Universidade do Minho
Escola de Engenharia

Mariana Fernandes Gonçalves

Quantification of Virulence Factors and Evaluation of Interleukin Degradation by Supernatant of *Pseudomonas aeruginosa* Clinical Cystic Fibrosis Isolates

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Master's Dissertation
Master Degree in Biomedical Engineering
Clinical Engineering

Dissertation Supervised by
Professor Doutor Joana Azeredo
Professor Doutor Tom Coenye
Professor Doutor Tom Van de Wiele

September 2024

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity.

I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, Braga, September 2024

Mariana Fernandes Gonçalves

RESUMO

A infeção por *Pseudomonas aeruginosa* em doentes de fibrose quística é uma das principais causas de inflamação nos pulmões. Este patógeno coloniza as vias aéreas do hospedeiro e adapta-se ao ambiente destas, desenvolvendo um vasto leque de diferentes fenótipos e genótipos. Os fatores de virulência segragados pela *P. aeruginosa* favorecem a adaptação ao ambiente crónico dos pulmões do doente e a sua expressão pode diferir no mesmo doente e entre doentes. Assim, têm sido observadas diferentes respostas inflamatórias em diferentes pacientes crónicos infetados com este patógeno.

Atualmente, os antibióticos não são eficazes contra uma grande extensão de microrganismos, incluindo a *P. aeruginosa.* Com isto, o desenvolvimento de terapias alternativas torna-se ainda mais imperativo. Terapias anti virulência destacam-se como alternativas significativas contra a virulência bacteriana podendo constituir um novo tratamento para a infeção crónica nos pulmões pela *P. aeruginosa*.

O trabalho descrito nesta dissertação teve como objetivo caracterizar o impacto da *P. aeruginosa* na resposta inflamatória desencadeada em dois doentes crónicos de fibrose quística.

Estirpes de *P. aeruginosa*, com propriedades anti-, pro- e não inflamatórias, isoladas de doentes de fibrose quística foram cultivadas no meio synthetic sputum medium 2 e os fatores de virulência, pyoverdine, pyocyanin, ramnolípidos, atividade proteolítica e elastolítica foram quantificados. A degradação das interleuquinas IL-8 e IL-1β por sobrenadante destas estirpes clínicas da *P. aeruginosa* foi avaliada.

Diversidade na produção de fatores de virulência foi observada em dois doentes crónicos com fibrose quística. Não foi verificada produção dos fatores de virulência pyoverdine e pyocyanin. Apenas uma estirpe clínica mostrou produção significativa de ramnolípidos. As estirpes clínicas anti-inflamatórias mostraram atividade proteolítica e elastolítica elevada e capacidade de degradação das interleucinas IL-8 e IL-1β, enquanto que as estirpes pro-inflamatórias não mostraram atividade proteolítica nem elastolítica e não foi verificada degradação de IL-8 ou IL-1β. Assim, as proteases, e especificamente, elastase secretadas pela *P.aeruginosa* demonstram ter um efeito na redução da inflamação na fibrose quística.

Palavras-chave: Fibrose Quística; Fatores de Virulência; Inflamação; *Pseudomonas aeruginosa*

ABSTRACT

Pseudomonas aeruginosa infects Cystic Fibrosis patients causing chronic lung inflammation. This opportunistic pathogen is able to colonize the host airways and adapt to the lung environment, developing a wide range of phenotypes and genotypes.

P. aeruginosa expresses various virulence factors that enable this adaptation to the chronic lung environment and causes different inflammatory responses in the infected lungs. Variations in phenotype, virulence factor expression and inflammatory response have been observed among patients and within a single CF patient.

Antibiotics are no longer effective against an extensive range of bacteria, including *P. aeruginosa*. Consequently, the development of alternative therapies is even more imperative. Anti-virulence therapies stand out as a significant alternative against bacterial virulence that could enable a new treatment for chronic lung infection with *P. aeruginosa.* Therefore, the aim of this work was to explore the influence of *P. aeruginosa* virulence in the host inflammatory response.

P. aeruginosa sputum isolates, with anti-, non-, and pro-inflammatory character, originating from two chronic CF patients were cultured in synthetic sputum medium 2 and 5 virulence factors secreted by this pathogen, namely pyoverdine, pyocyanin, rhamnolipids, elastolytic and proteolytic activity were quantified. Degradation of the interleukins IL-8 and IL-1β by supernatant of clinical *P. aeruginosa* isolates was evaluated.

Heterogenicity of virulence factors production was observed within and between two chronically infected CF patients. No significant production of pyoverdine or pyocyanin was observed. Only one clinical isolate showed significant rhamnolipid production. The anti-inflammatory clinical isolates showed significant proteolytic and elastolytic activity and degradation of IL-8 and IL-1β, while the pro-inflammatory isolates did not show proteolytic or elastolytic activity and no degradation of IL-8 and IL-1β. Therefore, *P. aeruginosa* proteases and elastase appear to have an effect on the reduction of inflammation in CF.

Keywords: Cystic Fibrosis; Inflammation, *Pseudomonas aeruginosa*, Virulence Factors.

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ABREVIATIONS

AprA	Alkaline Protease
ASL	Airway Surface Liquid
BSA	Bovine Serum Albumin
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CFU	Colony-Forming Unit
CM	Conditioned Medium
CTA B	Hexadecyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
EDTA	Ethylenediaminetetraacetic acid solution
ELISA	Enzyme-linked Immunosorbent Assay
HRP	Avidin-Horseradish Peroxidase
IL	Interleukine
INF	Interferon
LAF	Laminar Air Flow
LB	Luria Bertani
LPM	Laboratory of Pharmaceutical Microbiology
LPS	Lipopolysacharide
LasA	Elastase A
LasB	Elastase B
MC	Medium Control
MB	Methylene Blue
MMPs	Matrix Metalloproteases
MOPS	Morpholinopropanesulfonic acid
MPO	Myeloperoxidase
MRSA	Methicillin-resistant *Staphylococcus aureus*
MQ	Milli-Q
OD	Optical Density

PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate Buffered Saline
PIV	Protease IV
PvdS	Sigma Factor Protein
QS	Quorum Sensing
rpm	Rotations per minute
SCFM2	Synthetic Cystic Fibrosis Sputum Medium 2
T1SS	Type 1 Secretion System
T2SS	Type 2 Secretion System
T3SS	Type 3 Secretion System
T4SS	Type 4 Secretion System
TCA	Trichloroacetic Acid
TLRs	Toll-like receptors
TMB	3,3',5,5' – tetramethylbenzidine
TNF	Tumour Necrosis Factor
TSA	Trypsone Soy Agar
V	Volume

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1. PRELIMINARY THESIS STATEMENT

1.1 Motivation and Context

Pseudomonas aeruginosa is an opportunistic pathogen that causes lung infection and inflammation in Cystic Fibrosis (CF) patients. *P. aeruginosa* is able to colonize the CF lungs during which its genotype and phenotype can drastically change (Workentine et al., 2013).

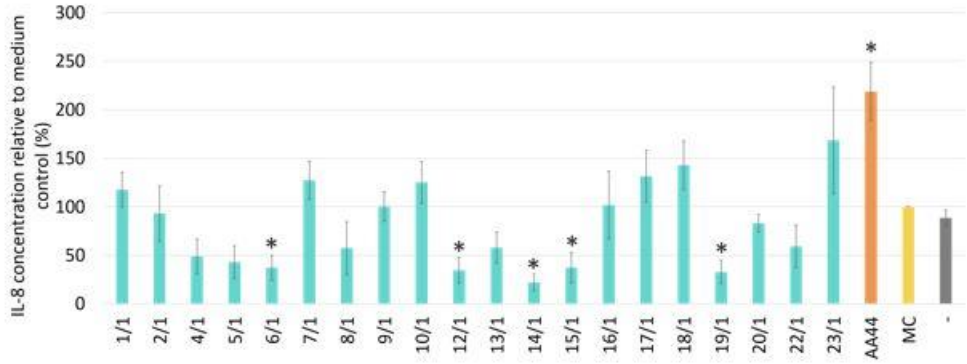
This results in a wide range of diverse isolates with, for example, different antibiotic resistance and virulence factor production (Workentine et al., 2013). The influence of these phenotypic variations on the inflammatory response of the host is not clear yet. Therefore, the project initiated by Wauters et al., 2023 aims to study the pro-inflammatory properties of *P. aeruginosa* isolates within and between CF patients.

To this aim, a previous selection of *P. aeruginosa* clinical isolates was made. The pro-inflammatory properties of one hundred clinical isolates of *P. aeruginosa* from five different CF patients (20 isolates/patient) were tested under conditions that mimic the chronic *in vivo* disease environment. The 100 isolates were incubated microaerophilically for 48h in Synthetic Cystic Fibrosis Sputum Medium 2 (SCFM2), which is nutritionally similar to authentic CF sputum. 3-D alveolar epithelial cells (A549) were exposed to the isolate's supernatants and 4 hours later the immune response was evaluated by quantifying the concentration of the cytokine interleukin-8 (IL-8) through an Enzyme-linked Immunosorbent Assay (ELISA) (Barrila et al., 2010; Turner et al., 2015; Wauters et al., 2023).

High variability was observed in the inflammatory response after exposure of the 3-D A549 cells to the supernatants of the clinical isolates between and within patients. The inflammatory response triggered after exposure to supernatants of the clinical isolates originating from Patient BS1 and patient BS6 was very different, i.e. being anti- and pro-inflammatory, respectively (**Figure 1**).

A selection of 15 isolates was made in which isolates that caused a statistically significant anti- and pro-inflammatory response and isolates that triggered no response were selected from patient BS1 and BS6 (Wauters et al., 2023).

The selected clinical isolates were further studied in the scope of this MSc thesis. A quantification of the virulence factors was performed, under conditions that mimic the chronic disease environment. Furthermore, an IL-8 and IL-1β degradation assay was performed to have more insights about how the virulence factors can influence the inflammatory processes.



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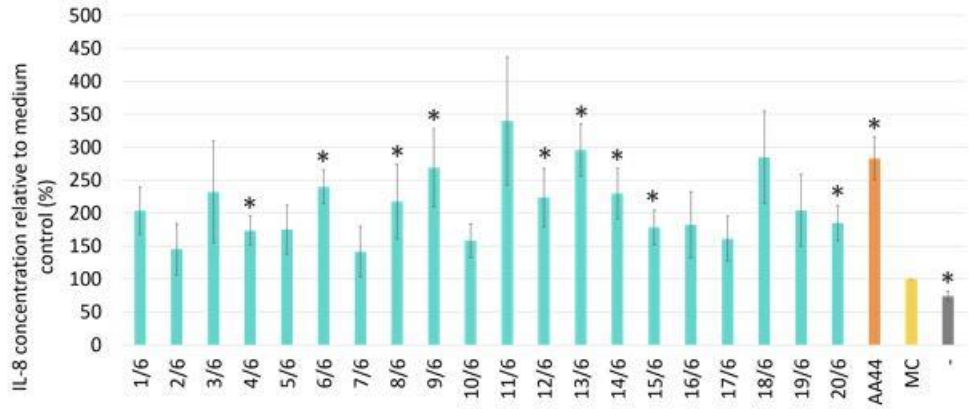

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Figure 1: The inflammatory response of an in vivo-like 3-D lung epithelial model to supernatants from individual *P. aeruginosa* sputum isolates after culturing in SCFM2 originating from patients BS1 (a) and BS6 (b) quantified by an IL-8 ELISA assay. AA44 in SCFM2 was used as the positive control, the medium control (MC) corresponds to SCFM2 alone. The blank (-) represents A549 cells in cell medium, i.e. G2 medium without FBS. The y-axis shows the relative IL-8 concentration compared to the MC. Error bars represent standard error. (n=3-7, * $p < 0.05$).

1.2 Aims and Contributions

It has been observed that the virulence factors produced by *P. aeruginosa* contribute to the pathogenicity of this pathogen in chronic lung infection in CF, enabling the bacterial adhesion to the host, tissue damage and drug resistance (Sun et al., 2020). *P. aeruginosa* triggers strong inflammatory responses contributing to the decrease in lung function (Lin & Kazmierczak, 2017). Hence, the quantification of these factors produced by the clinical isolates can contribute to the identification of the key factors that trigger higher or lower inflammation.

The aim of this dissertation is to quantify the production of virulence factors by *P. aeruginosa* namely, elastase, pyoverdine, pyocyanin, rhamnolipids and general proteases, to further study the inflammatory properties of this pathogen. In addition, the degradation of the inflammatory cytokines IL-8 and IL-1β by *P. aeruginosa* clinical isolates will be studied in order to have more insights into the inflammatory character of these clinical isolates.

2. INTRODUCTION

2.1 Cystic Fibrosis and Current Therapies

CF is a life-shortening genetic disease with a high rate of morbidity and mortality (Blanchard & Waters, 2022). It is estimated that this condition affects over 100,000 people globally. According to Singh et al., 2023, in the year of 2020 the annual number of deaths for people with CF was 342 in the United States (Singh et al., 2023). Also in the United States, the median survival age was 59 years in 2020 (Blanchard & Waters, 2022). Moreover, the median age of death ranges from 29 to 35.6 years, whereas the median surviving age ranges from 44 to 53 years, according to summarized data from the registries of the United Kingdom, Canada, Belgium, Australia, France, and Ireland, based on data from 2019 (McBennett et al., 2022).

In addition to the lungs, CF also affects the pancreas and other organs. This genetic disease is caused by a mutation in the CF transmembrane conductance regulator (CFTR) gene, which can result in dysfunction in the CFTR protein (Ribeiro & Gentzsch, 2022). The most common mutation in CF results in the deletion of phenylalanine in the nucleotide-binding domain NBD1 at the position 508 (F508del). This mutation causes the CFTR proteins to be misprocessed, reducing the transportation of CFTR to the cell surface (Habib et al., 2019; J. A. Lee et al., 2021).

CFTR is expressed in the kidneys, pancreas, intestine, heart, vas deferens, and lungs. The main function of this protein is the regulation of chloride channels. In addition, CFTR also plays various important roles, for example controlling the expression of the cytokines IL-8 and IL-10, aquaporin channels, vesicle trafficking, and ATP release. In cystic fibrosis, the defected CFTR proteins do not make the correct transport of chloride ions and water in cells, causing imbalance of these substances in the cells (Cystic Fibrosis Foundation, 2024) (**Figure 2**). This imbalance leads to the dehydration of airway surface liquid (ASL), leading to dehydration. Consequently, the compromised ciliary function and slower mucociliary transport provoke a reduction in the speed of moving mucus and trapped substances out of the respiratory tract. Moreover, the CFTR mutation leads to the increase of viscosity of the mucus and acification of the respiratory tract, leading to the obstruction of the airways, which favours an easier colonization by pathogens as they accumulate in the lungs (Goss & Burns, 2007; Bhagirath et al., 2016; Farinha & Callebaut, 2022).

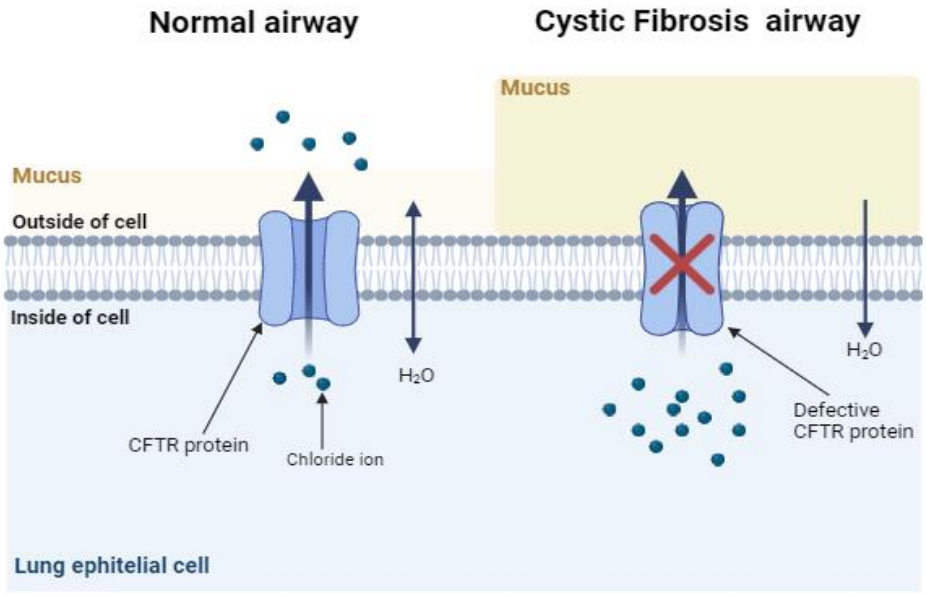

Figure 2: Effect of CFTR mutation in CFTR protein function.

In CF, the most common colonizers include the opportunistic pathogens *P. aeruginosa, Burkholderia* spp*., Achromobacter* spp., *Haemophilus influenzae* and *Staphylococcus aureus* (Goss & Burns, 2007; Bhatt, 2013; Blanchard & Waters, 2022). In healthy people*, P. aeruginosa* rarely causes disease, but it is one of the most frequent bacteria causing CF lung infections (Blanchard & Waters, 2022). Apart from people with CF, this opportunistic pathogen can also affect bloodstream and the renal system. In addition, infection with *P.aeruginosa* is associated with a descrease in life expectancy of 30 years, comparing with the life expectancy of 40 years in patients that are not colonized (Reece et al., 2021). As this thesis project focuses on studying clinical isolates of *P. aeruginosa*, its role in cystic fibrosis (CF) will be further explored in the following sections.

Burkholderia cepacian are gram-negative bacterium that have can adapt to environmental variations. *Burkholderia* spp. Can provoke tissue damage by the expression of several virulence factors, such as, pili allowing bacterial attachment and extracellular proteases. CF infection with *Burkholderia cenocepacia* is characterized by a great transmissibility and leads to worst clinical outcomes by accelerating decline in lung function (Blanchard & Waters, 2022).

Furthermore, in some older CF patients, *Achromobacter* infection can co-exist with chronic infection by *P. aeruginosa*. *Achromobacter* ssp. Are equipped with multiple efflux pumps, produce toxins, express binding factors to collagen and mucin which allows them to adhere to epithelial cells (Blanchard & Waters, 2022).

Staphylococcus aureus (S. aureus) is a gram-positive bacterium that can infect people with CF mostly during childhood (Ahlgren et al., 2015; Xu et al., 2024). This pathogen contributes to the alteration of the lung environment, since it expresses various virulence factors, for example, adhesins and toxins (Esposito et al., 2019; Rumpf et al., 2021). Infection with S*. aureus* constitutes a special concern for CF patients due to the appearance and development of Methicillin-resistant *S.aureus* strains (MRSA). This term (MRSA) is used to describe *S.aerus* strains that are resistant to all penicillnis, but now the antibiotic Methicillin was replaced by flucloxacillin and dicloxacillin to treat gram-positive bacterial infections (Tracey A. Taylor; Chandrashekhar G. Unakal., 2023; PubGhem, 2024).

Another pathogen that is common to infect CF patients during childhood is *Haemophilus influenzae*. It has been suggested that *H. influenzae* infection leads to inflammation and damage in the airways and can facilitate post colonization by *P. aeruginosa* (Starner et al., 2006).

During the course of CF disease, pulmonary exacerbations constitute an impactful event for CF patients, as these events are responsible for morbidity and mortality in CF (Goss & Burns, 2007; Bhatt, 2013). Some symptoms characteristic of pulmonary exacerbations include aggravated cough, greater production of sputum and decreasing in lung function (Caverly & Vandevanter, 2022). A viral infection, the acquisition of new organisms, like the opportunistic pathogens described above, or a change in bacterial density can initiate a pulmonary exacerbation. These events are associated with an increase in inflammation with a greater expression of inflammatory substances, such as, IL-8, neutrophil elastase, white cells and matrix metalloproteins (Goss & Burns, 2007; Bhatt, 2013). The treatment and prevention of these events can improve and maintain lung function and prolonging survival of people with CF (Bhatt, 2013).

There has been an impressive range of new developments in CF care (Pier, 2012). Currently, standard CF care includes several antibiotics and therapies.

Standard CF therapies are based on chest physiotherapy, oxygen therapy, nutritional support, and interventional therapy and medications, such as mucolytics, corticosteroids and bronchodilators (Bhagirath et al., 2016). However, there are concerns regarding the complexity of these therapies due to the number of various medications required and their time-consuming nature for both patients and physicians (Sawicki & Goss, 2015).

Standard antibiotics for CF infection treatment, such as colistin, tobramycin, aztreonam lysinate and levofloxacin, are used in order to improve lung function and reduce pulmonary exacerbations (Smyth et al., 2014; Taccetti et al., 2021). Furthermore, azithromycin is a macrolide antibiotic that is used as a long-term treatment for CF. Azithromycin will not only reduce inflammation caused by neutrophils and

decrease pulmonary exacerbations, but it will improve lung function, as well (Bethesda (MD): National Institute of Diabetes and Digestive and Kidney Diseases, 2012); Smyth et al., 2014; Bhagirath et al., 2016).

Inhaled antibiotics like colistin, tobramycin and aztreonam are efficient in managing *P. aeruginosa* chronic infection in CF. It has been established that these antibiotics reduce the bacterial density and increase lung function (Van de Kerkhove et al., 2016). Therefore, these drugs have significantly changed how CF is managed (Quon et al., 2014).

In addition to these standard care treatments, continuous alternating antibiotic therapy, in which two or more inhaled antibiotics are alternated monthly, has been studied and used to treat CF (Van de Kerkhove et al., 2016). In a study performed by Flume et al, the continuous alternating antibiotic therapy showed favourable outcomes in CF patients, leading to less pulmonary exacerbations and a reduction in the number of hospitalizations (Flume et al., 2016).

Nevertheless, personalized treatment according to the patients' needs with these drugs continues to be difficult to manage (Smyth et al., 2014). In addition, these therapies have side effects, including transient bronchoconstriction, throat irritation, cough, dysphonia, and altered taste (Quon et al., 2014).

There has been great interest in gene therapy as an alternative to the treatments mentioned above. In gene therapy, the mutated CFTR gene is replaced by a non-mutated version, by placing an DNA sequence of the corrected version of the CFTR protein into the patient's cells so that functional CFTR proteins could be produced (Cystic Fibrosis Foundation, 2024). This therapy is considered to be a permanent cure for this condition. However, there are still many concerns regarding gene therapy, for example, highly inflammed CF airways reduce the efficacy of the delivery. Moreover, it is also difficult to deliver large DNA fragments with existing delivery methods (J. A. Lee et al., 2021).

Phage therapy has shown great potential for treating bacterial infections, especially infections by multidrug-resistant bacteria (Bhagirath et al., 2016; Hibstu et al., 2022). It has been reported the use of phage therapy against *S. aureus*, *Streptococcus* spp*., P. aeruginosa, Salmonella* spp., *Enterococcus* spp. and *Escherichia coli* (Lin et al., 2017). However, a rising concern is that bacteria can develop resistance to phages (Bhagirath et al., 2016). There has been research in new alternatives to deal with the bacterial resistance to phages. One of these alternatives involve pre-adapting the phages to the bacterial strains. This strategy has shown the reduction of bacterial density in chronic infections, however, there is the need to personalize this therapy to the patient because of the different responses of the different strains' phenotypes to the phages (Friman et al., 2016). In a study review performed by Singh et al, it was concluded that the treatment of CF with bacteriophages was beneficial to CF patients infected with *P.*

aeruginosa (Singh et al., 2023). Several improvements in the disease outcome were observed, such as a decrease in pulmonary exacerbations, lower bacterial density and a reduction in inflammation. The need for medical care and antibiotic therapy was reduced, as well. However, *P. aeruginosa* showed resistance against the phage treatment in two of the studied cases. These results show a promising future for phage therapy in CF, however, there will be the need for more clinical trials to assess the efficiency and safety of this therapy.

Finally, CFTR modulators, that target the mutated CFTR protein, are being intensively researched (J. A. Lee et al., 2021). The CFTR therapy has significantly improved the clinical outcomes in CF patients (Lopes-Pacheco, 2020; Gaschignard et al., 2023).

Currently, there are two main classes of CFTR modulators for the treatment of CF, potentiators and correctors (Zaher et al., 2021). The potentiators are drugs that can restore CFRT's chloride channel function by targeting the CFTR mutated protein. These modulators maintain the CFTR channel's gate open, allowing chloride to pass through the cell membrane (Cystic Fibrosis Foundation). Ivacafor is an example of a potentiator used in CF treatment (Gentzsch & Mall, 2018). Lumacaftor is one of the CFTR correctors that target the F508del mutation and therefore is supports the CFTR protein folding resulting in the right protein shape and restore its function (Cystic Fibrosis Foundation; Rowe & Verkman, 2013; Shaughnessy et al., 2021).

Recently, the Food and Drug Administration approved Trikafta as a CFTR modulator for CF treatment for cases when patients have at least one F508del mutation (Food and Drug Administration , 2019). This drug is the combination of three CFTR modulators: elexacaftor, tezacaftor and ivacaftor (Cystic Fibrosis Foundation, 2024). This new modulator has the capacity to target the CFTR not only in the lungs but also in the gastrointestinal tract and pancreas (Zaher et al., 2021). As it is the combination of three CFTR modulators, Trikafta helps the protein to fold correctly and increases the opening time of the chloride channel of CFTR (Maia, 2023). However, some of these modulators are ineffective or not applicable to all the CF variants. Also, the full potential side effects of this therapy are still unknown (Bell et al., 2020).

Despite all these recent developments in CF treatment, further understanding *P. aeruginosa* pathogenicity in CF and the adaptive mechanisms of pathogens to the infected CF respiratory tract will potentially provide new insights of how to cure this condition (Faure et al., 2018).

2.2 *Pseudomonas aeruginosa* in the CF Lung

2.2.1 *Pseudomonas aeruginosa*

In 2017, the World Health Organization listed *P. aeruginosa* in the high priority category of pathogens with urgent need to discover new treatments to eliminate this pathogen (World Health Organization, 2024).

P. aeruginosa has been considered a "super bug" and is part of the ESKAPE *(Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter)* species pathogens, six pathogens that show resistance to various drugs (Scoffone et al., 2019; Mulani et al., 2019).

P. aeruginosa is a Gram-negative bacterium that can be found in many different environments, including in hospital environments, where it can survive for long periods of time. This pathogen has multiple mechanisms that enables its survival and adaptation in a wide range of environments, for example, the respiratory tract. This opportunistic pathogen can cause chronic lung infection in CF patients, representing a major health threat to patients because of its high drug resistance strains (Faure et al., 2018).

2.2.2 Initiation of Infection

The entry of *P. aeruginosa* into the upper respiratory tract initiates the bacterial pulmonary infection.

Firstly, *P. aeruginosa* replicates and colonizes the airways' lumen. Lung residual macrophages eliminate part of this pathogen. The bacteria which are not cleared by the macrophages reaches the lung epithelial cells. Then, by attaching to the membrane of the epithelial cells, *P. aeruginosa* replicates and colonizes the host cells (Deshpande & Zou, 2020).

One important defence mechanism against pathogens is the mucociliary system, as mucus acts like a trap for invasive molecules and pathogens that are inhaled (Petrocheilou et al., 2022).

The thick mucus caused by the CFTR mutation is responsible for creating a microaerobic environment, which promotes the prevalence of infection (Bhagirath et al., 2016; Blanchard & Waters, 2022). Additionally, this viscous mucus causes airway obstruction, leading to local hypoxia which activates the synthesis of inflammatory cytokines, such as IL-1β and IL-1α by the immune system (Petrocheilou et al., 2022).

The deficient mucociliary clearance benefits the colonization of the airways by *P. aeruginosa* (Lin & Kazmierczak, 2017). According to previous research, Malhotra et al, proposes that the anomalies in the innate immune system caused by the CFTR mutation makes the patient susceptible to infection, which is followed by inflammation (Malhotra et al., 2019). The CF lungs' pro-inflammatory environment and the difficulty in bacterial clearance promotes the evolution to the chronic inflammation, that damages the lung and overstimulates the innate immune cells (**Figure 3**) (Bhagirath et al., 2016).

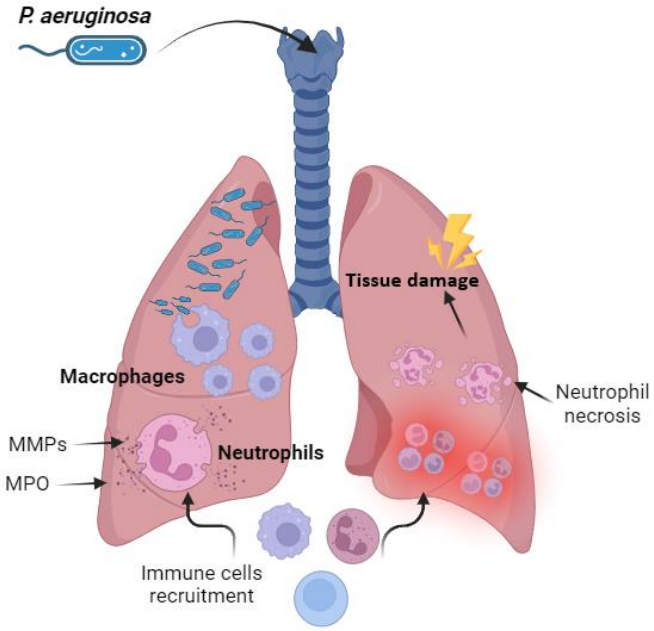

Figure 3: Scheme of *P. aeruginosa* pathogenesis. The immune cells are activated in the presence of *P. aeruginosa*. Neutrophils are recruited as a mechanism of the host inflammatory response. The excessive immune cells recruitment can cause inflammation in the lungs resulting in tissue damage. Image based on (Qin et al., 2022).

2.2.2.1 Inflammation

P. aeruginosa can be recognized by the immune system due to its pathogen-associated molecular patterns (PAMPs), which triggers a response by the innate immune receptors (Lin & Kazmierczak, 2017).

Macrophages are able to detect the presence of pathogens, including *P. aeruginosa*, by Toll-like receptors (TLRs). These receptors are activated after recognition of PAMPs. TLRs activate the pro-inflammatory cytokine IL-8, resulting in neutrophil recruitment. The unsuccessful bacterial clearance by

these immune molecules leads to the accumulation of neutrophils in the host airways, releasing reactive oxygen and nitrogen species, leading to oxidative stress in the airways (Bhagirath et al., 2016).

The dysfunctional CFTR is not able to transport anti-oxidant molecules which also contributes to the increase of oxidative stress in the CF airways, and activates the pro-inflammatory cytokine IL-8, contributing to an increased inflammation in the airways (Bhagirath et al., 2016).

IL-8 and IL-1β, produced by alveolar macrophages are responsible for initiating the inflammatory process in the chronic lung ((Lin & Kazmierczak, 2017; Liao et al., 2022). The inflammasome is a multiprotein complex that is formed upon the recognition of PAMPs, such as flagellin, type IV pili, Type 3 secretion system (T3SS), the exotoxin A and LPS (Sun et al., 2020). The inflammasome formation activates the inflammatory Capase-1, which is able to cleave pro-IL-1β into its mature active form (Molla et al., 2020; Sun et al., 2020). The pro-inflammatory cytokine IL-1 β is secreted by the cells of the innate immune system and plays an essential role in the regulation of inflammation as it regulates various processes of the innate immune response (Lopez-Castejon & Brough, 2011; Kaneko et al., 2019). This cytokine promotes the recruitment of immune cells and contributes to the local inflammation, once neutrophil proteases are able to produce active IL-1β. Furthermore, IL-1β production can be induced by IL-1α and IL-1β, contributing to a continuous inflammation (Patankar et al., 2015; Balázs & Mall, 2019).

Neutrophils are found in high levels in the CF airways and are strongly connected to the inflammation in CF. The major function of these immune cells is to protect the host from invasive pathogens; hence, neutrophils have various mechanisms to kill and remove pathogens, mainly by phagocytosis (Laval et al., 2016).

To perform its protective role, neutrophils release a wide range of molecules and proteases, for example, elastase, myeloperoxidase (MPO) and metalloproteases (MMPs) in the presence of pathogens like *P. aeruginosa*. In CF, neutrophils release a higher amount of these proteases, comparing to neutrophils from healthy individuals, which results in a major inflammatory response (Brockbank et al., 2005; Downey et al., 2009).

The function of the neutrophil elastase is to degrade phagocytized components, but in the CF airways this protease can also increase lung damage by degrading elastin, collagen, fibronectin and proteoglycans which are important structural components of the lungs.

The neutrophil release of MPO, which is essential for killing pathogens, also causes damage to epithelial cells by the formation of the cytotoxic oxidant HOCl, contributing to CF inflammation, lung damage, and impairment of lung function (Van Der Vliet et al., 2000).

In infected CF airways, the phagocytosis capacity of neutrophils is compromised by the excessive bacterial load, as demonstrated previously by (Morris et al., 2005).

Furthermore, the neutrophil function is influenced by intracellular and extracellular environments. In the acidic CF airways, the bacterial ingestion can lead to neutrophil necrosis, provoking the degradation of the lung tissue (Downey et al., 2009).

2.2.3 Evolution from Acute to Chronic Infection

Due to failed bacterial clearance and persistence of the inflammatory response, the infection evolves from an acute stage to a chronic stage.

During the acute stage of infection, there is a higher cytotoxicity and virulence caused by *P. aeruginosa* isolates, which contributes to the establishment of the infection (Sousa & Pereira, 2014).

The chronic condition is characterized by continuous neutrophil recruitment, failed phagocytosis and production of antimicrobial molecules by neutrophils. This continuous cycle of inflammatory processes leads to pulmonary exacerbations, lung tissue damage and pulmonary failure, which can result in the patient's death (Malhotra et al., 2019). Furthermore, at this stage of the infection, *P. aeruginosa* expresses efflux pumps that export drugs from the bacterial cells. This strongly contributes to *P. aeruginosa* drug resistance (Sousa & Pereira, 2014).

During chronic infection, the CF lung environment undergoes constant changes, such as osmotic and oxidative stress, presence of antibiotics, competitive interactions with other bacteria, host immune response and low O_2 availability (Sousa & Pereira, 2014; Bhagirath et al., 2016; La Rosa et al., 2021).

P. aeruginosa is able to adapt, phenotypically and genotypically, to the host environment (Faure et al., 2018). The acute phenotype of *P. aeruginosa* is mostly characterized by the production of toxins, the secretion of the virulence factors, such as rhamnolipids, pyoverdine and proteases, and motility (**Figure 4**) (A. M. Sousa & Pereira, 2014; Winstanley et al., 2016). As the infection evolves to chronic condition, *P. aeruginosa* loses its motility and represses the secretion of various virulence factors, for example, T3SS, exotoxin A and proteases (Faure et al., 2018). *P. aeruginosa* chooses to save the energy needed to produce the virulence factors for alternative metabolic pathways, that are more beneficial to its survival in the chronic CF environment. In addition, the switching from acute to chronic infection is associated with the switch to a biofilm lifestyle (Bhagirath et al., 2016). It has been reported by Worlitzsch et al. 2002 that *P. aeruginosa* can grow mostly in a biofilm lifestyle in the region from the trachea to the bronchiolus of the patient. (Worlitzsch et al., 2002; Sousa & Pereira, 2014). In CF lungs, bacteria

preferentially develop multicellular clusters or microcolonies within the sputum rather than on the epithelium surface, contrarily to what is observed in device (catheters, prostheses, pacemakers, stents) - associated infections that bacteria colonize the surface directly (Sousa & Pereira, 2014).

Biofilms are complex structures of bacteria embedded in a polymeric matrix that is composed of exopolysaccharides, proteins and DNA (Høiby et al., 2010). *P. aeruginosa* can produce the exopolysaccharides alginate, which is the major component of the matrix, Pel and Psl that play various functions including attachment to other cells, maintenance of biofilm structure and as a protective barrier against antimicrobials and host defences (Colvin et al., 2012; Jennings et al., 2021). Indeed, the chronic phenotype is characterized by the overproduction of alginate, that is essential for the formation and development of the biofilm matrix (Winstanley et al., 2016). Biofilms are well coordinated structures and are developed in response to environmental alterations, such as, bacterial density, nutrient availability and energy sources (Moreau-Marquis et al., 2008). Biofilm development is associated to a great capacity of mutations and to a strong diversity of phenotypes, constituting an important adaptative mechanism (Høiby et al., 2010; Sousa & Pereira, 2014). This great diversity can lead to the existence of persister cells with more resistance to antibiotics (Martin et al., 2021). The *P. aeruginosa* communication system Quorum-sensing (QS) is essential to the complex regulation of biofilms and the QS dependent production of lipases LipA and LipC contribute to the thickness and robustness of biofilms (Liao et al., 2022).

The constrained penetration of antimicrobial substances through the exopolysaccharide matrix strongly contributes to the elevated resistance of biofilms to antibiotics and to their ineffectiveness against P. aeruginosa (Sousa & Pereira, 2014). In addition, it has been reported that P. aeruginosa can express efflux pumps and β-lactamases enzymes that function as a resistance mechanism for antibiotics and antimicrobial substances (Sousa & Pereira, 2014; Martin et al., 2021). *P. aeruginosa* biofilms show more than1.000-fold increase in antibiotic resistance, greater tolerance to phagocytosis and to host immune system (Starner et al., 2006; Høiby et al., 2010). Biofilms strongly contribute to bacterial persistence and long-term infection in CF and therefore constitute an important concern to people with CF (Starner et al., 2006; Høiby et al., 2010).

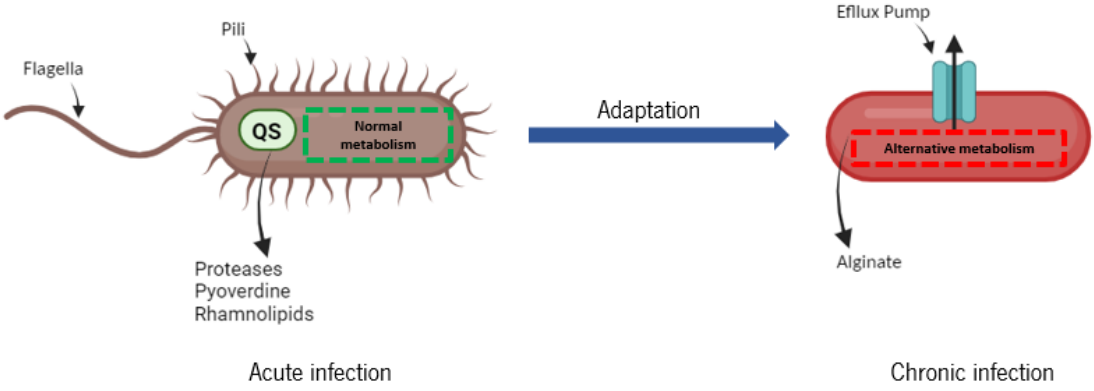

Figure 4: *P. aeruginosa* adaptation during CF infection. In acute infection *P. aeruginosa* is armed with flagella and pili and produces various virulence factors, for example, proteases, pyoverdine and rhamnolipids. In chronic infection *P. aeruginosa* uses an alternative metabolism, represses the production of virulence factors, losses its motility, overproduces alginate and expresses efflux pumps resulting in drug resistance. Image based on (Sousa & Pereira, 2014).

2.2.4 Phenotypic Heterogenicity

Recently, it was reported that a high diversity of *P. aeruginosa* phenotypes can be found in infected CF lungs (Feliziani et al., 2014).

The different zones of the respiratory tract have distinct environmental conditions due to different exposures to antibiotics, oxidative stress, host defences and O_2 availability. *P. aeruginosa* strains that can resist these environmental conditions undergo genotypic and phenotypic changes, leading to a wide diversity of strains (Tai et al., 2015).

Research performed by Feliziani et al, suggests that in the CF lungs there is a high number of *P. aeruginosa* strains with genomic mutations that confer to these strains the most beneficial phenotypic characteristics to survive in that environment (Feliziani et al., 2014)

Tai et al studied the association between specific gene mutations, antibiotic resistance and poor clinical outcomes. Mutations in the *lasR* and *mexZ* genes (key regulatory genes for virulence factors and multi-drug efflux pumps, respectively) were found in 188 *P. aeruginosa* isolates from 105 different patients, suggesting genomic intra-strain diversity in *P. aeruginosa* isolates. These findings also suggest that infections with different strain subtypes of *P. aeruginosa* may lead to different clinical outcomes (Tai et al., 2015).

The high mutagenic capacity of *P. aeruginosa* and the long-term use of antibiotics can contribute to this wide heterogeneity of phenotypes and to drug-resistant strains (Pang et al., 2019; Jurado-Martín et

al., 2021). For example, mutations in the *mexZ* gene of *P. aeruginosa* clinical isolates cause overexpression of efflux pumps, leading to increased antibiotic resistance (Pang et al., 2019).

Moreover, within isolates, the existence of typical chronic phenotypes was observed with, for example, a low virulence factor production. The existence of typical wild-type phenotypes was also studied, with for example, an intensive biofilm production (Clark et al., 2015). These findings are in line with a study performed by Workentine at al, that found a wide heterogeneity between different phenotypes within isolates, such as colony morphology, motility, QS, proteases, auxotrophy, siderophores, growth rates and antibiotic resistance (Workentine et al., 2013).

The study of the *P. aeruginosa* population diversity between patients contributes to the comprehension of how *P. aeruginosa* evolves to different genotypes and phenotypes in the CF lungs. In addition, more understanding of this heterogeneity is important to defining clinical interventions, as it can lead to differences in disease progression and to different clinical outcomes (Feliziani et al., 2014; Bhagirath et al., 2016).

2.3 Virulence Factors

P. aeruginosa capacity to invade and colonize immune compromised CF patients is mainly due to the production of a wide set of virulence factors (Malhotra et al., 2019). These factors enable invasion, bacterial attachment and colonization, causing damage to the host (Sharma et al., 2017).

In addition to this, *P. aeruginosa* virulence factors compromise the host immune response to infections, promote tissue destruction and can consume nutrients that are present in the host (Liao et al., 2022).

Virulence factors can be divided in two major groups according to their properties: cell-associated and extracellular factors. The cell-associated factors contribute to bacterial colonization and include LPS, flagella, type IV pili and the secretion systems (T1SS, T2SS, T3SS, T4SS) (Williams et al., 2010; Malhotra et al., 2019). The extracellular factors, include cytotoxins injected by the T3SS, i.e., (ExoU, ExoT, ExoS and ExoY), the siderophores pyoverdine and pyochelin and various proteases (Williams et al., 2010). These factors are secreted into the extracellular medium once the lungs are colonized (T. de Sousa et al., 2021).

2.3.1 Quorum-Sensing

The QS system is an inter-cellular communication system found in *P. aeruginosa*, as well as in other bacteria, that controls gene expression in response to external alterations in the environment and to bacterial density. QS regulates the secretion of virulence factors and biofilm formation (J. Lee & Zhang, 2015; Li et al., 2022).

P. aeruginosa QS consists of four hierarchically organized and complex circuits, i.e., LasI/LasR, RhlI/RhlR, Pps/PsR and Iqs/IqsR, which use the following four autoinducers, respectively: N-(3-oxododecanoyl)-1-HSL (OdDHL), N-butanoyl-L-homoserine lactone (BHL), 2-heptyl-3-hydroxy-4(1H)-quinole (PQS) and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) (Li et al., 2022) .

The four QS systems are interconnected and on the top of the hierarchy is the LasI/LasR system, which influences the three other systems. The RhlI/RhlR system is also regulated by the Pqs system (**Figure 5**) (Scoffone et al., 2019; Li et al., 2022).

At low bacterial density, autoinducers diffuse at low levels, which are not detected. However, when the cell density reaches higher levels, autoinducers accumulate in higher levels which promotes signal detection and response by the expression of major growth genes in high bacterial density and also production of more autoinducers (Rutherford & Bassler, 2012). It is generally presumed that QS also regulates the mobility of *P. aeruginosa* and the production of various virulence factors, like elastase, exotoxin A and alkaline protease (J. Lee & Zhang, 2015; T. de Sousa et al., 2021). Furthermore, QS also contributes to biofilm formation by regulating the secretion of rhamnolipids and siderophores (Rutherford & Bassler, 2012). By way of example, in the LasI/LasR system, the autoinducer synthase LasI regulates the synthesis of the N-(3-oxododecanoyl) homoserine lactone. LasI, when bound to the transcriptional activator LasR allows the regulation of the transcription of the virulence gene LasB, resulting in the production of Elastase B (LasB) (Pearson et al., 1997; Jurado-Martín et al., 2021).

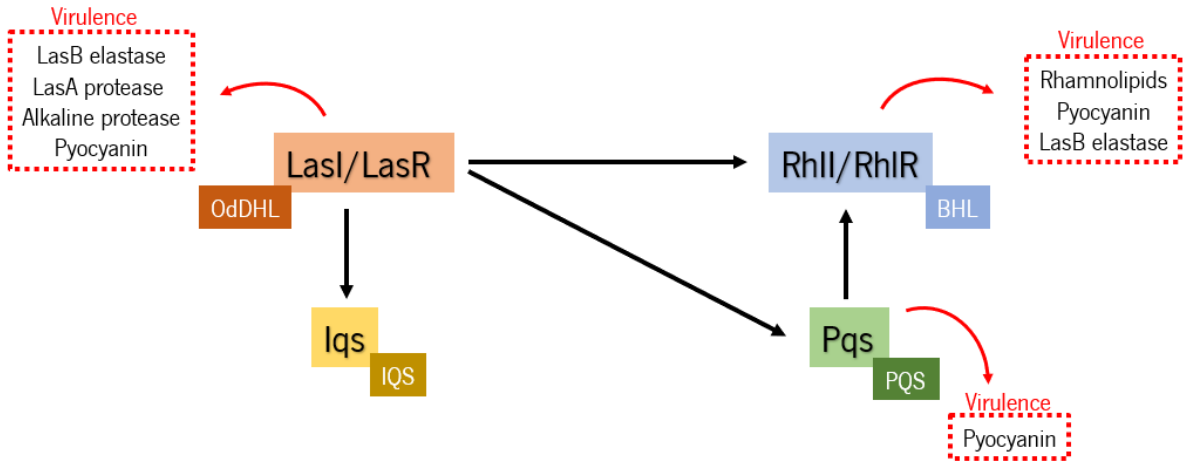

Figure 5: *P. aeruginosa* QS systems LasI/LasR, RhlI/RhlR, Pps and Iqs, and their autoinducers OdDHL, BHL, PQS and IQS, respectively. The LasI/LasR system controls the RhlI/RhlR, Pps and Iqs systems. The Pqs system controls the RhlI/RhlR system. Image based on (Li et al., 2022).

2.3.2 Secreted Proteases

P. aeruginosa releases various secreted proteases that play a crucial role in acute infections by this pathogen and promote pathogen-host interactions through their capacity to interact with a wide range of host molecules contributing to tissue damage by degrading host proteins (Faure et al., 2018; Jurado-Martín et al., 2021; Qin et al., 2022).

The secreted proteases that contribute to *P. aeruginosa* virulence include the alkaline protease (AprA), protease IV (PIV), LasB and elastase A (LasA) (Faure et al., 2018; Jurado-Martín et al., 2021)

AprA targets components of the endothelium such as the fibronectin and laminin. In addition, this protease degrades flagellin monomers, complement components, namely C1Q, C2, and C3, and also cytokines, such as IL-6, TNF-α, and IFN-γ. Therefore, AprA interferes with the host immune response to infections (Faure et al., 2018; Jurado-Martín et al., 2021; Liao et al., 2022).

PIV contributes to the evolution of infections in the respiratory tract as it is capable of degrading various host molecules, including immunoglobulins, complement components and lung's surfactant, which is important not only for the maintenance of lung function, but also for the innate host defence (Malloy et al., 2005; O'callaghan et al., 2019). In addition to this, PIV also targets the elastin, fibrinogen, transferrin and lactoferrin promoting damage to host tissues (Liao et al., 2022).

LasB and LasA reduce lung function by degrading host proteins (Kessler et al., 1998; Liao et al., 2022). LasA is capable of degrading host tissues' proteins contributing to tissue damage and consequent

impairment of lung function (Liao et al., 2022). LasB is capable of degrading the elastin of the host's lungs' tissue, contributing to the decrease of lung function, compromising the integrity of the endothelial barrier and causing the rupture of epithelial tight-junctions and pulmonary haemorrhages (Faure et al., 2018; Alfiniyah et al., 2019). The virulence of this protease is also marked by its capacity to degrade the surfactant proteins A (SP-A) and D (SP-D), which have anti-infectious properties contributing to the maintenance of the lung homeostasis and modulate the inflammatory response, as well as other host proteins as immunoglobulins and the cytokines IL-6, IL-2, TNF-α and IFN-γ (Jurado-Martín et al., 2021; Watson et al., 2021).

2.3.3 Siderophores

Iron is an essential component to the survival of all living organisms and humans are no exception to this, as iron is a major metal in the human body because of its role in various metabolic processes (Abbaspour et al., 2014). Despite this, this insoluble element can be cytotoxic if free. Therefore, iron is presented bound to metalloproteins (Abbaspour et al., 2014; Schalk & Perraud, 2023).

For *P. aeruginosa*, this element is crucial for growth. During chronic infections, *P. aeruginosa* competes against the host for iron by producing two different siderophores, pyoverdine and pyochelin. These siderophores are responsible for chelating irons, preferably Fe^{3+}, from the host and consequently, responsible for *P. aeruginosa* virulence (Sun et al., 2020).

Pyochelin produces hydroxyl radicals, promoting oxidative damage in host's tissues and consequently contributing to inflammation (Jeong et al., 2023). Pyoverdine can regulate the secretion of various toxins, for example, exotoxin A of *P. aeruginosa* (Kang et al., 2019; Jeong et al., 2023).

In a study carried out by Dumas et al, it was concluded that *P. aeruginosa* switches the production of these two siderophores according to the amount of iron available in the environment (Dumas et al., 2013). Pyoverdine is produced when there is limited iron, whereas, when there is a higher availability of iron, the production of pyochelin is preferred. This switching of siderophore produced shows advantages for the bacteria regarding the synthesis cost benefit, since pyoverdine has a higher molecular mass than pyochelin and requires a larger number of genes to be expressed for its synthesis.

2.3.4 Rhamnolipids

Rhamnolipids are extracellular biosurfactants that have been found in the sputum of CF patients infected with *P. aeruginosa* (Soberón-Chávez et al., 2005).

In the respiratory tract, these biosurfactants strongly contribute to *P. aeruginosa* infection because of their capacity to disrupt tight junctions, which affects the epithelial barrier. The epithelial barrier is responsible for denying invasive microorganisms access to the host airways, protecting the host from infections (Zulianello et al., 2006; Jurado-Martín et al., 2021; T. de Sousa et al., 2021). In addition, rhamnolipids solubilize lung surfactant and promote lung tissue degradation by phospholipase C (Soberón-Chávez et al., 2005; Alfiniyah et al., 2019; T. de Sousa et al., 2021).

Furthermore, rhamnolipids contribute to the formation and persistence of *P. aeruginosa* biofilms by promoting the lysis of monocyte-derived macrophages and polymorphonuclear leucocytes (PMNs) (Van Gennip et al., 2009; Yan et al., 2014).

2.3.5 Pyocyanin

Pyocyanin is a released product of *P. aeruginosa*. This blue secondary metabolite, with redox activity, is capable of increasing oxidative stress by increasing intracellular ROS and H_2O_2 and it is cytotoxic to host cells. In this way, pyocyanin contributes to impaired lung function and disease severity. In addition, this molecule damages various components of host cells, which leads to cell lysis (Jurado-Martín et al., 2021; Liao et al., 2022).

2.4 Quantification of Virulence Factors and Interleukins

2.4.1 Azocasein Assay for Proteolytic Activity Quantification

The azocasein assay was first proposed by Charney & Tomarelli, 1947.

The azocasein solution, when hydrolysed, releases a chromophoric group and a red-orange colour is originated from the solubilization of the chromophoric group in trichloroacetic acid (TCA) (Côlho et al., 2016). In the presence of TCA, the proteins that are present in the sample precipitate, including the azocasein. Therefore, the measured absorbance at 420 nm corresponds to the quantity of the protein azocasein digested (Kessler et al., 1993; Côlho et al., 2016).

2.4.2 Elastin-Congo Red Assay for Elastolytic Activity Quantification

The elastolytic activity of *P. aeruginosa* can be evaluated by the Elastin-Congo Red assay.

The presence of Elastase B in *P. aeruginosa* supernatant promotes the cleavage of the dye from the elastin-congo red substrate. The amount of released dye can be measured spectrophotometrically ay 492 nm to determine the quantity of Elastase B produced by *P. aeruginosa* (Cigana et al., 2021).

2.4.3 Pyoverdine Quantification

The spectrophotometric quantification of pyoverdine was firstly reported by Meyer, 1978.

The absorbance of pyoverdine can be measured at 230 nm or 400 nm and detected directly in bacterial cultures (Filloux & Ramos, 2014).

2.4.4 Methylene Blue Agar Plates Method for Rhamnolipid Quantification

The methylene blue agar plates are agar plates with methylene blue (MB) and cetyl trimethylammonium bromide (CTAB) that can be used to visualize and measure rhamnolipid production. This method was firstly introduced by Siegmund & Wagner, 1991.

The Methylene Blue Agar Plates Method is a semi-quantitative method that relies on the complexation between rhamnolipids, MB and CTAB. Rhamnolipid producers can be identified on the plates by the formation of a dark blue halo around the colony; rhamnolipid production can be semi-quantified by measuring the area of the dark blue halo (Siegmund & Wagner, 1991; Pinzon & Ju, 2009). For this method the surfactant Tween-80 can be used as a positive control.

2.4.5 Chloroform-HCl Extraction for Pyocyanin Quantification

Pyocyanin can be quantified by performing the Chloroform-HCl extraction as described previously by (Kurachi', 1958). In this method, pyocyanin is extracted with chloroform. The organic phase, containing chloroform and pyocyanin is separated from the aqueous phase. Then, the organic phase is centrifuged with HCl and the aqueous layer, containing pyocyanin develops a pink color. (Nawas, 2018; Shouman et al., 2023).

It was firstly discovered by (Sheldon Wolff & Von Borstel, 1954) that the quantity of pyocyanin production can be determined by measuring this pink layer spectrophotometrically at 520 nm.

2.4.6 Enzyme-linked Immunosorbent Assay for Interleukin Quantification

The ELISA was originally proposed by Engvall & Perlmann, 1971. ELISA is a quantitative method that determines the concentration of proteins or peptides in biological fluids.

There are four types of ELISA: direct, indirect, sandwich and competitive ELISA, with sandwich ELISA being the most sensitive method (Alhajj, Zubair, & Farhana., 2022). This type of ELISA detects the desired antigen which is immobilized between two antibodies. The method starts with the wells of a microtiter plate being coated with a capture antibody, targeting the protein of interest. Afterwards, the microtiter wells are blocked to prevent non-specific binding of the detection antibodies to the plate surface itself. During the next step, the samples are added, which contain the protein of interest, i.e., the antigen, to the capture antibody. An enzyme-labelled antibody is added to colour development (**Figure 6**). The absorbance in the wells can be obtain by subtracting the absorbance of the background at 570 nm from the absorbance at 450 nm (Sakamoto et al., 2018; BioLengend, 2022).

There are two more alternatives for the sandwich ELISA method. The indirect sandwich ELISA, instead of the enzyme-labelled antibody, uses a primary unlabelled antibody and a second enzyme-labelled antibody (Creative Biolabs, 2024). The sandwich ELISA with Avidin-biotin detection uses a biotin-labelled detection antibody. Hence, this method requires the use of avidin-horseradish peroxidase (HRP), which has a high affinity to biotin (Thermo Fisher Scientific, 2009). In the avidin-HRP conjugate, the avidin protein is conjugated to the enzyme HRP, that provides the enzyme activity needed for detection, in the presence of the TMB-substrate (Thermo Fisher Scientific, 2009). This substrate contains 3,3´,5,5´-tetramentylbenzidine (TMB), a substrate for the HRP. When this product is oxidised by HRP it develops a blue colour that when adding sulfuric acid, changes the colour to yellow, which is spectrophotometrically measured at 450 nm (Avantor, 2024).

In this thesis, the sandwich ELISA with Avidin-biotin detection was used.

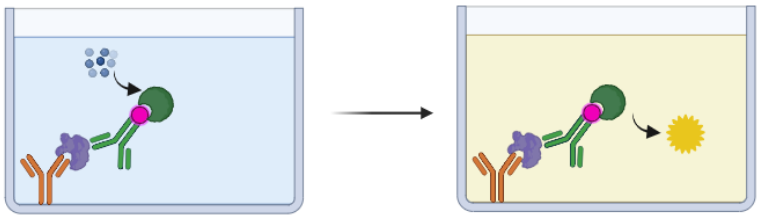

Figure 6: Sandwich ELISA method. The antigen binds to the immobilized antibody. A biotin-labelled detection antibody is added and an antibody-antigen-antibody "sandwich" is formed. The Avidin-HRP and TMB substrate are added next and a blue colour is produced in proportion to the concentration of antigen present. To terminate the reaction, sulfuric acid is added and the reaction colour changes from blue to yellow (BioLengend, 2022).

3. MATERIALS AND METHODS

3.1 Solutions and Growth Media

3.1.1 Milli-Q Water

Milli-Q (MQ) water is obtained by performing multiple filtration and ionization steps starting from tap water. For these steps, a water purification system was used: Milli-Q Reference System (Merk - Millipore, Burlington). The MQ water was then autoclaved for 20 minutes at 121 °C.

3.1.2 Physiological Saline

0.9% physiological saline is obtained by dissolving 9 g of sodium chloride (NaCl) (Chem-lab Zedelgem, Belgium) in 1 L of MQ water. The solution was sterilized by autoclaving for 20 minutes at 121 °C.

3.1.3 Luria Bertani broth

Luria Bertani broth (LB) is a widely used medium for bacterial growth (Bertani, 1951). LB was prepared by dissolving 25 g of LB powder (Neogen Company - Heywood, UK) in 1 L of MQ water. Sterile LB was obtained by autoclaving for 20 minutes at 121 °C.

3.1.4 Trypsone Soy Agar

Trypsone Soy Agar (TSA) is a multi-purpose agar that allows the growth of a wide range of microorganisms (Neogen Culture Media, 2022). TSA medium was prepared by dissolving 37 g of TSA powder (Neogen Cultute Media - Heywood, UK) in 1 L of MQ water. The solution was then sterilized by autoclaving for 20 minutes at 121 °C. The TSA medium was poured into sterile petri dishes and left to solidify before use.

3.1.5 Synthetic Cystic Fibrosis Sputum Medium 2

SCFM2 is a relevant *in vitro* model to study *P. aeruginosa* CF infection that mimics the chemistry and viscosity of authentic CF sputum (Turner et al., 2015; Cornforth et al., 2020). This medium is complemented with several substances, similar to those known to be present in CF sputum, such as, N-acetyl glucosamine (GlcNAc), mucin, (salmon sperm) DNA and DOPC (Turner et al., 2015).

For the buffer base, the first step was to weigh 0.145314 g of tyrosine powder (N. V. Mettler - Toledo S.A., Switzerland) and dissolve in 8 mL of filter sterilized 1M sodium hydroxide (NaOH) (Sigma-Aldrich - Saint Louis, UK). Next, 8 mL of MQ water was added along with 0.110074 g of asparagine. An additional 16 mL of MQ water was added followed by 0,002655 g of tryptophan. The next step was to weigh the rest of the buffer components, present in **Table 1**, and dissolve them in 400 mL of MQ water. Subsequently, the amino acids present in **Table 2**, were weighed and dissolved. The final volume was adjusted to 800 mL with MQ water using a graduate cylinder and the solution was sterilized by filtering with a vacuum filter (0.22 μm).

Table 1: Buffer components required for 800 mL of Buffered Base.

Component	Concentration (mg/mL)
NaH_2PO_4	0.2242
Na_2HPO_4	0.2218
KNO_3	0.04398
K_2SO_4	0.05903
NH_4Cl	0.1525
KCl	1.3925
NaCl	3.7875
MOPS	2.6158

Table 2: Amino acids required for 800 mL of Buffered Base.

Amino acid	Concentration (mg/mL)
Serine	0.1900
Glutamic acid hydrochloride	0.3555
Proline	0.2390
Glycine	0.1129
Alanine	0.1982
Valine	0.1636
Methionine	0.1181
Isoleucine	0.1838
Leucine	0.2638
Ornithine hydrochloride	0.1425
Lysine hydrochloride	0.4857
Arginine hydrochloride	0.0806
Threonine	0.1596
Cysteine hydrochloride	0.0315
Phenylalanine	0.1094
Histidine hydrochloride monohydrate	0.1360

Afterwards, 1 g of mucin (Sigma-Aldrich - Saint Louis, UK) was weighed and dissolved in 40 mL of MQ. A stir bar was added. Then, the mucin was autoclaved for 20 minutes at 121 °C. Next, 160 mL of the buffered base along with 120 mg of salmon sperm DNA (Sigma-Aldrich - Saint Louis, UK) were added and dissolved using a stirrer.

Following that, the stock solutions were prepared by weighing the components listed in **Table 3** and dissolve in the correspondent volume of MQ water. All the stock solutions were sterilized using a syringe filter. The stock solutions were then added to the mucin in the correspondent concentrations listed in **Table 4**.

Table 3: Preparation of the stock solutions.

Component	Concentration (g/mL)
Dextrose (D-glucose)	0.18
Calcium chloridde dihydrate ($CaCl_2$ * $2H_2O$)	0.147
Magnesium chloride hexahydrate ($MgCl_2$ * 6 H_2O)	0.203
Iron sulfate heptahydrate ($FeSO_4$ * 7 H_4O)	0.001
N-acetylglucosamine	0.055
L-Lactic acid 85% solution	0.106

Table 4: Final concentration of the stock solutions.

Amino acid	Concentration (mg/mL)
Dextrose (D-glucose)	2.7
L-lactic acid	4.93
Calcium chloridde dihydrate ($CaCl_2$ * $2H_2O$)	6.84
Magnesium chloride hexahydrate ($MgCl_2$ * 6 H_2O)	1.79
Iron sulfate heptahydrate ($FeSO_4$ * 7 H_4O)	0.003045
N-acetylglucosamine	0.3316

The next step was to add the 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids - Alabama, UK) to the mixture. DOPC was freshly prepared because of the evaporation of the chloroform over time. In the fume hood, 80 µL of DOPC was added to the mixture.

After addition of the DOPC, the medium was allowed to be stirred in the fume hood for 1 hour to evaporate the toxic solvent from the medium.

Finally, the pH of the final medium was adjusted to 6,8 with 1M NaOH and using a pH meter.

3.1.6 ELISA Reagents

3.1.6.1 Coating Buffer

Coating buffer was prepared by dissolving 8.4 g of sodium hydrogen carbonate ($NaHCO_3$) (Sigma-Aldrich, USA) and 3.56 g sodium carbonate (Na_2CO_3) (Fluka Chemicals) in 1 L of MQ water. Next, the

solution was filtered with a 0.2 µm pore size filter (Merck KGaA, Darmstadt, Germany). Finally, the pH was adjusted to 9,5.

3.1.6.2 Assay Diluent

Assay diluent was used to block non-specific binding. This solution was also used to dilute the Detection Antibody (200X) (BioLegend, USA) and the HRP (200X) (BioLegend, USA) (BioLengend, 2022).

The assay diluent was prepared by dissolving 1 g of bovine serum albumin (BSA) in 1 L of phosphate-buffered saline (PBS) (Sigma-Aldrich - Saint Louis, UK). The solution was then vacuum filtered with a 0.2 µm pore size filter.

3.1.6.3 Washing Buffer

Washing buffer was made by adding 0.05% non-ionic detergent Tween-20 (Sigma-Aldrich, France) to 1 L of PBS (BioLengend, 2022).

3.1.6.4 TMB Substrate Solution

The TMB solution (BioLegend, USA) generates a blue colour in the presence of HRP. Prior to performing the ELISA, the TMB Substrate working solution was made by mixing 6 mL of TMB substrate A with 6 mL of TMB substrate B. The blue product can be measured at 650 nm (AAT Bioquest, 2014).

3.1.6.5 Stop Solution

The stop solution is a 2N sulphuric acid (H_2SO_4) solution. This solution terminates the oxidation of the TMB substrate. The addition of the stop solution changes the reaction colour to yellow, which is measurable at a wavelength of 450 nm (Avantor, 2024).

3.1.7 Preparation of Methylene Blue Agar Plates

To prepare the methylene blue agar plates 0.2 g of hexadecyltrimethylammonium bromide (CTA B) was weighed and dissolved in 100 mL of MQ water. Subsequently, the components listed in **Table 5** were weighed and dissolved in 6 L of MQ water, to make 6 L of medium. Next, 20 mL of glycerol was added. After that, the pH of the medium was adjusted to 6.9 using 0.5 M HCl.

After pH adjustment, 15 g of bacteriological agar (Neogen Culture Media, UK) and 0.05 g of methylene blue powder (VWR International PROLAB) was added to the mixture. The medium was then autoclaved for 20 minutes at 121 °C. Finally, 25 mL of medium was poured into sterile petri dishes and was left to dry in the Laminar air flow (LAF) cabinet.

Table 5: Components for the methylene blue agar medium per liter.

Component	Concentration (g/L)
$Na_2HPO_4 7H_2O$	2.1333
KH_2PO_4	0.5000
NaCl	0.0833
$MgSO_4$	0.0401
KNO_3	0.0843

3.2 Collection of Patient Samples and *P. aeruginosa* Isolates

The collection of patient samples and *P. aeruginosa* isolates was performed by a former PhD student at the Laboratory of Pharmaceutical Microbiology (LPM), Ghent University. Sputum samples of 50 patients with CF, infected with *P. aeruginosa* and treated at the Ghent University Hospital, were collected through spontaneous expectoration. The samples were then liquefied with sputasol and stored at -20 °C. The isolates were recovered by performing standard protocols, such as plating on McConkey agar and storage at -20 °C. At the moment, this collection consists of more than 100 isolates with, at least 20 *P. aeruginosa* isolates recovered from 5 of the sputum samples (Wauters et al., 2023).

The *P. aeruginosa* clinical isolates studied in this thesis and the label used are listed in **Table 6**.

Table 6: *P. aeruginosa* clinical isolates studied in this thesis.

Patient	Clinical Isolate	Label
BS1	6	6/1
	9	9/1
	12	12/1
	14	14/1
	15	15/1
	16	16/1
	19	19/1
BS6	2	2/6
	6	6/6
	7	7/6
	9	9/6
	12	12/6
	13	13/6
	14	14/6

In addition to the clinical isolates, the *P. aeruginosa* strains PAO1 and AMT2330 were also used for this thesis as control strains in the performed assays. The strain AMT2330 was used as a positive control for the quantification of pyocyanin. In addition, the high inflammatory strain AA44 was included in this study (Wauters et all, 2023).

3.3 General Techniques

3.3.1 Stock cultures

Stock cultures of each *P. aeruginosa* isolate were prepared using Microbank™ cryovials (Pro-Lab Diagnostics, Ontaria, Canada). Each cryovial contains a cryopreservative and approximately 25 sterile beads, to which bacteria can adhere. Using a sterile micro-loop, bacterial colonies from a pure culture were transferred into the cryovial. Next, the cryopreservative fluid was removed using a sterile pipette and the cryovials were stored at -80 °C.

3.3.2 Pure cultures

Fresh pure cultures of each *P. aeruginosa* isolate were prepared every week.

One bead from the stock culture cryovial was transferred to a sterile TSA plate using a sterile micro-loop. Firstly, the bead was spread out on one-third of the TSA plate. Then, parallel lines were drawn. Next, the TSA plate was rotated to streak parallel lines in another direction (**Figure 7**). The pure cultures were incubated under aerophilic conditions at 37 °C.

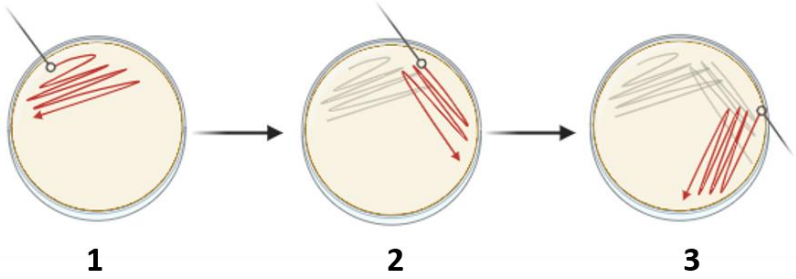

Figure 7: Preparation of pure cultures.

3.3.3 Overnight culture

Overnight cultures of each *P. aeruginosa* isolate were prepared in 24-well plates (Bioswisstec, Schaffhausen, Switzerland) by inoculating one bacterial colony from a pure culture, using a sterile micro-loop, in 1 mL of LB medium. A blank of 1 mL of LB medium was also added to the well plate. To prevent evaporation, the exterior wells were filled with 1 mL of MQ water. Subsequently, the overnight cultures were incubated aerophilically at 37 °C under shaking conditions (250 rotations per minute (rpm)).

3.4 Incubation of Conditioned Medium

For the incubation of conditioned medium (CM), the first step was to measure the optical density (OD) of the overnight culture at a wavelength of 590 nm, using the Envision plate reader (PerkinElmer - Waltham, USA). Secondly, a bacterial suspension with a concentration of 5×10^7 colony-forming units (CFU)/mL was prepared by adding the required volume of bacterial suspension (V) to 500 µL of SCFM2. Previously, it was determined that a concentration of 5×10^7 CFU/mL corresponds to an optical density of 0,005. The required volume was calculated using the **Equation 3-1**.

$$500\ \mu L\ .\ OD_{0{,}005} = V.\ (OD_{590\ nm} - OD_{blank})$$

Equation 3-1

Subsequently, the CM plate was prepared by filling the interior wells of a 48-well plate (Cellstar Cell Culture Plate, Greiner Bio-One) with 1 mL of SCFM2 and the exterior wells with 1 mL of MQ water, to prevent evaporation. The medium control (MC), 1 mL of SCFM2, was also included in the plate. Next, 10 µL of the 5×10^7CFU/mL bacterial suspension was added to 1 mL of SCFM2, making a 1:100 dilution of the initial bacterial suspension. The CM plate was incubated for 48 hours under stationary microaerophilic conditions (5% CO_2, 3% O_2) at 37 °C (Elscolab - Kruibeke, Belgium).

3.5 Preparation of Supernatants

After 48 h of incubation, the content of the CM plate was centrifuged for 10 minutes at 3700 rpm. The *P. aeruginosa* supernatant was collected and filtered by a 0.22 µm pore size filter in order to obtain cell-free supernatant (**Figure 8**). The MC was also filtered as described.

The filtered supernatant was used for the quantification of virulence factors and for the interleukin degradation assays.

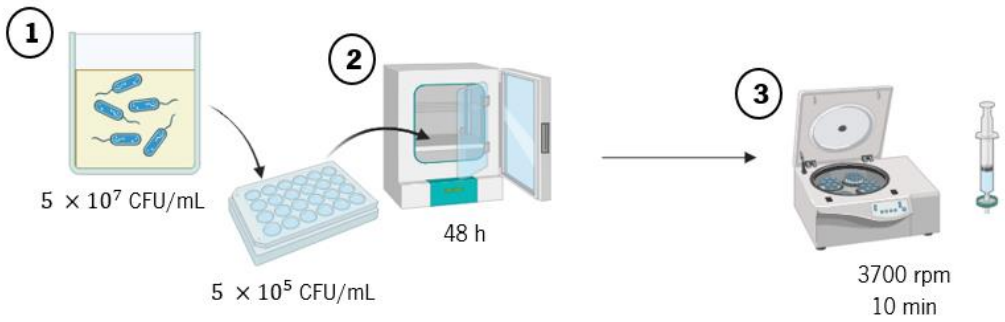

Figure 8: Workflow of incubation of CM and preparation of *P. aeruginosa* supernatants for quantification of virulence factors and interleukin degradation assays. 1) Preparation of the CM plate. 2) Incubation in microaerophilic conditions for 48 h. 3) Collection of supernatants by centrifugation and filtering.

3.5.1 Microdilutions

The microdilution plating method was performed to assess the bacterial growth of the 5×10^5 CFU/mL bacterial suspension, i.e., starting conditions, and of the bacterial culture after 48 h of incubation in the CM.

For the starting conditions, the microdilutions were prepared by filling the rows B to H of a 96-well plate (SPL Life Sciences, Pocheon-si, Korea) with 180 µL of physiological saline. Next, 200 µL of the 5×10^7 CFU/mL bacterial suspension was transferred to the first row (row A) of the plate. The dilutions were made by transferring, after homogenization, 20 µL of the previous row to the following row of the plate (i.e. 20 µL of row A was transferred to row B) until a 10^{-7} dilution was reached. Subsequently, 10 µL of the dilutions (10^0 to 10^{-7}) were plated in triplicate onto a TSA agar plate (**Figure 9**). The plates were incubated overnight at 37 °C, under aerophilic conditions, until small and separate colonies could be counted.

For the microdilutions after 48 h of incubation in the CM, the same volume (800 µL) of supernatant removed from the CM plate was replenished with physiological saline. The plate was covered and placed on the shaker for 10 minutes at 500 rpm in order to resuspend the bacterial culture. Following that, all the rows (rows A to H) of a 96-well plate were filled with 180 µL of physiological saline using a Handystep. Next, 20 µL of the resuspended bacterial culture were transferred to the first row of the plate (row A).

The dilutions were performed as described previously until a 10^{-8} dilution was reached. Subsequently, 10 µL of the dilutions (10^{-1} to 10^{-8}) were plated in triplicate onto a TSA agar plate (**Figure 10**). The plates were incubated overnight at 37 °C, under aerophilic conditions, until small and separate colonies could be counted.

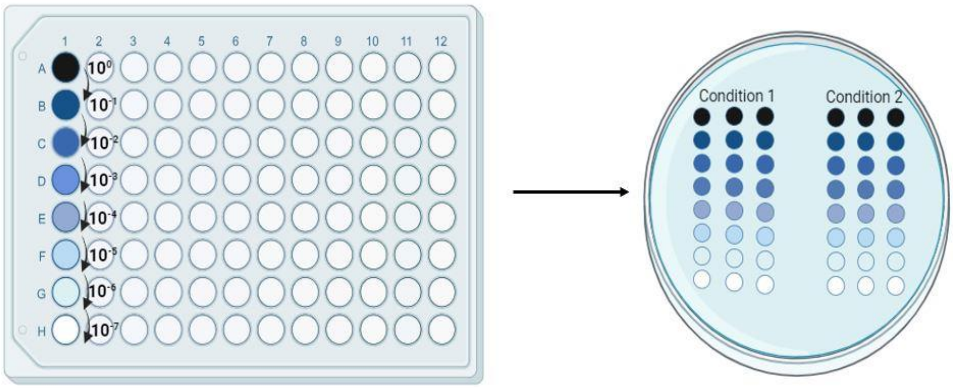

Figure 9: Microdilution plating of starting conditions.

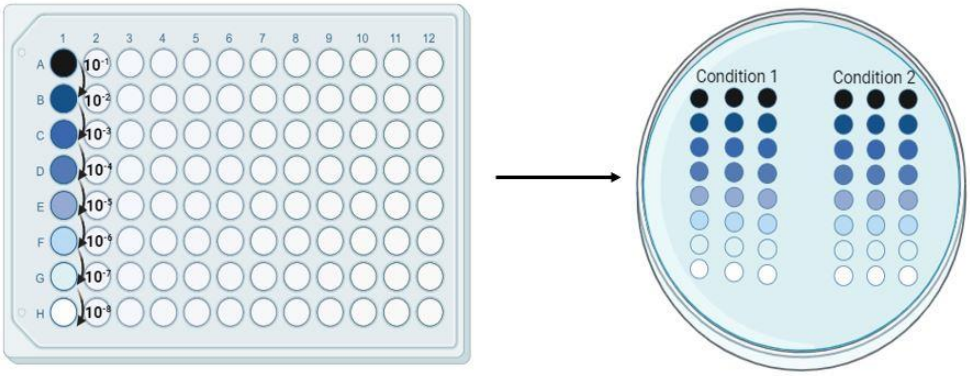

Figure 10: Microdilution plating after incubation of conditioned medium.

3.6 Quantification of Virulence Factors in Cell-free Supernatants of *Pseudomonas aeruginosa*

The first step for the quantification of virulence factors was to collect the *P. aeruginosa* supernatants as described in section 3.5.

For all quantification assays, filtered MC supernatant was included as a negative control and PAO1 supernatant as a positive control.

3.6.1 Azocasein Assay

The proteolytic activity was quantified by performing the azocasein assay (Charney & Tomarelli, 1947).

The first step was to prepare a fresh azocasein (Sigma Aldrich, St Louis, USA) solution (5 mg/mL in 0.1 M Tris-HCl buffer, pH=8) (Sigma-Aldrich). 400 µL of *P. aeruginosa* supernatant was mixed with 400 µL of azocasein solution in an Eppendorf tube (Greenpia Technology, Gyonggido, Korea). For each condition, two technical replicates were made. After incubating the samples for 1 hour at 37 °C under shaking (250 rpm), the protease reaction was stopped by the addition of 100 µL 10% (w/v) TCA solution. Next, the samples were centrifuged for 15 minutes at 13000 rpm. Finally, 100 µL of 625mM NaOH was mixed with 100 µL of supernatant in a 96-well plate in duplicate and the OD was measured at a wavelength of 420 nm using the Envision reader (**Figure 11**).

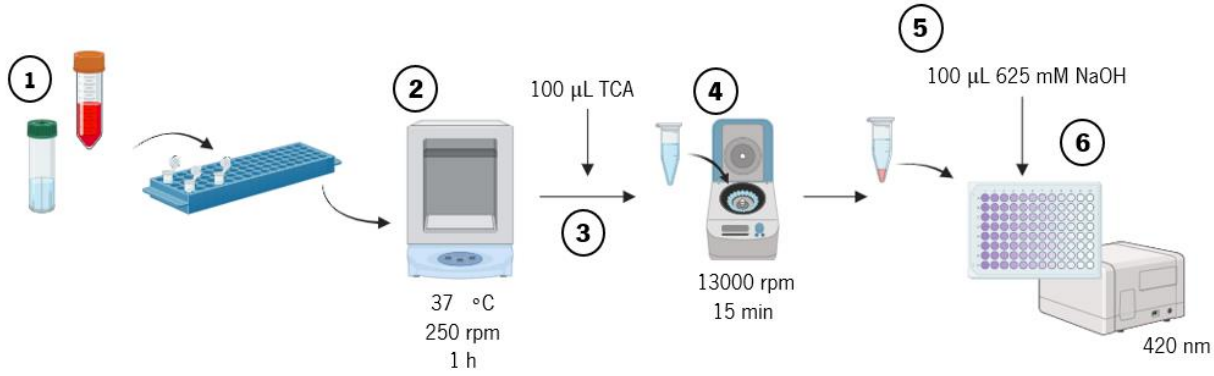

Figure 11: Workflow of the azocasein assay. 1) Mixing the *P. aeruginosa* supernatant with the azocasein solution. 2) Incubation of the samples. 3) Addition of TCA. 4) Centrifugation of the samples. 5) Addition of 625mM NaOH. 6) Measurement of the OD of the samples at 420 nm.

3.6.2 Elastin-Congo Red Assay

For the quantification of the elastolytic activity, the Elastin-Congo Red assay was performed (Cigana et al., 2021).

Initially, an Elastin-Congo Red (Elastin Products Co., Owensville, Missouri) suspension was prepared (20 mg/mL in 0,1 M Tris-HCl buffer, pH=7,6) (Sigma Aldrich). Next, 400 µL of *P. aeruginosa* supernatant was mixed with 400 µL of Elastin-Congo Red suspension in an Eppendorf tube. Two technical replicates for each condition were made. The samples were then incubated for 24 hours at 37 °C under shaking (250 rpm). The next step was to centrifuge the samples for 15 minutes at 13000 rpm. Subsequently, 200 µL of supernatant was transferred to a 96-well plate in duplicates and the OD was measured at 492 nm (**Figure 12**).

To prevent oversaturation of the signal, the samples were diluted 1:10 in MQ water when necessary.

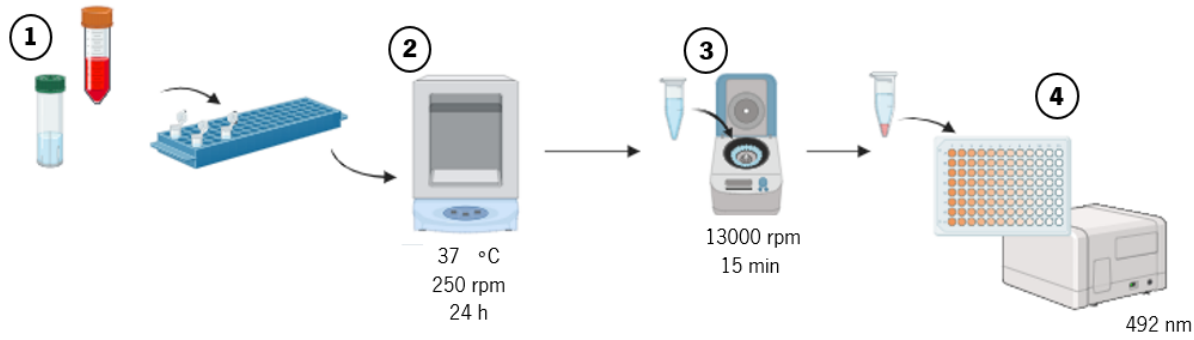

Figure 12: Workflow of the Elastin-Congo Red assay. 1) Mixing the *P. aeruginosa* supernatant with the Elastin-Congo Red suspension. 2) Incubation of the samples. 3) Centrifugation of the samples. 4) Measurement of the OD of the samples at 492 nm.

3.6.3 Pyoverdine Assay

To quantify pyoverdine, 200 µL of filtered *P. aeruginosa* supernatant was transferred to a 96-well plate in duplicate and the OD was measured at 400 nm using the Victor Nivo (Perkin Elmer, Waltham, USA) (Meyer, 1978).

3.6.4 Methylene Blue Agar Method

The production of rhamnolipids was evaluated by using Methylene Blue Agar Plates Method (Siegmund & Wagner, 1991).

After preparing the methylene blue agar plates as described in Section 3.1.7, using a sterile 1000 µL pipette tip, a hole in the center of the plates was made. Then, 100 µL of 5×10^7 CFU/mL bacterial culture was transferred into the hole in the plate. The plates were incubated for 48 hours, at 37 °C, under microaerophilic conditions (**Figure 13**).

In addition to the MC negative control, the surfactant Tween-20 was used as a positive control.

The semi-quantification of rhamnolipids was determined by measuring the zone of the solubilized blue in the plates. (**Figure 14**).

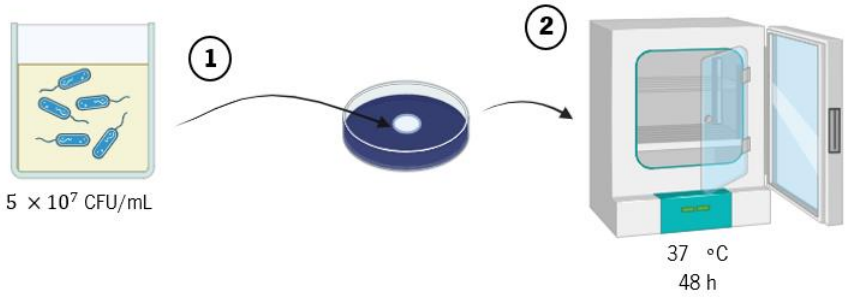

Figure 13: Workflow of the methylene blue agar plates assay. 1) Plating the 5×10^7 CFU/mL bacterial culture. 2) Incubation of the plates for 48h under microaerophilic conditions.

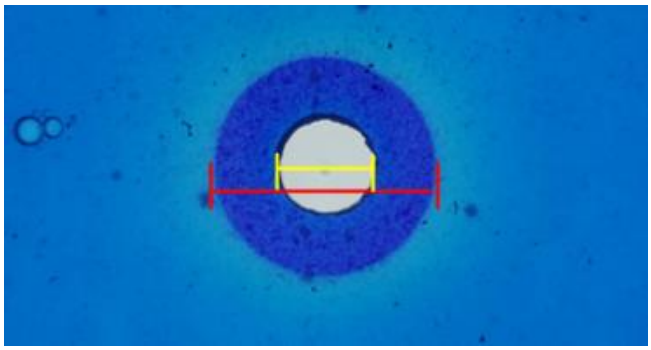

Figure 14: Measurement of the solubilized blue zone in the methylene blue plates. The diameter of the halo (yellow) was subtracted to the solubilized zone (red).

3.6.5 Chloroform-HCl Extraction

For the quantification of pyocyanin, the Chloroform-HCl extraction was performed (Kurachi', 1958).

Firstly, 1.5 mL of *P. aeruginosa* supernatant was mixed with 1.5 mL of chloroform in Falcon tubes (nerbe plus GmbH & Co. KG, Winsen/Luhe, Germany), under the fume hood. The tubes were vortexed vigorously for 1 minute. Next, the tubes were centrifuged for 10 minutes at 5000 rpm to separate the aqueous and organic phase. Subsequently, 1 mL of the chloroform layer was transferred to a new Falcon tube and 1 mL of 0.2N HCl (Sigma Aldrich) was added. The tubes were again vortexed for 1 minute. The next step was to centrifuge the tubes for 10 minutes at 5000 rpm. Finally, 300 µL of the aqueous fraction were transferred to a 96-well plate in duplicate and the OD was measured at 530 nm (**Figure 15**).

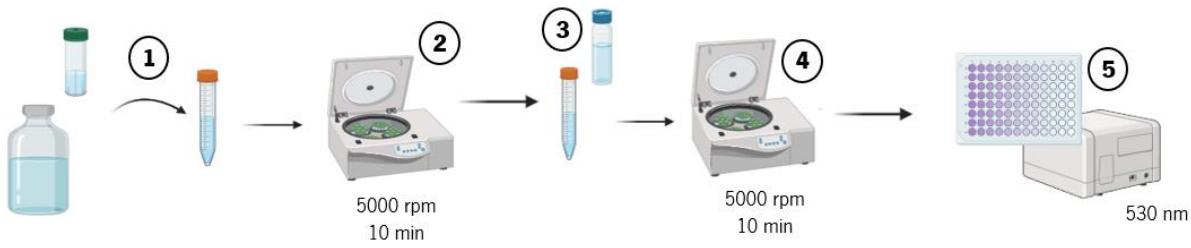

Figure 15: Workflow of the Chloroform-HCl extraction assay. 1) Mixing *P. aeruginosa* supernatant with chloroform. 2) Centrifuge the samples. 3) Transfer the chloroform layer. 4) Centrifuge the samples. 5) Measurement of the OD at 530 nm.

3.7 Interleukins Degradation Assay

In order to evaluate the degradation of IL-8 and IL-1β by supernatants of *P. aeruginosa* clinical isolates, a degradation assay for each interleukin was performed (Wauters et al., 2023).

3.7.1 IL-8 Degradation Assay

First, 200 µL of *P. aeruginosa* supernatant was transferred from the centrifuged CM plate to a 96-well filter plate in duplicate. The filter plate was centrifuged for 10 minutes at 3700 rpm, in order to obtain filtered supernatant. Subsequently, 100 µL of filtered supernatant was transferred to a new 96-well plate. Following that, 100 µL of a 1 ng/mL IL-8 solution was added. This 1 ng/mL IL-8 solution was obtained by performing a 10-fold dilution of a 10 ng/mL IL-8 stock solution with MQ water.

To prepare the 10 ng/mL IL-8 stock solution, the lyophilized Human IL-8 standard (BioLegend, USA) was reconstituted by adding 1.84 mL of MQ water to 18.4 ng of IL-8 standard.

Next, the plate was incubated for 4 hours at 37 °C under microaerophilic conditions. Afterwards, 2 µL of Halt™ Protease Inhibitor Cocktail (100X) (Thermo Scientific, USA) and 2 µL of 0.5M Ethylenediaminetetraacetic acid solution (EDTA) (100X) (Thermo Scientific, USA) were added to the wells. The Halt™ Protease Inhibitor Cocktail inhibits a wide range of proteases and the EDTA inhibits metalloproteases (Thermo ScientificTM, 2014). The plate was then wrapped in parafilm and stored in the -80° C freezer to perform the ELISA assay in the next day (**Figure 16**). For this assay, 200 µL of MQ water was included as a negative control and 100 µL of 1 ng/mL IL-8 solution mixed with 100 µL of MQ water as a positive control.

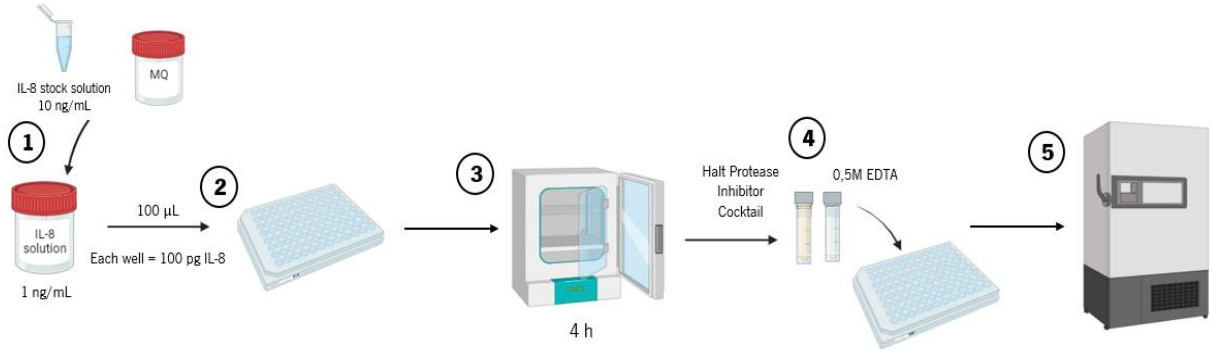

Figure 16: Workflow of the IL-8 degradation assay. 1) Dilution of the IL-8 10x stock solution. 2) Mixing IL-8 solution with *P. aeruginosa* supernatant. 3) Incubation for 4 hours in microaerophilic conditions. 4) Addition of Halt™ Protease Inhibitor Cocktail (100X) and EDTA (100X). 5) Storage in -80°C.

3.7.2 IL-1β Degradation Assay

Initially, *P. aeruginosa* supernatant was collected as described in section 3.7.1 and transferred to a new 96-well plate. Next, 100 µL of a 1 ng/mL IL-1β solution was added to the filtered supernatant.

The 1 ng/mL IL-1β solution was prepared by diluting a 10 ng/mL IL-1β stock solution. The 10 ng/mL IL-1β stock solution was made by reconstituting the lyophilized Human IL-1β standard (BioLegend, USA) by adding 2.52 mL of MQ water to 25.2 ng of IL-1β standard.

After 4 hours of incubation at 37 °C under microaerophilic conditions, 2 µL of Halt™ Protease Inhibitor Cocktail (100X) and 2 µL of 0,5M EDTA (100X) were added. 200 µL of MQ water was included as a negative control and 100 µL of 1 ng/mL IL-1β solution mixed with 100 µL of MQ water as a positive control. Finally, the plate was wrapped in parafilm and stored in the -80 °C freezer (**Figure 17**).

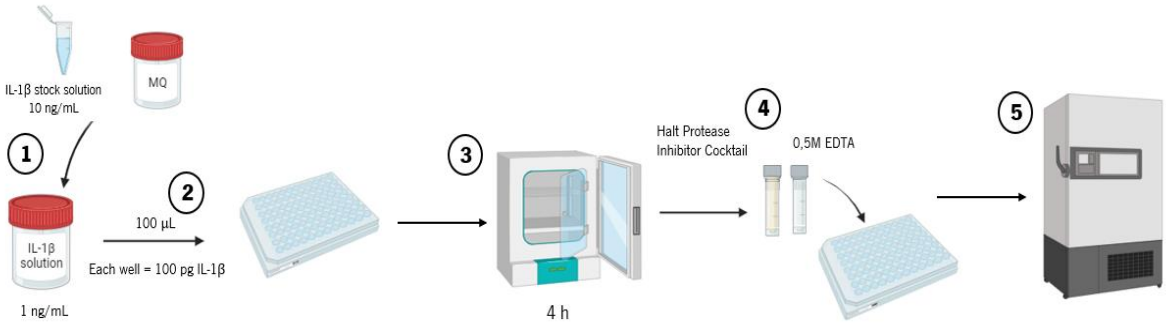

Figure 17: Workflow of the IL-1β degradation assay. 1) Dilution for the IL-1β stock solution. 2) Mixing IL-1β solution with *P. aeruginosa* supernatant. 3) Incubation for 4 hours in microaerophilic conditions. 4) Addition of Halt™ Protease Inhibitor Cocktail (100X) and EDTA (100X). 5) Storage in -80° C.

3.8 ELISA

The degradation of the interleukins IL-8 and IL-1β by *P. aeruginosa* supernatant was quantified by performing a Sandwich ELISA assay.

3.8.1 IL-8 ELISA

For the detection of IL-8, the Human IL-8 ELISA Max™ Standard Set (BioLegend, USA) was used.

One day prior to performing the ELISA, a high binding 96-well plate (Greiner bio one - Kremsmünster, Austria) was coated with 100 µL of capture antibody solution. 60 µL of capture antibody (200X) was diluted in 12 mL of coating buffer. The plate was then sealed and incubated overnight at 8 °C to immobilize the IL-8 capture antibodies on the surface of the plate. The next day, the first step was to wash the plate 4 times with 200 µL of wash buffer. All the next washing steps were performed similarly. Next, to block non-specific binding, i.e, to avoid the occurrence of an antibody binding to a unintended protein, and reduce background, 200 µL of assay diluent was added to every well of the plate. Subsequently, the plate was sealed and incubated for 1 hour with shaking (500 rpm) at room temperature. The next step was to wash the plate and to add 100 µL of the samples from the IL-8 degradation assay and the IL-8 standards.

The IL-8 standards were prepared by performing six two-folds serial dilutions of the 1000 pg/mL top standard with assay diluent in separated Eppendorf tubes. Assay diluent serves as the zero standard (0 pg/mL).

After that, the plate was sealed and incubated for 2 hours with shaking. Next, the plate was washed and 100 µL of detection antibody solution were added to the plate. 60 µL of detection antibody (200X) was diluted in 12 mL of assay diluent. The plate was incubated for 1 hour with shaking. Then, after washing the plate, 100 µL of Avidin-HRP solution were added to the plate, followed by 30 minutes of incubation with shaking. 12 µL of Avidin-HRP (1000X) was diluted in 12 mL of assay diluent. The next step was to wash the plate 5 times with 200 µL wash buffer letting the wells soak in wash buffer for 30 seconds to 1 minute. Afterwards, 100 µL of TMB Substrate Solution was added to each well and the plate was incubated in the dark for 15 minutes until development of a blue color in the positive wells. Next, the reaction is stopped by adding 100 µL of stop solution to the plate. Finally, the OD was measured at a wavelength of 450 nm and 570 nm.

For data analysis, the absorbance at 570 nm (background) was subtracted from the absorbance at 450 nm. The IL-8 concentrations were calculated using the program GainData - arigo's ELISA calculator (arigo biolaboratories, 2020).

3.8.2 IL-1β ELISA

For the detection of IL-1β the Human IL1-β ELISA MAX™ Deluxe Set (BioLegend, USA) was used.

Initially, 100 µL of capture antibody was added to each well of a high binding 96-well plate (Greiner bio one - Kremsmünster, Austria). 60 µL of capture antibody (200X) was previously diluted in 12 mL of coating buffer A (1X). The coating buffer A (1X) was made by mixing 2,4 mL of coating buffer A (5X) with 9,6 mL of MQ water.The plate was then incubated overnight between 2 °C and 8 °C. In the next day, the plate was washed 4 times with 200 µL of wash buffer. The following washing steps were performed similarly. Next, 200 µL of blocking buffer was added to block non-specific binding and reduce background. After 1 hour of incubation at room temperature with shaking (500 rpm), 50 µL of assay buffer was added to the wells. Then, 50 µL of the samples from the IL-1β degradation assay were added to the plate, as well as 50 µL of the IL-1β standard concentrations. The samples were diluted 1:5 with sterile MQ water.

The standards were prepared by performing six two-folds serial dilutions of the 125 pg/mL top standard with assay diluent A (1X) in separate Eppendorfs tubes. Assay diluent A (1X) was made by

mixing 12 mL of assay diluent A (5X) with 48 mL of PBS. Assay diluent A (1X) serves as the zero standard (0 pg/mL).

Next, the plate was incubated for 2 hours with shaking. Afterwards, the plate was washed and 100 µL of detection antibody solution was added to each well. The plate was incubated for 1 hour with shaking. 60 µL of detection antibody (200X) was diluted with 12 mL of assay diluent A (1X). Following that, after washing the plate, 100 µL of Avidin-HRP solution was added to the plate. 12 µL of Avidin-HRP (1000X) was diluted in 12 mL of assay diluent A (1X). Next, the plate was incubated for 30 minutes with shaking.

The next step was to wash the plate 5 times with 200 µL of wash buffer and allow the wells to soak in wash buffer for 30 seconds to 1 minute. After, 100 µL of Substrate Solution F was added to each well and the plate was incubated for 15 minutes in the dark. The positive wells will turn blue in color. Subsequently, 100 µL of stop solution was added. The last step was to measure the OD at 450 nm and at 570 nm.

For data analysis, the absorbance at 570 nm was subtracted from the absorbance at 450 nm. The concentration of IL-1β was calculated using the website GainData - arigo's ELISA calculator (arigo biolaboratories, 2020).

3.9 Statistical Analysis

At least 4 biological replicates of each assay were performed. The SPSS Statistics version 27 (IMB, Armonk, US) was used for the statistical analysis.

The data was evaluated for normal distribution by performing the Shaphiro-Wilk test. In case of not normally distributed data, the non-parametric Mann-Whitney U test was performed to access the difference between the groups. In case of normally distributed data, the one-way ANOVA was performed. The Holm-Bonferroni correction for multiple testing was used. Statistically significant data when p-value<0,05.

4. Results and Discussion

4.1 Bacterial Growth

For the study of *P. aeruginosa* virulence in CF infections, it is highly important to mimic the authentic CF sputum. In the composition of CF sputum is airway mucus, inflammatory substances and bacterial products. This sputum is likely to be nutritional for *P. aeruginosa*, since they can grow to high densities, ranging between 10^7 to 10^9 CFU/mL, during CF lung infection (Turner et al., 2015).

In addition, Cornforth et al, compared several laboratory infection model systems, such as, an acute mouse pneumonia model, planktonic growth in morpholinepropanesulfonic acid (MOPS)-succinate and in lysogeny broth and growth in a CF airway epithelial cell model and SCFM2 (Cornforth et al., 2020). It was observed that in SCFM2 *P. aeruginosa* gene expression was most similar to gene expression in expectorated CF sputum.

Aiming to mimic the chronic infection in CF, *P. aeruginosa* clinical isolates were incubated in SCFM2 medium for 48 h in microaerophilic conditions, to mimic the low level of O_2 characteristic of the CF lung environment, as described in Section 3.4.

Microdilutions of both the starting (initial inoculum) and after 48 h of incubation in CM were performed to evaluate the growth of each isolate (**Figure 18**).

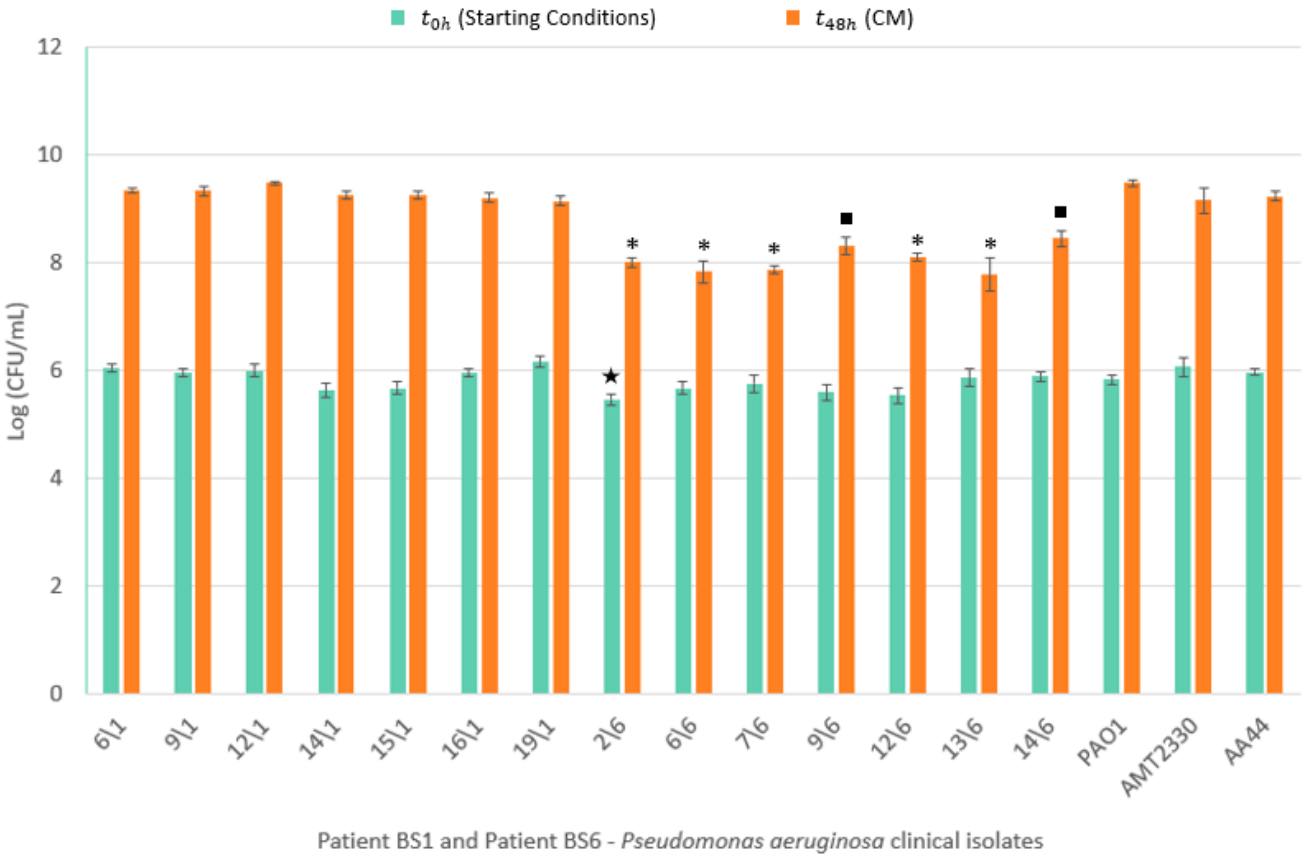

Figure 18: Number of culturable cells (measured as CFU/mL) of *P. aeruginosa* clinical isolates originating from patient BS1 and patient BS6, obtained after incubations at 37 °C in LB medium and 37 °C, 5% CO_2, 3% O_2 in SCFM2. PAO1 and AMT2330 were used as positive control for virulence factor quantification and pyocyanin quantification, respectively. AA44 was used as a positive control for the inflammatory experiment presented in Section 1.1. The green bars represent growth in the starting conditions and the orange bars represent growth after 48 h of incubation in CM. Error bars indicate the standard error. *p< 0,05: statistically significant comparing to isolates originating from patient BS1.★ p< 0,05: statistically significant comparing to isolates 9/6, 9/1 and 16/1. ■ p<0,05: statistically significant comparing to isolates 6/1, 9/1, 12/1, 14/1 and 15/1. (One-Way ANOVA with Holm-Bonferroni correction for multiple testing).

Bacterial numbers for the stating conditions are similar, however a slight variability can be observed. The isolate 2/6 originating from patient BS6 has statistically significant less growth than the isolates 9/6, 19/1 and 6/1.

After incubation in CM, it can be observed variability in growth. Isolates originating from patient BS6 show less growth comparing with isolates originating from patient BS1 and the control strains.

The control strains PAO1, AMT2330 and the pro-inflammatory strain AA44 grew to high densities in CM, similar to the clinical isolates, as expected.

4.2 Virulence Factor Quantification

The quantification of virulence factors was performed in the supernatant of *P. aeruginosa* clinical CF isolates with anti, pro and non-inflammatory properties, according to the inflammatory results in 3-D A549 cells presented in Section 1.1.

4.2.1 Pyoverdine

Pyoverdine production was assessed as described in Section 3.6.3.

Residual quantities of pyoverdine can be observed for the isolates originating from patient BS1 and for isolates 2/6 and 7/6. *P. aeruginosa* pro-inflammatory isolates originating from patient BS6 showed no production of pyoverdine. The control strain PAO1 showed statistically significant production of pyoverdine, comparing to the MC. (**Figure 19**). No statistical differences were observed between pyoverdine quantities from the *P. aeruginosa* clinical isolates and the MC. Low variability between patients was observed for pyoverdine production.

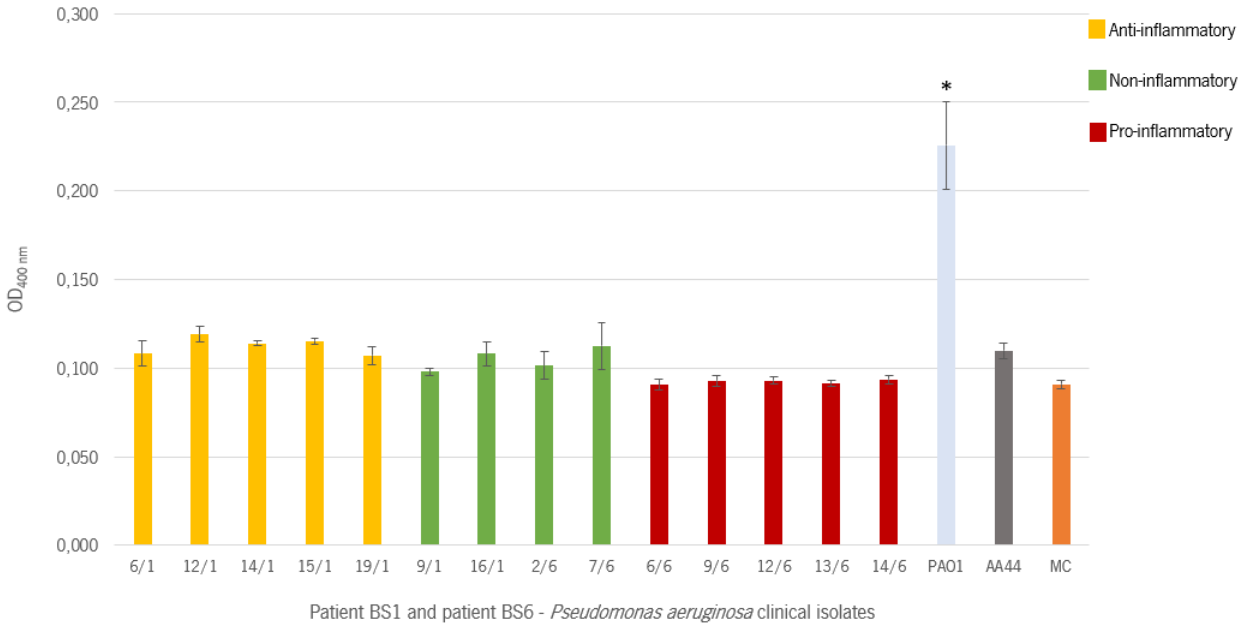

Figure 19: Pyoverdine production by *P. aeruginosa* clinical isolates assessed through the measurement of the OD at 400 nm of *P. aeruginosa* supernatants. Results are reported as average ± SE of at least four biological replicates. *p< 0,05 (One-Way ANOVA with Holm-Bonferroni correction for multiple testing). SE: Standard Error.

Low pyoverdine results were obtained by De Vos et al that concluded that *P. aeruginosa* pyoverdine-negative isolates accumulate in the CF sputum with colonization time, and are frequent among CF isolates (De Vos et al., 2001).

Another study reports that sputum samples from CF patients contain significant amounts of pyoverdine. However, the high mutation rate during the colonization of the CF lungs can lead to pyoverdine-deficient mutants that are able to obtain iron from other co-existing pyoverdine-producing strains (Visca et al., 2007).

Since no pyoverdine production was observed for the clinical isolates, it is hypothesized that these isolates are pyoverdine-deficient mutants.

Furthermore, it is suggested that the great iron availability in the CF lungs contributes to the permanent switch from pyoverdine producers to pyochelin producers, and therefore, *P. aeruginosa* avoids spending higher amounts of energy to synthetize pyoverdine, when the action of pyochelin is sufficient for iron acquisition (Dumas et al., 2013).

Moreover, it was reported by Visca et al that in low concentrations of O_2, the synthesis of the sigma factor protein PvdS, which activates the expression of pyoverdine synthesis genes, is reduced. (Visca et al., 2007). Therefore, pyoverdine production decreases under lower O_2 conditions. Since pyoverdine quantification was performed under microaerophilic conditions, the limited O_2 concentration could be another cause of the absence of pyoverdine production by these clinical isolates.

4.2.2 Pyocyanin

The quantification of pyocyanin was evaluated by performing the Chloroform-HCl extraction, as described in Section 3.6.5.

There are no statistically significant results for the production of pyocyanin by *P. aeruginosa* clinical isolates comparing to the MC (**Figure 20**). Only the positive control, strain AMT2330, showed significant pyocyanin production compared to the MC. There are no statistically significant differences between patients for the production of pyocyanin.

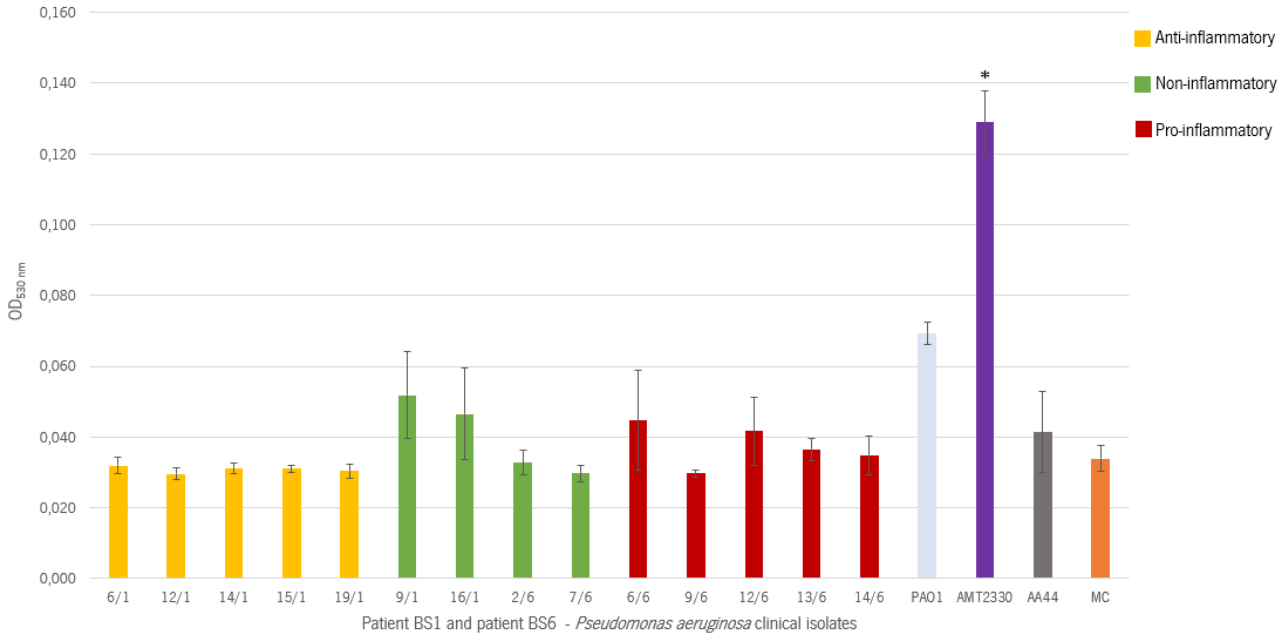

Figure 20: Pyocyanin production by *P. aeruginosa* clinical isolates analysed by Chloroform-HCl Extraction. Results are reported as average ± SE of at least four biological replicates. *p< 0,05 (One-Way ANOVA with Holm-Bonferroni correction for multiple testing). SE: Standard Error.

These results are in line with the findings of Forthergill et al who described that for 3 out of 6 CF patients, temporal loss of pyocyanin producing isolates was observed (Fothergill et al., 2007). In addition, 1 of the 6 patients, had no pyocyanin producers, with exception of one isolate. This indicates variability in the production of pyocyanin by *P. aeruginosa* CF isolates.

Furthermore, Papa et al reported that pyocyanin production is O_2 dependent. In this study, where the pyocyanin production by *P. aeruginosa* clinical CF isolates was evaluated in normoxia (20% O_2) and anoxia (0% O_2), it was observed that pyocyanin was produced in the presence of O_2. (Papa et al., 2024). Moreover, a higher quantity of pyocyanin was observed after 24 h of infection than 48 h of infection, concluding that the pyocyanin production decreases over time of infection. Additionally, they observed that *P. aeruginosa* wild-type strains produced more pyocyanin than *P. aeruginosa* clinical CF isolates.

The results for the pyocyanin quantification presented in this thesis are consistent with these findings, as little to no pyocyanin production was observed after 48 h of incubation under microaerophilic conditions.

4.2.3 Rhamnolipids

The production of rhamnolipids was assessed using Methylene Blue Agar Plates Method, as described in Section 3.6.4.

There is no production of rhamnolipids by clinical isolates originating from patient BS6 (**Figure 21**). Contrarily, rhamnolipid production by isolates originating from patient BS1 was observed, with only one isolate, i.e., 15/1 showing statistically significant rhamnolipid production comparing to the MC. The other isolates originating from patient BS1 were no statistically significant rhamnolipid producers. The surfactant Tween-80, used as a positive control and the strain PAO1 showed statistically significant rhamnolipid quantity, comparing to the MC.

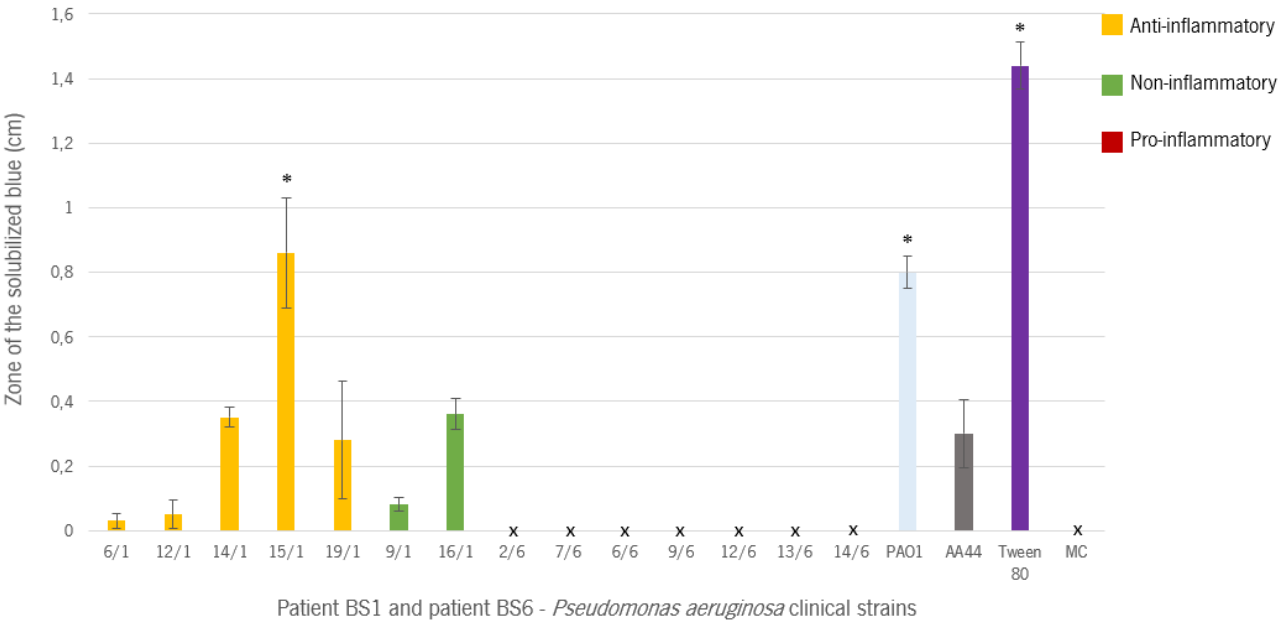

Figure 21: Rhamnolipid production (semi-quantification) by *P. aeruginosa* clinical isolates assessed by Methylene Blue Agar plates in *P. aeruginosa* 5×10^7 CFU/mL bacterial suspension after 48 h in incubation in microaerophilic conditions. Results are reported as average ± SE of at least four biological replicates. *p< 0,05 (One-Way ANOVA with Holm-Bonferroni correction for multiple testing). SE: Standard Error. The X indicates no zone of solubilized blue observed (Appendix I).

Similar results were obtained by Bjarnsholt et al. It was observed that isolates from intermittently colonized CF patients showed significantly higher levels of rhamnolipids compared to isolates from chronically colonized patients (Bjarnsholt et al., 2010). The rhamnolipid production results presented

above also show that there is no rhamnolipid production by the chronic isolates originating from patient BS1 and BS6, with exception of the isolate 15/1.

Rhamnolipids have the capacity to destroy tight junctions compromising the protective role of the epithelial barrier, making the host more susceptible to infections (Jurado-Martín et al., 2021).

In addition, rhamnolipids contribute to tissue damage by degrading lung's surfactant T. de Sousa et al., 2021). Therefore, isolate 15/1 could be contributing to infection in the lungs this way.

4.2.4 Proteolytic Activity

Proteolytic activity by *P.aeruginosa* clinical isolates was evaluated through the azocasein assay, described in detail in Section 3.6.1.

The results for the proteolytic activity showed great variability between the two patients (**Figure 22**). While isolates originating from patient BS6 showed a very low proteolytic activity, isolates originating from patient BS1 showed statistically significant proteolytic activity, similar to the positive control strain PAO1, comparing with the MC. Some variability could be observed within patient BS1, in which isolate 9/1, does not show proteolytic activity.

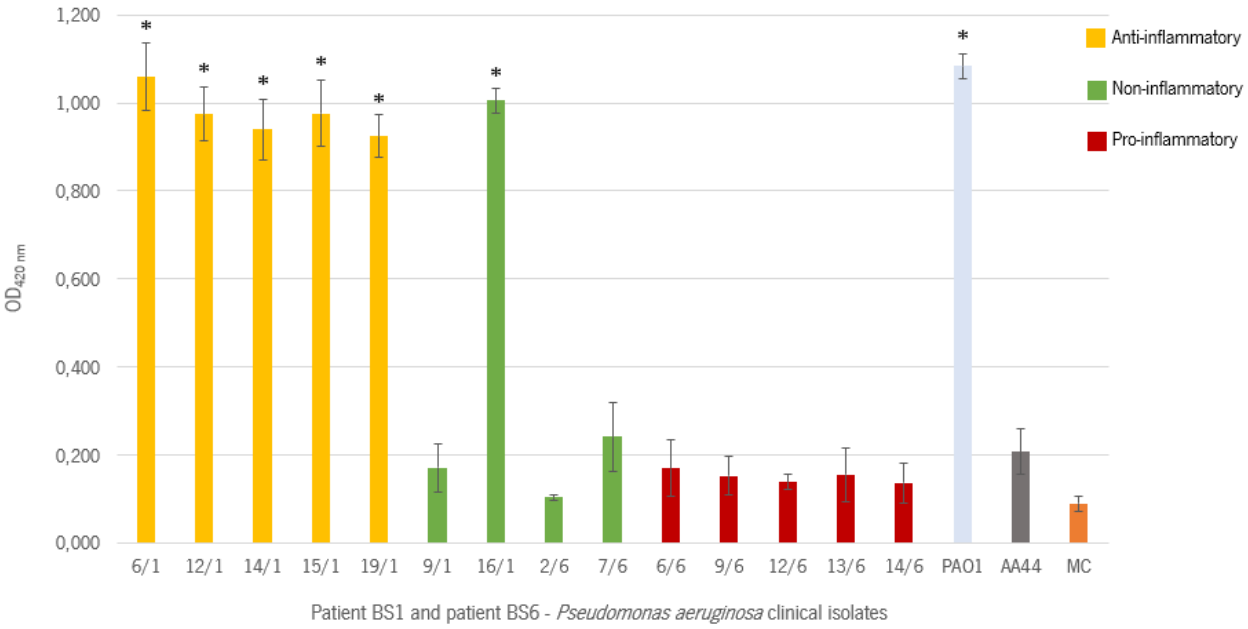

Figure 22: Proteolytic activity by *P. aeruginosa* clinical isolates assessed through the azocasein assay. Results are reported as average ± SE of at least four biological replicates. *p< 0,05 (One-Way ANOVA with Holm-Bonferroni correction for multiple testing). SE: Standard Error.

Moreover, it can be observed that the anti-inflammatory isolates originating from patient BS1 show high proteolytic activity contrarily to the pro-inflammatory isolates originating from patient BS6. This suggests that there could be a relation between the inflammatory results that were previously obtained by Wauters et al., 2023 and the hypothesis that *P. aeruginosa* proteases can have an effect in inflammation.

Jeukens et al studied the proteolytic activity of two *P. aeruginosa* chronic infection CF isolates, and four acute CF isolates using BHI agar plates with 1% skim milk (Jeukens et al., 2014). The chronic isolates did not show proteolytic activity. Similar results were obtained for the clinical isolates originating from patient BS6, as no proteolytic activity was found for these isolates.

Moreover, similar results were found by Smith et al, who observed that a 96-month CF isolate showed loss of proteolytic activity compared to the 6-month CF isolate originating from the same strain (Smith et al., 2006). In addition, previous finding by Snell Jagger at al showed that isolates from chronic infected CF patients have lower proteolytic activity than isolates originating from CF patients in early stages of infection (Snell Jagger et al., 1983).

These findings are in line with the results obtained for the isolates originating from patient BS6, as this patient is chronically colonized with *P. aeruginosa* and the isolates do not show significant proteolytic activity. On the other hand, the proteolytic activity results obtained for patient BS1 are not in line with literature as the isolates, except isolate 9/1, show high proteolytic activity.

4.2.5 Elastolytic Activity

Elastolytic activity was evaluated by performing the Elastin Congo Red assay, described in Section 3.6.2.

High variability can be observed for the elastolytic activity (**Figure 23**). There is no elastolytic activity by clinical isolates originating from patient BS6, as the results are not statistically different from the MC.

Isolates originating from patient BS1 show statistically significant elastolytic activity, with the exception of isolate 9/1, comparing to the MC.

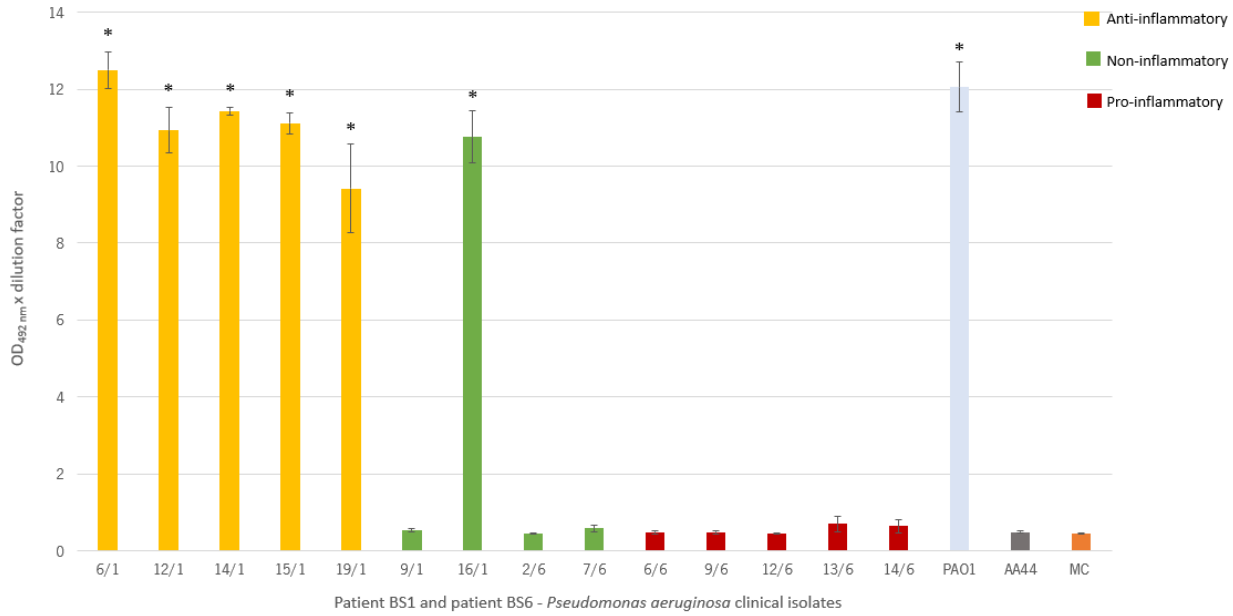

Figure 23: Elastolytic activity by *P. aeruginosa* clinical isolates assessed through the Elastin-Congo Red assay. Results are reported as average ± SE of at least four biological replicates. *p< 0,05 (One-Way ANOVA with Holm-Bonferroni correction for multiple testing). SE: Standard Error.

Furthermore, it can be observed that the anti-inflammatory clinical isolates originating from patient BS1 how high elastolytic activity while the pro-inflammatory isolates originating from patient BS6 do not show elastolytic activity. This suggests that elastase can have an effect in inflammation.

In the study carried out by Smith et al it was observed that a *P. aeruginosa* 96-month CF isolate showed loss of elastolytic activity compared to a 6-month CF isolate (Smith et al., 2006).

Furthermore, Jeukens et al also studied the elastolytic activity of *P. aeruginosa* clinical CF isolates by using the Elastin-Congo Red assay. Comparing to the reference strain PAO1, the chronic isolates showed no or low elastolytic activity (Jeukens et al., 2014).

Previously, Jagger et al also found that *P. aeruginosa* isolates from patients with *P. aeruginosa* infection showed higher elastase production than the chronically infected isolates (Snell Jagger et al., 1983).

These results are in line with elastolytic results presented in this thesis for patient BS6. Patient BS1 is chronically colonized with *P. aeruginosa* and the isolates, except isolate 9/1, show significant elastolytic activity.

Patient BS1's higher elastolytic activity may contribute to tissue damage due to the capacity of elastase to degrade elastin and surfactant proteins in lung's tissue (Faure et al., 2018; Liao et al., 2022).

Since the azocasein assay performed in this thesis to assess the proteolytic activity measures the total proteolytic activity, it would be interesting to test, as future work, the contribution of elastase in the proteolytic activity results in order to understand how much of elastase is being produced by the isolates.

4.3 Interleukin Degradation and Quantification

Degradation of the interleukins IL-8 and IL-1β by *P. aeruginosa* clinical CF isolates was quantified through an ELISA, as described in Section 3.8.1 and Section 3.8.2.

4.3.1 IL-8 Degradation

Great variability between patients in IL-8 degradation can be observed (**Figure 24**). The isolates originating from patient BS6 do not seem to be able to degrade IL-8, as well as isolate 9 originating from patient BS1. IL-8 appears to be strongly degraded by the supernatant of the *P. aeruginosa* clinical isolates originating from patient BS1, with the exception of isolate 9/1. Statistically significant results for the IL-8 degradation, comparing to the MC, were obtained for 4 of the isolates originating from patient BS1, namely, 6/1, 12/1, 14/1 and 15/1.

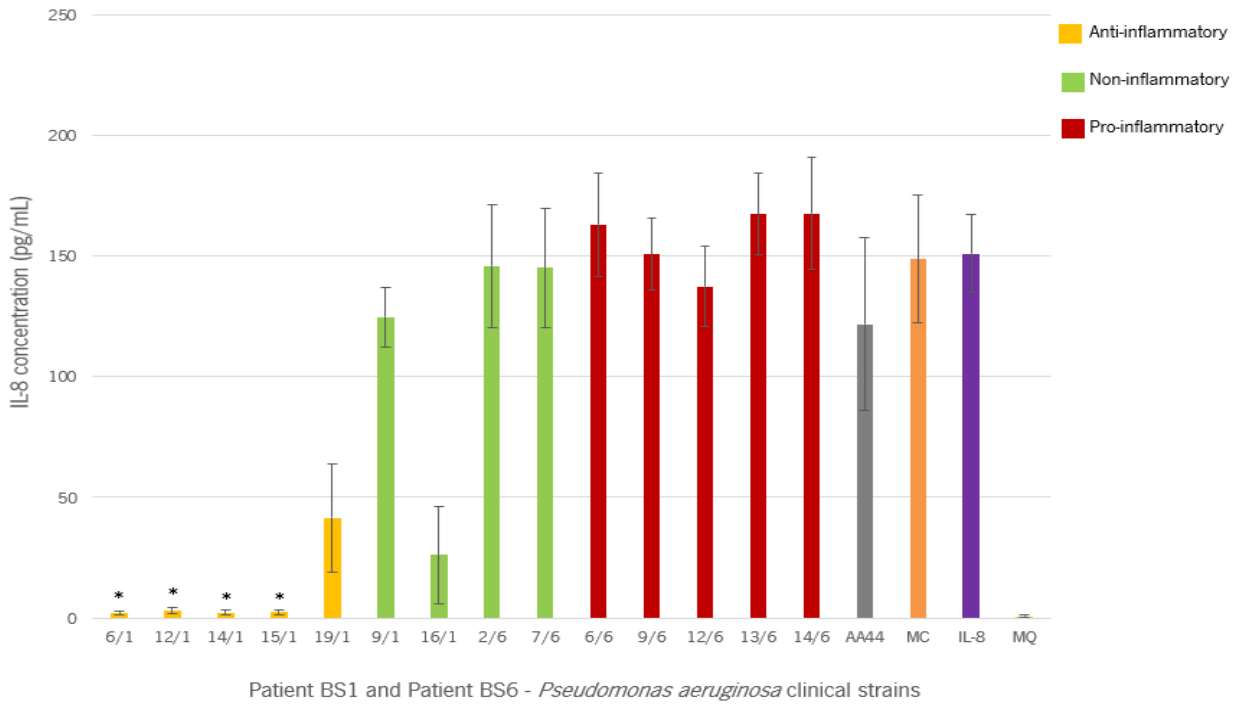

Figure 24: IL-8 ELISA results. Results are reported as average ± SE of at least four biological replicates. *p< 0,05 (One-Way ANOVA with Holm-Bonferroni correction for multiple testing). SE: Standard Error.

The anti-inflammatory isolates originating from patient BS1 and the non-inflammatory isolate 16/1 show significant degradation of IL-8.

Since IL-8 is a pro-inflammatory cytokine responsible for inducing neutrophil recruitment to the infection site, patient BS6 is more likely to show exaggerated inflammation in the lungs, as these isolates do not degrade IL-8.

In contrast, since patient BS1 is colonized with *P. aeruginosa* isolates capable of degrading IL-8, this patient could experience a reduced inflammatory response in the lungs, which is confirmed by previous results obtained after exposing 3-D lung epithelial cells to the supernatant, presented in Section 1.1 (Wauters et al., 2023).

Patient BS6 isolates contribute to an exaggerated inflammation in the CF lungs by not being able to degrade IL-8. This is in line with the classification of these isolates as pro-inflammatory. It is noteworthy that isolate 16/1 stands out as it shows a much different result for the degradation of IL-8 compared to the rest of the non-inflammatory isolates. This isolate shows a great capability to degrade IL-8, suggesting a more anti-inflammatory character. In addition, this isolate also shows higher proteolytic and elastolytic activity.

4.3.2 IL-1β Degradation

No statistically significant results were found for the degradation of IL-1β comparing to the MC (**Figure 25**). However, a clear difference between the concentration of IL-1β for the isolates originating from patient BS1 and from patient BS6 can be observed. Isolates originating from patient BS1 seem to the able to degrade IL-1β. Conversely, isolates originating from patient BS6, show no degradation of IL-1β similar as the pro-inflammatory strain AA44 (served as a positive control for the inflammation experiment presented in Section 1.1).

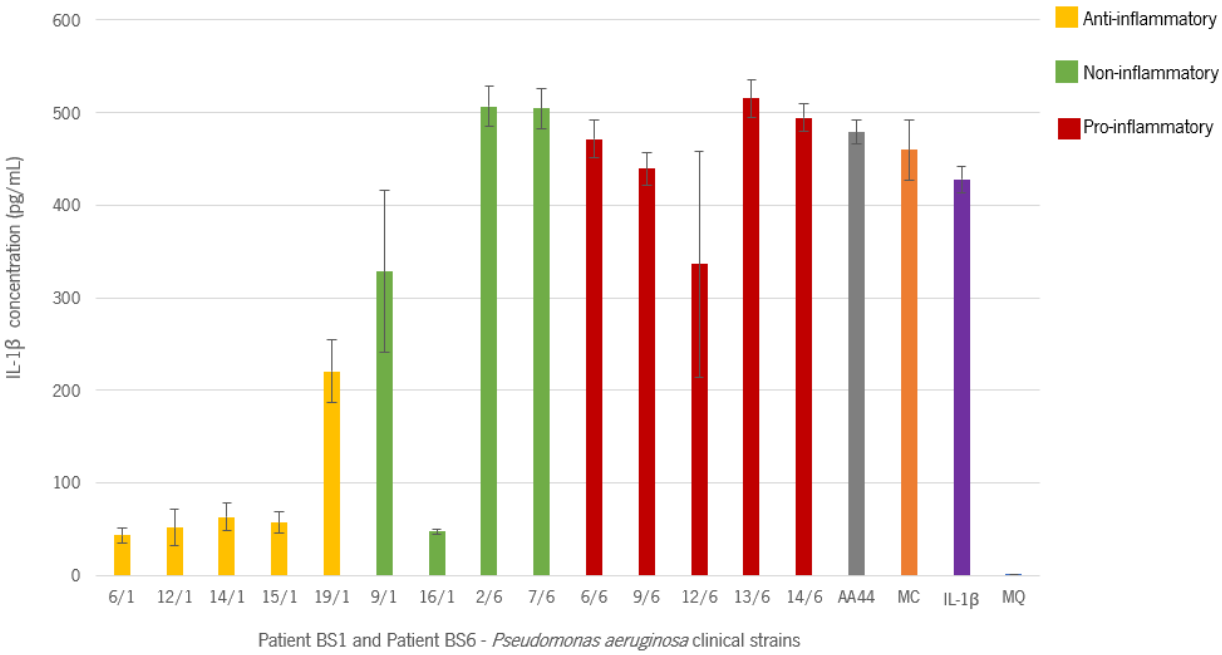

Figure 25: IL-1β ELISA results. Results are reported as average ± SE of at least four biological replicates. *p< 0,05 (One-Way ANOVA with Holm-Bonferroni correction for multiple testing). SE: Standard Error.

The interleukin IL-1β controls several innate immune response mechanisms. This pro-inflammatory cytokine induces neutrophil recruitment, starting the inflammatory process in the CF lung, (Kaneko et al., 2019; Lopes-Pacheco, 2020; Liao et al., 2022).

Patient BS1's isolates ability to degrade IL-1β could promote a decrease in lung inflammation by reducing IL-1β concentration, and therefore, reducing the neutrophil recruitment to the infection site. Isolate 16/1 shows an unexpected result, low IL-1β concentration, similar to the anti-inflammatory isolates. Similar to the BS6 *P. aeruginosa* clinical isolates, the high inflammatory AA44 strain also showed no proteolytic or elastolytic activity and no capability to degrade IL-8 or IL-1β.

4.4 Relation between Proteolytic and Elastolytic Activity and Interleukin Degradation

4.4.1 Proteolytic activity and Interleukin Degradation

A correlation between the proteolytic activity results, presented in Section 4.2.4, and the degradation of IL-8 and IL-1β can be observed. A higher proteolytic activity corresponds to a higher interleukin degradation (**Figure 26**).

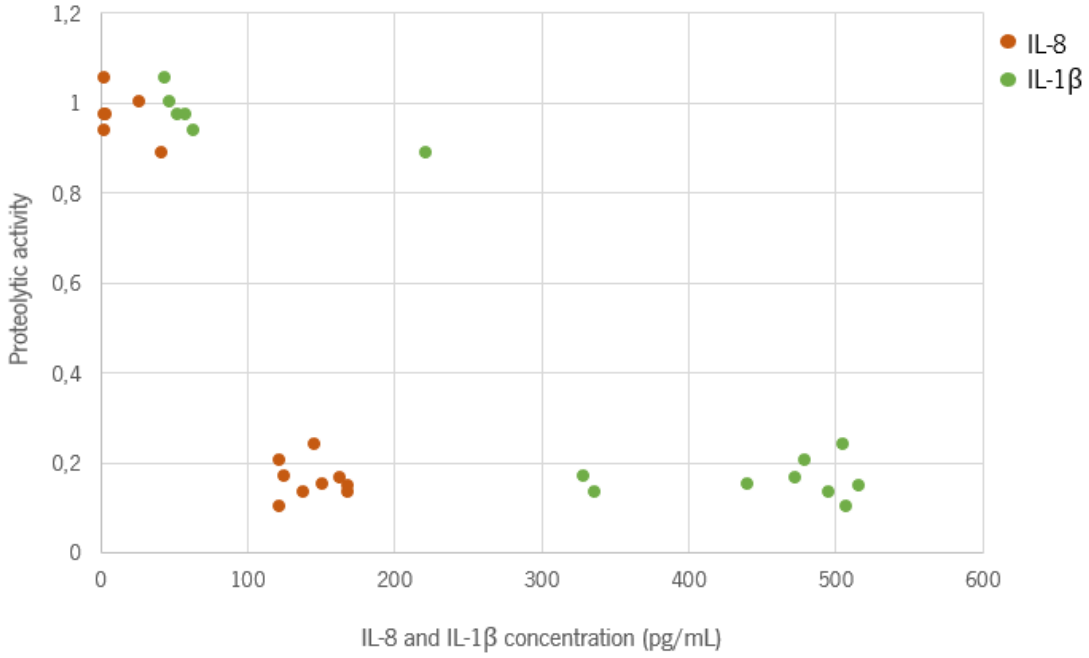

Figure 26: Correlation between proteolytic activity and degradation of IL-8 and IL-1β.

Isolates originating from patient BS1 that show a higher proteolytic activity, and low interleukin degradation, i.e., interleukin degradation. Contrarily, isolates originating from patient BS6, as well as, the isolate 9/1, show a minor proteolytic activity and do not seem capable of degrading IL-8 or IL-1β.

This suggests that there could be other *P. aeruginosa* proteases, apart from elastase, that also targets both interleukins. For example, alkaline protease secreted by *P. aeruginosa* is able to degrade IL-6, TNK-α and IFN-γ and therefore, contributing to a lower lung inflammation. However, since *P. aeruginosa* protease IV has the ability to degrade surfactant proteins and increase bacterial infection by degrading several host proteins, such as fibrinogen, transferrin, lactoferrin and elastin, patient BS1 may be more

subject to tissue damage and decline in lung function (Liao et al., 2022). Further research could be done to confirm which *P. aeruginosa* proteases are contributing to the degradation of the interleukins.

In contrast, patient BS6 may show a higher concentration of inflammatory cytokines, as all the strains isolated from this patient show no proteolytic activity. Therefore, in this patient, lung inflammation may be caused by the action of these cytokines rather than *P. aeruginosa* proteases.

4.4.2 Elastolytic activity and Interleukin Degradation

Analysing the elastolytic activity, presented in Section 4.2.5 and the IL-8 and IL-1β degradation, a correlation between these results can be observed. It is clear that to a high elastolytic activity corresponds a low concentration of IL-8 and IL-1β (**Figure 27**).

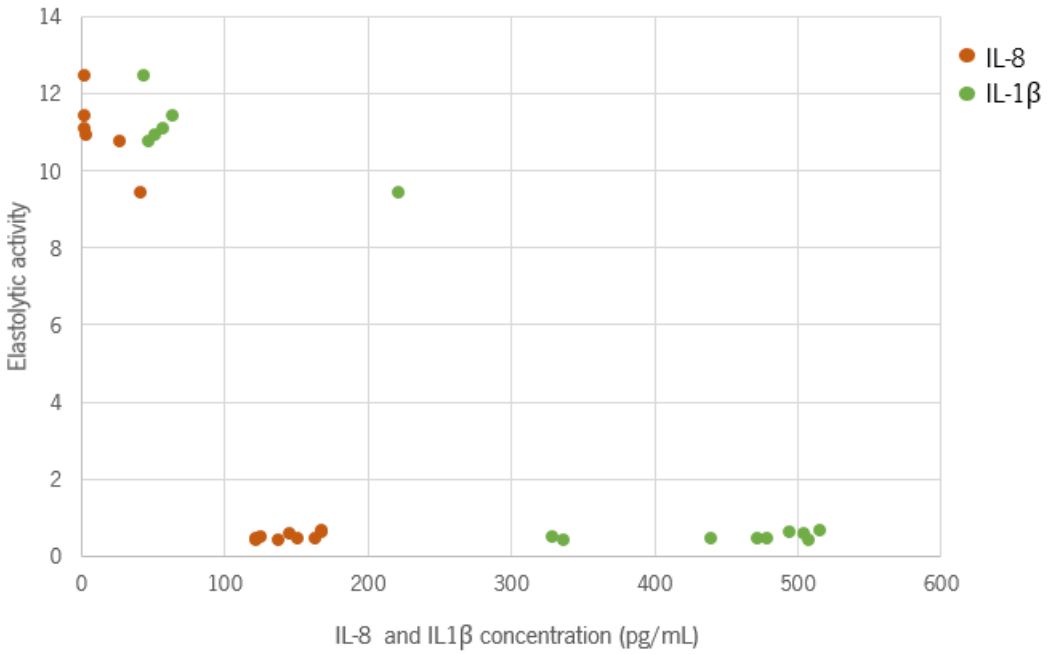

Figure 27: Correlation between elastolytic activity and degradation of IL-8 and IL-1 β.

In detail, regarding the isolates originating patient BS1, these show a high elastolytic activity and higher degradation of both interleukins. Isolates originating from patient BS6 and the isolate 9/1, contrarily, show no elastolytic activity and no degradation of IL-8 or IL-1β.

These findings suggest the possibility that elastase produced by *P. aeruginosa* CF isolates is able to degrade IL-8 and IL-1β in the infected lungs.

Elastase capacity to degrade IL-8 was previously suggested by LaFayette, who studied the inflammatory response caused by *P. aeruginosa lasR* mutants isolated from CF patients (LaFayette et al., 2015). Comparing *P. aeruginosa* isolates with wild-type *lasR* gene and *P. aeruginosa lasR* mutants isolates, the *lasR* mutants were not able to degrade IL-8 or IL-6 showing high concentrations of these interleukins. In addition, it was observed that three *P. aeruginosa* isolates originating from three chronic CF patients produced less elastase and induced a higher concentration of IL-8 and IL-6.

Moreover, it was observed that infection with elastase negative isolates lead to a greater neutrophil recruitment and an exaggerated inflammatory response in the airways.

This supports the hypothesis that elastase is able to degrade inflammatory cytokines and therefore contribute to reduce lung inflammation.

Previously, Sun et al concluded that elastase can cleave pro-IL-1β, resulting in active IL-1β, and there is no further cleavage by elastase (Sun et al., 2020). This would increase neutrophil recruitment and lead to tissue damage in the lungs. These results presented in this thesis propose otherwise as the results suggest the hypothesis that elastase can cleave mature IL-1β.

4.4.3 Effects of Interleukin Degradation in CF Lung Inflammation

The degradation of inflammatory cytokines plays an important role in the evolution of the *P. aeruginosa* infection in CF.

The degradation of IL-8 and IL-1β could lead to a lower neutrophil recruitment, contributing to a lower inflammation in the CF lungs and, consequently, to less tissue damage.

On the other hand, inflammation is important to clear *P. aeruginosa* from the lungs. Hence, the reduction of neutrophil recruitment can contribute to the persistence of this pathogen in the lungs, leading to a chronic infection (Lin & Kazmierczak, 2017).

The chronic infection would be characterized with down-regulation of virulence factors, reduced expression of proteases and elastase, promoting an increase in cytokine concentration, promoting a more intense inflammatory response by the host (A. M. Sousa & Pereira, 2014). This could lead to more tissue damage, contributing to the impairment of lung function. Therefore, the inflammatory response in the beginning of the *P. aeruginosa* infection, in the acute stage of CF, can contribute to bacterial clearance and control the infection (Lin & Kazmierczak, 2017).

5. Conclusion and Future Work

5.1 Conclusion

Due to the increasing antibiotic resistance, it is crucial to develop effective strategies against *P. aeruginosa* infections. This pathogen infects CF patients causing lung infections different inflammatory responses. Moreover, diverse virulence factors production among strains isolated from patients have been observed. Therefore, it is of most importance to understand how the inflammatory response and bacterial virulence

factors interact in order to effectively combat *P. aeruginosa* associated infections.

In the chronic stage of CF there is a down-regulation of virulence factors and thus, since the expression of these factors was evaluated in chronic CF isolates under conditions that mimic the chronic infection, a minor production of virulence factors was expected (A. M. Sousa & Pereira, 2014). In this work, heterogenicity in virulence factor production of clinical isolates within and between chronically infected CF patients was observed. No significant production of pyoverdine or pyocyanin was found for the clinical isolates originating from patient BS1 and patient BS6. Rhamnolipid production was only observed for one isolate originating from patient BS1. The isolates originating from patient BS1 showed high proteolytic and elastolytic activity, while the isolates originating from patient BS6 did not show proteolytic or elastolytic activity. For the strain AA44, a pro-inflammatory strain, no production of pyoverdine, pyocyanin or rhamnolipids was observed.

Degradation of the interleukins IL-8 and IL-1β was observed for all the anti-inflammatory clinical isolates and for one non-inflammatory clinical isolate originating from patient BS1. A correlation between proteolytic and elastolytic activity and interleukin degradation was observed. The pro-inflammatory isolates originating from patient BS6, that do not show significant proteolytic or elastolytic activity, do not seem to be able to degrade IL-8 or IL-1β. This suggest that *P. aeruginosa* proteases, and specifically, elastase have an effect on the reduction of inflammation. However, on the other hand, the ability of *P. aeruginosa* proteases to degrade host proteins could lead to tissue damage and consequently decreasing lung function.

Pyoverdine and pyocyanin do not seem to be directly related to the inflammatory action of the studied clinical isolates, since these factors were not produced by the anti or pro-inflammatory isolates. The cytotoxicity effects of pyocyanin may not be contributing to the disease severity in patient BS1 and patient

BS6. Additionally, there might be occurring less competition for the host's iron in these patients, since pyoverdine is not being produced.

Lastly, in spite of rhamnolipids' strong contribution to infection in CF, the virulence properties do not appear to be connected with the inflammation character of the clinical isolates, since only one isolate originating from patient BS1 showed significant rhamnolipid production.

5.2 Prospect for Future Work

As future work, degradation assays could be performed for other interleukins. This could contribute to the discovery of potential new pro and anti-inflammatory targets for chronic CF treatment.

Pro-inflammatory interleukins, such as, IL-6, IL-1 and TNF- α could be further studied (Courtney et al., 2004). These have been found in sputum of CF patients in high concentrations. IL-1 amplifies the neutrophil response and promotes the neutrophil adhesion to the endothelium. IL-6 plays a role in the initiation and the resolution of inflammation and TNF-α contributes to the adhesion of neutrophils to the endothelium and enhances chemotaxis of neutrophils to site of inflammation (Courtney et al., 2004; (McGreal et al., 2010). IL-17 is another pro-inflammatory interleukin that is found in high concentrations in CF lungs and stimulates inflammatory cell signalling (Roesch et al., 2018). In addition, it would be interesting to study the levels of IL-10 in CF patient's sputum, as this is an anti-inflammatory cytokine that is capable of inhibiting the secretion of TNF-α (Courtney et al., 2004).

Quantification of elastase and the other *P. aeruginosa* secreted proteases could provide more insights about the proteolytic activity of *P. aeruginosa* and contribute to the identification of a key factor for CF treatment.

Finally, *P. aeruginosa* isolates with mutation in the *lasR* gene, the gene that encodes *P. aeruginosa* LasB, can persist within a patient for several years (Fothergill et al., 2007). Hence, as future experimental work, identification of *lasR* mutation in the selected isolates could be done with the aim to understand if the lack of elastase production by the isolates originating from patient BS6 is due to a mutation in the *lasR* gene or if there is another mechanism by which elastase is not being produced.

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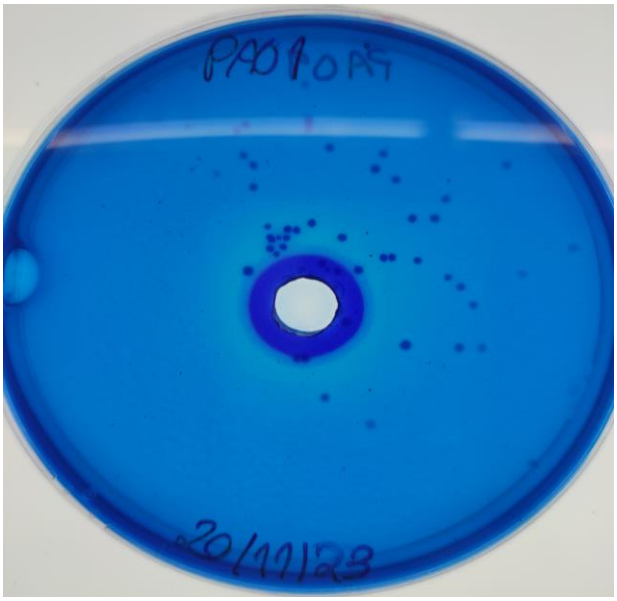
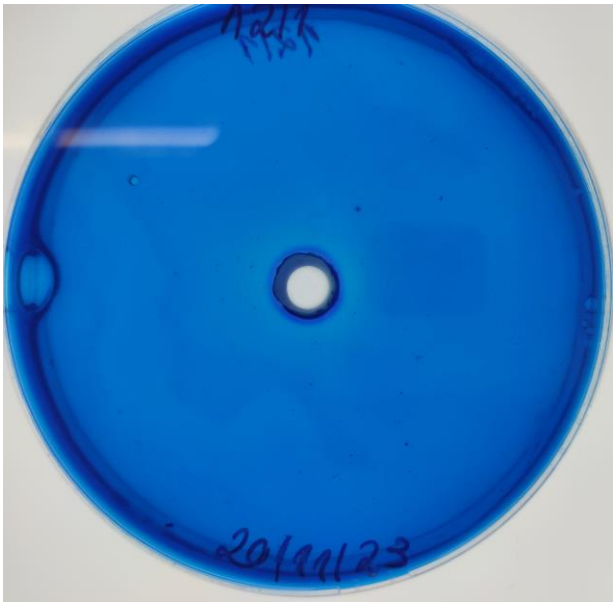
SUPPLEMENTARY MATERIAL I – SOLUBILIZED BLUE ZONE



Supplementary Figure 1: Solubilized blue zone for PAO1, indicating rhamnolipid production.



Supplementary Figure 2: No solubilized blue zone for isolate 12/1, indicating no rhamnolipid production.