Insights into *Pseudomonas aeruginosa* and *Burkholderia contaminans* interactions in cystic fibrosis-associated biofilms infections

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Universidade do Minho
Escola de Engenharia

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Master Dissertation
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This work was realized under supervision of:
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Professor Doctor Maria Olívia Baptista de Oliveira Pereira

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STATEMENT OF INTEGRITY

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ABSTRACT

Insights into *Pseudomonas aeruginosa* and *Burkholderia contaminans* interactions in cystic fibrosis-associated biofilm infections

The Gram-negative *Pseudomonas aeruginosa* and the *Burkholderia cepacia* complex are opportunistic pathogenic bacteria commonly responsible for severe infections in Cystic Fibrosis (CF) patients. These bacteria form biofilms in the airways, making infections challenging to treat. Recently, some advances have been made to understand the complex interaction between microorganisms and their implications for disease prognosis and therapy response in CF. Therefore, this study aimed to contribute to the knowledge of the potential role of interactions between *P. aeruginosa* and the emerging *B. contaminans* during infection, as well as their impact on the action of antibiotics frequently used to control *P. aeruginosa*. Regarding their biofilm formation ability, the presence of *B. contaminans* resulted in a decrease in biomass and the number of cultivable cells in dual-species biofilms compared to *P. aeruginosa* single biofilms after 72h, suggesting an antagonistic interaction between the bacteria.
Concerning the role of these microbial interactions in susceptibility to colistin and ciprofloxacin, the Minimum Inhibitory Concentration (MIC) was first determined. For ciprofloxacin, it was determined to be 0.25mg/L for both, while for colistin, the MIC varied between 0.5 and 1mg/L for *P. aeruginosa*, and it was not possible to determine the MIC of this antibiotic in *B. contaminans*. In biofilms, *P. aeruginosa* exhibited higher susceptibility to ciprofloxacin treatment, in the presence of *B. contaminans,* at concentrations starting at 2 × MIC. Several genes associated with virulence in *P. aeruginosa* were analyzed, and the specificity of the primer pairs corresponding to the genes *pqsE*, *lasI*, and *mucA* in *P. aeruginosa* were confirmed by PCR and qPCR. Additionally, RNA extraction was optimized for *B. contaminans* biofilms, concluding that using 10 biofilms yielded a greater RNA quantity. However, qPCR results indicated that *16S rRNA*, the gene chosen for relative expression normalization, was not a suitable reference gene due to significant variation in cycle quantification across different conditions. Thus, further optimizations will be necessary to quantify the expression of the genes of interest.

In summary, this study demonstrates how interspecies interactions can influence antibiotic susceptibility, enhancing our understanding of *P. aeruginosa* and *B. contaminans* behavior in dual-species biofilms.

Keywords: Biofilms, *P. aeruginosa*, *B. contaminans*, Interactions, Cystic Fibrosis

RESUMO

Estudo das interações entre *Pseudomonas aeruginosa* e *Burkholderia contaminans* em infeções associadas a biofilmes em ambiente de fibrose cística

As Gram-negativas *Pseudomonas aeruginosa* e do complexo *Burkholderia cepacia* são bactérias patogénicas oportunistas responsáveis por infeções graves em pacientes com Fibrose Cística (FC). Estas bactérias formam biofilmes nas vias aéreas, tornando as infeções difíceis de tratar. Recentemente, foram feitos alguns avanços para compreender a complexa interação entre diferentes microrganismos e as suas implicações no prognóstico da doença e na resposta à terapia em FC. Assim, este estudo teve como objetivo contribuir para o conhecimento do possível papel das interações entre *P. aeruginosa* e a emergente *B. contaminans* durante a infeção bem como o seu impacto na ação de antibióticos frequentemente utilizados no controlo de *P. aeruginosa*.

No que refere a capacidade de formação de biofilme, a presença de *B. contaminans* resultou numa diminuição da biomassa e no número de células cultiváveis em biofilmes duplos em comparação com os biofilmes simples de *P. aeruginosa* após 72h, sugerindo uma interação antagonista entre as bactérias. No que diz respeito ao papel destas interações microbianas na suscetibilidade à colistina e ciprofloxacina, primeiro foi determinada a concentração Inibitória Mínima (CIM). No caso da ciprofloxacina foi determinada como sendo 0,25mg/L para ambas, enquanto a CIM para a colistina variou entre 0,5 e 1mg/L para *P. aeruginosa*, não sendo possível determinar o MIC deste antibiótico em *B. contaminans*. Em biofilmes, *P. aeruginosa* mostrou maior suscetibilidade ao tratamento com ciprofloxacina na presença de *B. contaminans* a concentrações a partir de 2× CIM. Para avaliar a expressão de alguns genes relacionados com a virulência em *P. aeruginosa*, a especificidade dos *primers* correspondentes aos genes *pqsE*, *lasI* e *mucA* foram confirmados por PCR e qPCR. Adicionalmente, a extração de RNA foi otimizada para biofilmes de *B. contaminans*, concluindo-se que a utilização de 10 biofilmes proporcionava uma maior quantidade de RNA. No entanto, os resultados da qPCR indicaram que o *16S rRNA*, gene escolhido para a normalização da expressão relativa, não era um gene de referência adequado devido a variações significativas nos ciclos de quantificação detetados nas diferentes condições sob estudo. Assim, mais otimizações serão necessárias para quantificar a expressão dos genes de interesse.

Em resumo, este estudo demonstra como as interações entre espécies podem influenciar a suscetibilidade aos antibióticos, melhorando a nossa compreensão do comportamento de *P. aeruginosa* e *B. contaminans* em biofilmes de espécies duplas.

Palavras-chave: Biofilmes, *P. aeruginosa*, *B. contaminans*, Interações, Fibrose cística

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ABBREVIATIONS LIST

AHL	N-Acyl-Homoserine Lactone
ANOVA	One-Way Analysis of Variance
Bcc	*Burkholderia cepacia* complex
BCSA	*B. cepacia* Selective Agar
C4-HSL	N-Butanoyl-L-Homoserine Lactone
cDNA	complementary DNA
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CFUs	Colony Forming Units
C_q	Cycle Quantification
CV	Crystal Violet
EDTA	Ethylene Diamine Tetra Acetic Acid
eNaC	Amiloride-Sensitive Epithelial Sodium Channel
EPS	Exopolysaccharide
EUCAST	European Committee on Antimicrobial Susceptibility Testing
gDNA	genomic DNA
HSL	Homoserine Lactone
IQS	2-(2-Hydroxyphenyl)-Thiazole-4-Carbaldehyde
LPS	Lipopolysaccharide
MHB	Mueller-Hinton Broth
MIC	Minimum Inhibitory Concentration
NaCl	Sodium Chloride
NRT	No Reverse Transcriptase
NTC	No Template Control
OD	Optical Density
$OD_{595\ nm}$	Optical Density at 595 Nm
$OD_{620\ nm}$	Optical Density at 620 Nm
OdDHL	N-(3-Oxododecanoyl)-L-HSL
PCR	Polymerase Chain Reaction

PIA	Pseudomonas Isolation Agar
PQS	2-Heptyl-3-Hydroxy-4(1H)-Quinolone
qPCR	quantitative Polymerase Chain Reaction
QS	Quorum Sensing
RF	Reference Gene
ROS	Reactive Oxygen Species
RV	Reverse Transcriptase
SD	Standard Deviation
T3SS	Type III Secretion System
T6SS	Type VI Secretion System
TAE	Tris Acetate Ethylene Diamine Tetra Acetic Acid Buffer
TCA	Tricarboxylic Acid
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
RT	Room temperature

1. INTRODUCTION

1.1. Background

Cystic Fibrosis (CF) is a genetic disease caused by a mutation in the CF transmembrane conductance regulator (CFTR) gene, responsible for the expression of a chloride channel in apical cell membranes of multiple organs [1]–[3]. Impairment of this function can have severe consequences, with the respiratory system being the most commonly affected, resulting in higher morbidity and mortality rates for CF patients [4]. In the lungs, a defective chloride ion transport across the epithelial cell surface often results in the decrease of the volume of the periciliary fluid in the lower respiratory tract [5]. Consequently, the lungs of CF patients are characterized by the accumulation of thick, dehydrated mucus that obstructs the airways [5], [6]. This creates a welcoming environment for microorganisms to thrive [7]. The respiratory microbiome is often dominated by opportunistic pathogens, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia cepacia* species, and others known to cause respiratory infections in CF patients [8]. *P. aeruginosa* is one of the organisms most commonly isolated from the airway and due to its adaptive mechanisms, can survive and persist in the respiratory tract of CF patients [9]–[11]. CF patients can also become infected with species from the *B. cepacia* complex (Bcc), a group of bacteria that are characterized by its high transmissibility and are often resistant to most used antibiotics [12]. In CF, Bcc infections give rise to a highly variable and unpredictable outcome, which can range from an asymptomatic manifestation to necrotizing pneumonia known as "cepacia syndrome", resulting in a rapid clinical deterioration that can lead to early death [13], [14]. Therefore, infections with Bcc, alone or in co-infection with other species, constitute an important concern for the CF population. Among individuals with CF, the most common isolated species are *B. cenocepacia* and *B. multivorans* [16]–[18]. However, *Burkholderia contaminans* has been recovered from respiratory samples from Portuguese [15] and Italian patients [16] and, currently, is reported to be the most frequent Bcc species in Spanish [17] and Argentinean [18] CF patients. In some cases, infection with *B. contaminans* can result in unfavorable clinical outcomes, including death [15].

Biofilm formation allows microorganisms to survive in hostile environments [19], constituting microorganisms' preferred mode of growth [20]. The clinical setting is no different, and it has been demonstrated that most bacteria are in biofilms in both chronic and acute infections [21]. Aside from the protective role played by the biofilm matrix, bacteria within biofilms can utilize various survival tactics to

evade host defenses [22], [23] and often exhibit reduced susceptibility to antibiotics [24]. Consequently, biofilm-related infections are difficult to eradicate as is the case of infections caused by *P. aeruginosa* [9]–[11]. In the particular case of CF patients, it was shown that biofilm-grown *P. aeruginosa* or a member of Bcc*, B. cepacia*, were less susceptible than their planktonic counterparts to either single or multiple combinations of antibiotics [25]. As such, biofilm-based infections pose an important threat to our ability to treat infection with the current antimicrobial therapy available.

1.2. Motivation

The understanding of CF infection has evolved, and it is now known that CF airways are not solely affected by a single pathogen [26]. Instead, it is recognized that various microorganisms coexist in the airways of individuals with CF, forming complex ecological communities. These interactions can be synergistic or antagonistic and can also involve nutritional dependencies among others [11], [27]. Nevertheless, the influence of these interactions on the behavior of the individual species and the activity of the polymicrobial community is poorly understood [26]. The majority of the studies about interactions in the CF community focus on the traditional and prevalent *P. aeruginosa* and other common pathogens in CF such as *S. aureus* [11], [28]–[30]. However, despite the recent emergence of *B. contaminans* in several countries, practically nothing is known about the interactions between *P. aeruginosa* and *B. contaminans,* and their impact on CF pathogenesis. Therefore, understanding the interactions between these two pathogens could provide valuable insights to help pave the way to design more effective treatments.

1.3. Aims

Taking into consideration the lack of literature about the interactions between the well-known *P. aeruginosa* and the less-studied *B. contaminans,* this dissertation aimed to understand the ecological interactions between these two pathogens, under the biofilm mode of growth, and provide insight into the underlying mechanisms of airway microbial ecology.

To achieve the goal proposed, the first task was to assess the ability of *B. contaminans* and *P. aeruginosa* to develop biofilms, in mono (in the case of *B. contaminans*) and dual-species populations. The biofilms were characterized based on the quantification of total biomass and by culturable cells (Colony Forming Units (CFU) counts). Subsequently, the susceptibility to clinically relevant antibiotics used for *P. aeruginosa* therapy when administered in a dual- versus a mono-species biofilm was

addressed. To accomplish this, the antibiotic that presented the lowest minimum inhibitory concentration (MIC) was chosen and administered to biofilms, at different concentrations, followed by cell counting to understand the effect of the antibiotics in this mode of growth. To gain a more comprehensive understanding of how *B. contaminans* influences *P. aeruginosa* virulence in the presence of antibiotics and without, the final task involved the utilization of quantitative PCR (qPCR) to determine the expression levels of genes associated with *P. aeruginosa* virulence.

2. LITERATURE REVIEW

2.1. Cystic Fibrosis

CF, one of the most prevalent fatal hereditary diseases worldwide, is a monogenic disorder caused by an autosomal recessive mutation in the CFTR gene, which codes for the CFTR protein [31]. Currently, for individuals born between 2017 and 2021, the median predicted survival age is 53.1 years [8]. CFTR protein is typically expressed in the surface epithelium of the airways, submucosal glands and other epithelial organs (e.g., digestive and reproductive systems) [32]. Therefore, CF is considered a multi-organ disease characterized by heterogeneous clinical manifestations and the potential to affect individuals over their lifetime [33]. Its malfunction contributes to growth retardation, pancreatic insufficiency, male infertility, asthma and others [34], [35]. Complications in the respiratory system are the principal cause of morbidity and mortality among affected patients, with recurrent chronic bronchopulmonary infections leading to worsening cough, tachypnea, and wheezing are among the most prominent symptoms [5], [36].

Currently, over 2000 mutations have been identified in the gene *cftr*, although functional consequences have not yet been defined [31], [37]. The most common mutation (*ΔF508*) produces a deletion of three nucleotides in the *cftr* gene on chromosome 7 causing the loss of a single codon for phenylalanine [38]. As a result, an abnormal misfolded protein lacking a phenylalanine residue is generated [39]. This misfolded protein is retained within the endoplasmic reticulum and does not undergo further processing, resulting in a loss of function. CFTR mainly functions as a chloride channel but is also known as a regulator of the amiloride-sensitive epithelial sodium channel (ENaC) [40]. In the absence of functional CFTR, chloride secretion is reduced, and sodium absorption is increased, leading to an osmotic gradient that causes water reabsorption in the apical membranes of lung epithelial cells [40] (**Figure 1**). Loss of the channel leads to a reduction in the volume of the lubricating layer between the epithelium and the mucus in the lower respiratory tract, thereby compromising mucociliary clearance [41]. Thus, CF lung disease is characterized by the accumulation of thick dense mucus, which remains tethered to the epithelium [42]. As a result, the viscous mucus obstructs the airways and leads to infection [42]. This results in a vicious cycle of defective clearance, obstruction, infection, and inflammation, rendering patients vulnerable to chronic respiratory infections and acute exacerbations, progressively damaging the airway tissue, and eventually resulting in respiratory failure and death (**Figure 1**) [35].

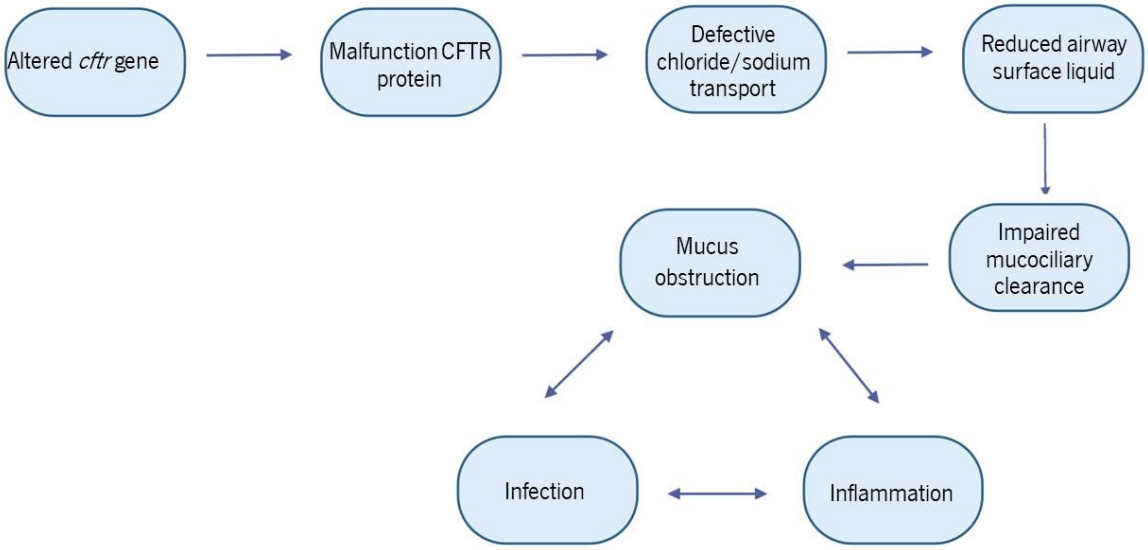


Figure 1 | Pathophysiological cascade of CF lung disease. A mutation on the *cftr* gene leads to a defective chloride ion transport increasing sodium and, consequently, water absorption. Together, these effects are hypothesized to decrease the volume of the periciliary fluid, compromising the mucociliary clearance and triggering the overproduction of dehydrated and viscous mucus. This leads to the persistent colonization of bacteria in the lungs, and the physiologic consequences are persistent inflammatory responses, obstructive lung physiology, and respiratory insufficiency, which ultimately results in death from chronic respiratory failure. CFTR: Cystic Fibrosis transmembrane conductance regulator.

2.2. The Microbiome of CF in the Lungs

In CF lung disease, the combination of thickened mucus and decreased clearance promotes the formation of mucus plugs. These plugs can obstruct the airways and establish a sheltered environment for microorganisms [43]. The microflora of airways in CF patients is characterized by their diversity, being often polymicrobial and constantly evolving, triggering acute and chronic inflammation, and posing a significant threat to CF patients [19]. As a result, microbial lung infections are recognized as the leading cause of morbidity and mortality in individuals with CF [44].

For many years, the detection and identification of microbial species relied on culture-based techniques with *S. aureus*, and Gram-negative bacteria such as *P.aeruginosa*, Bcc, *Stenotrophomonas maltophilia* and *Achromobacter* spp., as the most commonly identified pathogens in CF airway samples [45]. However, recent advances in microbial diagnosis have revealed a much more diverse polymicrobial community in the airways of CF patients. Among the bacteria increasingly identified in the sputum of CF patients are the anaerobic bacteria from the genera *Prevotella*, *Fusobacterium*, *Veillonella*, and *Porphyromonas* [18][46].

A limited number of species are recognized to significantly contribute to CF lung disease, with prevalence dependent on patient age. The most phylogenetically diverse CF lung microbiome is observed during childhood, with the diversity declining from adolescence to adulthood (**Figure 2**) [26]. This decrease is typically associated with antimicrobial use and with worsened lung function [37]. *S. aureus*, one of the first pathogens isolated from CF samples, is the most prevalent pathogen in children and adolescents, reaching its peak value in the early teenage years at a prevalence of almost 80% [26]. Methicillin-resistant *S. aureus* (MRSA) is an increasing threat that occurs in up to 30% of patients in some US centres, although its prevalence in Europe is below 10% in most countries [47]. *Haemophilus. influenzae* is also involved in chronic lung infections in CF children and adolescent patients [48].

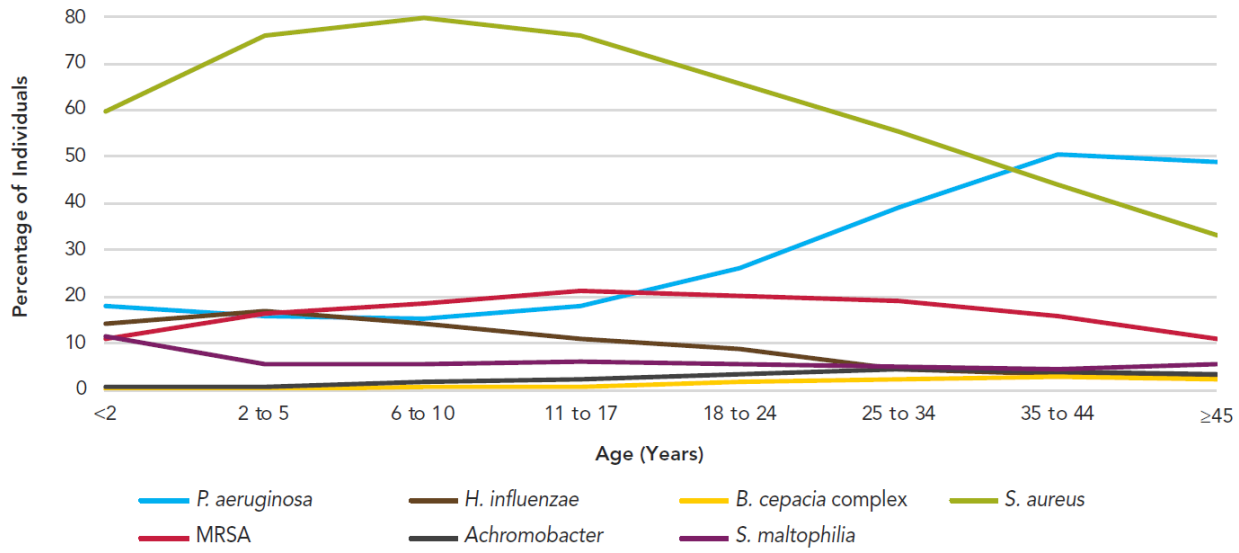


Figure 2| Prevalence of respiratory microorganisms by age cohort. Image retrieved from [8].

P. aeruginosa is the dominant pathogen in older individuals. With increasing age, infections with other bacteria, including *Achromobacter xylosoxidans, S. maltophilia* and Bcc, become increasingly common (**Figure 2**) [5]. *S. maltophilia* and *A. xylosoxidans* are more common than Bcc but are generally less virulent. The incidence of each of these pathogens varies between CF patients, although they generally occur in <15% of patients and rates increase with disease severity [35].

In light of the current understanding of the intricate nature of polymicrobial infections, the focus should be to understand their role within the context of the larger microbial community [43]. To establish the significance of the microbiome in CF lung disease, it is essential to correlate the microbial community composition and disease severity, such as exacerbation and lung function [11].

2.3. *P. aeruginosa*

2.3.1. Distribution and general characteristics

P. aeruginosa, a Gram-negative opportunistic pathogen, is a heterotrophic, motile, rod-shaped bacterium about 1–5μm in length and 0.5–1.0μm in width [49]. This bacterium is heterotrophic and functions as a facultative aerobe, capable of growing through both aerobic and anaerobic respiration using nitrate as the terminal electron acceptor [50]. Additionally, *P. aeruginosa* can grow anaerobically utilizing arginine and exhibits limited fermentative capabilities, resulting in slow or no growth [50]. It has the potential to cause a broad spectrum of infections including pneumonia, wound infections (burns, diabetic wounds, etc.) and urinary tract infections, especially in hospitalized and immune-compromised patients [51]. This versatile bacterium is equipped with a large genome containing nearly 6,000 genes, including genes responsible for metabolic pathways, substance transport and virulence factors enabling *P. aeruginosa* to adapt to various environments [52], [53].

P. aeruginosa is widely distributed in different environments, including terrestrial, aquatic, animal and human [54]. Most *P. aeruginosa* strains produce one or more pigments, such as pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent) and pyorubin (red-brown) [55].

2.3.2. *P. aeruginosa* in CF airways

The presence of *P. aeruginosa* in CF is associated with chronic infections that lead to a decline in pulmonary function, increased frequency of daily cough and worse chest radiological imaging scores [56]. Consequently, this pathogen is the major target for antibiotic treatment. Despite the long and aggressive antibiotic treatments administered to CF patients and the constant attack of the host immune system, *P. aeruginosa* infections are extremely difficult to eradicate, leading to increased morbidity rates and even death [33], [34].

Acute infection of the CF airways caused by *P. aeruginosa* takes place in the early stages of the disease, and it can be acquired from the environment or the healthcare setting [57]. In contrast to the natural environment, CF airways present numerous different conditions and selective pressures, including variations in oxygen concentration, nutrient availability (e.g., iron and zinc), presence of antibiotics, and other microbial species that could drive interspecies competition [58], [59]. Therefore, bacterial adaptation is essential to establish infection in the CF airways.

In an initial phase, *P. aeruginosa* isolates are frequently non-mucoid, susceptible to antibiotic treatments and still express several virulence factors such as pyocyanin, type III secretion system (T3SS), which is responsible for the secretion of various exotoxins and proteases (**Figure 3**) [60], [61]. This phenotype, together with the higher function of the lung when compared with the later stage of the disease, facilitates the eradication of the bacterium if antibiotic treatment is quickly started [60].

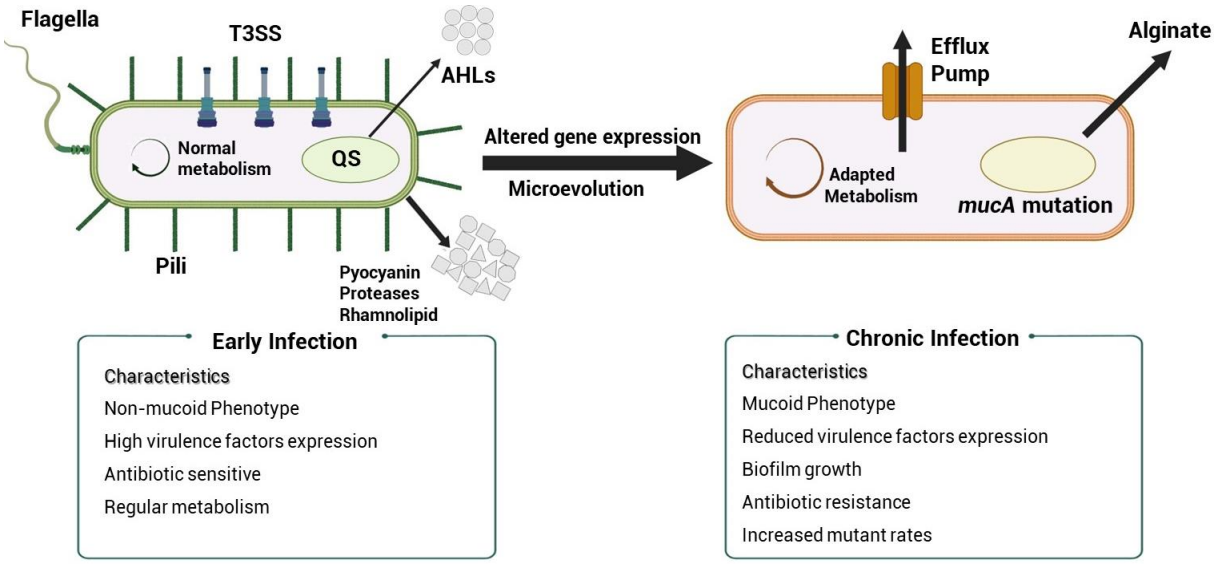


Figure 3| Representation of *P. aeruginosa* evolution during infection in CF airways. In the early stages of a *P. aeruginosa* infection, the bacterium expresses several virulence factors, including flagella, pili, proteases, pyocyanin, and rhamnolipid, and is susceptible to antibiotics. However, during the chronic stage of the infection, the bacterium undergoes several adaptations, such as increased production of alginate, loss of some of the virulence factors that are necessary for establishing the initial infection and developing resistance to antibiotics while adapting its metabolism to the changing conditions. This image is adapted from [62]. T3SS: Type 3 Secretion System, AHLs: N-acylated-L-homoserine lactones; QS: Quorum Sensing.

The continuous and selective pressure on the population leads to the emergence of diverse genetic and phenotypic variants. *P. aeruginosa* strains become less inflammatory and less cytotoxic and lose the structures responsible for adherence and motility, like flagellum and pili (**Figure 3**). Other characteristics include (i) the appearance of mucoid, (ii) the emergence of highly antibiotic-resistant variants, (iii) changes in lipopolysaccharide (LPS) structure and (iv) loss of T3SS, and the quorum sensing (QS) systems that control numerous important secreted components including pyocyanin, elastase, cyanide, and rhamnolipids [62], [63].

P. aeruginosa transition into a mucoid form is mainly due to the mutational inactivation of the *mucA* gene (**Figure 3**). This gene encodes for a membrane-bound cytoplasmatic protein that negatively controls the transcription of the *algD* gene [64]. Inactivation of this gene leads to the upregulation of *algT* and the production of alginate [65]. Furthermore, this upregulation promotes the conversion into a mucoid

phenotype characterized by the biofilm mode of growth [66]. The finding that *P. aeruginosa* increases the expression of genes involved in the production of alginate and certain exopolysaccharides (EPS), as well as biofilm determinants in CF airways, suggests that *P. aeruginosa* lives under the biofilm lifestyle [66]. These alterations reduce the efficacy of the immune system to recognize *P. aeruginosa*, aiding in its presence in CF airways [67]. *P. aeruginosa* isolates are hardly phagocytosed by alveolar macrophages and neutrophils, helping bacteria to evade host immune defenses and persist in CF airways [68].

To survive and adapt, *P. aeruginosa* also has to adjust its metabolic pathways. In CF lungs, the dehydration of the mucus layer that covers the epithelium surface creates a tight mucin mesh that restricts the movement of *P. aeruginosa* and other microorganisms leading to the accumulation of bacteria in the mucus [69]. Additionally, *P. aeruginosa* encounters low oxygen concentrations or even anaerobic conditions [58]. When oxygen dissolves in the mucus layer, a gradient is created and, thus, the surface presents a higher oxygen concentration and below the surface the oxygen concentration diminishes, and microaerophilic growth is possible [58], [69]. To survive and grow in anoxic niches, *P. aeruginosa* can adapt its metabolic pathways, obtaining energy from the fermentation of pyruvate and arginine, in environments with high amounts of amino acids [70]. Anaerobic environments can increase *P. aeruginosa* antibiotic tolerance and the robustness of biofilms through the increased production of alginate [71].

2.4. *Burkholderia cepacia* Complex

2.4.1. Diversity and Taxonomy

The Bcc is a group of 24 Gram-negative beta proteobacteria that are closely related and phenotypically similar. These obligate aerobic, non-spore-forming bacilli were first described in 1950 by Burkholder, as a plant pathogen and a cause of "sour skin" onion rot [72]. They carry complex genomes (with sizes ranging from 6 to 9 Mb) and can rapidly adapt through mutations, which translates into remarkable genotypic and phenotypic plasticity [73]. Initially, they were referred to as *Pseudomonas cepacia*, although in 1992 they were transferred to the new genus *Burkholderia* based on *16S rRNA* sequences, cellular lipid and fatty acid composition and phenotypic characteristics [74]. Later, Vandamme *et al*. [75], using *Burkholderia cepacia* isolates from CF patients, demonstrated that the bacteria initially identified as *B. cepacia* comprised several distinct species which led to the subdivision in genomovars, referring to phenotypically similar but genetically distinct species, forming a group of closely related species denominated as Bcc (**Table 1**).

Table 1 | *Burkholderia cepacia* complex (Bcc) species and their main sources. CF: Cystic Fibrosis

Bcc species	Genomovar	Natural Environment	General Characteristics	References
B. cepacia	I	Rhizosphere, soil, water	Highly represented in the Portuguese CF population	[74]
B. multivorans	II	Rhizosfere, soil, water	The second most common Bcc species in CF infection; Can infect non-CF humans	[75]
B. cenocepacia	III	Rhizosphere, plant, soil, water	Major CF pathogen; possesses several highly transmissible lineages and is often associated with poor prognosis	[76]
B. stabilis	IV	Rhizosphere	Isolated from CF and non-CF patients	[77]
B. vietnamiensis	V	Rhizosphere, plant, soil, water	Limited prevalence as a CF pathogen	[78]
B. dolosa	VI	Maize, rhizosphere, plant	Almost exclusively cultured in CF infection; patient-to-patient transmission	[79]
B. ambifaria	VII	Rhizosphere, soil	Rarely encountered as a CF pathogen	[80]
B. anthina	VIII	Rhizosphere, soil	Infections in CF patients (low prevalence) and animals	[81]
B. pyrrocinia	IX	Rhizosphere, soil, water, plant	Infections in humans (CF and non-CF)	[82]
B. ubonensis	[a]	Soil	Infection in non-CF patients, isolated from nosocomial infections	[83]
B. arboris	[a]	Rhizosphere, soil, water	Infections in CF and non-CF humans	[84]
B. diffusa	[a]	Soil, water	Infections in CF and non-CF humans	[84]
B. latens	[a]	Unknown	Infections in CF patients	[84]
B. metallica	[a]	Unknown	Infections in CF patients	[84]
B. seminalis	[a]	Rice, rhizosphere, soil	Infections in CF and non-CF humans	[84]
B. contaminans	[a]	Soil, water	Infections in humans and animals	[85]
B. lata	[a]	Soil, water	Infections in humans (CF and non-CF)	[85]
B. pseudomultivorans	[a]	Rhizosphere	Present in humans and the environment	[85]

[a] Conventionally, the organisms within the Bcc group were initially termed genomovars until distinct phenotypic traits were identified, allowing for the formal adoption of binomial nomenclature. Subsequently, the advancement of more precise phenotypic and genotypic tools facilitated the differentiation of these bacteria, thus enabling the direct naming of newly discovered bacteria within this complex and, thus, no genomovars are indicated herein [79]

2.4.2. Bcc as Opportunistic Pathogens in Humans

Bcc have been associated with serious respiratory infections among immunocompromised, chronic granulomatous disease and CF patients [86]. Unlike most opportunistic pathogens, members of the Bcc are not prone to commensal carriage, and infections are typically acquired directly from the environment or person-to-person [87].

The infection of Bcc in CF patients, although less common than *P. aeruginosa* or *S. aureus* infections, is still considered one of the most dangerous due to the accelerated decline of pulmonary function and unpredictable clinical outcomes [88]. In CF mouse models, it was shown that members of the Bcc can induce a robust immune response [89], [90] and can invade and replicate in cultured respiratory epithelial cells [91], [92]. More recent work in transplanted or autopsied CF lungs suggests that Bcc species are found associated with host cells, particularly macrophages, or mucus [93]. However, it is still unknown how exactly bacteria survive in this environment. Even though a smaller portion of CF patients are infected with Bcc, most of those infected patients experience a reduction of pulmonary function caused by chronic infection and exacerbation events, and a subset of about 20% develop the life-threatening "cepacia syndrome", which has not been observed in other CF pathogens [35]. This syndrome is characterized by high fevers, necrotizing pneumonia and septicemia, ultimately leading to early patient death [94]. The most prevalent Bcc species in the CF community are *B. cenocepacia* and *B. multivorans*, which have caused epidemics worldwide and can be transmitted from patient-to-patient [95]. Besides their high virulence, at least five species within the Bcc group, *B. cepacia*, *B. cenocepacia*, B*. multivorans*, *B. dolosa*, and *B. contaminans* can be transmitted through aerosol droplets. This ability allows these pathogens to quickly spread among vulnerable patients in healthcare environments, both through direct and indirect contact with infected individuals [88]. Particularly, *B. contaminans* has also been recovered from contaminated pharmaceutical products and healthcare facilities, where it has caused outbreaks [96]. Currently, it is the most prevalent Bcc species in Spanish [17] and Argentinian [18] patients, with a high representation in Portuguese [15] and Italian CF patients [16]. Infections caused by this species have been linked to adverse clinical outcomes, occasionally leading to fatalities [15].

2.5. Microbial biofilms

2.5.1. *P. aeruginosa* biofilms

Biofilms can be defined as a complex aggregate of microorganisms encased in a matrix of polymers attached to a surface and/or to each other [97]. The development of biofilms is regarded as a key adaptive strategy of *P. aeruginosa* within the CF sputum, and evidence indicates its growth predominantly occurs in the region spanning from the trachea to the terminal bronchioles [92], [112][98].

The biofilm matrix is composed primarily of polysaccharides, extracellular DNA, proteins, and lipids [99]. This matrix accounts for over 90% of the biofilm biomass and provides a protective shield for the embedded bacteria against hostile environmental conditions, including antibiotics and host immune responses [100]. The three EPS produced, namely Psl, Pel, and alginate, play a crucial role in surface attachment, formation, and stability of the biofilm architecture [99], [101], [102]. Depending on the strain, the importance and contribution of the EPS to the matrix varies. Psl is necessary for the adhesion of sessile cells to surfaces and cell interactions during biofilm initiation [103]. Similarly to Psl, Pel is involved in surface attachment and maintenance of biofilm integrity [99]. Notably, chronic infection in CF patients is often associated with elevated levels of alginate, since this EPS is predominantly produced in *Pseudomonas* mucoid biofilms [104], [105].

Generally, biofilms of *P. aeruginosa* can be developed on abiotic surfaces, such as medical implants or industrial equipment [99]. However, the development of biofilms in CF airways differs from those on abiotic surfaces in terms of colonization patterns, as the bacteria tend to form multicellular clusters [106]. The process of biofilm development requires the coordination of individual cells, generally involving the action of QS systems [107]. QS is a cell-to-cell communication system that facilitates the rapid adaptation of bacteria to their surroundings [108]. Bacteria can identify the self-secreted small molecules known as autoinducers, which then coordinate the expression of numerous genes implicated in bacterial community behavior, virulence, and the formation of biofilms [109], [110]. *P. aeruginosa* harbors four distinct QS pathways systems namely Las, Rhl, Iqs, and Pqs, each employing a specific autoinducer, respectively, N-(3-oxododecanoyl)-l-HSL (OdDHL), N-butanoyl-L-homoserine lactone (C4-HSL), 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS), and 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS) [111].

Biofilm formation can be divided into five distinct stages, as illustrated in **Figure 4**. In the first stage, bacterial cells adhere to a surface through the use of supporting structures such as flagella and

type IV pili [112], [113] (**Figure 4, stage 1**), followed by irreversible attachment in the second stage (**Figure 4, stage 2**). In chronic infections, *P. aeruginosa'* s cells downregulate flagellum and type IV pili since they are no longer needed to move across sputum and along epithelial cell surfaces [114]. The subsequent stages involve the formation of the matrix and the propagation of attached bacteria into microcolonies (**Figure 4, stage 3**), which eventually develop into mature biofilms characterized by three-dimensional mushroom-like structures (**Figure 4, stage 4)**. The EPS, Psl, forms a peripheral meshwork helping to maintain structural stability [22][121]. In the final stages of biofilm development, the matrix is disrupted through cell autolysis, releasing cells (**Figure 4, stage 5**)[115]. This event triggers a transition into a planktonic growth mode, providing *P. aeruginosa* with an opportunity to colonize new niches or zones within the CF sputum, thereby perpetuating the infection (**Figure 4, stage 6**)[116].

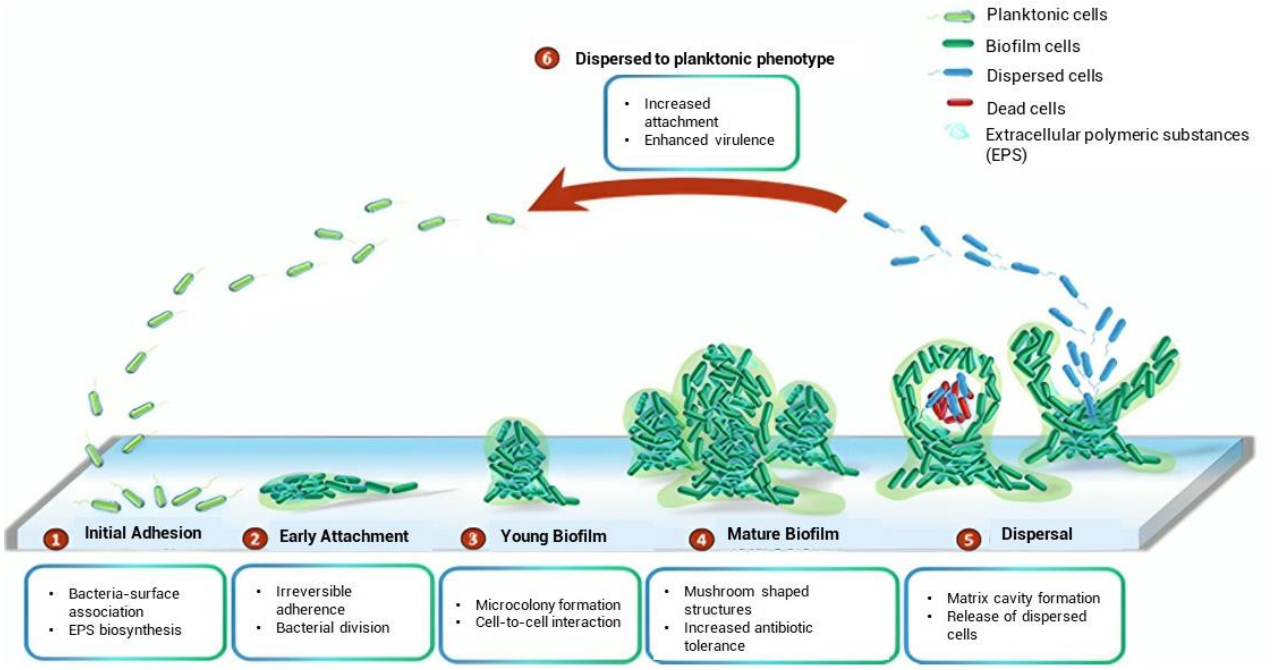


Figure 4| Cycle of *P. aeruginosa* biofilm development. Initially, the bacteria associates with the surface and produces extracellular polymeric substances (EPS) (**Stage 1**). Next, cell division and the transition of reversible attachment into irreversible take place (**Stage 2**). The following steps are the formation of multicellular clusters (**Stage 3**) and the further development into mushroom-shaped structures (**Stage 4**). Cell-to-cell interaction and production of virulence factors play essential roles in the maturation and robustness of biofilms. The matrix is disrupted for the release of the cells (**Stage 5**). Finally, the released cells undergo a transition into the planktonic phenotype (**Stage 6**). Image retrieved from [117].

2.5.2. Bcc biofilms

The precise mechanisms underlying biofilm formation are not fully understood. However, the process of biofilm formation in bacteria from the Bcc group is known to be mediated by the N-acyl-homoserine lactone (AHL) QS system and the initial adhesion is facilitated by fimbriae, pili, and flagella [118]. Furthermore, EPS constitute a significant component of the biofilm matrix, contributing to cell attachment and providing mechanical stability [118], [119].

Bacteria from the Bcc, when in a biofilm, are often less susceptible than planktonic cells to antimicrobials [120]. According to Caraher *et al.* [121] the formation of biofilms by Bcc bacteria results in an increased *in vitro* antibiotic tolerance, specifically to ceftazidime and ciprofloxacin. The three-dimensional structure of biofilms promotes gradients of nutrients and oxygen, which promote areas of dormant bacteria [122]. Van Acker and colleagues [123] found that biofilms formed by *B. multivorans, B. cenocepacia* and *B. dolosa* harbor a subset of dormant cells denominated as persister cells. Nowadays, it is generally accepted that persister cells are included in a state known as dormancy, which is triggered due to exposure to antibiotics or environmental stresses [124]. The concept of dormancy pertains to populations of cells that exhibit a decrease in their metabolic activity, though not its complete cessation [124]. After antibiotic treatment, these cells begin to grow again, leading to the formation of a population that is as susceptible to antibiotics as the original one (**Figure 5**) [125], [126]. The formation of persister cells in biofilms has been investigated in several Bcc bacteria, such as *B. cenocepacia* and *B. multivorans*, which have been shown to retain persister cells even after treatment with tobramycin, an aminoglycoside antibiotic commonly used to treat CF-infected patients [120], [122], [123]. The formation of persister cells in biofilms allows them to withstand the presence of high antibiotic concentrations by avoiding the production of reactive oxygen species (ROS). Transcriptomic analysis revealed that the surviving persister cells displayed downregulation of genes involved in the major metabolic pathways, including the tricarboxylic acid (TCA) cycle and the electron transport chain [120]. However, other proteins related to the oxidative stress response were upregulated, namely those involved in alternative metabolic pathways, such as the glyoxylate shunt. These results suggest that persister cells can redirect their metabolism to survive and protect themselves against environmental stresses [123].

The definitions of the terms persister, resistant and tolerant cells and their distinction are often confused. Antibiotics typically require actively growing cells to achieve an antibacterial effect. Compared to normally dividing cells, persisters are metabolically dormant and, therefore, antibacterial drugs are ineffective on these cells [127]. Interestingly, persister cells are genetically similar to non-persister cells,

while resistant bacterial cells are a result of an altered genotype due to the acquisition of new antibiotic-resistance genes [124]. Persister cells differ from tolerant since the first has the added attribute of affecting only a subpopulation of cells, whereas tolerance is the general ability of a population to survive longer treatments by, for example, having a lower killing rate [136][137], [138].

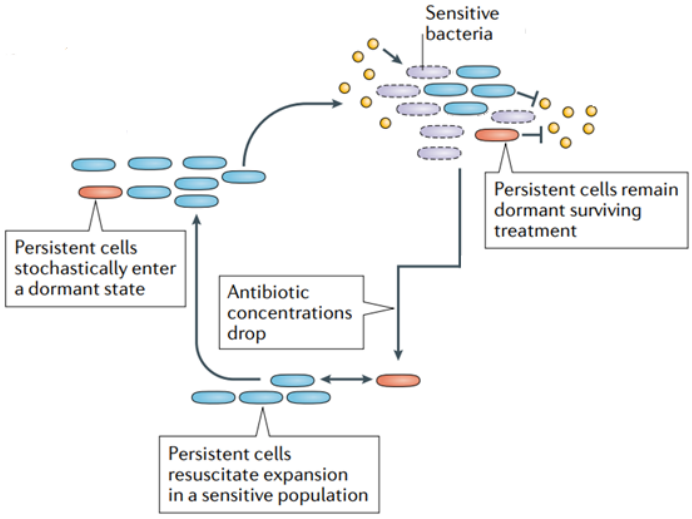


Figure 5| Persister cell formation during antibiotic treatment. When antibiotic exposure commences, a subset of persister cells emerges, which remain in a dormant but viable state. At the end of the antibiotic regimen, susceptible cells are eliminated, while persister cells persevere and retain their capability to revive and propagate a new population of cells. Image adapted from [128].

2.6. Interactions of Bcc and *P. aeruginosa*

As stated previously, individuals with CF exhibit colonization of the lungs by several bacterial species, which results in complex interspecies interactions [59]. These interactions are believed to significantly influence and, in some cases, determine the progression of the disease [129]. In recent years, several studies have attempted to elucidate the mechanisms by which these pathogens interact and, more importantly, to determine the impact of these interactions on disease progression and response to antimicrobial therapies [130]. **Table 2** summarizes some of the main interactions reported in the literature regarding *P. aeruginosa* and Bcc bacteria.

Table 2 | The main interactions reported in the literature between *P. aeruginosa* and Bcc bacteria. Bcc: *Burkholderia cepacia* complex; AHL: N-acyl-homoserine lactone; QS: Quorum sensing; T6SS: Type VI secretion system

Interaction	Mechanism	Mode of Growth	Reference
P. aeruginosa* enhanced virulence factors production by *B. cepacia	Adding the supernatants of *P. aeruginosa,* siderophore, lipase and protease production was increased in *B. cepacia*	Planktonic	[131]
Bcc bacteria can recognize and respond to *P. aeruginosa* QS AHL	*P. aeruginosa* AHL´s molecules can stimulate the production of extracellular proteases, activated by the QS of *B. cepacia*.	Biofilm	[132]
***P. aeruginosa* inhibited the growth of Bcc bacteria**	The production of pyocyanin is suggested to inhibit the growth of Bcc bacteria	Biofilm and planktonic	[133]
***P. aeruginosa* PAO1 inhibits *B. cepacia'* s biofilm**	The inhibition of *B. cepacia* growth by *P. aeruginosa* PA01 prevented the formation of stable mixed biofilms	Biofilm and planktonic	[134]
Co-infection of *P. aeruginosa* and *B. cenocepacia* leads to increased biofilm formation and increased host inflammatory response in an *in vitro* biofilm model and a mouse model of chronic infection	*B. cenocepacia* influenced biofilm formations by *P. aeruginosa* resulting in increased biomass. Co-infection by both species increased the production of several virulence factors	Biofilm and planktonic	[135]
Bcc bacteria inhibit *P. aeruginosa* growth in an anaerobic environment	Possibly, fermenting Bcc bacteria exhibit unique mechanisms of resistance to pyocyanins or the pigment may not be active. Additionally, Bcc bacteria may produce bacteriocins under anaerobic conditions	Planktonic	[93]
The competitive efficacy of *P. aeruginosa* to *B. cenocepacia* from Bcc is altered by several factors	The modification of the culture medium, the genetic adaptation of *P. aeruginosa* to the CF lung and the addition of another bacterial species can alter the direct competition and maintain higher levels of bacterial diversity in the community	Planktonic	[136]

Table 2| (Continued) The main interactions reported in the literature between *P. aeruginosa* and Bcc bacteria

Interaction	Mechanism	Mode of Growth	Reference
Bcc bacteria can outcompete *P. aeruginosa* isolated from older CF patients but not against PA01	The loss of T6SS by *P. aeruginosa*, a result of its adaptation to the host lungs, constitutes an advantage for Bcc strains	Planktonic	[137]

Initially, Bcc and *P. aeruginosa* interaction was believed to be synergistic [138]. Both of these bacteria utilize the same chemical language to control biofilm formation, the HSL-mediated QS system, suggesting that they were not only able to communicate but there was a possible role for inter-species communication in the course of the disease of CF patients co-infected with bacteria of both genera [73], [139]. Mckenney *et al.*, in 1995 [131] showed that when adding culture supernatants of *P. aeruginosa* to *B. cepacia* cultures, the latter enhanced the production of siderophores, lipases and proteases. Moreover, Riedl and colleagues [132] showed that Bcc bacteria can recognize and respond to *P. aeruginosa* QS molecules, although the latter did not respond to the molecules produced by *B. cepacia*.

An important feature of *P. aeruginosa* and Bcc bacteria is their ability to form biofilms, which is one of the contributing factors to reduced antibiotic efficacy and poor patient prognosis [10], [118]. As such, Tomlin *et al.* demonstrated these bacteria were able to form mixed-species biofilms and substantial concentrations of both species were observed [133]. Interestingly, *P. aeruginosa* strain PA01 inhibited the growth of several Bcc strains (*B. multivorans* ATCC 17616, and *B. cepacia* strains K56-2 and CEP509) [133]. It was suggested that the cause of this inhibition was related to the secretion of pyocyanin, since *P. aeruginosa* strain 6106, which does not produce observable levels of pyocyanin, resulted in markedly less inhibition when compared to strain PA01. In CF, *B. cepacia* infections rarely precede a *P. aeruginosa* infection [140]. Rather, *B. cepacia* seems to colonize existing *P. aeruginosa* biofilms [134]. Therefore, to better understand these interactions, Al-Bakr and colleagues [134] challenged *P. aeruginosa* or *B. cepacia* biofilms with planktonic cells of the other species using the Sorbarod model of biofilm growth [134]. In short, using a syringe, the bacterial cultures are dropwise transferred onto a sorbarod filter and, then, perfused with culture media at a controlled flow rate [141]. Using this technique, it is possible to obtain a quasi-steady state long-term culture and control the growth rate at which the biofilms are formed [142].

Regardless of which organism was the primary colonizer, dual-species biofilms were formed when co-inoculated. However, when *B. cepacia* was added to an established *P. aeruginosa* biofilm and vice-versa, a stable mixed community was not established [141]. These authors also suggested that various *P. aeruginosa* secondary metabolites were functioning as inhibitory factors to *B. cepacia* growth. Based on these results, the presence of a primary *P. aeruginosa* biofilm does not seem to facilitate the attachment of *B. cepacia* as a secondary, successional colonizer. Nevertheless, *B. cepacia* and *P. aeruginosa* strains, when simultaneously introduced into an un-colonized surface, produced stable mixed biofilms.

The development of biofilms and interaction between microorganisms can influence not only the virulence potential but also the pathogenicity of each bacterium. Bragonzi *et al.* [141] demonstrated, using clinical isolates, that *P. aeruginosa* had a negative effect on *B. cenocepacia* growth. In a mouse model mimicking a bronchopulmonary chronic infection in CF, Bragonzi and colleagues [135] showed that challenging with a single or mixed inoculum induced the development of chronic infections after two weeks. Furthermore, bacteremia in co-infected mice was induced by both pathogens as indicated by a similar bacterial load found in the liver, kidney, spleen, and lungs. However, inflammation markers were increased in co-infection, suggesting that the greater inflammation response in dual-infection murine airways may be influenced by the expression of particular virulence factors rather than the number of bacteria [135].

All the previously mentioned studies examined *P. aeruginosa* and Bcc bacteria interaction *in vitro* and/or in murine models, but no studies explored the interaction in humans. Schwab *et al*. were the first to examine the interaction of these two bacteria in excised lungs from CF patients [93]. They showed that the number of *P. aeruginosa* was reduced in tissue sections and sputum samples in Bcc co-infected lungs versus lungs infected with *P. aeruginosa* only. As CF lung disease progresses, the airway mucus becomes more anaerobic [143]. Under these conditions, Shwab used *in vitro* co-cultures with Bcc bacteria (*B. multivorans* and *B.cepacia*) and found that it reduced the competitive advantage of *P. aeruginosa* and consequently increased Bcc survival compared to aerobic conditions [93]. The latter supported the hypothesis that environmental conditions might impact competitive interactions between *P. aeruginosa* and the Bcc [136]. Bernier and colleagues also showed that the growth medium influenced the advantage of *P. aeruginosa* over *B. cenocepacia* [136].

The epidemiology of these two Gram-negative bacteria is distinct as *P. aeruginosa* infects many patients during childhood and becomes the most common pathogen in adults [137]. In contrast, Bcc infections mainly occur in older CF patients [137]. Bernier *et al*. [136], showed that using *P. aeruginosa* clinical isolates collected over 10 months, the adaptation that *P. aeruginosa* undergoes in the CF lung had profound effects on the viability of *B. cenocepcia*, where the latter became the dominant microorganism. Perault *et al.* [137] postulated that these differences may be partially attributed to *P. aeruginosa* loss of the type 6 SS (T6SS) in its host adaptation process. The T6SS is one of several mechanisms in which bacteria secrete toxic effector proteins into prokaryotic or eukaryotic cells [144]. These authors reported that *B. cenocepacia* BcAU1054 and other Bcc clinical isolates failed to outcompete any *P. aeruginosa* strains isolated from young children but successfully outcompeted about half of the *P. aeruginosa* isolates from CF patients aged 11–31 years by a T6SS-dependent mechanism. Indeed, mutations in regulatory genes that abrogate T6SS activity were found in several clinical isolates' sequences [137].

Over the years, *in vitro* studies have shown that *P. aeruginosa* dominates over Bcc bacteria in both planktonic and biofilm modes of growth [133]. This may be attributed to the production of pyocyanin or other virulence factors. However, in anaerobic regions, Bcc bacteria can thrive and survive over the other pathogens. In conclusion, it is hoped that a better understanding of the physiology of Bcc bacteria and their interactions with *P. aeruginosa* will stimulate the development of more rational approaches for therapeutic interventions.

2.7. Antibiotic treatments for *P. aeruginosa* and Bcc

P. aeruginosa is the main pathogen in people with CF and significantly contributes to low life expectancy [50]. *P. aeruginosa* is, therefore, one of the main targets in therapeutic programs for CF [57]. Currently, the standard care for managing persistent *P. aeruginosa* infection in CF patients involves the regular inhalation of antibiotics [145]. This approach is supported by substantial evidence indicating a reduction in *P. aeruginosa* levels in sputum, along with better respiratory symptoms, enhanced quality of life, and improved lung function [146]. This success is attributed to the targeted delivery of potent antibiotics directly to the bronchial lumen, minimizing systemic toxicity [146]. Tobramycin inhalation solution, for instance, has been instrumental in managing persistent and chronic bacterial infections, leading to improved lung function, prolonged resistance against reinfection, and reduced mortality rates [147]. Research has explored various inhaled antibiotics, including colistin, gentamicin, ceftazidime,

cephaloridine, aztreonam lysine, taurolidine, and a combination of gentamicin and carbenicillin, all displaying efficacy against microbial infections [146].

Combination therapies, employing both oral and inhaled antibiotics with distinct mechanisms, are routine in treating CF-associated infections. Quinolones, especially ciprofloxacin and levofloxacin, are frequently utilized orally alongside inhaled tobramycin, aztreonam, or colistin [148]. Moreover, interest has grown in oral and intravenous antibiotics, including intravenous administration of β-lactams like piperacillin or ticarcillin, third- and fourth-generation cephalosporins such as ceftazidime and cefepime, carbapenems like meropenem or imipenem, and monobactams like aztreonam [149]. These intravenous antibiotics are often combined with oral aminoglycosides such as amikacin, gentamicin, or tobramycin [150].

As previously mentioned, infections caused by bacteria belonging to the Bcc group can lead to diverse clinical outcomes, and the presence of specific bacterial strains may lead to rapid deterioration in pulmonary function [151]. Despite the potential for these infections to become fatal, their management is frequently neglected, and there is limited clinical data available to establish a safe and effective therapeutic approach for eradicating Bcc in individuals with CF [152]. Frost *et al.* [153] found no single randomized trial investigating Bcc eradication, only case series or reports. Consequently, the treatment of these infections is primarily individualized, taking into consideration *in vitro* antibiotic susceptibility data and past clinical responses [154]. Therefore, clinical trials are highly needed to assess the effectiveness of different antibiotic regimens in patients infected with Bcc.

Numerous antimicrobial agents spanning various classes have undergone testing against Bcc bacteria, mainly through *in vitro* testing [149]. However, only a few have exhibited antibacterial efficacy. For example, *B. cenocepacia* is naturally resistant to most polymixins, aminoglycosides and β-lactams [155], [156]. In general, Bcc bacteria have been observed to display susceptibility when exposed to semisynthetic penicillins, carbapenems, and ceftazidime, all of which target cell wall synthesis, as well as fluoroquinolones, which inhibit DNA replication [25]. According to *in vitro* susceptibility data, trimethoprim–sulfamethoxazole, ceftazidime, meropenem, and doripenem continue to stand out as the most effective treatments for Bcc infections [150]. Nevertheless, *in vitro* resistance to trimethoprim–sulfamethoxazole and ceftazidime range from 10% to 40% and 30% to 40%, respectively [151]. In addition to the fading effectiveness of first-line options due to increased resistance and tolerance, particularly towards trimethoprim–sulfamethoxazole, can further restrict the available therapeutic choices [153].

Exploring the role of polymicrobial dynamics in CF lung disease may provide alternate therapeutic targets. This is because these bacteria are either directly harmful or can enhance the virulence of other pathogens [157]. Additionally, they might inhibit antibiotics, thereby reducing their impact on *P. aeruginosa* [158]. For example, if an organism, such as *P. aeruginosa*, becomes less susceptible to antibiotic therapy, an alternative treatment avenue may mediate the desired clinical response by effectively managing the composition of the microbial community in such a way that removes bacterial species responsible for exacerbating the state of a principal pathogen [37].

3. MATERIALS AND METHODS

3.1. Bacteria and growth conditions

In this study, *Pseudomonas aeruginosa,* strain UCBPP-PA14, a highly virulent clinical isolate isolated from burn wound patients at a hospital in Pennsylvania [159] and *Burkholderia contaminans* 293K04B [160], were used. *B. contaminans* and *P. aeruginosa* were stored at -20ºC in Tryptic Soy Broth (TSB, BD Biosciences, Franklin Lakes, NJ, USA), supplemented with 20% glycerol (Panreac, Barcelona, Spain). *P. aeruginosa* was sub-cultured from frozen stock preparations onto Tryptic Soy Agar (TSA) plates, which were prepared with TSB (VWR, Radnor, PA, USA) supplemented with 1.5% (w/v) agar (Liofilchem, Abruzzi, Italy). For each experiment, isolated colonies were picked from TSA plates, inoculated into TSB, and incubated overnight at 37ºC and 150rpm (ES-80 Shaker-Incubator, BioSan, Riga, Latvia). For the inoculation of *B. contaminans*, this bacterium was grown directly from stock solutions stored at -20ºC.

3.2. Determination of calibration curves

B. contaminans calibration curves were determined by correlating the number of culturable cells with optical density (OD). Briefly, overnight cultures' OD, at 620nm (OD_{620}), was assessed using an ELISA microtiter plate reader (EZ Read 800 Plus, Biochrom, Cambridge, UK) and then diluted to obtain an OD_{620nm} of 1. Afterwards, this suspension was serially diluted in TSB, to achieve OD_{620nm} of 0.5, 0.1 and 0.05 (±0.05) and, then, the number of culturable cells was determined by Colony Forming Units (CFU) counting. To do so, the suspensions with different ODs were serially diluted (1:10) in 0.9% sodium chloride (NaCl) and 10µL of dilutions, ranging from 10^{-4} to 10^{-7}, were spotted onto TSA plates and incubated aerobically at 37ºC for 24h. The number of culturable cells was expressed as CFU per mL and represented as the average of three biological replicates.

3.3. Formation of single- and dual-species biofilms

Biofilm formation was performed as previously described [161], with some modifications. To start biofilm formation, overnight cultures of each species were diluted, in TSB, to an OD_{620nm} of ~ 0.5 to obtain a final concentration of ~$2{,}3 \times 10^9$ CFU/mL. Thereafter, a 1:20 dilution was performed in TSB to achieve ~ 1×10^8 CFU/mL. For dual-species cultures, the inoculum of each species ($2{,}3 \times 10^9$ CFU/mL) was diluted (1:10) and combined in a 1:1 ratio (to achieve ~ 1×10^8 CFU/mL). Subsequently, 10µL of each of the *B. contaminans*, *P. aeruginosa* and *B. contaminans* + *P. aeruginosa* suspensions were

dispensed in 24 well-tissue culture plates (Orange Scientific, Braine L'Alleud, Belgium) containing 1mL of TSB. The plate was incubated at 37ºC at 120rpm (ES-20 Shaker-Incubator, BioSan, Riga, Latvia) for up to 72h, with medium replacement every 24h. TSB without bacteria was used as a negative control to exclude any possible contamination.

3.4. Methodologies for biofilms analysis

3.4.1. Quantification of biofilm biomass

Biofilm biomass quantification was determined by the crystal violet (CV) staining method as described before [161], with some alterations. In brief, after biofilm formation, the biofilm bulk fluid was carefully removed, biofilms washed once with 1mL of 0.9% NaCl and then incubated for 15min at 37ºC to dry the attached biofilms. Afterwards, biofilms were fixed with 1mL of 100% (vol/vol) methanol (Fisher Scientific, Leicestershire, UK) for 20min at room temperature (RT) and, then, incubated for 5min in the hood to evaporate the remaining methanol. Subsequently, 1mL of 1% (vol/vol) CV (Merk, Germany) was added and left to incubate for 20min. Excess stain was removed by washing the biofilms at least three times with 1mL of distilled water and the stain bound to the biofilm was then solubilized with 33% (v/v) acetic acid (Fisher Scientific, UK). After well homogenized, the absorbance of the solution was determined, at 595nm (OD_{595nm}), using an ELISA microtiter plate reader. When the absorbance was higher than 1, samples were diluted with 33% (v/v) of acetic acid. Wells with only TSB were also stained and used as a control. Experiments were run four times with technical duplicates for each condition tested.

3.4.2. Biofilm Cells Culturability

After biofilm growth, the bulk fluid was removed, the biofilms were washed once with 1mL of 0.9% NaCl and then suspended in 1mL of the same solution by scraping the cells from the plastic surface. Thereafter, the biofilm cells were sonicated for 10s at 30% amplitude (Cole-Parmer 750-W Ultrasonic Homogenizer 230 VAC, IL, USA) to break cell clusters. Subsequently, bacterial suspensions were serially diluted (1:10) in 0.9% NaCl and plated onto different selective agar media to discriminate the two species: Cetrimide agar (Liofilchem) was used to assess *P. aeruginosa,* while *B. cepacia* Selective Agar (BCSA; Oxoid, Basingstoke, Hampshire, UK), supplemented with *Burkholderia cepacia* Selective supplement (Oxoid) containing 30mg/L polymyxin B, 50mg/L ticarcillin and 2.5mg/L of gentamycin, was used to discriminate *B. contaminans*. TSA plates were also used as controls. Afterwards, selective agar plates were incubated at 37ºC for 16-24h and the number of CFU/mL was determined and expressed as Log_{10}

CFU/mL. Experiments were repeated three to four independent times with technical duplicates for each species and condition tested.

3.5. Antibiotic Susceptibility

3.5.1. Antibiotics

In this study, the effectiveness of two antibiotics in inhibiting the growth of *P. aeruginosa* in the absence and presence of *B. contaminans* was assessed. Ciprofloxacin (Sigma-Aldrich, St. Louis, MO, USA) is a fluoroquinolone that interferes with DNA replication by inhibiting DNA gyrase and topoisomerase IV [162], while colistin (Sigma-Aldrich) is a polymyxin that act as a detergent on cell membranes [163]. The range of concentrations used for each antibiotic is detailed in **Table 3**. Antimicrobials were prepared by dissolving the powder in either water (colistin) or a solution of 0.015M of hydrochloric acid (ciprofloxacin). Of note, the solution of hydrochloric acid, at the used concentration, is not toxic for these bacteria as determined earlier in our research group. Antibiotic working solutions were prepared, in TSB, on the day of the experiment, with a concentration two-fold higher than the concentration necessary to test to be later diluted with the addition of bacteria.

Table 3 | Range of antibiotic concentrations tested in this study to determine the minimum inhibitory concentration (MIC) of each antibiotic under test

Antimicrobial agent	Range of concentrations tested (mg/L)		
	B. contaminans	*P. aeruginosa PA14*	*P. aeruginosa ATCC 27853*
Ciprofloxacin	0.00775-2	0.00775-2	0.00775-2
Colistin or polymyxin C	0.0155-64	0.0155-4	0.0155-4

3.5.2. Minimum Inhibitory Concentration (MIC)

The MIC of the antibiotics under study was determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST), using the broth microdilution method [164]. For quality control purposes, *P. aeruginosa* ATCC 27853 was used.

First, antibiotics were prepared in Mueller-Hinton Broth (MHB) (Liofilchem) and then serial diluted, 1:2, in 96-well polystyrene microtiter plates (OrangeScientific), to obtain the range of

concentrations shown in **Table 3**. Thereafter, 100µL of bacterial suspension, with a previously adjusted concentration of 1×10^6 CFU/mL, was added to the antibiotic solution, obtaining a final concentration of 1×10^5CFU/mL. As negative and positive controls, MHB only and bacteria without antibiotics were used, respectively. A control with MHB supplemented with each antibiotic was also performed. Subsequently, the plates were incubated at 37ºC for 18-24h. The MIC was defined as the lowest concentration where no turbidity was observed. To aid with this assessment, the OD_{620nm} of all wells was also recorded. Two to three independent assays were performed for each species and antibiotic.

3.6. Ciprofloxacin Activity against Single and Dual-Species Biofilms

The antimicrobial action of ciprofloxacin was evaluated in 72h single and dual-species biofilms of *P. aeruginosa* and *B. contaminans*. Briefly, biofilms were formed as described in **section 1.3** for 72h in 24-well plates. At that time point, the medium was removed and 1mL of ciprofloxacin at MIC, 2× MIC, 4× MIC, 8× MIC, 16× MIC, 64× MIC and 128× MIC, prepared in TSB, were added to the biofilms. Negative controls were also performed by adding 1mL of TSB instead of the antibiotic. The plates were then incubated at 37ºC, 120 rpm for 24h. After 24h, the number of culturable bacteria within the biofilms was determined by CFU counting following the procedure in **section 1.4.2**. Three independent assays were performed for each species and condition.

3.7. Gene expression quantification

3.7.1. Polymerase Chain Reaction for primer specificity testing

To obtain genomic DNA (gDNA) to use as a template for PCR, biomass of both *B. contaminans* and *P. aeruginosa*s was collected from TSA plates and suspended in 100µL of DNase/RNase-free water. The suspension was incubated at 100ºC, for 10min, and then cooled down on ice for an additional 10min. Finally, the suspension was centrifuged at 4ºC for 10min at 16000×*g* and the supernatant recoved and stored at -20ºC until further use. Thereafter, gDNA was amplified with NZYTaq II 2× Green (Nzytech, Lisbon Portugal), in a 10µL reaction volume, using 0.5µL of forward and reverse primers (10µM), 1µL of template gDNA and 5µL of the NZYTaq II 2× Green master mix. As a positive control, *Staphylococcus epidermis* RP62A gDNA and primers for the amplification of the genes *icaA* and *tagO* were used. In addition, a negative control that contained every component except for the gDNA was also prepared. The samples were placed in a T100 Thermal Cycler (Bio-Rad, CA, USA) with the following parameters: 95ºC for 3min, followed by 34 cycles of 94ºC for 30sec, 60ºC for 30sec and 72ºC for 15sec. Thereafter, a final extension step at 72ºC for 10min was also performed.

Confirmation of PCR products was performed through agarose gel electrophoresis. The gels contained 1% (w/v) agarose (AGTC biproducts, Potters Bar, UK) dissolved in 1× TAE buffer (Tris–acetate–ethylenediaminetetraacetic acid (EDTA)) and were stained with GreenSafe Premium (Nzytech). The NZYDNA ladder VI (Nzytech) was used as a marker. Electrophoresis was performed in 1× TAE buffer at 80V for 1h in a runVIEW gel system (Cleaver Scientific, Rugby, UK) and the gels were visualized using a ChemiDoc™ XRS (Bio-Rad) equipment with Image Lab™ 5.1 software (Bio-Rad).

3.7.2. RNA Extraction

RNA extraction was conducted was conducted from single and dual-species biofilm pools as previously optimized by our research group [165]. The RNA isolation method used combines mechanical lysis (glass beads) with a silica membrane-based RNA separation. In brief, biofilms were washed once with 0.9% NaCl and then suspended in 1mL of the same solution by scraping the cells from the bottom and the wall of the plates. Then, biofilm cells were harvested by centrifugation at 16000 × *g* (10min, 4ºC) and total RNA was extracted using the E.Z.N.A® Total RNA Kit I (Omega Bio-Tek, Norcross, GA, USA). In short, bacterial pellets were suspended in 600µL lysis buffer and the entire suspension was transferred to a safe lock tube (2mL) with 0.5g of acid-washed 150–212µm silica beads (Sigma-Aldrich). Cells were disrupted in a BeadBug6 cell disruptor (Benchmark Scientific, Sayreville, NJ, USA) at 4350 rpm for 35s. The samples were then cooled on ice, for 5min, and the lysis step was repeated three times more. Afterwards, the samples were centrifuged at 16000 ×*g* for 5min at 4ºC. Of note, all the following steps were done at RT unless otherwise stated. The supernatants were transferred into a new RNase-free tube, mixed with an equal volume of 70% ethanol (Fisher Scientific), prepared in RNase-free water (Grisp, Lisbon, Portugal). Next, 700µL of the lysate (including any remaining precipitate) was transferred into the RNA isolation column and centrifuged for 1min at 12000 ×*g*. The flow-through was discarded and the column was placed back into the collection tube. This procedure was performed until all the lysate was passed through the column. Then, each column was washed once with 500µL of wash buffer I and then washed twice with 500µL of wash buffer II. Afterwards, the columns were centrifuged for 2min to remove any trace of ethanol. The collection tube was discarded, and each column was inserted into a recovery tube. Finally, RNA was eluted by adding 45µL of RNase-free water to the centre of the membrane and centrifuged for 2min at 12000 ×*g*.

3.7.3. DNase treatment

To degrade any possible gDNA co-purified with total RNA, the samples were treated with a DNase I kit (Thermo Fisher Scientific). Briefly, 2µL of DNase I and 5µL of DNase buffer were added to 45µL of RNA samples and incubated at 37°C for 30min. Subsequently, 5µL of 25mM EDTA was added, and the DNase I enzyme was inactivated by heating the samples at 65°C for 10min. The samples were immediately placed on ice or frozen at -20°C until further use.

3.7.4. RNA concentration and purity determination

The concentration and purity of the total RNA were spectrometrically determined using a NanoDropOne™ (Thermo Fisher Scientific). The absorbance ratios A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm} were used as indicators of protein contamination, and polysaccharide, phenol, and/or chaotropic salts contamination, respectively [166]. The samples were immediately placed on ice or frozen at -20°C until further use.

3.7.5. complementary DNA synthesis

Complementary DNA (cDNA) synthesis was performed using the Xpert cDNA synthesis kit (Grisp) following the manufacturer's instructions. Briefly, the same amount of total RNA in each sample (~500-1000ng) was reverse transcribed, in a 20µL reaction volume, using random primers as a priming strategy. Control reactions lacking the reverse transcriptase enzyme (NRT control) were prepared for each RNA sample to later determine the level of gDNA contamination. The samples were placed into the T100 Thermal Cycler (Bio-Rad) with the following parameters: 25°C for 10min, 55°C for 15min, 85°C for 5min and kept at 4-8°C until further use.

3.7.6. Quantitative PCR (qPCR)

qPCR analysis was performed using the Xpert Fast SYBR (Grisp) kit in a 10µL reaction volume as optimized earlier [165]. All samples, including the NRT control, were diluted 200× in DNase-free water and, then, 2µL of these solutions were mixed with 5µL Xpert Fast SYBR 2× Mastermix, 1µL of primer mixture (at 10µM) and 2µL of DNase-free water. The qPCR runs were performed in a CFX96 (Bio-Rad) with the following cycle parameters: 95°C for 2min, followed by 40 cycles of 95°C for 5s, and 60°C for 20s. Melt analysis was performed at the end to assess the presence of unspecific products and primer

dimers. The gene *16S rRNA* was tested as a reference gene (RG), for posterior normalization and relative gene expression quantification.

3.8. Statistical Analysis

Data were analyzed with GraphPad Prism version 8 (GraphPad Software, California, USA) using one-way analysis of variance (ANOVA) and subsequent comparisons among different groups were performed using the Tukey multiple-comparisons test, as all data followed a normal distribution (according to the Kolmogorov-Smirnov's test). Differences were considered statistically significant when $p < 0.05$. Results are presented as the mean ± standard deviation (SD).

4. RESULTS AND DISCUSSION

4.1. Calibration Curves

To be able to adjust the concentration of bacterial cells for all the experiments presented below, a calibration curve correlating the OD with the number of CFU/mL was performed for *B. contaminans*. As can be seen in **Figure 6**, a high correlation between the values of OD_{620nm} and CFU/mL was established, obtaining a calibration curve with a coefficient of determination near 1 (R^2= 0.9963).

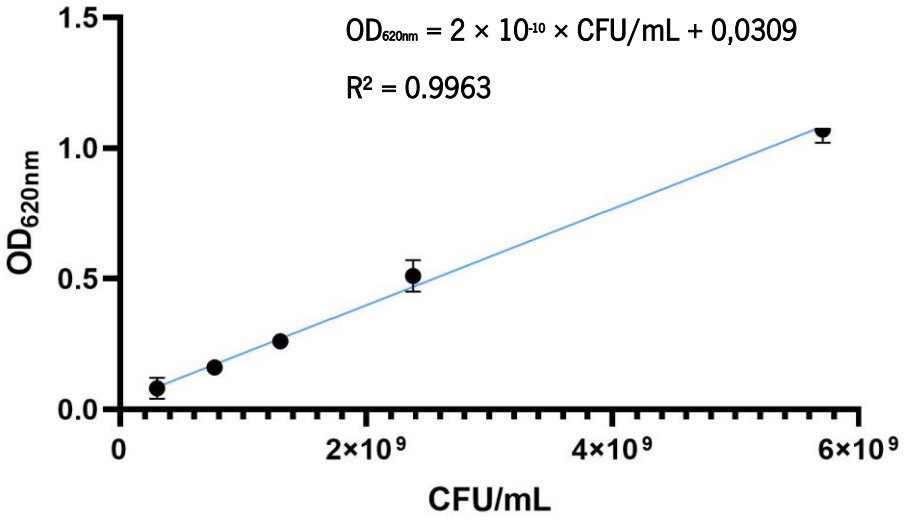


Figure 6| Calibration curve of optical density (OD_{620nm}) vs. colony forming units (CFU) for *B. contaminans*. The data are presented as mean ± SD for 3 independent assays.

In the case of *P. aeruginosa*, a calibration curve previously established by members of our research team was used [167]. However, considering that the laboratory conditions may have changed over time, to ensure that the calibration curve was still applicable, the data was validated by adjusting the OD_{620nm} of a suspension and confirming if the number of CFU/mL was the one expected using the *P. aeruginosa* calibration curve. As shown in **Table 4**, the number of CFU/mL calculated using the calibration curve and the ones experimentally obtained were very similar, validating the previous *P. aeruginosa* calibration curve ([CFU/mL] = $5 \times 10^9 \times OD_{620nm} - 2 \times 10^8$).

Table 4| Validation of the calibration curve for *P. aeruginosa*. The bacterial suspensions were adjusted to a specific OD_{620nm} value and the result in experimental counts of CFU/mL are presented as observed values. The CFU/mL values obtained from the calibration curves are referred to as expected values. The mean of 2-3 experimental assays is presented. OD: optical density at 620nm; CFU: Colony Forming Units

Microorganism	OD_{620nm}	Expected Value (CFU/mL)	Observed value (CFU/mL)
P. aeruginosa	0.5	2.30×10^9	2.15×10^9
	0.1	3.00×10^8	3.42×10^8

4.2. Selective Media Testing

To study the influence of *B. contaminans* on *P. aeruginosa*'s fitness, it was necessary to first ensure that we were able to discriminate between the two bacteria. Herein, for *B. contaminans* selection, *B. cepacia* selective agar (BCSA) was chosen. Henry *et al*. [168] showed that BCSA, in comparison with two other media used for Bcc discrimination, was superior in terms of the quickness and quality of recovery of Bcc organisms from CF respiratory specimens. In addition, it was shown that BCSA inhibits the growth of other organisms, such as *P. aeruginosa*, yeasts, and fungi [169]. BCSA contains polymyxin, gentamicin and vancomycin as selective agents along with lactose, sucrose and phenol red to help differentiate Bcc microorganisms [170]. For *P. aeruginosa* selection, there are a few commercial media such as Pseudomonas Isolation Agar (PIA) and cetrimide. Previously, it was demonstrated that the PIA medium was able to support the growth of certain Bcc organisms, and it was further confirmed with this specific strain of *B. contaminans* [5] (**Figure 7**).

As such, we used cetrimide agar, a medium that enhances fluorescein and pyocyanin pigment production by *P. aeruginosa* [171]. However, to ensure that these media worked properly under our experimental conditions, suspensions with 2×10^9CFU/mL of either *B. contaminans*, *P. aeruginosa* or a combination of the two bacteria were plated on TSA (as a control), BSCA or/and cetrimide. As depicted in **Figure 8**, *B. contaminans* did not exhibit growth in cetrimide, both in single and mixed suspensions, while it was able, as expected, to grow in both TSA and BCSA media. *P. aeruginosa* only showed growth in TSA or cetrimide medium in single or dual-species planktonic suspensions. Therefore, this assay was able to demonstrate that *B. contaminans* is not able to grow in a cetrimide medium and *P. aeruginosa* cannot grow in the BCSA selective medium, which in combination, allows for the discrimination of these two bacteria in mixed cultures. Noteworthy, these findings indicate that the quantification of bacterial load

does not significantly vary whether a rich or a selective medium is utilized, as shown by the similar numbers of cells.

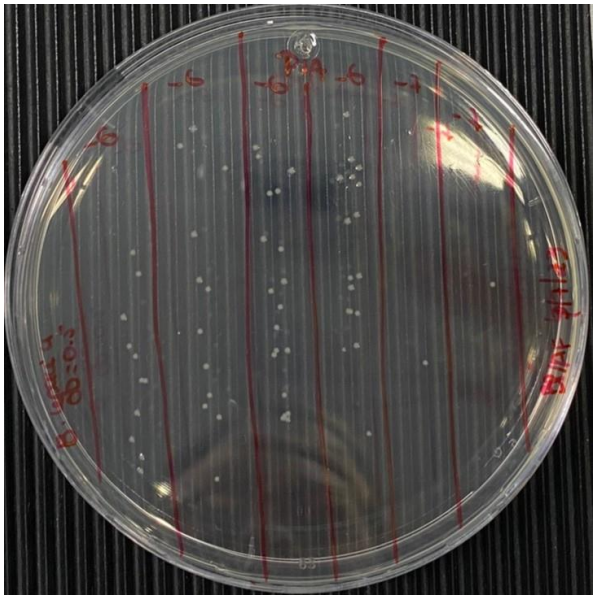


Figure 7| Growth of *B. contaminans* in PIA medium after about 24h.

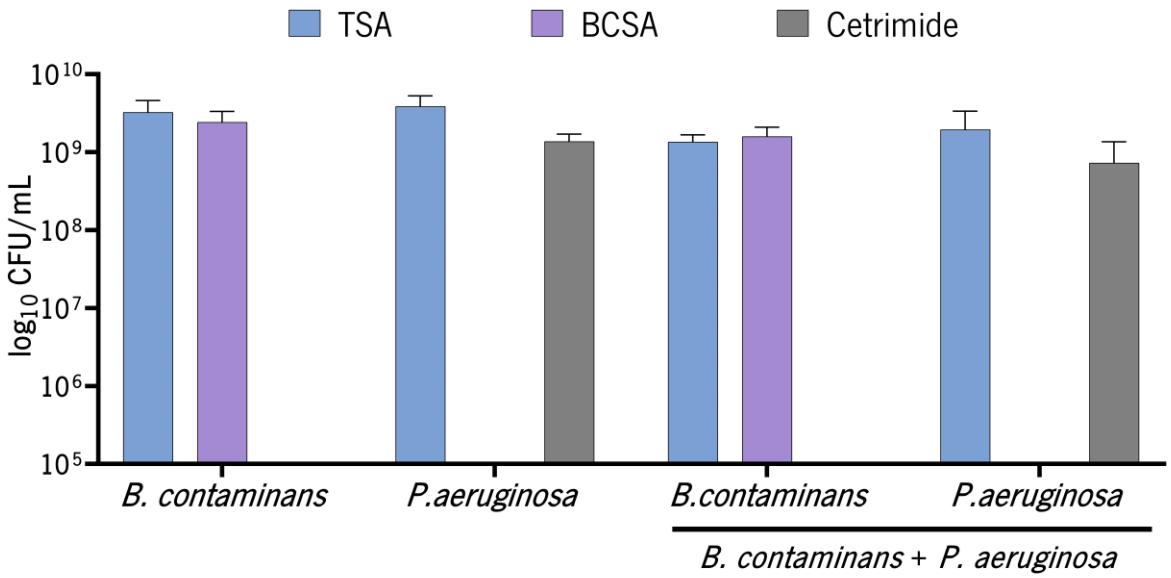


Figure 8| Growth of *B. contaminans*, *P. aeruginosa* and suspensions containing the two bacteria in two selective (BCSA and cetrimide) and one rich medium (TSA). The mean + SD for 3 independent assays is presented. BCSA: *B. cepacia* selective agar; TSA: Tryptic Soy Agar.

4.3. Effects of sonication on *B. contaminans* aggregation and culturability

While planktonic suspensions of bacteria typically require only vortexing before analysis, biofilms present a more intricate structure, comprising additional components such as proteins, lipids, and extracellular DNA [142]. Consequently, traditional methods for the preparation of planktonic suspensions fall short when it comes to effectively harvesting biofilms into a uniform, single-cell suspension [172]. To accurately enumerate biofilm cells, biofilms must be disaggregated into individual cells as, for instance, CFU counts rely on the assumption that a CFU originates from a single bacterium. If biofilm aggregates are directly plated, distinguishing individual cells becomes impossible, leading to inaccurate estimates [172]. To circumvent this issue, sonication is employed as one of the methods to disintegrate cell clusters [173]. The previous optimization of the sonication cycle for biofilms of *P. aeruginosa* involved a 30% amplitude cycle for 10s [167]. To assess the best sonication conditions to disaggregate *B. contaminans* aggregates without resulting in cell death, three different sonication cycles were selected: 30% amplitude for 5, 10 or 15s using cells from 72h-old biofilms. As shown in **Figure 9**, varying the duration of sonication appears to have minimal impact on *B. contaminans* culturability. The number of CFU/mL remained similar across different sonication durations tested. These findings suggest that *B. contaminans* does not form substantial aggregates, and sonication does not significantly affect cell culturability, as evidenced by consistent cultivable cell counts between biofilms subjected to sonication and those without. Consequently, based on these results a 10s sonication duration, consistent with that used for *P. aeruginosa*, was chosen for subsequent assays.

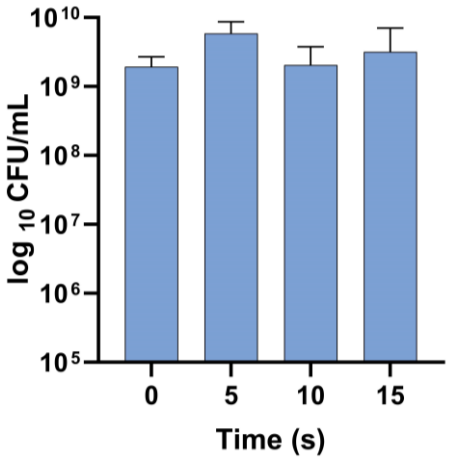


Figure 9| Quantification of culturable cells from 72h-old biofilms of *B. contaminans* after sonication at 30% amplitude for different times (0, 5, 10 and 15s). The mean + SD for 1-3 independent assays is presented.

4.4. Single and dual-species biofilms analysis: biomass and culturable cells enumeration

4.4.1. Biofilm biomass

As described in the literature, *P. aeruginosa* and Bcc bacteria have been shown to coexist in CF lung airways; however, it is not clear whether they benefit from being together or not and no information has been shown to address the emergent member of Bcc, *B. contaminans* [93]. To better understand how these two bacteria may influence each other's biofilm formation ability, the total biofilm biomass was determined using the CV method initially described by Stepanovic *et al.* [161]. CV is a basic dye that binds negatively charged molecules and stains both bacteria and the surrounding biofilm matrix [174].

As shown in **Figure 10**, *B. contaminans* maintained their biomass amount throughout the three-time points evaluated, while *P. aeruginosa* showed an increase in biomass after 48h. In the case of dual-species biofilms, the developed biomass was significantly smaller than the one formed by *P. aeruginosa* alone after 48h of biofilm growth. The same observation was also seen in 72h biofilms, where *P. aeruginosa* produced more biomass than the dual-bacteria biofilm, whereas *B. contaminans* biofilms presented less biomass than their dual-species counterparts.

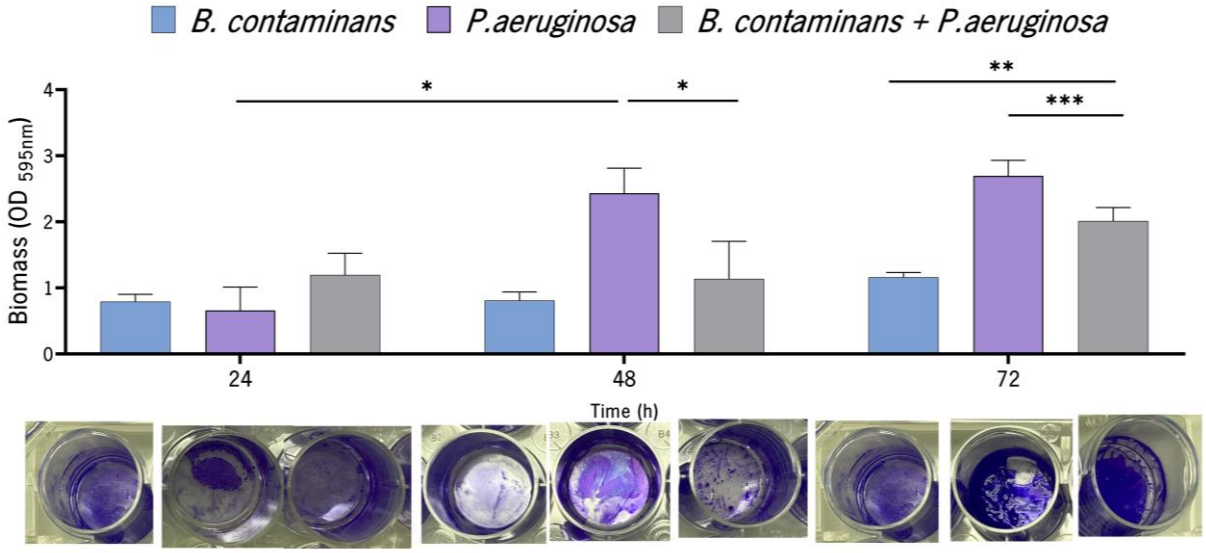


Figure 10| Biofilm biomass obtained for 24-, 48- and 72h-old single- and dual-species biofilms formed by *P. aeruginosa* and *B. contaminans*. The mean ± SD of 4 independent assays is presented. Statistical significance was determined by performing one-way ANOVA followed by Tukey's multiple comparison tests with a 95% confidence level. * $p<0.05$, ** $p<0.01$; *** $p<0.001$.

These results suggest that after 48 and 72h of growth, there is an effect on the biomass produced in dual-species biofilms, which may be due to an antagonistic interaction between these two bacteria. However, it remains unclear, with these results alone, which bacteria are affected by this relationship. Nevertheless, *P. aeruginosa* appears to be impacted by the dual-species consortia, leading to a decrease in biofilm biomass after 48h and 72h of growth when compared with the single-species biofilm. Bragonzi and colleagues [135] also investigated the biofilm formation ability of clinical and environmental *P. aeruginosa* RP73 strains and another Bcc bacterium, *B. cenocepacia* LMG16656. In mixed biofilms, the biomass increased only at the 24h mark when compared to their pure culture counterparts [135]. However, this study was performed under different conditions, namely the medium used, which could contribute to the different results. Furthermore, the biofilms were grown in flow chambers and continuously perfused with medium, while this study uses static fed-batch biofilm growth [161]. Analyzing other studies involving *P. aeruginosa* and bacteria associated with CF, particularly *S. aureus,* in dual-species biofilms encompassing *P. aeruginosa* PA14 and *S. aureus* ATCC 25923, both produced similar values of biomass in comparison with their individual biofilms [29]. In addition, biofilms generally increased their biomass until they reached 48h of growth in all conditions [29].

4.4.2. Biofilm Population Discrimination

To determine the prevailing species in dual-species consortia, the number of CFU was evaluated for each species using cetrimide and BCSA media. It was observed that in the biofilm mode of growth, *P. aeruginosa* displayed a different morphology compared to planktonic suspensions (**Figure 11B and D**). The two types of morphologies observed were one with round white colonies and the other with the typical green, large circular colonies in both TSA (**Figure 11B**) and cetrimide media (**Figure 11D**). To confirm the identification of the various colonies as *P. aeruginosa*, each colony was isolated and cultured on TSA and cetrimide plates. As shown in **Figures 12A and B**, both colonies exhibited growth on cetrimide agar and TSA. Notably, only green biomass was observed in both cultures, indicating that both colonies were indeed P*. aeruginosa* and not a result of contamination by *B. contaminans* or other bacterial species. In addition, cells from biofilms required a longer incubation period at 37◦C (about 72-96h) to grow in cetrimide (**Figure 11C and D**). These discrepancies in colony appearances made the discrimination between *P. aeruginosa* and *B. contaminans* challenging. Therefore, to aid in differentiating the bacteria in dual-species biofilms, TSA media was used in conjunction with the other selective medium, and the number of *P. aeruginosa* was estimated by counting the cells that exhibited the usual morphology. It is acknowledged that this

methodology has its limitations since it may not accurately estimate the number of *P. aeruginosa* colonies and could potentially misidentify them as *B. contaminans* cells.

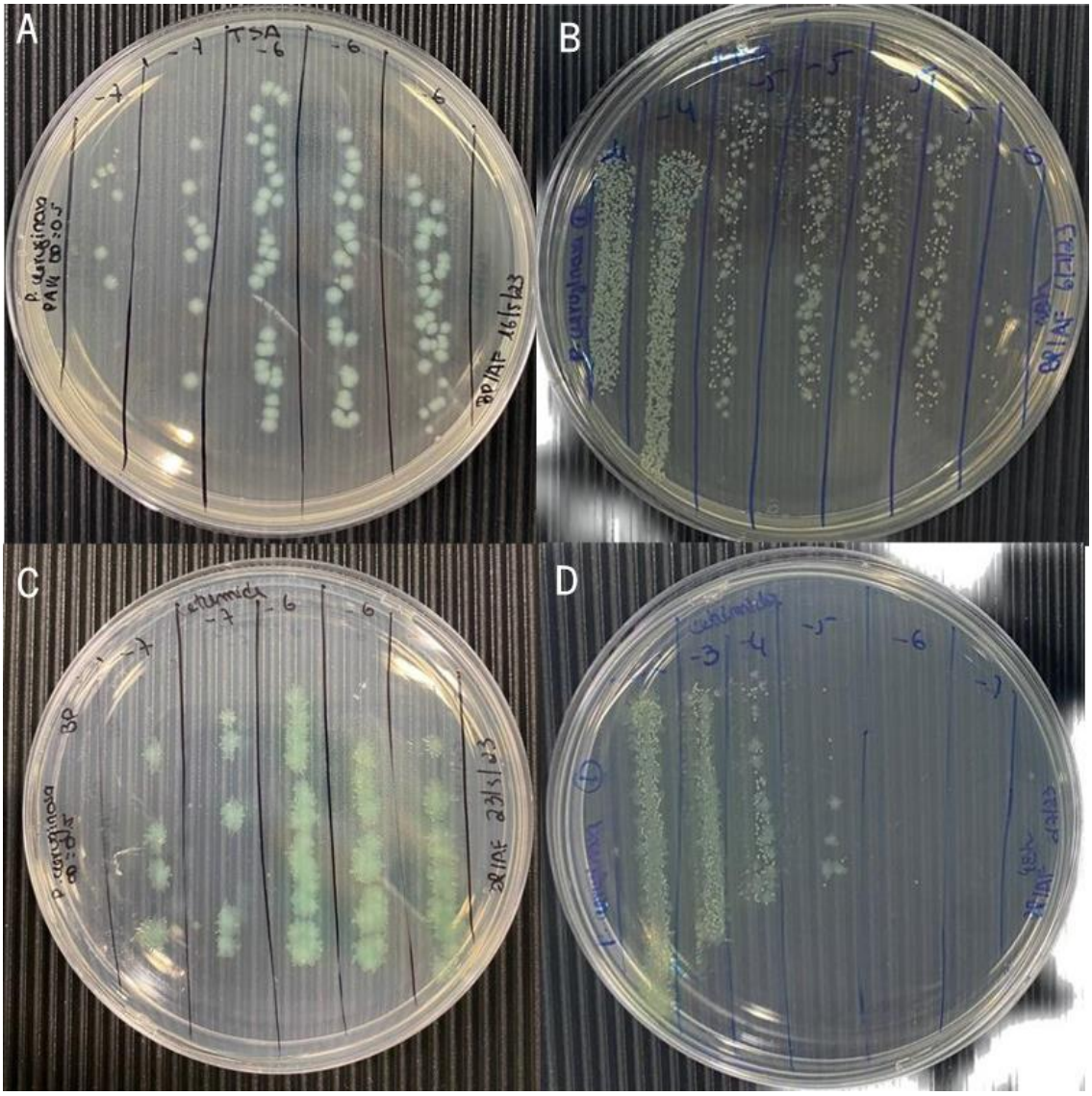


Figure 11 | Representative images of *P. aeruginosa* colony morphology of **(A)** planktonic cells at approximately 16h of incubation on TSA, **(B)** 48h-old biofilm cells after about 16h of incubation on TSA, **(C)** planktonic cells after approximately 24h of incubation in cetrimide, and **(D)** 48h-old biofilms after about 72h of incubation in cetrimide medium.

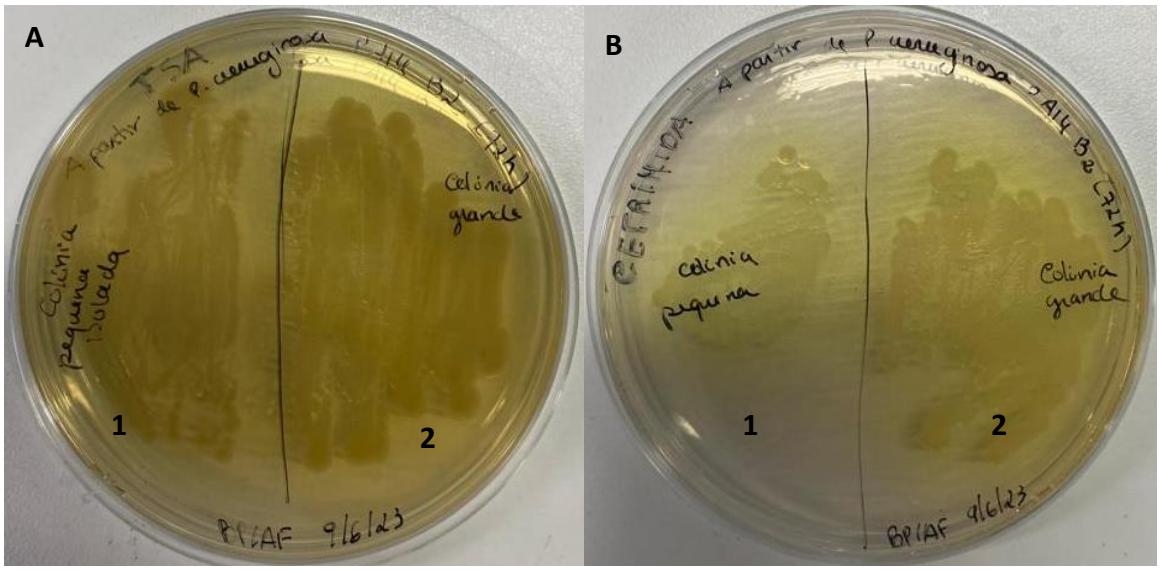


Figure 12 | Isolation of colonies originating from 72h *P. aeruginosa* biofilms. From TSA plates, a small white colony and a large green colony were selected and subsequently incubated in **(A)** TSA plates and **(B)** cetrimide plates. The notation **(1)** corresponds to the growth derived from the small white colony, while **(2)** represents the growth originating from the large green colony.

Cell counts indicate that *B. contaminans* maintained consistent cell numbers throughout all time points (**Figure 13**). In contrast, *P. aeruginosa* increased its cell numbers from 24h to 48h of growth. The analysis of dual-species biofilms revealed that *P. aeruginosa*'s culturability significantly decreased from 48 to 72h in the presence of *B. contaminans*. On the other hand, *B. contaminans* was affected by the presence of *P. aeruginosa*, as it showed decreased CFU counts, after 24h and 48h. However, the reduction was less pronounced in 48h biofilms, and by the 72h timepoint, the number of cultivable cells was higher in the dual-species consortia. These results are in agreement with the results obtained in biofilm biomass quantification, demonstrating that *B. contaminans* is the dominant species, capable of outcompeting *P. aeruginosa* after 48h of growth, without its growth being significantly affected.

Co-infections involving both *P. aeruginosa* and Bcc infections have been documented in the lower airways of patients suffering from CF [93], [138], in which disease outcomes were reported to be worse than mono-infections in both humans [138] and mice [135] studies, suggesting synergistic interactions. Despite their coexistence, several studies have reported that *P. aeruginosa* outcompetes and/or eliminates species of the Bcc in various environments, including *in vitro* [93], [133]–[135], [175], [176] and in a chronic lung infection mouse model [135]. Al-Bakri *et al.* [134] showed that in clinical *P. aeruginosa* 313113 and *B. cepacia* 313113 strains, both were able to reach similar levels of CFUs in single and dual-species biofilms. However, when compared with this study, Al-Bakri and colleagues used a semi-defined medium, and the method for biofilm formation employed was also distinct, as those

authors used the Sorbarod model [134]. On the other hand, using *P. aeruginosa* PA01 and environmental Bcc strains, Tomlin *et al*. [133] showed that *P. aeruginosa* inhibited the formation of mixed biofilms. This inhibition was suggested to be due to the secretion of pyocyanin, an antimicrobial exoproduct produced by *P. aeruginosa*. Other *in vitro* studies suggest that phenazines, rhamnolipids, hydrogen cyanide, and other molecules yet to be identified are released by *P. aeruginosa* [133], [175], [176]. However, mixed infections where Bcc species outnumber *P. aeruginosa* in CF lungs [93], [177] have been reported, challenging the idea that Bcc is always dominated by *P. aeruginosa*.

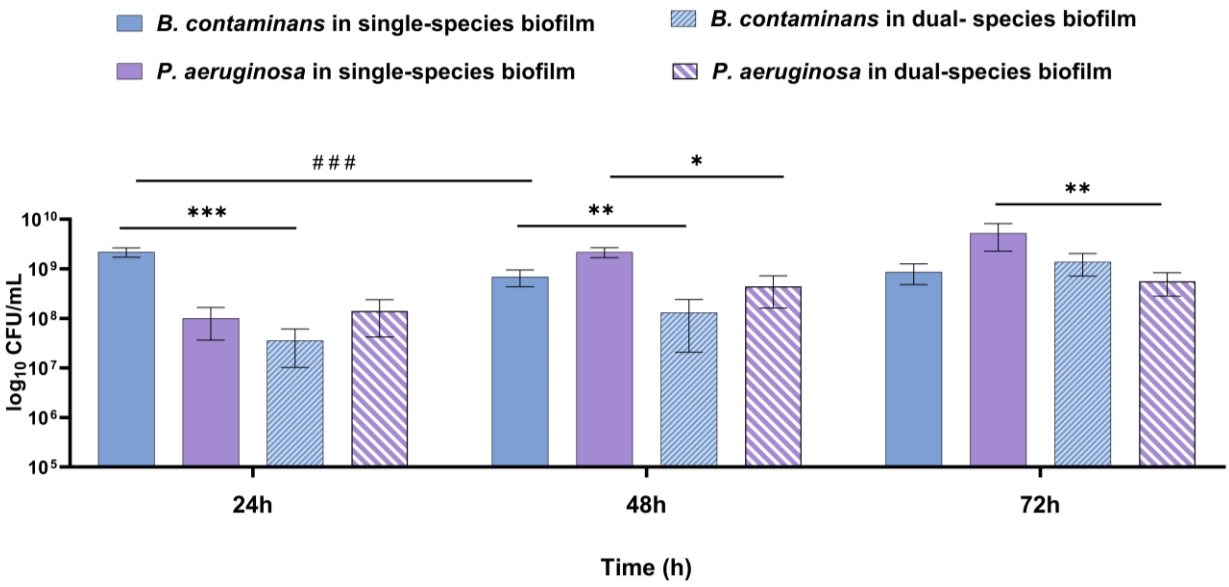


Figure 13 | Biofilm quantification by CFU counting of single- and dual-species biofilms of *P. aeruginosa* and *B. contaminans* growing for 24-,48- and 72h. The mean ± SD for 3-4 independent assays is presented. Statistical significance was determined by performing one- way ANOVA followed by a Tukey's multiple comparison test to compare: (#) differences between 24, 48 or 72h single-species biofilms of *P. aeruginosa* or *B. contaminans* (### p <0.001); (*) and between single and dual-species biofilms (*p<0.05, **p<0.01; ***p<0.001).

Our study introduces a novel perspective on these interactions, specifically concerning *P. aeruginosa* and *B. contaminans*. A hypothesis that might explain the observed results is that *B. contaminans* might produce inhibitory substances that impact *P. aeruginosa* cultivability or viability. To investigate this hypothesis, challenging *P. aeruginosa* with *B. contaminans* supernatant from a 72h biofilm, a time point where noticeable differences in the number of culturable cells occurred. This approach aims to provide clarity on whether secreted compounds from *B. contaminans* influence the growth of *P. aeruginosa*. Additionally, evaluating cell viability using flow cytometry, employing propidium

iodide and SYBER green staining would yield valuable insights, elucidating the state of the cells during these interactions [178].

4.5. Antimicrobial Susceptibility of planktonic and biofilm populations

4.5.1. Susceptibility profiles of planktonic populations towards antibiotic agents

Given the complex bacterial community present in CF airways, there is a growing interest in studying the behavior and antimicrobial susceptibility of different CF pathogens in the presence of each other [37]. Interactions within this community can lead to alterations in biofilm formation, metabolism, virulence factors production, and antibiotic susceptibility [179]. For instance, chloramphenicol-resistant strains of *S. pneumoniae* and *S. aureus*, expressing chloramphenicol acetyltransferase, were able to protect susceptible *S. pneumoniae* cells by intracellular degradation of chloramphenicol [180]. Conversely, competitive, or antagonistic interactions could enhance the pathogen's response to antibiotics by increasing antibiotic efficacy and disrupting cross-feeding networks, among other mechanisms [37], [181]. Therefore, this study aimed to explore how *B. contaminans* impact the susceptibility of *P. aeruginosa* to two commonly used antibiotics for *P. aeruginosa* infection treatment. Initially, the MIC of each antibiotic had to be determined, and the results are presented in **Table 5**.

Table 5 | *In vitro* susceptibility patterns of planktonic cultures for *P. aeruginosa*, *B. contaminans* and a control strain (*P. aeruginosa* ATCC 27853), against ciprofloxacin and colistin. S: Susceptible

	Ciprofloxacin (mg/L)	Colistin (mg/L)
B. contaminans	0.25	>64
P.aeruginosa PA14	0.25 (S)[a]	0.5-1 (S)[a]
P. aeruginosa ATCC 27853	0.25-0.5	1-2

[a] According to the EUCAST breakpoint table [164]

In general, ciprofloxacin was effective at inhibiting the growth of *P. aeruginosa* and *B. contaminans* at low concentrations. In contrast, colistin failed to inhibit the growth of *B . contaminans* at high concentrations, while it was effective against *P.aeruginosa* at low concentrations. These results were validated using a control strain, *P. aeruginosa* ATCC 27853, in which results were within the accepted intervals as per the EUCAST guidelines [164]. Members of the genus *Burkholderia* are inherently unsusceptible to many antimicrobial agents, including polymixins, a class of antibiotics to which colistin

belongs [182]. Resistance to polymixins has been explored in various *Burkholderia* species [183]. These antibiotics function through the self-promoted uptake pathway, where a cationic antibiotic displaces cations bridging the LPS molecules, facilitating the uptake of other substances and reducing membrane permeability [163]. In these bacteria, alterations in LPS hinder the antibiotic's access to the inner membrane [183]. Hence, this might explain why determining the MIC for *B. contaminans* proved difficult.

4.5.2. Impact of *B. contaminans* on *P. aeruginosa* response to ciprofloxacin treatment

A significant characteristic of *P. aeruginosa* and Bcc species infections lies in their ability to form biofilms, a factor contributing to reduced antibiotic effectiveness and unfavorable patient outcomes [23], [100], [117]. Within these biofilms, the structural features and constituent bacteria play a crucial role in the development of antibiotic resistance [19], [22]. In fact, bacteria residing in biofilms exhibit a 10 to 1000-fold increase in drug resistance, compared to their free-floating (planktonic) state [19], [121].

One limitation of MIC testing is its lack of consideration for the microbial context, such as the impact of interactions between different species within a bacterial community [181]. Therefore, to gain insights into the effect of ciprofloxacin in a polymicrobial setting, specifically under the biofilm growth mode, various concentrations (MIC, 2 × MIC, 4 × MIC, 8 × MIC, 16 × MIC, 64 × MIC, 128 × MIC) were administered to 72h-old biofilms and the number of biofilm-cells assessed by CFU counting. This time point was chosen since this was when it was most noticeable the changes that the introduction of a second species made on the culturable cell count of *P. aeruginosa* and *B. contaminans*. Overall, increasing concentration of the antibiotic resulted in gradual reductions of biofilm cell culturability in both single and dual-species cultures, when compared to untreated biofilms (the highest log reduction being 3.36 in the dual-species biofilm of *B. contaminans*) (**Figure 14**).

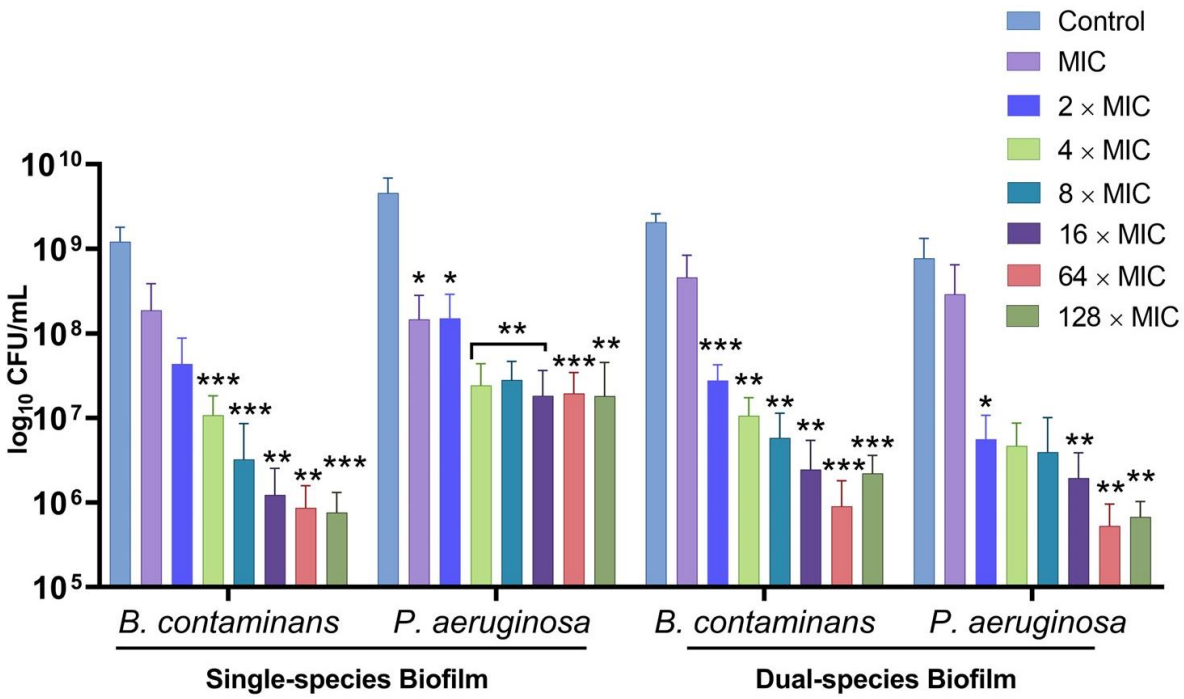


Figure 14| Biofilm quantification by CFU counting of 72h-old single- and dual-species biofilms of *P. aeruginosa* and *B. contaminans* followed by 24h treatment with ciprofloxacin at different concentrations. The mean + SD for 3 independent assays is presented. Statistical significance was determined by performing one-way ANOVA followed by a Tukey's multiple comparison test to compare the differences between the untreated control and the biofilms treated with the antibiotic with a 95% confidence interval. *p<0.05, **p<0.01; ***p<0.001.

As seen in **Figure 14**, a dose-dependent response was observed in single-species biofilms for *B. contaminans*; the higher the concentration of ciprofloxacin, the higher the log reduction. Conversely, *P. aeruginosa* showed an initial log reduction with MIC and 2× MIC concentrations of ciprofloxacin; however, beyond 4× MIC, further increases in concentration did not result in changes in the count cells.

In the context of dual-species biofilms, *B. contaminans* did not alter its susceptibility to the antibiotic when combined with *P. aeruginosa*. On the other hand, *P. aeruginosa*, when coexisting with *B. contaminans*, displayed a higher susceptibility to ciprofloxacin at concentrations 2× MIC and higher. Consequently, this interspecies interaction appears to enhance the susceptibility of *P. aeruginosa* to ciprofloxacin, although the specific mechanisms underlying this interaction remain unclear.

4.6. Analyzing the expression of genes related to *P. aeruginosa* virulence in Single and Dual-Species Biofilms

The analysis of the genes expression using qPCR aimed to determine how the presence of *B. contaminans* might impact *P. aeruginosa* in terms of (i) the expression of virulent factors and (ii) the effectiveness of ciprofloxacin treatment, in both dual and single-species biofilms.

The genes chosen to accomplish this goal were: *mucA*, *pqsA*, *rhlA*, *rhlR*, *pqsE* and *lasI*. MucA is an anti-σ-factor, responsible for the repression of alginate production [70]. Mutations in this gene are a hallmark of mucoid conversion in *P. aeruginosa*, leading to excessive alginate production [71]. Alginate enhances *P. aeruginosa* biofilm formation and elevates antibiotic tolerance by impairing the penetration of certain antibiotics [72]. Rhla is crucial for rhamnolipid synthesis, which is a biosurfactant produced by *P. aeruginosa* [203]. Due to their surfactant properties, rhamnolipids enhance the solubility of hydrophobic molecules, aiding the uptake of nutrients like hydrocarbons [204]. Additionally, they promote biofilm formation and inhibit host immune response [205]. *P. aeruginosa* has three major QS systems: Rhl, Las and Pqs. Each depends on specific transcriptional regulators activated by corresponding autoinducers, such as AHLs, which function as inter-cellular signals [73]. QS systems comprise an AHL synthase (LasI) and its cognate transcriptional regulator (LasR or RhlR), modulating gene expression in response to increasing AHL concentrations. RhlR, when bound to its autoinducer, controls the transcription of various genes, including those encoding virulence factors like pyocyanin, elastases, and rhamnolipids [206]. PqsA is another transcriptional regulator responsible for the production of several virulence factors, such as elastase, pyocyanin, and rhamnolipids [207]. The LasI also regulates key virulence factors, including exotoxin A, elastase, alkaline protease, and pyocyanin, all pivotal in the pathogenicity of *P. aeruginosa* infections [208].

4.6.1. Assessing Primers Specificity

Primer specificity is crucial to obtain accurate results, especially in the case of mixed-species cultures. Additionally, primer specificity is essential in qPCR assays, where the quantification of the product is determined by DNA intercalating dyes, which relies on the quantification of fluorescence intensity of all DNA molecules produced [184]. As such, any unintended target amplification could significantly impact the accuracy of the measurements [185]. Given the similarity between *P. aeruginosa* and *B. contaminans*, as the latter was part of the Pseudomonas genus before 1992 [74], it was essential to assess any cross-amplification. To address this concern, we conducted a PCR analysis to evaluate the specificity of each primer pair, ensuring that only the intended genetic material was amplified.

Regarding the samples with *P. aeruginosa* gDNA as a template, single bands, with the expected size, were observed (**Figure 15A**, lanes 1 through 7). However, when using *B. contaminans* gDNA as a template (**Figure 15B**, lanes 10 to 16), a stronger band appeared for the *rhlR* gene primer (lane 14), while fainter bands were visible for the *rhlA* and *pqsA* gene primers (lanes 10 and 12). For all other genes,

no bands were observed. Hence, it appears that only the primers designed for the amplification of the genes *16S rRNA*, *mucA*, *pqsE*, and *lasI* are specific to the *P. aeruginosa* genome. Of note, no contamination was detected in the reactions performed as can be seen in **Figure 15B** (lanes 17 to 23), despite the faint bands observed in lanes 20 and 21. Considering their small size (~50 bp), these bands are probably the result of primer dimers. Finally, bands 8 and 9, are relative to positive control samples, where the gDNA of *S. epidermidis* was used to amplify genes *tagO* and *icaA.*

To further validate these findings, a qPCR run was performed using 48h biofilms of *B. contaminans* and *psqE, mucA, lasI* and *16S rRNA* primers, since this method is highly sensitive [185]. The qPCR analysis revealed that the *pqsE*, *lasI*, and *mucA* primers exhibited cycle quantification (C_q) around 35, whereas the *16S rRNA* primers showed an average C_q value of 26.17 (**Figure 16**). Generally, C_q values equal to or greater than 35 are considered not significant [186]. Consequently, it was assumed that there was no significant amplification for the primers corresponding to *pqsE*, *lasI*, and *mucA*,. However, there was amplification for the *16S rRNA* gene primers using *B. contaminans* cDNA.

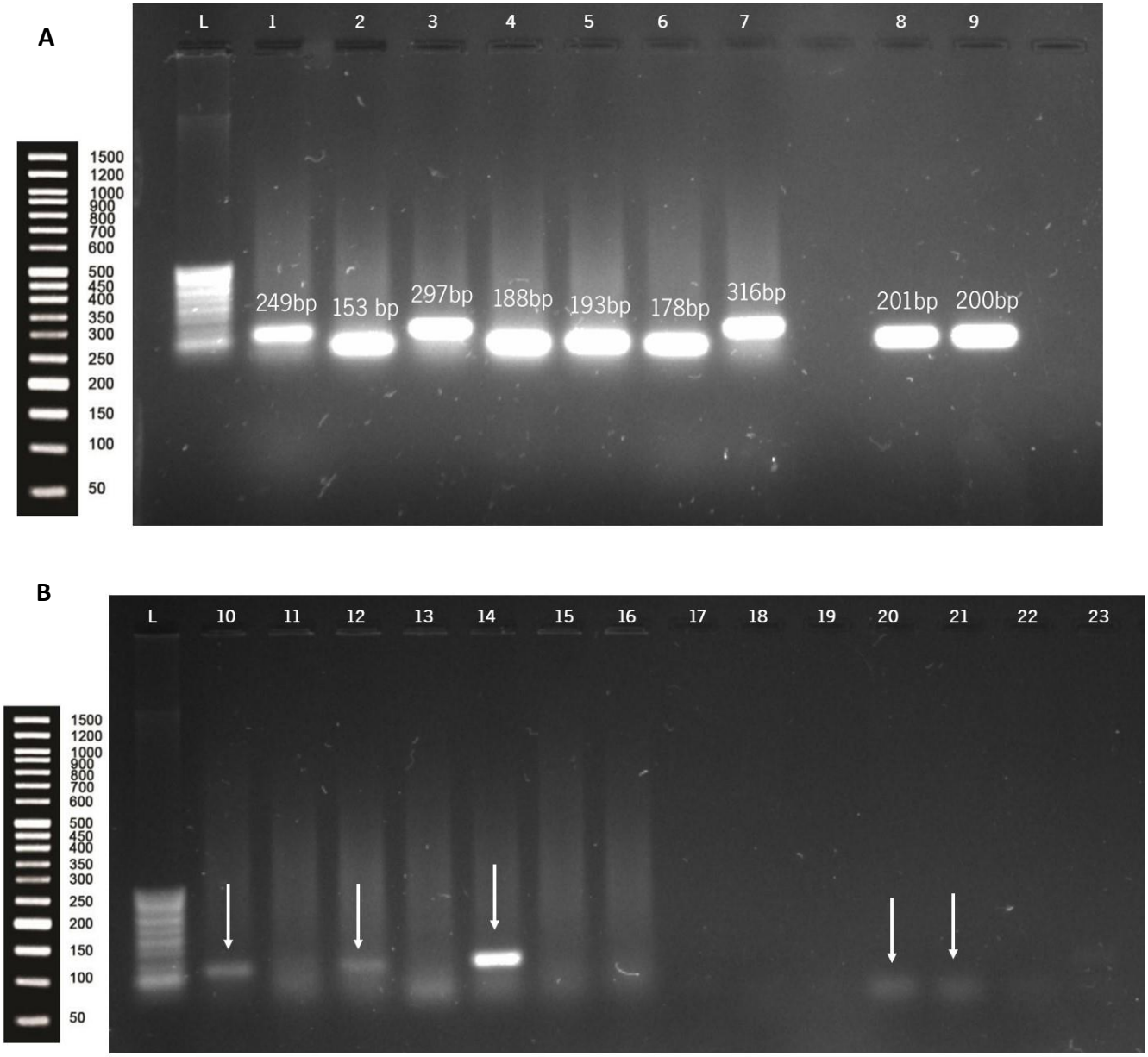


Figure 15| Agarose gel electrophoresis showing the PCR amplifications with the primer pairs of *P. aeruginosa*, (A) Lane 1-7: Amplification using gDNA of *P. aeruginosa* with the primer pairs for the genes **(1)** *rhla*, **(2)** *mucA*, **(3)** *pqsA*, **(4)** *pqsE*, **(5)** *rhlr*, **(6)** *lasI*, **(7)** *16s rRNA*, and the positive control using gDNA of *S. epidermis* RP62A and the primers for the genes **(8)** *icaA* and **(9)** *tagO*. **(B)** Lane 10-16: Amplification using gDNA of *B. contaminans* with the primer pairs for **(10)** *rhla*, **(11)** *mucA*, **(12)** *pqsA*, **(13)** *pqsE*, **(14)** *rhlr*, **(15)** *lasI*, **(16)** *16s rRNA* and from lanes 17 to 23 the NTC (No Template control), corresponding to **(17)** *rhla*, **(18)** *mucA*, **(19)** *pqsA*, **(20)** *pqsE*, **(21)** *rhlr*, **(22)** *lasI* and **(23)** *16s rRNA*. **L** in (A,B): NZYDNA ladder VI with seventeen DNA fragments from 50 to 1500 bp.

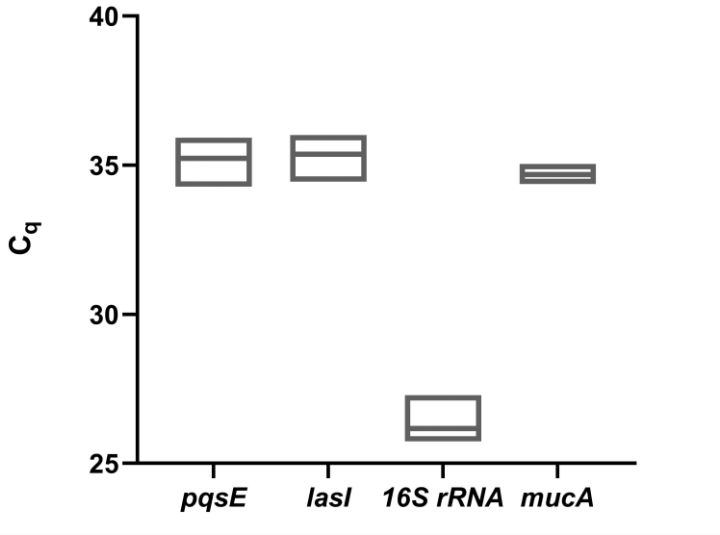


Figure 16| Cycle quantification (C_q) values for *pqsE*, *lasI*, *16S rRNA* and *mucA* genes from *B. contaminans* 48h biofilms sample. Three technical replicates from the same biological sample were used. The median is depicted as a horizontal line.

4.6.2. Optimizing RNA extraction for *B. contaminans* biofilms

The first step in a qPCR is RNA extraction. This step is often considered the most important since poor-quality RNA will influence the accuracy and reproducibility of the downstream applications [187]. Common indicators of RNA extraction success include the concentration, purity and integrity of RNA [187]. Additionally, biofilms present a heterogeneous nature that can also contribute to the variability of the procedure [100]. Optimal RNA concentrations are important since low concentrations may not be sufficient to detect low-expressed genes [188]. Hence, to find the ideal conditions meeting all the specified criteria, three distinct conditions were tested for extracting RNA from *B. contaminans* biofilms (**Table 6**). It is important to note that optimizing the RNA extraction protocol was not required for *P. aeruginosa*, as this bacterium is extensively studied within our research group, and the extraction process has already been optimized.

Table 6| Tested conditions to optimize RNA extraction for biofilms of *B. contaminans* using different numbers of biofilms and maturation ages. The concentration and the parameters A_{260}/A_{280}, A_{260}/A_{230} are also presented

Biofilm age	N° biofilms	RNA (µg/µL)	A_{260}/A_{280}	A_{260}/A_{230}
24h-old	2	34.7 ± 3.3	1.82 ± 0.13	0.45 ± 0.02
48h-old	6	21.1 ± 1.7	1.6 ± 0.15	0.39 ± 0.03
48h-old	10	94.6 ± 0.2	1.65 ± 0.01	0.93 ± 0.00

Pooling 10 biofilms resulted in the highest RNA yield. Interestingly, with an intermediate number of biofilms, and with an older biofilm age, the quantity of extracted RNA was lower than that obtained when using a pool of two 24h old biofilms. This could be due to the number of cultivable cells in these time points, as previously assessed, that showed an average of 2.18×10^{9} CFU/mL for 24h biofilms vs. a 2.56×10^{8} CFU/mL for 48h biofilms, equivalent to a 0.933 log reduction. Thus, the initial number of bacterial cells used for the RNA extraction influenced the total RNA quantity.

Concerning the higher RNA yield observed with the pool of 10 biofilms, some modifications to the isolation protocol were employed. In this condition, the 10 biofilms were resuspended in the same 1mL of NaCl 0.9%, thereby concentrating the number of cells in the liquid. In contrast, for the condition with 2 biofilms, each was individually suspended in 1mL of NaCl 0.9%, which was then vortexed and a 1mL sample was used for RNA extraction. It is worth noting that using a pool of biofilms has the added advantage of reducing the variability associated with biofilm growth. This reduction in variability enhances the accuracy of gene expression analysis, a phenomenon demonstrated by Sousa and colleagues [189], where increasing the number of biofilms from 1 to 10 resulted in a 3.5-fold reduction in variability associated with *S. epidermidis* biofilms [189].

In addition to the importance of RNA quantity, ensuring sample purity is crucial in RNA extraction, as the presence of inhibitory compounds can affect downstream applications [187]. RNA purity is typically assessed using absorbance ratios, namely A_{260}/A_{280} and A_{260}/A_{230}. Pure RNA is expected to have ratios above 1.8 [190]. A high A_{260}/A_{280} ratio indicates minimal protein contamination, while a high A_{260}/A_{230} ratio indicates a low level of polysaccharides, phenol, and/or chaotropic salt contamination [166]. Only the 24h-old biofilm condition exhibited an A_{260}/A_{280} ratio above 1.8. However, for all conditions, the A_{260}/A_{230} value was significantly lower than 1.8. Given the nature of the samples, i.e., biofilms, RNA purity might have been compromised due to the presence of proteins and polysaccharides from the matrix, potentially explaining the low values in these measurements.

Nevertheless, RNA quality indicators do not always correlate with reliable gene expression quantification. Cicinnati *et al.* [191] concluded that a low A_{260}/A_{230} ratio (0.43) did not compromise the reliability of qPCR. Furthermore, QIAGEN showed that high concentrations of guanidine thiocyanate (100mM), which is present in lysis buffers of RNA isolation kits and is one of the main contaminants that contribute to low A_{260}/A_{230} ratios, does not affect the accuracy of qPCR results [192].

4.6.3. Assessing *16S rRNA* as a Reference Gene in Mono- and Dual-Species Biofilms

To be able to quantify gene expression using the relative quantification method, a constitutive gene, a gene whose expression levels remain unaffected by experimental factors [191], is required for accurate quantification. These genes are referred as to RG and are internal reaction controls. The gene *16S rRNA* is a well-known constitutive gene that is commonly used as an RG. Thus, its stability was evaluated herein using cDNA synthesized from a pool of 10 72h-old biofilms of single (*P. aeruginosa*) and dual-species, treated or not with 2 × MIC of ciprofloxacin.

The C_q variations of *16S rRNA* in all tested conditions are depicted in **Figure 17**. There was a significant variation in the number of cycles between the conditions, with the highest variation being approximately 9 cycles. These results show that under these particular experimental conditions, *16S rRNA*, by itself, is not an adequate RG as its expression was influenced by the experimental conditions. The variation observed might be related to the fact that the primers used do not seem to be specific to *P. aeruginosa*. They could be amplifying cDNA from *B. contaminans*, causing nonspecific amplification and potentially leading to different C_q values (**Figure 16**).

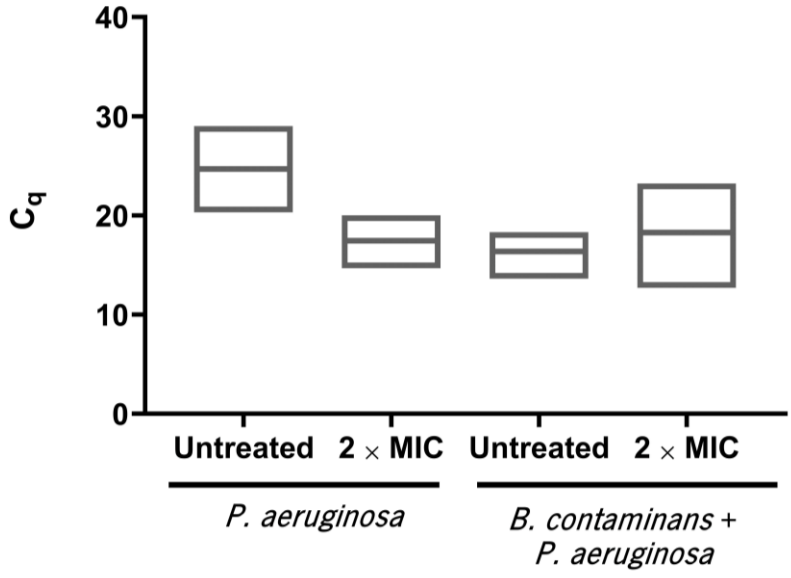


Figure 17 | Variability in cycle threshold (C_q) values for *16S rRNA* gene using cDNA synthesized with RNA isolated from 72h *P. aeruginosa* and *P. aeruginosa* co-cultured *B. contaminans* biofilms. For each condition, 2 independent experiments were performed (n=2), each consisting of three technical replicates. The median is depicted as a horizontal line. MIC: Minimum Inhibitory Concentration.

Therefore, more genes with constitutive expression must be tested under our experimental conditions. Meng *et al*. [193], using *P. aeruginosa* PA01 mid-exponential planktonic suspensions grown in Lysogeny-Broth, found that *nadB* was the best RG in a selection of 10 candidates (*algD*, *gyrA*, *anr*, *recA*, *fabD*, *proC*, *ampC*, *rpoS*, and *rpsL*). They also analyzed, with the same strain of *P. aeruginosa,* the stability of RGs under the pressure of different antibiotics at ½ × MIC. For antibiotics such as kanamycin or chloramphenicol *algD* was the best RG, while for gentamycin and tetracycline *nadB* was the best internal control. Similarly, Savli *et al*. [194] collected 23 strains of *P. aeruginosa* with different resistance profiles to antibiotics namely meropenem, ceftazidime, ciprofloxacin, amikacin and imipenem, and evaluated the stability of the six commonly used RG and found that only *proC* and *rpoD* met the requirements, while *ampC*, *fabD*, *pbp-2* and *rpoS* were not recommended due to their poor stability. Several other genes have been used to normalize qPCR experiments with *P. aeruginosa* including *rpoS*, *proC*, *recA*, *rpsL*, *rho*, *oprL*, *anr*, *tipA*, *nadB*, *fabD*, *ampC*, *algD* and *gyrA* [195].

Ideally, normalization against a single reference gene is not advised since variations in the expression of the RG have significant implications for gene expression quantification. Thus, as mentioned by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines, unless there is clear evidence that the expression of that gene does not vary under the experimental conditions being used [196], at least three RG should be used.

5. CONCLUSION AND FUTURE PERSPECTIVE

5.1. Main conclusions

The complex network of microorganisms in the CF airway microbiome has been demonstrated to engage in complex interactions, potentially influencing clinical outcomes. These interactions might enhance a species' ability to withstand antibiotic treatment if neighboring species offer protection or, conversely, competitive interactions could enhance susceptibility to antibiotics. Hence, our work aimed to uncover the mechanisms underlying interactions between *P. aeruginosa* and *B. contaminans* within the biofilm environment and shed light on their role in antimicrobial responses.

Our results showed reduced biomass and CFU counts in dual-species biofilms compared to *P. aeruginosa* single biofilms. In contrast, an increase in biofilm biomass was observed compared to *B. contaminans* biofilms. Analyzing *B. contaminans* CFUs over three days, an increase was noted in dual-species biofilms, while in single-species biofilms remained consistent. Taken together, these results suggest that *B. contaminans* might outcompete *P. aeruginosa* in the biofilm mode of growth.

To assess *P. aeruginosa*'s response to colistin and ciprofloxacin when combined with *B. contaminans*, we determined the MIC for each antibiotic. *P. aeruginosa* exhibited MICs of 0.25mg/L for ciprofloxacin and a maximum of 1 mg/L for colistin, whereas *B. contaminans* had a MIC of 0.25mg/L for ciprofloxacin. Determining the MIC for colistin in *B. contaminans* proved challenging, being consistent with previous reports indicating resistance to polymixins in several Bcc species. When ciprofloxacin was administered at different concentrations in biofilms with both species, *B. contaminans* appeared unaffected. In contrast, *P. aeruginosa* displayed reduced culturability at higher concentrations of ciprofloxacin, compared to the single-species biofilms counterparts, suggesting altered susceptibility in the presence of *B. contaminans*.

To understand the underlying molecular mechanisms that were responsible for this altered susceptibility, an analysis of genes related to *P. aeruginosa* virulence was conducted for that purpose. To do so, we first evaluated the specificity of the primers and *mucA*, *pqsA* and *lasI* were shown to be specific to *P. aeruginosa*. However, qPCR proved challenging due to the lack of specificity and unsuitability of *16S rRNA* as an RG. Identifying new RGs is crucial for future experiments.

Overall, this study provided insights into interactions between these pathogens and shed light on *P. aeruginosa*'s response to treatment with a commonly used antibiotic in the presence of *B. contaminans*.

5.2. Future Perspectives

This dissertation highlights the potential impact of *B. contaminans* on *P. aeruginosa*. However, there are certain limitations in this research. Firstly, only one strain of *P. aeruginosa* and *B. contaminans* was utilized. To draw more robust conclusions, it is important to include multiple strains, including clinical isolates.

It's also crucial to acknowledge that equal co-infections of *P. aeruginosa* and Bcc bacteria are unlikely to happen in CF lungs, where longstanding *P. aeruginosa* infections often exist at high levels when Bcc bacteria enter [93]. Future experiments should explore scenarios where *B. contaminans* is introduced into established chronic *P. aeruginosa* infections, simulating the progression of CF infection.

Furthermore, only the antimicrobial susceptibility of antibiotics commonly used for *P. aeruginosa* was assessed, specifically focusing on ciprofloxacin. This analysis should be extended to antibiotics effective against *B. contaminans* or explore combinations of antibiotics, a strategy proven successful in treating *in vitro* biofilms of *P. aeruginosa* and *B. cepacia* [25].

Considering the diverse bacterial strains associated with CF airway colonization, further investigations should involve various strains/species combinations, especially those residing in the airway for different durations by using another prevalent pathogen such as *S. aureus* or other Bcc bacteria namely *B. cenocepacia* or *B. multivorans*.

Considering the anaerobic conditions present in certain areas of CF airways, it would be interesting to investigate whether the interactions observed between *B. contaminans* and *P. aeruginosa* persist under different conditions. In another study, using planktonic populations, an anaerobic environment provided a competitive advantage to Bcc bacteria [93]. Exploring this scenario with *B. contaminans* in the biofilm mode of growth may reveal if similar advantages exist and what factors contribute to these advantages. Additionally, previous research noted a competitive advantage for *B. cenocepacia* in older clinical isolates of *P. aeruginosa* [137]. Investigating this phenomenon using *P.*

aeruginosa chronic and non-mucoid phenotypes in biofilms with *B. contaminans* would provide valuable insights.

Exploring these concepts would contribute to a deeper understanding of the interactions between these two bacteria in the context of CF and could aid in predicting clinical outcomes and appraising novel antimicrobial combinations.

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