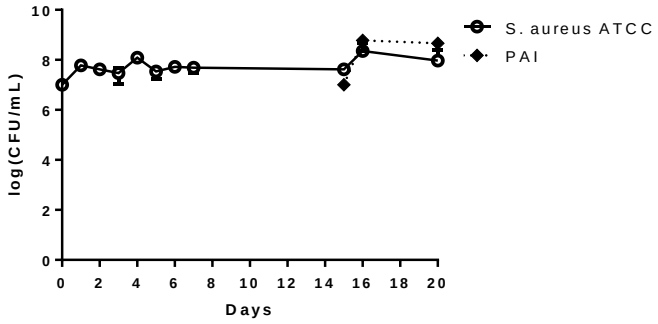
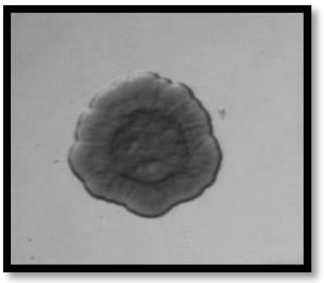


Figure 3.8. Growth kinetics of *S. aureus* and PAI grown in ASM over 20 days. *P. aeruginosa* was added to ASM at a concentration of 10^7 CFU/mL after day 15 of *S. aureus* adaptation.

Despite the *P. aeruginosa* growth profile was identical to the previously observed, the population diversity was clearly different from the previous results obtained in this study. After 5 days of *P. aeruginosa* co-culture with *S. aureus*, *P. aeruginosa* SCV were observed (Figure 3.9). These SCV were previously detected when PAI was cultured alone in ASM being well characterized in in-house libraries [184]. This phenotype was described as resistant to ciprofloxacin, susceptible to ceftazidime, cefepime, imipenem, gentamicin, tobramycin and intermediate to ticarcillin/clavulanic acid and aztreonam [186].

A



B

Colony features	
Form	Irregular
Margin	Undulate
Surface	Homogeneous
Texture	Wrinkled
Surface elements	None
Sheath	Absent
Opacity	Iridescent
Elevation	Flat
Consistency	Dry
Size	Small
Colour	Blue-green

Figure 3.9. (A) *P. aeruginosa* SCV detected after 5 days of co-culture with CF-adapted *S. aureus* (B) Characterization of the SCV accordingly the morphological criteria established by Sousa *et al.* (2013) [186].

Although this result was at this point concordant with the literature studies [164], it should be refer that PAI SCV were also detected when PAI was cultured alone in ASM [184]. This evidence led to discard the idea that *S. aureus* influence the *P. aeruginosa* adaptation to CF environment, reinforcing the first results obtained. Therefore, different experimental conditions such as medium used for bacterial growth should be the cause of the differences found between this study and the literature. These contradictory evidences highlight the importance of the *in vitro* conditions, which could lead to different results. ASM closely approximates to the CF sputum and furthermore, the different inoculation timing of each bacterial species provides to this study the most "realistic" *in vitro* study performed so far. Taking into account the deficit of literature regarding this theme, more studies focusing on *S. aureus* importance on

P. aeruginosa adaptation are needed. Still, the results obtained in this study needed to be strengthened by the use of different *S. aureus* and *P. aeruginosa* consortia.

Nevertheless, *S. aureus* may play a role in *P. aeruginosa* antibiotic resistance. This hypothesis was not tested in this study, but it is of upmost importance to clarify this issue because can directly impact on the current antimicrobial stewardship.

Given the fact that in this scenario *P. aeruginosa* exhibited SCV, probably the time of *S. aureus* adaptation to the ASM needs to be higher to the one used in the first assays, reaching as consequence a higher approximation to the existing conditions in CF.

The results herein presented provide relevant information related to the interactions between these two main pathogens and gives valuable insights about the interspecies interaction in CF lungs.

3.4 *P. aeruginosa* proteomic profile

Proteomics is the study of protein expression from cells, tissue or organs measuring protein expression in terms of their presence and relative abundance [210,211]. Indeed, proteomics has been a key tool in system biology and in the chemical field detecting important disease markers and biological processes underlying antibiotic resistance [210,211].

The use of this technique has been of high importance for the comprehension of bacterial infectious disease because it allows the reproducible fractionation of complex protein mixtures, retaining the qualitative-quantitative relationship [212,213]. Currently, two-dimensional polyacrylamide gel electrophoresis (2-DE) is the only method that can handle this task and, thus it has gained special importance in the clinical field [213,214]. According the results obtained in this study, it was verified that the two *P. aeruginosa* strains exhibit distinct behavior in ASM. PAI resisted against aggressive ciprofloxacin treatments in the presence of *S. aureus* in opposite to PA01 that was. As so, given the similar behavior of PAI against antibiotics in comparison with *P. aeruginosa* chronic isolates, it was considered important to find out the biological processes that were being expressed. Since 2-DE can provide an overview of the global protein expression and further allows identifying the respective biological processes, this method was used for this study [211].

3.4.1 Optimization of sample preparation

Sample preparation is a key step for an efficient and reproducible 2-DE [215]. The quality of the results is determined by the quality of the samples that, in turn, is determined by the sample preparation method. Therefore, the sample preparation is one of the most important steps of 2-DE analysis.

There are several methods of sample preparation described in literature applied to *P. aeruginosa* cells that vary in the lysis method, sonication time and lysis buffer [216–218]. Based on the literature review, cell disruption, in particular, sonication and lysis buffer were the most variable parameters of sample preparation and, therefore, they were optimized in this study before 2-DE analysis. The first approach to optimize the extraction protocol was made using 4 colonies of *P. aeruginosa* PAO1 and the lysis buffer I and buffer II (Table 2.1), varying the sonication time. Buffer I and II were chosen to be tested given its simple composition and extensive use in proteomics. For the optimization procedure *P. aeruginosa* PAO1 was the chosen strain. This choice was made only given the availability of colonies of this strain at the moment of the extraction protocol.

During the whole sonication time of 5 min, several samples were collected during the "off" periods of sonication (every 30 s) being the results presented in Figure 3.10.

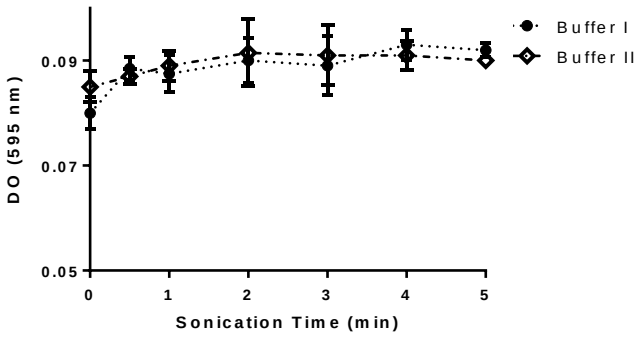


Figure 3.10 Effect of sonication time of *P. aeruginosa* colonies on protein yield measured by optical density (OD) read at 595 nm.

The amount of protein obtained during sonication using the lysis buffer I and II was not significantly different ($p \geq 0.05$) despite of theoretically, longer time of sonication should increase the amount of protein until a critical point [219]. One factor that could be influencing the results obtained is the volume of sample collected to quantify the protein extracted. Given the volume of the main sample was approximately low (≈ 2 mL) it was mandatory to recover a small volume for protein quantification to

maintain a volume of main sample adequate to proceed with sonication. Furthermore, samples were recovered without agitation not ensuring homogeneity.

Given the nonexistence of significant differences ($p \geq 0.05$) between the tested time of sonication and protein concentration obtained during sonication time, 5 min of sonication was the chosen as the "optimal" time of sonication. This timing is one of the most used for protein extraction procedures for proteomic studies of *P. aeruginosa* [220,221].

After the establishment of the optimum sonication time and using the lysis buffer I, a 2-DE was made in an attempt to infer about the quality of the samples prepared. A low-quality sample is responsible for smear appearance that difficult the visualization of spots. The causes of a sample with poor quality could be related to a inadequate precipitation or to the presence of sugars and salts that requires a specific buffer. Staining method has also impact on 2-DE analysis. The choice of staining method is determined by several factors, including sensitivity needed, linear range, ease of use, cost and complexity of the protein sample. Currently, there are several methods, including BlueSafe, Coomassie and silver staining.

At first stage, 2-DE gels were stained with BlueSafe (Figure 3.11). BlueSafe is a sensitive, safe and the staining process could be performed with a single step. It is safer than Coomassie Blue staining since it has not methanol or acetic acid in its procedure and, therefore, it was considered a good option for gel staining [222].

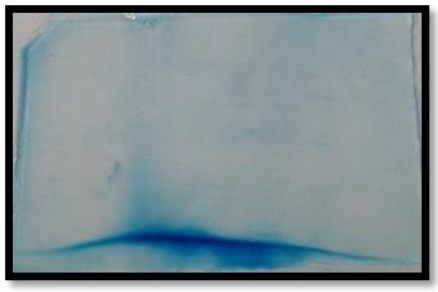


Figure 3.11 2-DE -gel stained with BlueSafe. This gel was loaded with protein sample treated with lysis buffer I.

Despite the high concentration of protein, the gel of the 2-DE (stained with BlueSafe) did not reveal spots. The absence of spots and detection of soft smear lead to hypothesize the poor quality of the protein sample probably due to the presence of lipids, salts or sugars or the lack of resolution of the staining method [223]. First, it was attempted to eliminate lipids and salts from the sample. For that,

two new lysis buffers (III and IV) were tested (Table 2.1) and TCA/Acetone precipitation was executed twice. The lysis buffers III and IV contain CHAPS in their composition, which was added to disrupt hydrophobic interactions and increase solubility of proteins at their pI and DTT to disrupt disulfide bonds. The buffer III differ from buffer IV in EDTA. EDTA is a chelating agent that chelates the magnesium ions in solution, which are cofactors for some degrading enzymes.

After protein extraction procedure using the two new lysis buffers, protein quantification was performed. The results obtained revealed higher protein yield in comparison to the previous extractions methods using buffer I and II (Table 3.3). However, these results showed to be "false-positive" because some components of buffer III and IV interfere with the quantification method used (BCA kit).

Table 3.3. Protein yield of the extraction procedure using four different lysis buffer.

Lysis Buffer	Protein concentration (µg/mL)
I	856
II	671
III	2466,7
IV	1505,6

Concerning the hypothesis that the absence of spots was caused by the lack of sensitivity of the staining method, two other staining methods more sensitive, Coomassie blue and silver nitrate, were tested. At this point, only SDS-PAGE gels were performed in order to obtain more quickly the results. To strength this analysis the same sample was used to run SDS-PAGE ensuring that the results were not influenced by the variability of new protein extraction procedure and different sample quality.

Coomassie is a more sensitive method than BlueSafe and also allows an easy detection [224]. As it is a simpler method than silver nitrate staining method, Coomassie blue was first tested (Figure 3.12).

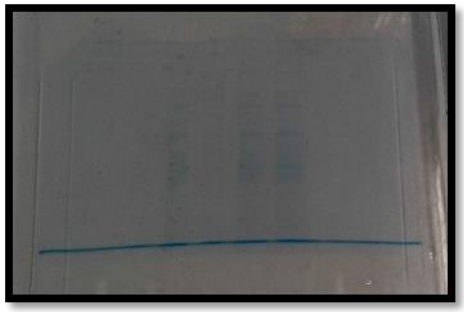


Figure 3.12. SDS gel obtained using Coomassie blue dye. It is possible to visualize some soft bands, revealing the need of use a more sensitive method.

The results demonstrated that in fact Coomassie blue improved protein detection in comparison to BlueSafe. Some soft bands appeared which allow visualizing that proteins were separated and, more importantly, that samples had good quality. Although this improvement, the bands were still soft and, thus, a more sensitive staining method was required to clearly visualize and differentiate the protein bands. Therefore, silver nitrate staining method was tested (Figure 3.13). This method offers the highest sensitivity, but protocol is time-consuming and more complex that Coomassie blue and BlueSafe [225].

Figure 3.13. SDS gel obtained using silver nitrate staining. It is notorious the sensibility of this method to stain proteins in comparison with the previous SDS-gels.

The results demonstrated that there were a lot of protein bands that were not detected by the BlueSafe and Coomassie methods. Therefore, it was concluded that silver staining was the most adequate method to detect proteins, reinforcing its detrimental advantage of being a high sensitive method. Next, all gels were stained with silver nitrate to detect differences in protein expression.

3.4.2 Profiling of *P. aeruginosa* strains grown in ASM

After the optimization procedures, SDS-PAGE gels were performed for both *P. aeruginosa* strains in order to evaluate if they exhibited differences in protein expression.

After staining with silver nitrate, a lot of bands were detected being hardly to distinguish them (Figure 3.14). Despite it was used a standard protein load to perform SDS gels, high protein load may be the cause for this difficult bands differentiation. Unfortunately, the influence of protein load was not possible to include in this study.

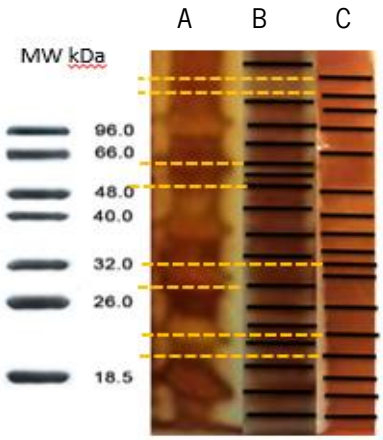


Figure 3.14. Protein detected on SDS-PAGE stained with silver nitrate extracted from PAI (B) from *P. aeruginosa* PAO1 colonies (C); and molecular weight protein marker (A).

Due to technical limitations, SDS-PAGE gels had to be analyzed manually, in particular band detection and band matching steps. To facilitate this manual analysis, a black line was used to identify the bands detected in each proteomic profile. Both *P. aeruginosa* strains exhibited a proteome composed with proteins ranging from 18.5 kDa and 96.0 kDa. From the manual analysis, it seemed that PAO1 differentiated from PAI in 7 protein bands of approximately 19, 22, 33, 40, 45, 66 and 96 kDa. Proteins with these molecular weights could be related with a wide range of biological functions, such as iron transport, lipids biosynthesis, carbon catabolism, acid metabolism, type IV pili biosynthesis and peptidoglycan binding [226–228]. Based on these results, it was concluded that more resolution was needed to better identify the differences between the two *P. aeruginosa* strains and, thus, 2-DE was performed (Figure 3.15). Unfortunately, 2-DE gels were not analyzed by software to detect and match spot due to the same technical limitations. The analysis of the 2-DE profiles was performed manually.

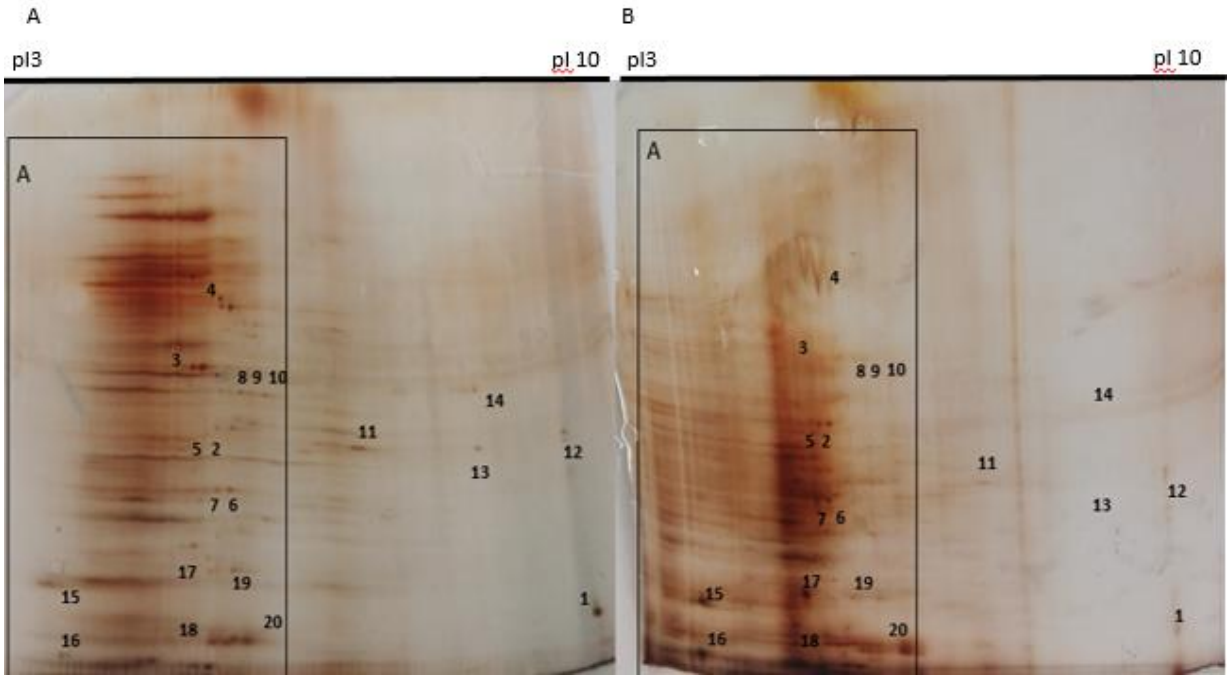


Figure 3.15.Proteomic profiles of *P. aeruginosa* PAI (A) and PAO1 (B) obtained by 2-DE. The numbers identify some of the spots detected.

After staining with silver nitrate, 60 and 48 spots were clearly identified in PAI and in PAO1, respectively. It was possible to observe that both strains shared similarities, mainly the expression of a high amount of proteins with identical range of pI and molecular weight, highlighted in Figure 3.15 as zone A. Due to this high density of protein, more resolution is required in order to better identify all spots contained in this zone. Increased resolution could be achieved using a tighter isoelectric point range, for instance of 4-7. It is expected to identify more spots clearly discriminate and consequently to find more differences between these *P. aeruginosa* strains. Unfortunately, this analysis was not included in this study.

Analyzing the differences between the spots detected in both strains, approximately, 26 differences were found, including 17 presence/absence of spots and 6 proteins up-expressed in PAI and 3 proteins up-expressed in PAO1. In order to estimate the molecular weight of the spots detected and the respective isoelectric point and being not possible to perform mass spectrometry (MS) to identify the proteins, the gels were compared with those presented by Sauer *et al.* (2002) [226]. Some proteins could be "identified", in specific, some conversed domains and, further, the isoelectric point and molecular weight of some surrounding proteins of these conversed domains were predicted. Comparing the proteomic profiles, it seemed that *P. aeruginosa* strains had differed in protein expression of proteins with an average molecular weight between 14 to 60 kDa and isoelectric point between 5 and

8. Proteins with this molecular weight and isoelectric point could be involved in iron transport, lipid biosynthesis amino acid metabolism, carbon catabolism and cofactor biosynthesis [226].

In overall, *P. aeruginosa* strains studied exhibited differences in protein expression that must be identify urgently by MS approaches to verify if they are involved in antibiotic resistance mechanisms. These proteins could be important for the development of new therapies once their blockage could seriously reduce antibiotic resistance.

4. FINAL REMARKS

4.1 Conclusions

P. aeruginosa is the main cause of chronic infections development in CF lungs provoking increased mortality rates. Although the administration of aggressive and long antimicrobial treatments directed to *P. aeruginosa*, bacteria still persist and still killing patients. Therefore, clinical community must be focused on understanding *P. aeruginosa* mechanisms underlying persistence and antibiotic resistance. Some advances were made about *P. aeruginosa* mechanisms of adaptation, but this knowledge is still not enough to help the development of new antimicrobial strategies. Therefore, it was hypothesized that other bacteria resident with *P. aeruginosa* in CF lungs could be the key in *P. aeruginosa* persistence and resistance. These bacteria could enhance *P. aeruginosa* resistance to antibiotic by, for instance, triggering biofilm formation and conversion to SCV. The mechanisms by which pathogens interact and the implications of these interactions in the disease progression are poorly understood.

The understanding of the interspecies interactions between *P. aeruginosa* and other pathogens are valuable because they could be used to develop new treatments effective in *P. aeruginosa* eradication. Treatments focused on other pathogens especially the early colonizers could weaken *P. aeruginosa* or decreased its pathogenicity and, thus, provide a therapeutic window of opportunity to eradicate *P. aeruginosa*. Therefore, the main goal of this study was to investigate the interactions established between *P. aeruginosa* and two early CF colonizers - *S. aureus* and *S. maltophilia* -, in attempt to understand how these interactions affected *P. aeruginosa* adaptation to the CF environment and its resistance to antibiotic treatments. To achieve this goal, co-culture of *P. aeruginosa* and early colonizers in ASM was performed using a new *in vitro* CF model. The early colonizing species was grown alone during 3 days in order to adapt to ASM. After that, *P. aeruginosa* was co-cultured with the early colonizing species and samples were recovered every 24 h to study the interactions established between pathogens. This experimental setup is the most similar to the *in vivo* colonizing profile of CF lungs so far described.

The study of the interactions between *P. aeruginosa* and *S. maltophilia* revealed that *P. aeruginosa* seemed outcompete *S. maltophilia*. The lack of a selective medium for *S. maltophilia* forced that growth profiles of both species were constructed based on cell counting in TSA (a generic medium). Therefore, the outcompetition of *S. maltophilia* could occur indeed in ASM, but there was still the possibility that

outcompetition could occur in TSA. A selective medium for *S. maltophilia* must be developed to confirm the kind of interaction between *S. maltophilia* and *P. aeruginosa*. Without an effective differentiation between species, the results did not led to take consistent conclusions about *S. maltophilia* impact on *P. aeruginosa* pathogenicity.

Interestingly, the results obtained from the study of *S. aureus* interactions with *P. aeruginosa* evidenced that *S. aureus* did not induce the formation of *P. aeruginosa* SCV, or enhanced its growth in ASM or even helped to resist to ciprofloxacin treatments. Therefore, it was concluded that *S. aureus* seemed not contribute for *P. aeruginosa* pathogenicity. As these results do not corroborate which is described in literature, several hypotheses were formulated to explain these differences. First, it was hypothesized that initial concentration of *P. aeruginosa* of the experiments could be high allowing *P. aeruginosa* to easily adapt to the ASM. Second, that *S. aureus* could need longer period of adaptation to provide adaptive advantages to *P. aeruginosa*. *P. aeruginosa* concentration hypothesis was discarded because the results showed no differences in growth profiles and population diversity between both tested concentrations. Regarding the second hypothesis, it was concluded that *S. aureus* needed indeed a longer period of adaptation to the medium, because other colony variants, in particular SCV were detected. However, its impact on *P. aeruginosa* was not noticed. Therefore, it seemed that in fact *S. aureus* did not play a role in *P. aeruginosa* persistence and resistance in CF lungs. Given the two hypotheses discard, the differences between this study and the other studies of the literature may be on the new *in vitro* CF model developed. This model simulated much closer to the complex nutritional conditions in CF lungs using ASM, as well as the different CF lungs colonization timings. ASM seemed to detrimental requisite for CF studies that the majority of studies do not use. Also, the adaptation time given to the early colonizer species approximates to the general CF infection profile where first species like *S. aureus* and *S. maltophilia* colonize the lungs with *P. aeruginosa* appearing in a latter stage of infection. This adaptation time given to early colonizers was not seen in other studies.

The two *P. aeruginosa* strains demonstrated different responses to ciprofloxacin treatments. Therefore, a proteomic analysis was performed in order to identify the proteins differentially expressed that may be involved in persistence and resistance mechanisms. The 2-DE profiles allowed concluding that the strains differentially expressed several proteins that could be involved in biological processes including iron transport, lipid biosynthesis amino acid metabolism, carbon catabolism and cofactor biosynthesis.
The findings of this study will have great impact on the study of CF-associated infections because they pointed out that *S. aureus* could be not the key for *P. aeruginosa* adaptation, therefore other factors

must be investigated. Moreover, the findings of this study demonstrated the importance of approximating experimental conditions to the *in vivo* scenario. The use of ASM that mimics lungs sputum and the prolonged adaptation time of *S. aureus* that mimics the early phase of infection strength the assumption that *S. aureus* did not play a role in *P. aeruginosa* adaptation.

4.2 Future work

The interspecies interactions that occur in CF lungs are certainly important for disease progression impacting thus in patient quality of life and more importantly in his life expectancy. Despite the findings of this project pointed that the earlier colonizer *S. aureus* seemed not play a role in *P. aeruginosa* persistence and resistance against antibiotics, it does not mean that other earlier colonizer have not impact. Therefore, the study involving *S. maltophilia* must be continuing overcoming the technical difficulties. The optimization of the *S. maltophilia* culture mediums or the change of the selected method to obtain growth rates is needed. Flow cytometry could be used to evaluate the growth rate using different fluorescent dyes being a good alternative for this study.

Identical investigation as this study should be addressed the interactions between other *P. aeruginosa* co-colonizers, as *H. influenzae*, *Achromobacter* and *Burkholderia cepacia* maintaining the experimental setup as this study evidenced its relevance.

The study of the impact of CF microbiome on *P. aeruginosa* will be more closely mimicked if mixed cultures including several earlier colonizers would co-culture with *P. aeruginosa*. *P. aeruginosa* pathogenicity could be modulated not by an individual bacterial species but by complex consortia of several species. This issue should be clarified as soon as possible. The validation of this hypothesizes could be greatly change the current design of the antibiotic treatments.

More studies concerning the effect of early antibiotic treatment on the CF pathogens is also important. Several antibiotics, such as tobramycin and colistin are used in CF treatment and, as so, should be tested in the diverse co-culture assays.

The detection and quantification of the produced exoproducts by HPLC during co-infection in an early phase of infection and their implication in the disease progress is also a future task. Exoproducts are known to be involved in several interspecies interactions as the induction of SCV of *P. aeruginosa* by *S. aureus* and as so it is important to quantify them as a marker for the occurrence of interactions.

Proteome profile characterization of the strains and species used in this and in further co-culture studies is also a promising task, allowing the comparison of biological processes occurring. The identification of the different proteins expresses by mass spectrometry is also in mind.

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SUPPLEMENTAL MATERIAL

I- MORPHOLOGICAL CHARACTERISTICS THE SPECIES IN STUDY

Table S 1. Morphological characteristic of the *S. maltophilia* colonies accordingly to Sousa *et al*. (2015) system. The colonies were grown in TSA during 48h

	FORM	MARGIN	SURFACE	TEXTURE	SURFACE ELEMENTS	SHEALTH	OPACITY	ELEVATION	CONSISTENCY	SIZE	COLOUR
S. MALTOPHILIA	circular	entire	absent	homogeneous	none	smooth	opaque	flat	dry	medium	beige

Table S 2. Morphological characteristic of each colony morphotype accordingly to Sousa *et al.* (2015) system. The colonies were grown in TSA during 48h.

	FORM	MARGIN	SURFACE	TEXTURE	SURFACE ELEMENTS	SHEALTH	OPACITY	ELEVATION	CONSISTENCY	SIZE	COLOUR
MT 22	circular	entire	homogenous	Smooth	None	Absent	Opaque	Flat	Dry	Large	Beije
MT 23	Circular	Entire	homogenous	Smooth	None	Absent	Opaque	Flat	Dry	Small	Beije
MT14	circular	entire	homogenous	Rough	Holes	Absent	Iridiscent	Flat	Dry	Large	Beije
MT02	Circular	Undolate	homogenous	Rough	none	Present	Opaque	Flat	dry	Large	Yellow

II- RELATIVE INCREASED RATION AND COMPETITIVE INDEX

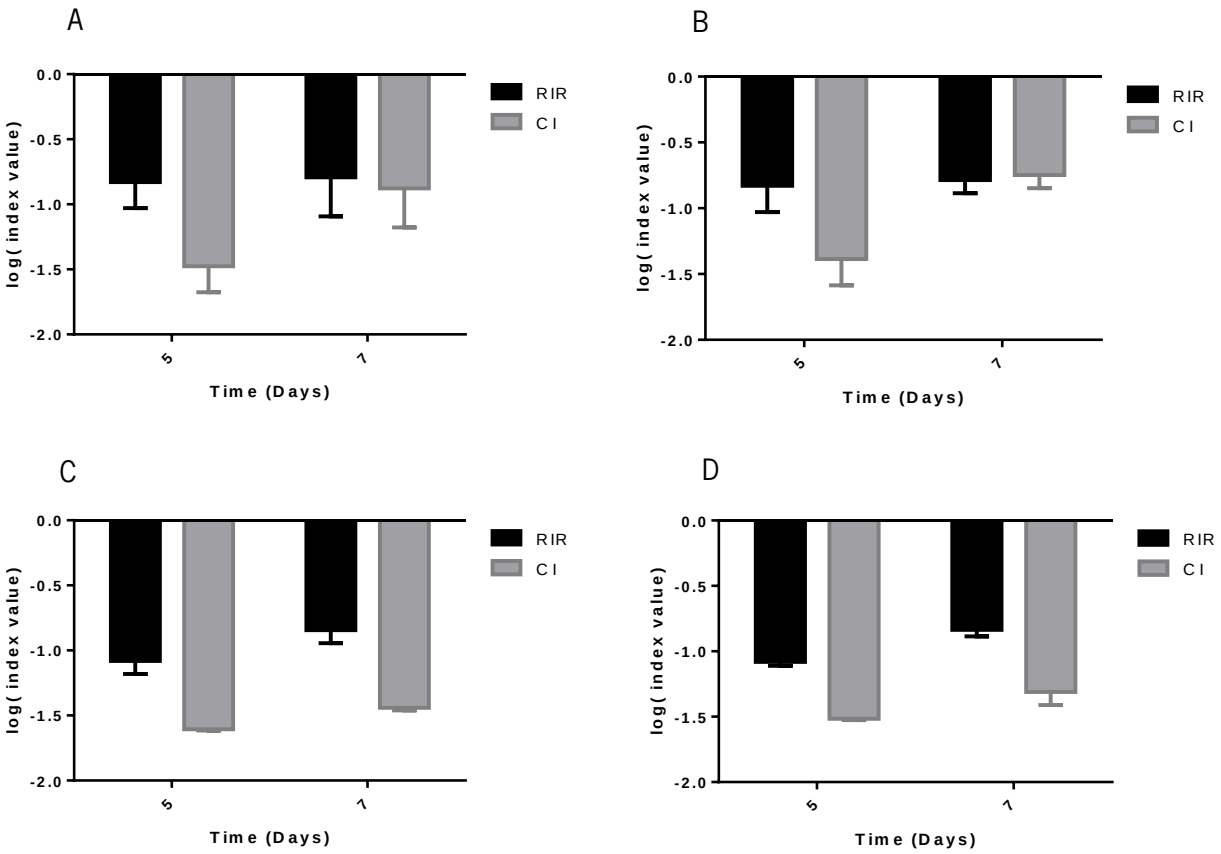
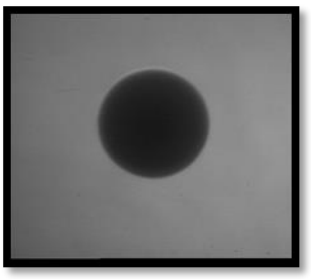


Figure S 1. Competitive index (grey bars) and relative increase ratio (black bars), for co-culture of SAI and *P. aeruginosa* PAO1 (A); SAI and PAI (B); *S. aureus* ATCC and *P. aeruginosa* PAO1 (C) and *S. aureus* ATCC and PAI (D)

III- *S. AUREUS* YELLOW COLONY



Colony features	
Form	Circular
Margin	Entire
Surface	Homogeneous
Texture	Wrinkled
Surface elements	None
Sheath	Absent
Opacity	Opaque
Elevation	Flat
Consistency	Dry
Size	Large
Colour	Yellow

Figure S 2. *Staphylococcus aureus* yellow colony. This new morphotype appeared in co-culture with *P. aeruginosa* PAO1 at day 7 after ciprofloxacin treatment.