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Achromobacter spp. in Cystic Fibrosis: Investigating Pathogenicity, Host Response, and Molecular Identification

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Achromobacter spp. In Cystic Fibrosis:
Investigating Pathogenicity, Host Response,
and Molecular Identification- Giulia Maria Saitta
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Background and contents of the thesis

Abstract

Achromobacter spp. are non-lactose fermenting, catalase and oxidase-positive Gram-negative bacilli commonly found in various environments. They play a significant role in human infections, particularly in people with cystic fibrosis (CF) where they can cause chronic respiratory infections. Notably, these bacteria exhibit considerable antibiotic resistance due to both innate and adaptive mechanisms.

The *Achromobacter* genus includes 22 species, with *A. xylosoxidans* being the most prevalent in CF clinical samples, linked to increased pulmonary exacerbations and hospitalizations. Other relevant species include *A. dolens* and *A. insuavis*. Current identification methods, such as VITEK 2 and MALDI-TOF MS, often struggle to accurately differentiate between species.

This research includes several studies examining the virulence, antimicrobial resistance, host immune response, and species identification of *Achromobacter* spp. The first study compared phenotypic characteristics of CF clinical isolates, revealing critical differences in virulence and antibiotic resistance, which may correlate with bacterial persistence in the lungs.

A second study investigated the host inflammatory response in CFTR-knockout and wild-type mice exposed to selected isolates, finding that virulent strains induced higher lung inflammation and mortality, particularly in knockout mice. This highlights the importance of understanding the pathogenic mechanisms of *Achromobacter* spp.

Further research utilized an in vivo zebrafish model to explore host interactions, revealing varying levels of host mortality, bacterial persistence, and pro-inflammatory gene expression. We observed that macrophages played a crucial role in controlling bacterial growth and reducing host damage, as their depletion led to increased bacterial burden and mortality in embryos infected with the less virulent strain.

Finally, the development of a quantitative PCR (qPCR) protocol for species-level identification addressed challenges in accurately diagnosing *Achromobacter* infections.

This research enhances our understanding of *Achromobacter* spp. in CF and other infections, informing clinical management and future therapeutic strategies.

Introduction

1. Cystic fibrosis

Epidemiology

Cystic fibrosis (CF) is one of the most common life-shortening multi-systemic disease with an autosomal recessive inheritance pattern in Caucasian population, affecting 70.000 to 100.000 people worldwide, with a lower prevalence in African Americans, Hispanic and Asians [1–3]. In Europe the incidence ranges from 1:2800 to 1:6500 live births (Figure 1), with a wide geographic variation [4].

The disease is also widespread in other regions, particularly in the United States (1 in 4.000) and Australasia (1 in 3.000), while it remains infrequent in Asia, especially in Japan and China [4].

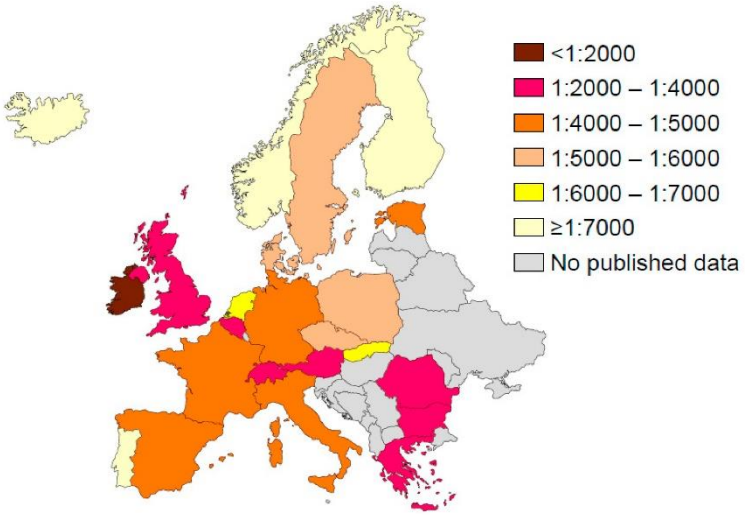

Figure 1. Incidence of cystic fibrosis in Europe: estimated incidence of CF in many regions. There is a wide geographic distribution, ranging from 1 in 2800 to 6500 live births. In the central area of Europe, the insidence varies from 1:5200 to 1:6500; it appears lower than 1:7000 in Portugal, Norway and Russia, while it is very low in Finland (1:25.000). (Scotet et al., 2020)

Molecular basis of CF

CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, situated in the long arm of chromosome 7, and encoding CFTR protein [5–7]. The CFTR protein is member of the ATP-binding cassette (ABC) transmembrane transporter superfamily and is composed of five domains (Figure 2): two membrane-spanning domains (MSDs), each characterised by six transmembrane sections; two nucleotide-binding domains (NBDs), each containing

conserved amino acid sequences that interact with ATP; and a unique regulatory domain (RD), linking the two halves of the protein, with multiple consensus phosphorylation sites[7–9].

Negatively charged ions like chloride (Cl^-) gather around the positively charged areas at both ends of the pore in MSDs. When the gate opens, these ions flow through. The phosphorylation of PKA activates the channel; when two ATP molecules bind to the NBDs, the two domains move closer together. Subsequently, ATP hydrolysis breaks the interface between NBD1 and NBD2, resulting in the opening of the gate (Figure 2, Figure 3).

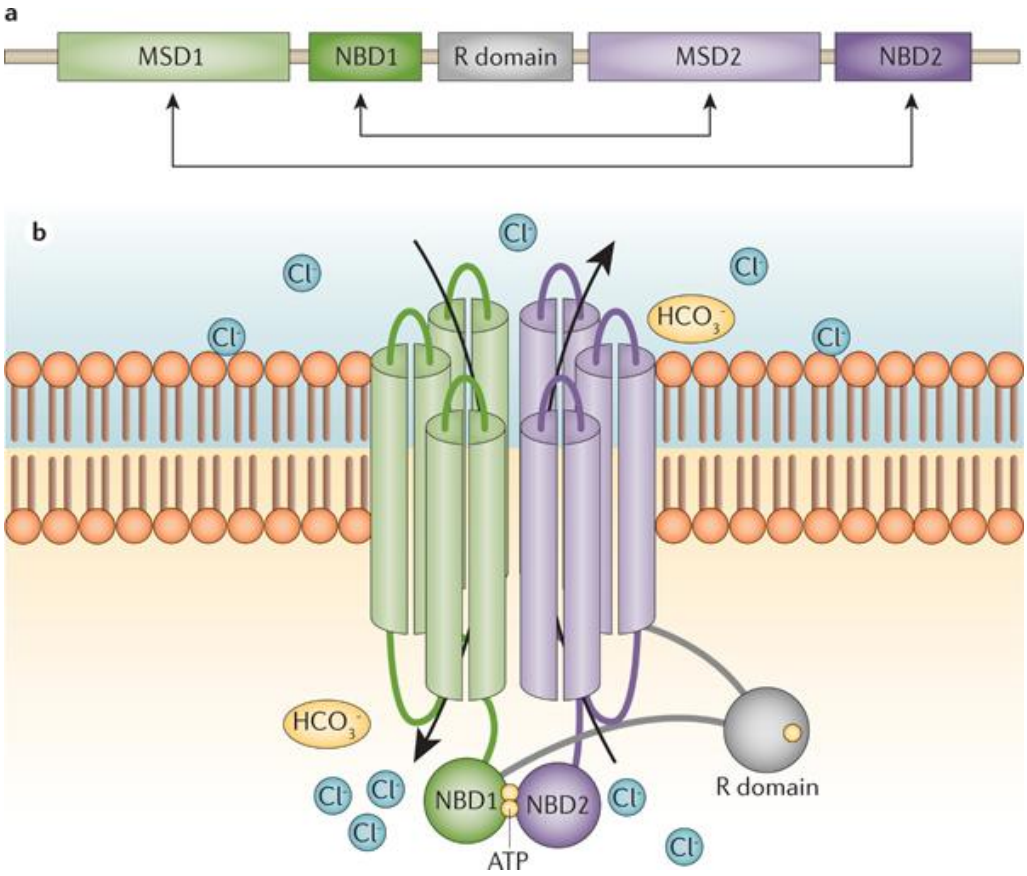

Figure 2. CFTR domains: linear structure (a) of CFTR protein and its structure (b). (Ratjien et al., 2015)

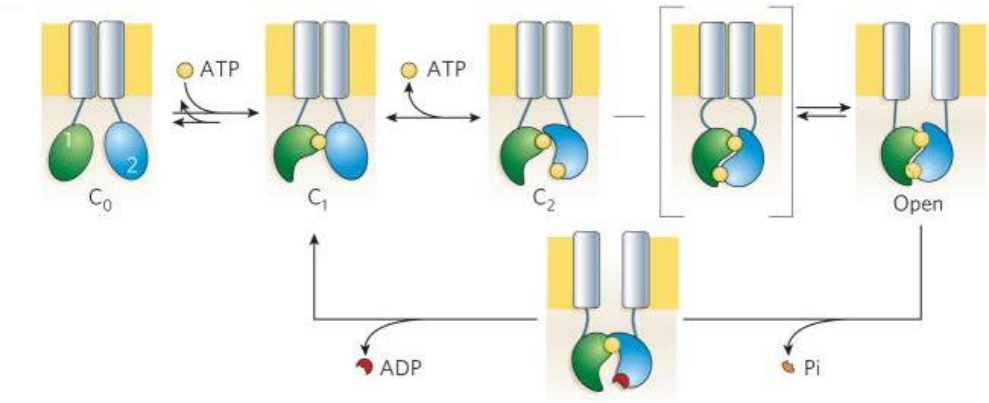

Figure 3. Opening and closing of CFTR channels: the R domain is omitted. ATP (yellow) remains tightly bound to NBD1 (green) motifs and many closed–open– closed gating cycles occur. ATP binding to NBD2 (blue) is followed by a slow channel opening step (C2-to-Open) that proceeds through a transition state (square brackets) in which the intramolecular NBD1–NBD2 tight heterodimer is formed but the transmembrane pore (grey rectangles) has not yet opened. The relatively stable open state becomes destabilized by hydrolysis of the ATP bound at the NBD2 composite catalytic site and loss of the hydrolysis product, inorganic phosphate (Pi). The ensuing disruption of the tight dimer interface leads to channel closure (Gadsby et al., 2006).

Approximately 2.000 variants or mutations of the CFTR gene have been identified, with only 360 of these variants known to cause the disease [10]. Typically, CF mutations are divided into six classes (I-VI) based on their effect on the CFTR protein function (Figure 4), synthesis or transport within the cell [11–14].

Following, the traditional classification of CF mutations, based on their phenotype:

- Class I mutations are characterized by reduced or absent CFTR protein, as a result of several mutation types, including frameshift, nonsense and splicing mutations [14].

- Class II mutations lead to misfolding or premature degradation of the protein, severely reducing the number of CFTR molecules at the cell surface. The predominant mutation, known as F508del, belongs to this class [15].

- Class III mutations impair the regulations of CFTR channel, causing an abnormal gating activity. The major causes of this gating dysfunction are abnormal ATP binding to the NBD1/2 and the lack of ATP hydrolysis [16].

- Class IV mutations usually take part in membrane-spanning regions critical for channel pore formation, altering the channel conductance[16].

- Class V mutations alter protein abundance by introducing promoter or splicing abnormalities, usually leading to a decrease in functional CFTR at the apical membrane[10,16].

- Class VI mutations can reduce conformational stability, or generate additional internalization signals, resulting in accelerated turnover [17].

A new classification proposed by Kris De Boeck and Margarida Amaral introduces a class VII of mutations [18]. Some researchers have suggested renaming this new class to class I subtype A, as it leads to the complete absence of mRNA production and severe disease manifestations similar to those seen in class I mutations [10,14].

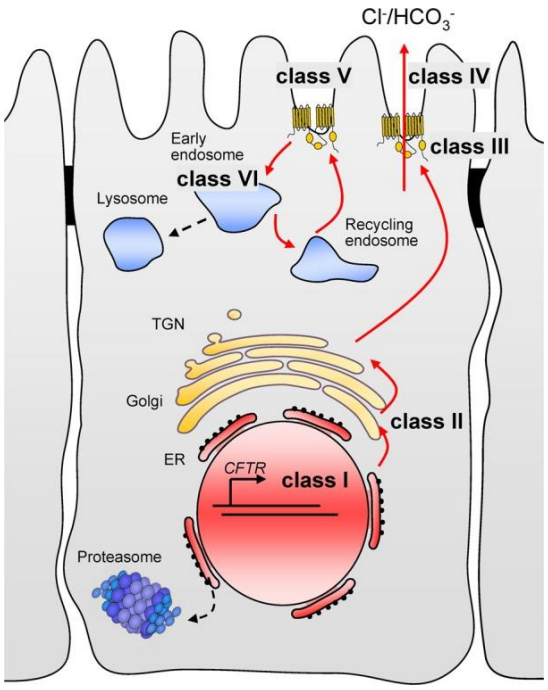

Figure 4. Classification of CFTR mutations: class I: lack of CFTR synthesis; class II: defective protein processing; class III: defective channel regulation or gating; class IV: defective chloride conductance; class V: reduced amount of CFTR protein; class VI: increased turnover of CFTR channel at the cell surface. (Veit et al., 2016)

Clinical manifestation

CFTR is predominantly expressed in epithelial tissues, particularly on the apical membrane of cells in various organ systems, including the respiratory tract, gastrointestinal system, reproductive system, and sweat glands. This protein plays a crucial role in chloride and bicarbonate transport; dysfunctional CFTR results in defective ion transport occurs, leading to dehydration and thickening of secretions.

An exception is found in the sweat glands, where the function of the CFTR channel is inverted, leading to dysfunction that produces sweat with elevated chloride (>60 mmol/L) and sodium levels[19]. CFTR protein dysregulation contributes to a range of complications in different body areas (Figure 5) [20].

In the pancreas, severe CFTR mutations (classes I-III) are primarily associated with pancreatic insufficiency (PI) [21]. This condition is characterized by dramatically reduced pancreatic ductal flow and the absence of digestive enzymes. As a result, proenzymes can become trapped in the ducts, leading to their premature activation, which causes inflammation and damage to pancreatic tissue. People with CF (pwCF) often experience symptoms such as steatorrhea, malabsorption, malnutrition, and deficiencies in fat-soluble vitamins [22,23]. Furthermore, milder CFTR mutations (classes IV-V) can increase the risk of developing pancreatitis, further complicating the potential for pancreatic insufficiency and the emergence of cystic fibrosis-related diabetes [1,20,24]. In the gastrointestinal tract, CFTR-mediated bicarbonate secretion is essential for buffering gastric acidity and ensuring proper expansion and hydration of intestinal mucus. It is reported that approximately 20% of neonates with cystic fibrosis experience meconium ileus, while adults may face constipation or distal intestinal obstruction [22].

The hepatobiliary system also suffers significantly due to CFTR dysfunction. CFTR protein is absent in hepatocytes and its function leads to the formation of inspissated bile, resulting in obstructive liver disease, multilobar biliary cirrhosis, and portal hypertension [25,26].

Infertility is another serious concern, with congenital bilateral absence of the vas deferens (CBAVD) leading to infertility in nearly all males with CF, while females may experience reduced fertility due to factors such as thick cervical mucus, acidic uterine environment, and impaired gonadotropin secretion [24,26,27].

Pulmonary involvement is the primary cause of morbidity and mortality in pwCF. Abnormal or absent CFTR lead to acidification and dehydration of airway surface liquid, causing mucus to become excessively viscous and thick. This impairs mucociliary clearance, leading to chronic bacterial infections and bronchiectasis[1]. The resulting inflammatory response, characterized by intense neutrophil recruitment, accelerates lung tissue damage and contributes to a rapid decline in lung function, ultimately leading to pulmonary insufficiency and a significantly worse prognosis for those affected [26].

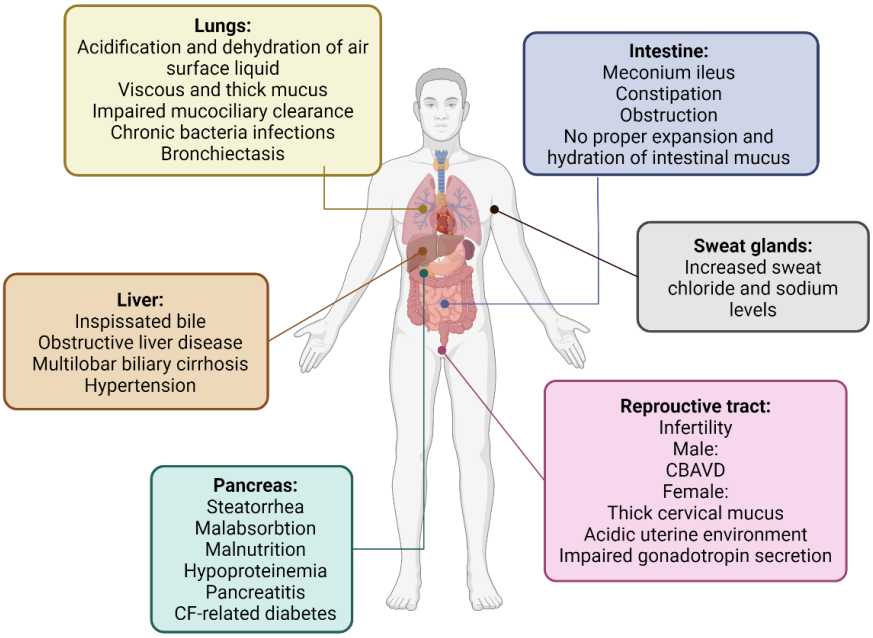

Figure 5. Impaired CFTR protein manifestations in main body districts: the figure illustrates the primary complication of the disease. Dysregulation of the CFTR protein leads to various manifestations in different body systems, which vary in severity depending on the mutations, just as the symptoms do (Realized with BioRender).

Overall, the complex interaction of CFTR dysfunction across various organ systems underscores the critical role of this protein in maintaining epithelial health and function.

CF modulator therapy and airway infections

The introduction of highly effective CFTR modulators, particularly the triple combination therapy of elexacaftor, tezacaftor, and ivacaftor (ETI), has marked a turning point in the treatment of CF. This therapy targets the underlying molecular defects caused by the most common CFTR mutation, F508del, and is currently approved for individuals with at least one copy of this mutation, accounting for over 85% of the CF population [28]. ETI works synergistically: elexacaftor and tezacaftor act as correctors, improving the folding and trafficking of the CFTR protein to the epithelial cell surface, while ivacaftor acts as a potentiator, enhancing the gating activity of the channel [28].

The clinical impact of this therapy has been substantial. Patients treated with ETI experience significant improvements in forced expiratory volume in one second (FEV_1), weight gain, and sweat chloride concentrations. More importantly, ETI has led to a notable reduction in the frequency of pulmonary exacerbations, hospitalizations, and the need for systemic and inhaled antibiotics. These outcomes reflect not only enhanced mucociliary clearance due to restored ion transport but also a modulation of the airway microenvironment that becomes less conducive to persistent infection [29].

In parallel, studies have begun to document shifts in the CF airway microbiome following ETI therapy. A decrease in the prevalence and load of *Pseudomonas aeruginosa* and *Staphylococcus aureus* has been observed, particularly in younger individuals and those with less advanced lung disease [29,30]. Nevertheless, patients with established chronic infections, especially those involving biofilm-forming pathogens, may continue to require long-term inhaled antibiotic regimens. The persistence of bacteria within hypoxic mucus plugs and biofilm aggregates limits the full eradication potential of modulator therapy alone [29,30].

It is also important to note that ETI is not universally effective. Approximately 10% of individuals with CF possess mutations that are not responsive to current CFTR modulators. This subset includes rare and nonsense mutations that result in absent or nonfunctional protein production. For these patients, ongoing research is

focusing on gene therapy, RNA-based approaches, and alternative strategies targeting epithelial ion channels or downstream effects of CFTR dysfunction [28].

In summary, while the advent of triple modulator therapy represents a paradigm shift in CF management, especially with respect to reducing the infectious burden, it has not eliminated the need for adjunctive antimicrobial therapies.

2. The pathophysiology of CF lungs

Pathogenesis

The CFTR protein is expressed in the glands and epithelial cells of various organs, playing a crucial role in regulating the transport of sodium, potassium, and water. In healthy individuals, the mucociliary system serves as the primary innate defence mechanism. This system comprises cilia, a mucus layer that coats the airways full of antimicrobial peptides, and an airway surface liquid (ASL) layer, which separates the mucus from the epithelial cells. Normally, the cilia work to clear irritants and microorganisms by trapping them in the thin fluid mucus, which is then cleared through ciliary beating, aided by the autoregulation of ASL volume [31]. However, in the airways of individuals with CF, the impaired CFTR protein disrupts chloride and bicarbonate transport (Figure 6). This leads to excessive reabsorption of sodium and water from the ASL, resulting in dehydration of the mucus layer and depletion of ASL, ultimately compromising cilia ability to remove the thickened mucus [32].

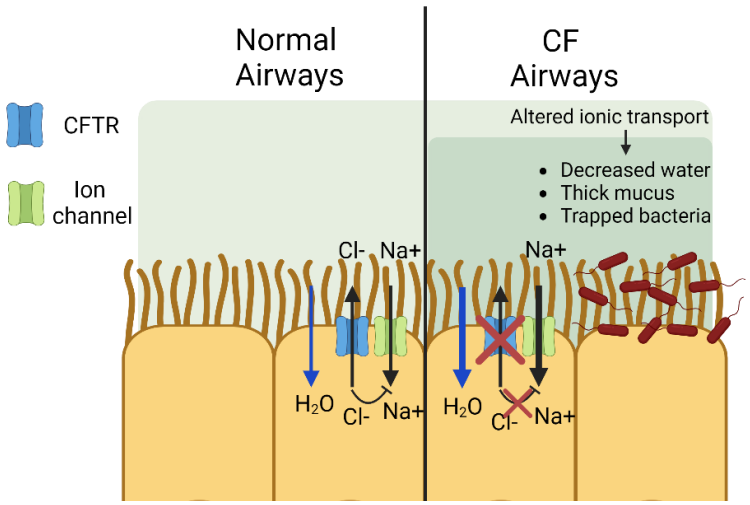

Figure 6. CF airways epithelium: the mechanism of CF dysfunction starts with the primary CFTR gene defect, which alters the ion transport, leading to decreased volume of water, thick mucus, and trapping bacteria (realized with BioRender).

The antimicrobials within the mucus become ineffective, the acidification of the environment increase the viscosity of the mucus, impairs host defences, and contributes to inflammation [33]. Finally, the thick, sticky, and viscous mucus traps bacteria, that are capable of proliferate and colonize the lung environment [34,35].

Inflammation

In the airways of pwCF, inflammation begins early in life and can occur independently of infection. The airways are continuously exposed to inhaled particles, varying from harmless antigens to harmful pathogens, prompting immune cells to shift from a neutral to a defensive role based on the type of threat present[36]. CF lung dysfunction results in exaggerated and ineffective airway inflammation that fails to eliminate pulmonary pathogens during infections [37]. The inflammation of the CF lung is marked by an excessive recruitment of neutrophils, resulting in the release of pro-inflammatory molecules, damaging lung tissue and perpetuating neutrophils and macrophages recruitment.

CF airways are characterized by elevated concentrations of proinflammatory mediators and cytokines, including interleukin 8 (IL-8), IL-6, IL-1ß, IL-17, IL-33, tumor necrosis factor α (TNF-α), granulocyte macrophage colony-stimulating factor (GM-CSF), Cathepsin S (CatS) and HMGB-1[38]. These mediators are involved in neutrophil stimulation and recruitment, regulation of bacterial clearance, oxidative stress response, and secretion of immune response molecules. Specifically, IL-8 and IL-17 are potent neutrophil chemoattractant, enhancing the neutrophils recruitment [39]. An important aspect of CF inflammatory response is the deficiency in regulatory mediators, that should terminate and resolve the inflammation, such as IL-10, nitric oxide and lipoxin-A4 [38,40–42].

The accumulation of phagocytic innate immune cells, primarily neutrophils and macrophages, in the airways of pwCF leads to ineffective pathogen clearance and persistent bacterial colonization [43]. Neutrophils show an imbalance in ion homeostasis, with increased chloride and sodium levels and decreased magnesium, which impairs their degranulation and phagocytic functions [43]. This accumulation triggers the release of potent antimicrobial compounds, such as neutrophil elastase

(NE), proteinase 3 (PR3), cathepsin G (CathG), and myeloperoxidase, contributing to heightened oxidative stress [43,44].

NE is central to CF pathophysiology; it increases mucus production, activates cathepsins and matrix metalloproteases, and upregulates key neutrophil chemoattractants like interleukin (IL)-8 and leukotriene B4. This cascade drives further neutrophil influx, airway obstruction, and tissue damage [44]. Elevated NE levels in bronchoalveolar lavage fluid (BALF) correlate with bronchiectasis and lung function decline, especially in children with CF (Houston). NE also interacts with cytokines, enhancing their potency and degrading pattern recognition receptors, which reduces sensitivity to bacterial lipopolysaccharide (LPS) and amplifies inflammation [45]. The accumulation of mucus, primarily due to NE, leads to degradation of pulmonary matrix components and is associated with tissue damage and remodelling [43,46].

The inflammatory response in CF creates a vicious cycle of neutrophilic inflammation and oxidative stress, resulting in irreversible airway destruction and fibrosis. This is worsened by an imbalance between proteases and antiproteases, with excessive protease activity leading to tissue degradation and impaired immune responses [37,45,47]. Elevated tumour necrosis factor (TNF-α) and neutrophil counts exacerbate the inflammatory response, with neutrophils remaining the predominant inflammatory cells in CF airways [38,48]. Interestingly, CF neutrophils often adopt a pro-survival phenotype that delays apoptosis, prolonging their presence and activity in the airways [38]. This persistence facilitates continuous release of pro-inflammatory mediators, contributing to sustained airway inflammation [37,49]. Neutrophil recruitment is driven by inflammatory signals such as IL-8, which correlates with disease severity [38,45]. Finally, the formation of neutrophil extracellular traps (NETs), while beneficial for trapping pathogens, can harbour NE and contribute to tissue damage [45]. As the burden of neutrophil-derived proteases and oxidants increases, the airway's ability to manage inflammation and clear pathogens becomes overwhelmed, leading to progressive lung disease in pwCF [45,46].

Macrophages also play a crucial role in the inflammatory response in the lungs of pwCF, significantly influencing both the onset and maintenance of inflammation. Their dysfunction is closely tied to CF pathology, as CFTR dysfunction leads to delayed phagolysosomal fusion, resulting in ineffective bacterial clearance [47].

Increased levels of alveolar macrophages and associated CC-chemokines have been detected in BALF from pwCF, highlighting intrinsic abnormalities [37]. In particular, IL-1α stimulates the production of IL-8 [36], while IL-1β further exacerbates inflammation [43]. This dysregulated cytokine production is intensified by an overactive NF-κB pathway in CF airway epithelial cells, leading to elevated levels of inflammatory mediators, including GM-CSF [32].

Additionally, NE cleaves phosphatidylserine receptors on alveolar macrophages, impairing their ability to clear apoptotic cells [46]. This impairment contributes to the inability to resolve inflammation, perpetuating the inflammatory cycle in the CF lung. CF macrophages also exhibit enhanced TLR4-dependent responses to LPS, resulting in exaggerated inflammatory reactions [36,47].

Furthermore, there is an imbalance in macrophage polarization between the "pro-inflammatory" M1 and "pro-resolution" M2 phenotypes, with a tendency towards the M1 phenotype, which is associated with chronic inflammation [32,36]. Overall, the interaction of neutrophil activity and macrophage dysfunction, coupled with aberrant cytokine production and the dysregulation of immune responses linked to CFTR deficiency, significantly contributes to the chronic inflammatory state observed in the lungs of pwCF.

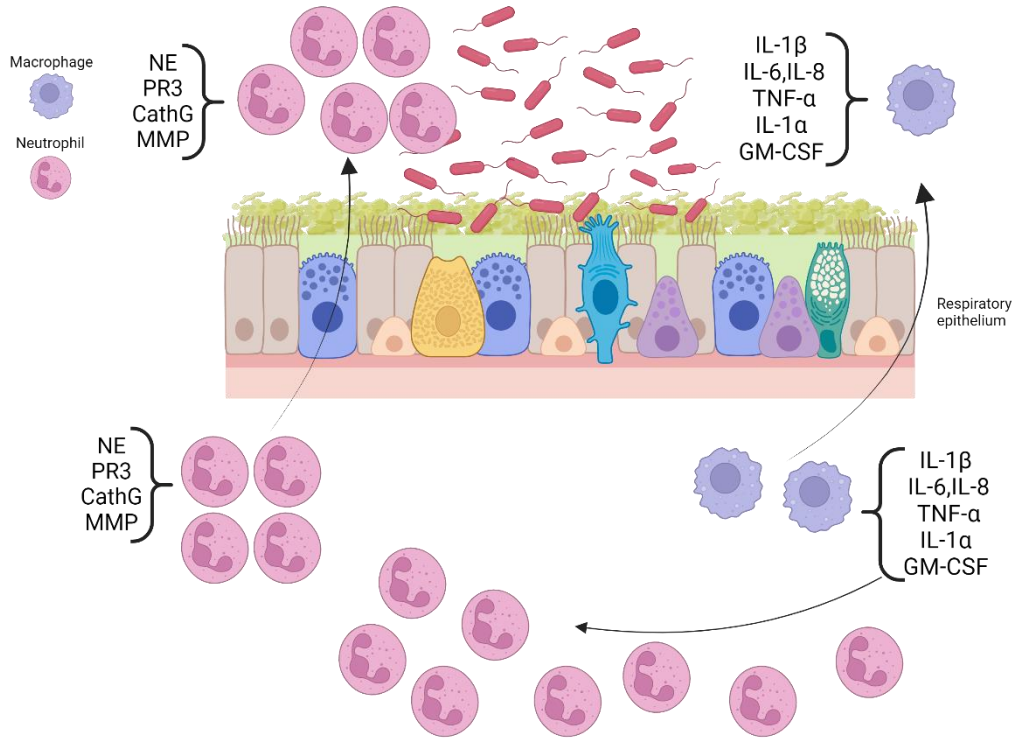

Figure 7. Semplified view of inflammation in the CF airway: in reaction to bacterial invasion, neutrophils move through the respiratory epithelium to the airway lumen, where they confront pathogens, secrete inflammatory mediators, and release proteases. This process damages the airway, and the released chemoattractants promote the recruitment of additional neutrophils. Macrophages also generate inflammatory mediators and play a crucial role in the host inflammatory response in CF (Realized with BioRender).

Lung infections

Impaired mucociliary clearance and ineffective innate immune defence results in complex lung infections caused by a variety of opportunistic pathogens, which can vary with age and disease progression. The predominant ones are *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, members of the *Burkholderia cepacia* complex (Bcc), *Stenotrophomonas maltophilia*, and *Achromobacter* spp. In younger children with CF, airway infections are frequently initiated by *S. aureus* and *H. influenzae* [50]. As patients age, particularly in older children and adults, *P. aeruginosa*, *S. maltophilia*, and *Achromobacter* spp. become more prevalent. Notably, *P. aeruginosa* is found in 60-80% of adult CF patients and is particularly difficult to eradicate, leading to significant declines in lung function and poorer prognosis [2,51,52]. Chronic infection with this pathogen can result in rapid progression to end-stage lung disease and increased mortality [2].

In addition to bacterial pathogens, fungal infections also play a critical role in CF lung disease. The most common filamentous fungi found in the lungs of pwCF are *Aspergillus* spp., which can cause allergic bronchopulmonary aspergillosis (ABPA), potentially leading to further pulmonary decline [53,54]. *Candida* spp. is another frequent isolate, associated with lower respiratory function [55].

Chronic infections caused by *S. maltophilia* are linked to adverse outcomes, including pulmonary exacerbations and deterioration of lung function, often necessitating lung transplantation or resulting in death [53,56–58]. The Bcc is particularly concerning due to its association with severe complications such as necrotizing pneumonia and Cepacia syndrome, which can lead to a rapid decline in lung function and fatal outcomes in 50% of cases [59–61].

Another pathogen of concern in CF is *Achromobacter* spp. [53,58,61]. This organism has gained recognition due to its potential to cause chronic lung infections, which can lead to significant respiratory inflammation and declining lung function. *Achromobacter* species exhibit innate and acquired multidrug resistance, complicating treatment efforts and making eradication challenging. Although infections with *Achromobacter* are less common than those caused by more established pathogens, the presence of A. *xylosoxidans* specie has been linked to adverse clinical outcomes, underscoring the need for vigilance in monitoring and managing infections caused by this emerging pathogen [62].

CF lung infections have been characterized as polymicrobial conditions, which are highly heterogeneous among patients and can fluctuate over time [63]. These pathogens can engage in intra- and inter-species interactions, potentially acting competitively or synergistically to gain an adaptive advantage. Such interactions may influence community composition, antibiotic resistance, and the progression of airway disease [64].

3. *Achromobacter* spp

Overview

*Achromobacter*ia are ubiquitous, lactose-non-fermentative, oxidase and catalase positive, motile, aerobic, gram-negative bacilli widely distributed in aquatic

environments and soil [65]. These opportunistic pathogens represent a significant risk to immunocompromised patients, including those with cancer, advanced HIV, diabetes mellitus, and chronic renal failure [66,67]. Notably, *Achromobacter* spp. are frequently isolated from sterile sources, including chlorhexidine solutions, ultrasound gel, and intravenous fluids and nosocomial outbreaks are often associated with contaminated disinfectant solutions[68–71].

Additionally, *Achromobacter* spp. exhibit resistance to various antibiotics, largely due to innate and adaptive mechanisms. Approximately 50 drug-resistance associated genes have been identified, along with a variety of mobile genetic elements carrying resistance genes [65,66,72–74].

Achromobacter spp. have been increasingly identified in respiratory secretions of pwCF, where they can persist for extended periods in both the upper and lower airways, contributing to disease progression and respiratory decline [65].

While in the past many species were referred to as *A. xylosoxidans* due to challenges in differentiation, today the genus *Achromobacter* includes twenty-two genetically distinct species and multiple genogroups [75,76]. *A. xylosoxidans* is the type species and the most prevalent one found in CF clinical samples. Other species associated with CF lung infections include *A. dolens* and *A. insuavis*, which are the other species most isolated in Europe, *A. ruhlandii*, *A. dentifricans*, *A. insolitus*, and *A. aegrifacens* [62,68,74,77].

Chronic colonization by *A. xylosoxidans* is linked to lung inflammation and declines in respiratory function, with infections resulting from both environmental acquisition and direct or indirect transmission [65,78–82].

Adaptation strategy

General view

The growing number of sequenced *Achromobacter* spp. genomes has allowed researchers to initially focus on genomic differences between clinical and environmental isolates, and later on the genomic factors associated with pathogenicity and adaptation during chronic infections, revealing various

adaptation mechanisms in the CF lung environment. A phylogenetic study designed to assess the differences between environmental and clinical strains showed that 35 genes involved in metabolism were positively selected in the clinical isolates and includes COG (Clusters of Orthologous Groups) functional categories, such as amino acid transport and metabolism, carbohydrate transport and metabolism, cell wall/membrane/envelope biogenesis, coenzyme transport and metabolism, energy production and conversion, inorganic ion transport and metabolism, secondary metabolite biosynthesis, transport and catabolism and lipid transport and metabolism. Additionally, these isolates carried a higher number of antibiotic resistance genes, particularly against aminoglycosides, β-lactams, chloramphenicol, and sulfonamides[83].

Indeed, during CF lung colonization, bacteria persist under the selective pressure exerted by the host immune system and antibiotic treatments by enhancing nutrient acquisition, developing mechanisms to avoid toxic compounds and evade immune responses, and facilitating the colonization of new niches[84].

Metabolism

Studies on the within-host evolution of *Achromobacter* spp. in pwCF have revealed that the most commonly mutated genes during adaptation are primarily involved in general metabolism, which play a crucial role in adapting to host conditions, outcompeting the resident microbiota, and overcoming other opportunistic pathogens [85–87]. These mutations often result in the attenuation of functions that are not essential for survival in the CF lung environment, such as amino acid synthesis. This might be due to the elevated amino acid concentrations in CF sputum, providing bacteria with an adequate supply[81]. One key adaptive mechanism identified in these studies is the bacterial ability to survive under limited oxygen conditions, which has been linked to its persistence in the CF airways [83]. The ability to grow in hypoxic or anaerobic conditions allows microorganisms to localize deeper within the mucous layer or within biofilm structures, or to colonize more hypoxic regions of the lung, where the effectiveness of antibiotics may be significantly reduced [88]. The use of denitrification for energy production in oxygen-deprived environments, such as CF mucus, has been demonstrated also in *P.*

aeruginosa[89]. Additionally, it has been shown that molybdenum uptake, which is necessary for denitrification, plays a crucial role in anaerobic proliferation and virulence in *P. aeruginosa* [90,91]. Studies have suggested that *Achromobacter* may share this adaptive strategy with *P. aeruginosa*, particularly in CF clinical isolates [83]. Furthermore, the ability to grow anaerobically has been proposed as a distinguishing factor between isolates from occasional infections and those from chronic infections [92].

Virulence factors

Despite the mechanisms underlying *Achromobacter* ability to colonize the CF lung are not being fully understood, several virulence factors have been identified that contribute to its survival and persistence in CF hostile environment. Similar to well-studied pathogens, biofilm formation, motility, tolerance to lower oxygen levels, and the secretion of virulence factors have been shown to play key roles in the infection, adaptation, and persistence of *Achromobacter* (primarily *A. xylosoxidans*).

Among these, biofilm formation plays a central role in bacterial adaptation. Biofilms, composed of bacterial microcolonies encased in a matrix of polysaccharides, proteins, and DNA, offer a protective environment that shields bacteria from environmental stress, including immune responses and antibiotics [93–96]. In fact, biofilm production is crucial for the chronic colonization of the CF lung, as it not only enhances bacterial survival but also contributes to antimicrobial resistance [81,94,95]. The genes involved in biofilm formation in *Achromobacter* spp. include the *flgB* gene, which encodes a flagellar basal body rod protein, implicated in biofilm formation in *Bordetella bronchiseptica* and *P. aeruginosa* [97,98], as well as the pgaABCD operon, which encodes a polysaccharide involved in cell-cell adhesion, also seen in other CF pathogens [82]. Additionally, a study conducted in vitro on *A. xylosoxidans* highlighted that the *echA* gene, which encodes a putative enoyl-CoA hydratase, plays an essential role in biofilm ultrastructure and antimicrobial resistance [96]. Members of the *Achromobacter* genus possess peritrichous flagella, which facilitate swimming motility and host cell invasion;

also, they contribute to the mechanism of biofilm formation, respiratory tract colonization and adaptation [99]. A study of 52 *A. xylosoxidans* strains isolated during an outbreak at a CF Centre in Rome (Italy) found that the majority of strains were motile and capable of biofilm production. Notably, a higher prevalence of strong biofilm-producing strains was observed in patients with severely impaired lung function [100]. These findings were attributed to an enhanced adaptation of *A. xylosoxidans* to the CF nosocomial environment. Biofilm production, in particular, appears to play a critical role in bacterial persistence, as gene expression profiles and antimicrobial susceptibility differ significantly between biofilm-associated and planktonic cells. In biofilms, genes associated with anaerobic respiration in *Achromobacter* spp. have been found to be upregulated, suggesting adaptation to the microaerobic and anaerobic conditions that prevail in the late stages of CF chronic infections [101]. Interestingly, a reduction in biofilm formation has been observed in chronic strains over time, which may be a result of within-host adaptation during prolonged colonization of the CF lung [94]. Furthermore, *Achromobacter* spp. has been shown to form mixed biofilms with *P. aeruginosa* in vitro, where it can influence *P. aeruginosa* biofilm formation [85,86].

Furthermore, *Achromobacter spp.* possess several well-known membrane-bound virulence factors, typical of Gram-negative pathogens that enhance their pathogenic potential. These include the Vi capsular polysaccharide, which aids in adhesion and protection against host defences and the O-antigen, involved in eliciting the host immune response [102]. The same effect is obtained by LPS, another important membrane-bound virulence factor, which triggers the production of inflammatory cytokines like IL-6, IL-8, and TNF-α [93,103]. It has been suggested that the accumulation of mutations in genes involved in LPS production might be involved in *Achromobacter* spp. persistence in CF lungs, probably leading to a reduced recognition by the host defence system[92].

Secretion systems are essential components of the bacterial cell membrane and play a critical role in bacterial pathogenicity by facilitating the release of toxins, proteases, and other virulence factors. In the *Achromobacter* genome, several genes encoding different types of secretion systems have been identified, including: the

type II secretion system (T2SS), widely conserved among γ-proteobacteria, which is responsible for releasing extracellular toxins and proteases; the type III secretion system (T3SS), which delivers virulence factors directly into host cells; the type VI secretion system (T6SS), which mediates factor transport through direct contact with target cells; and the type VII secretion system (T7SS), which includes genes encoding sigma-fimbriae[104]. Furthermore, research on the T6SS has revealed that *A. xylosoxidans* contains two T6SS-encoding gene clusters, TAX-1 and TAX-2. The presence of TAX-1 is associated with toxin secretion that helps eliminate competitive pathogens and facilitates bacterial internalization into alveolar basal epithelial cells [105].

The T3SS is one of the most clinically significant systems: it enhances the bacterial ability to infect host cells by injecting effector proteins and contributes to immune evasion [82,99,102]. A phospholipase A2 (PLA2), encoded by most *A. xylosoxidans* genomes and termed AxoU, has been identified as a T3SS substrate that induces cytotoxicity in macrophages, suggesting a pathogenic or inflammatory role in the CF lung [106], and recent studies have also shown that *Achromobacter* species can induce cell death in human macrophages via inflammasome-dependent pyroptosis, engaging NLRC4 or NLRP3 sensors in a T3SS-dependent manner [107]. The presence of T3SS has also been identified as a key distinguishing factor between clinical and environmental *Achromobacter* strains. Comparative genomics analyses revealed that virulence genes associated with T3SS are more common in CF isolates than in environmental strains [83,102], linking this feature to the bacterial ability to cause infection. Moreover, the presence of functional T3SS genes appears to be crucial for establishing chronic infections in the CF lung, whereas isolates from occasional infections tend to lack functional T3SS genes [74].

Genomic studies have identified several secreted virulence factors in *Achromobacter* spp. Among them, colicin V, a cytotoxic protein, likely provides environmental advantages by eliminating competing flora and promoting tissue invasion. Additionally, the gene encoding AepA facilitates the production of cellulases and proteases, which are key to mucosal invasion [73,82]. Moreover, the production of phospholipase C has been observed, which hydrolyses the

phospholipids of alveolar surfactants, leading to tissue disruption [90]. A heat-stable cytotoxic factor has also been identified, which is associated with an increase in pro-inflammatory cytokines *in vitro* [93].

Interestingly, some *Achromobacter* spp. strains have been shown to inactivate the *P. aeruginosa* quinolone signal (PQS), a key component of the Quorum Sensing (QS) mechanisms that coordinate gene expression when bacterial cells reach a critical density [108]. QS forms a global regulatory network that is thought to control the expression of up to 12% of the *P. aeruginosa* genome [109]. Therefore, *Achromobacter* spp. ability to disrupt QS may confer a competitive advantage over *P. aeruginosa* and potentially other CF pathogens, such as *S. maltophilia*, when coexisting in the same lung environment. This interference could affect various bacterial traits, including growth, motility, and biofilm formation [85,86,110].

Differences among the species

A genetic study by Veschetti et al. identified several key genes present in *A. xylosoxidans*, *A. aegrifaciens*, *A. insolitus*, *A. insuavis*, *A. dolens*, and other *Achromobacter* species that belong to non-associated genogroups (NG) [74] . These include genes for arginosuccinate synthase, carbamoyl phosphate synthase large chain, Hfq, and the large subunit ribosomal protein L36p. In contrast, *A. aegrifaciens*, *A. insolitus*, and *Achromobacter* NG lack genes for respiratory nitrate reductase, GDP mannose 4,6-dehydratase, and liN, which encodes a flagellar protein[74] . These species also lack type T3SS genes [74].

Moreover, in *A. aegrifaciens* and *A. insolitus*, the *fliP* and *fliC* genes, which encode flagellar proteins, are absent, as is the *yihY* gene, associated with serum resistance, in both these species and in *Achromobacter* NG isolates [74]. Notably, all *A. insuavis* strains exhibited a putative oxidoreductase gene, while endotoxin-related genes were identified in *A. xylosoxidans* [74]. In *A. dolens*, a gene encoding UDP 2-acetamido-3-amino-2,3-dideoxy D-glucuronic acid acetyltransferase, which is involved in O antigen biosynthesis, was found. Additionally, *A. aegrifaciens* and many *A. xylosoxidans* isolates contained the putative cysteine hydrolase *ycaC* gene, but only *A. aegrifaciens* had the *motA* gene, which is related to flagellar motility [74].

Interestingly, genetic differences were observed between occasional and chronic isolates of *A. xylosoxidans*: the former carried the *fliP* and NADP-dependent malic enzyme genes, whereas *A. insolitus* isolates—whether occasional or chronic—did not show significant genetic variation [74].

Antibiotic resistance

An important factor contributing to the survival of *Achromobacter* spp. within the host is its ability to resist antibiotics. This resistance is facilitated by both innate and acquired defense mechanisms, making infections particularly difficult to treat. Several factors contribute to the antibiotic resistance patterns of these bacteria, including the production of degrading enzymes, the activation of efflux pump systems, and alterations in the antibiotic target sites.

Achromobacter spp. exhibit innate resistance to a variety of antibiotic classes, including aminoglycosides, certain monobactams (such as aztreonam), tetracyclines, some penicillins (e.g., penicillin G and ticarcillin), and cephalosporins-antibiotics commonly used in the treatment of CF lung infections [99,100,111].

The most commonly conserved resistance genes in *Achromobacter* spp. can be classified into five groups: class B β-lactamase, group B chloramphenicol acetyltransferase, rRNA methylases, class A β-lactamase, and resistance-nodulation-cell division (RND) efflux pumps [72]. In particular, many members of the RND efflux pump group are linked to intrinsic resistance. Notably, AxyABM is capable of pumping out most cephalosporins, fluoroquinolones, aztreonam, and chloramphenicol, while AxyXY-Opr extrudes aminoglycosides and can also extrude ceftazidime, tetracyclines, and erythromycin [99,112,113].

Another key resistance mechanism in *Achromobacter* spp. is the production of β-lactamases. Interestingly, the distribution of bla_{OXA} genes is species-specific: while the presence of $bla_{OXA-114}$, $bla_{OXA-243}$, and $bla_{OXA-364}$ has been previously reported for *A. xylosoxidans*, *A. insuavis*, and *A. dolens*, respectively[113], recent studies found no bla_{OXA} genes in *A. aegrifaciens* and some *A. insolitus* isolates, while bla_{OXA-2} was identified in other *A. insolitus* strains [86]. OXA-114-like enzymes exhibit strong

activity in vitro against penicillin G, early cephalosporins, piperacillin, and ticarcillin [114]. However, *A. xylosoxidans* isolates positive for OXA-114 often show phenotypic susceptibility to piperacillin[114,115]. Furthermore, extended-spectrum β-lactamases, AmpC-type β-lactamases, and metallo-β-lactamases have been detected in *A. xylosoxidans* CF isolates, contributing to resistance against β-lactams, including carbapenems [66,116–120] .

Several studies have examined the role of antibiotic resistance in *Achromobacter* infections in pwCF. For example, a pan-genomic analysis of publicly available *Achromobacter* spp. genomes[83] revealed that clinical strains carry a higher number of resistance genes compared to non-clinical strains, including genes for resistance to aminoglycosides (six extra genes), β-lactams (six extra genes), chloramphenicol (three extra genes), and sulfonamides (two extra genes). These additional genes might contribute to acquired resistance, although the study did not provide information about their origin, such as whether they come from mobile genetic elements or recombination. Furthermore, the clinical strains showed a positive selection for three genes involved in the formation of efflux pump components: *emrA, macA*, and *mexW* [83]. The proteins encoded by these genes are part of different efflux pump systems: a major facilitator superfamily complex, an ABC-type pump responsible for exporting macrolides, and an RND-type pump respectively. The positive selection of these genes suggests that efflux pumps play a significant role in the adaptation of *Achromobacter* to a pathogenic lifestyle, as they are known to contribute to bacterial virulence [121] and tend to reduce specificity, leading to multi-drug resistance[122,123].

A recent study of 101 clinical isolates of *Achromobacter* revealed a strong link between the development of antibiotic resistance and chronic infections. The findings indicated that later strains (>1 year from 1st colonization event) were significantly less susceptible to antibiotics compared to earlier (<1 year from 1st colonization) or single isolates [124]. Furthermore, nearly all isolates showed resistance or susceptible increased exposure to several antibiotics, including aztreonam, ceftriaxone, cefuroxime, ciprofloxacin, moxifloxacin, penicillin, rifampicin, tobramycin, and trimethoprim. Notably, the study found that isolates

from a Danish epidemic strain of *A. ruhlandii* exhibited resistance or susceptible increased exposure to a median of 20 antibiotics, while *A. xylosoxidans* and *A. insuavis* isolates had a median resistance to 14 antibiotics. This higher level of resistance in the *A. ruhlandii* strain may help explain its prevalence among individuals with cystic fibrosis in Denmark.

In brief, the rise of acquired antimicrobial resistance in chronic *Achromobacter* infections in CF, via chromosomal mutations or horizontal gene transfer, is becoming an increasing concern. This is particularly troubling because such resistance can spread between genetically similar pathogens, such as *P. aeruginosa*, *Ralstonia* spp., and *Burkholderia* spp. Conducting more extensive antimicrobial susceptibility testing on *Achromobacter* clinical isolates could expand current knowledge and improve the diagnosis, helping to track its emergence and persistence.

CF infections prevalence

According to the ECFS Patient Registry of 2022, *Achromobacter* species can be found in up to 20% of the airways of pwCF in Europe, with a higher prevalence in adults compared with data regarding paediatric patients. Similarly to European data, the latest US annual registry showed that the prevalence of *Achromobacter* spp. varies by age group, with an increase of infections in adult patients (Patient Registry Annual Data Report, 2023). Unfortunately, European CF patients' annual registry has only reported *Achromobacter* spp. infection data starting from 2018, so a general comprehensive analysis of European data is not possible, but literature shows that at most centres around the world the prevalence of *Achromobacter* spp appears to be less than 10% [125].

Although the deficiency in *Achromobacter* spp. prevalence data, it has been observed a different distribution of *Achromobacter* species in pwCF among countries with available data. The most often isolated *Achromobacter* species in pwCF in all countries is *A. xylosoxidans* [73,77,124,126–129]. Denmark and France have similar rates of *A. insuavis* infections (20–24% and 19%, respectively), while the UK, Italy, and Argentina report lower rates (12%, 8%, and 5%, respectively). *A.*

dolens is most common in the United States (17%) and Argentina (10%), while the rates are lower in the United Kingdom, Italy, and Denmark (8%, 3%, and 0–2%, respectively). Additionally, the prevalence of *A. ruhlandii* ranges from 17 to 25% in Argentina, the United States, and Denmark, where two CF centers reported an outbreak[130,131]. *A. insolitus* appears to be more common in Italy (12%) than in France and the US (both 4%), whereas *A. aegrifaciens* is more common in Italy and France (12–15%) than in Denmark (<5%). Anyway, a more accurate estimation of prevalence is hampered by the small number of pwCF included in some of the studies, the still inadequate species-level identification methods and the evolving nomenclature[114,132–134].

In relation to data collected, *A. xylosoxidans, A. ruhlandii, A. dolens,* and *A. insuavis* are highly prevalent among clinical isolates; therefore, their phylogenetic clustering and capacity to cause chronic infections may suggest that they are better suited to cause opportunistic chronic infections [83].

Despite the fact that half of pwCF with airway colonization by *A. xylosoxidans* develop chronic infections, usually associated with decline in respiratory function and lung inflammation [75,78–80,128,135], the clinical impact of the other *Achromobacter* species is still poorly characterized. Based on the existing longitudinal studies [75,81,124], most individuals with cystic fibrosis who have a chronic lung infection caused by *Achromobacter* spp. appear to carry a distinct strain or clone type, as determined by whole genome sequencing (WGS) or pulsed-field gel electrophoresis (PFGE).

Notably, two of these studies [75,124] found that 20–24% of patients were infected with multiple *Achromobacter* species and/or clone types during the sampling period, indicating that not all strains of *Achromobacter* spp. are responsible for causing chronic lung infections. Instances of cross-infection among patients have been documented, including indirect transmission from one person to another [79,136]. For instance, the *A. ruhlandii* strain responsible for a Danish epidemic has been detected in several patients at the same cystic fibrosis center. More recently, transmission between patients has also been confirmed for *A. xylosoxidans* and *A. insuavis* strains. While some cases showed clear epidemiological links, such as between

siblings or during visits, others lacked identifiable connections to support the occurrence of cross-infection [124,137]. In all documented cases, whole genome sequencing (WGS) has been crucial for typing *Achromobacter* species and confirming patient-to-patient transmission.

Identification methods

Identification methods for *Achromobacter* genus determination in current use include biochemical testing (e.g. VITEK2), sequencing of nrdA or 16S rRNA gene, multi-locus sequence typing (MLST), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF).

One of the most common method used in clinical microbiology laboratories for routine identification of bacteria is the biochemical testing, which is usually a very rapid and reliable method, employed in clinical diagnostic for many years [138]. In the case of *Achromobacter* spp, it often leads to a less accurate species recognition and occasionally misidentification due to biochemical similarities with other Gram-negative bacilli [114,132,139] .

Another identification method commonly used for clinical diagnostic is MALDI-TOF MS, which enables precise identification at the genus level [132,139,140]. However, accurate species-level identification remains challenging due to the limited number of species represented in MALDI-TOF databases [114]. Efforts to enhance the accuracy of MALDI-TOF by expanding the species included in these databases have shown promising results [77,141]. Incorporating these species into commercial databases will facilitate more accurate identification at the species level.

Regarding sequence-based identification, *nrdA* gene sequencing or MLST (which consists of seven genes*: nusA*, *rpoB*, *eno*, *gltB*, *lepA*, *nuoL*, and *nrdA*-accessible via PubMLST[142]) provides more accurate species recognition[143]. In contrast, 16S rRNA gene sequencing has been shown to be inadequate for definitive species identification due to the gene's conserved nature [68]. With advancements in sequencing technologies, WGS has emerged as an appropriate method for accurately identifying bacteria while generating a wealth of data. The increasing application of WGS will facilitate correct species-level identification of

Achromobacter, including the reassignment of taxa that have historically been misidentified.

4. *Galleria mellonella* larvae as a model organism for CF infections

Advantages of Galleria mellonella larvae model

The larvae of *Galleria mellonella*, commonly known as the greater wax moth, have gained increasing attention as an alternative in vivo model for studying microbial infections, including those relevant to CF. Compared to traditional vertebrate models, *G. mellonella* offers several distinct advantages: larvae are inexpensive, easy to handle, do not require ethical approvals, and can be maintained at 37°C, a temperature that supports the growth of human pathogens [144]. Furthermore, their innate immune system shows remarkable functional similarities to that of mammals, including hemocyte-mediated phagocytosis, melanization, and the production of antimicrobial peptides (AMPs), opsonins, and phenoloxidase activity [145].

Additionally, the ability to breed large numbers of larvae enables statistically robust experimental designs, while their rapid development and short life cycle allow for quick generation of results. Their immune response is sufficiently complex to allow for meaningful insights into virulence mechanisms, yet simple enough to avoid the confounding effects of adaptive immunity [145,146].

Galleria mellonella larval model

The *G. mellonella* larvae infection model involves injecting pathogens directly into the larval hemocoel, typically through the last right proleg. Following infection, larvae are incubated at 37°C, and various parameters such as survival rate, melanization, and immune response markers are monitored over time [146]. The larvae's hemolymph acts analogously to vertebrate blood and hosts hemocytes that perform immune functions similar to mammalian neutrophils.

The immune responses assessed include hemocyte density, phenoloxidase (PO) activity, melanin production, and the expression of AMPs. Histological analyses are often conducted to observe tissue-level damage caused by infection. Larvae respond to microbial invasion with immune processes such as phagocytosis, nodulation, and encapsulation, followed by melanization, a key defense mechanism involving PO that results in the production of melanin to trap and neutralize pathogens [145].

Pathogens studied in *Galleria mellonella* larvae model

Numerous human pathogens have been studied using the *G. mellonella* larvae model, particularly those relevant to CF patients, such as *P. aeruginosa*, *S. aureus*, *C. albicans*, and *Aspergillus fumigatus* [145,147–150]. In fungal infection studies, for example, *C. albicans* and *A. fumigatus* induced strong immune responses in larvae, with observable differences in virulence reflected by mortality rates, hemocyte depletion, and increased melanization and PO activity [146,149,150].

The larvae have also been used in antibacterial and antifungal drug screening, offering predictive value for preclinical evaluations. The model has shown a good correlation with vertebrate outcomes, reinforcing its value as a surrogate host in early-stage studies [144,145].

5. Mouse as a model organism for CF infections

Although murine CFTR shares only 78% overall amino acid sequence homology with its human counterpart, the development of genetically engineered mouse models has significantly advanced our knowledge of CF pathogenesis, particularly with regard to pulmonary inflammation and infection [151]. Murine models represent a powerful tool for understanding the mechanisms underlying CF, offering several inherent advantages such as low maintenance costs, ease of handling, and rapid reproduction. A major advantage of murine models lies in the ability to perform targeted genetic manipulations and create CFTR-deficient lines carrying clinically relevant mutations, such as ΔF508, G551D, and R117H. These models allow

researchers to explore genotype-phenotype relationships, test mutation-specific therapies, and investigate modifier genes influencing disease severity.

The first CF mouse model (CFTRtm1UNC) was generated through the insertion of a premature stop codon (S489X) in exon 10 of the murine CFTR gene [152]. Subsequently, additional knockout models were developed (e.g., CFTRtm1CAM, CFTRtm1HSC, CFTRtm3BAY, CFTRtm3UTH, CFTRtm1HGU, CFTRtm1BAY) [153–157].

Although traditional CF mouse models have provided valuable insights, their ability to accurately replicate the pulmonary manifestations of human CF has been limited, largely due to species-specific anatomical and physiological differences. For example, to address early lethality caused by severe intestinal disease, "gut-corrected" mice were generated using transgenic expression of CFTR under an intestine-specific promoter [158].

Recent advances have introduced novel models that better reflect the genetic diversity observed in human populations. Bragonzi et al developed a line breeding CFTR-F508del mice on a Collaborative Cross (CC) genetic background, which exhibits variable phenotypic expression of CF-related traits, including intestinal disease severity and survival, depending on genetic context. These findings underscore the critical role of genetic modifiers in CF pathophysiology [159].

Lung Pathology in CF Mouse Models

While the upper respiratory tract of murine models mirrors that of humans with CF, the lower airways present a different scenario. No CFTR mouse model develops spontaneous lung inflammation without external challenge, limiting their utility in studying CF pulmonary disease progression [152–154,156,160–164].

This lack of severe spontaneous lung pathology has been partially attributed to the expression of alternative, non-CFTR calcium-activated chloride channel (CaCC), in murine tissues, which may compensate for CFTR deficiency and help maintain ion balance [165]. Notably, a congenic strain of the $CFTR^{tm1UNC}$ mouse model, termed

B6-CFTR^{tm1UNC}, lacking these alternative chloride channels, has been reported to develop spontaneous lung disease, including impaired mucociliary clearance and tissue fibrosis, even under pathogen-free conditions [166]. These mice also exhibit impaired control of *P. aeruginosa* infection. Furthermore, chronic pulmonary inflammation and bacterial persistence have been observed in CFTR^{tm1UNC} mice following intranasal challenge with *B. cepacia*, characterized by increased neutrophil counts and cytokine levels [167]. Significant pulmonary inflammation and associated pathology were also induced in CFTR^{tm1UNC} mice following intratracheal delivery of *P. aeruginosa*-embedded agarose beads [157].

Additionally, low expression levels of the ATP12A proton pump in CF mice may allow for normal ASL pH and unimpaired airway host defenses, potentially explaining the increased protection from pulmonary infection observed in these models [168].

Recent in vivo studies have further elucidated the role of CFTR in immune cell function. Loss of CFTR function in myeloid immune cells, particularly neutrophils, leads to persistent neutrophilic inflammation, even in the absence of infection, due to impaired resolution of inflammation and overproduction of proinflammatory cytokines[169]. Similarly, CFTR deficiency in macrophages alters their transcriptional program, resulting in delayed resolution of lung inflammation following *P. aeruginosa* infection [170]. Moreover, CFTR-deficient mice exposed to repeated LPS challenges develop persistent lung inflammation and remodeling, indicating that chronic inflammation alone can contribute to CF-like lung pathology [169].

Another relevant model is the β-ENaC transgenic mouse, which overexpresses the epithelial sodium channel, leading to airway surface dehydration, mucus obstruction, and spontaneous neutrophilic inflammation, thereby mimicking key features of CF lung disease[158].

Studying Lung Inflammation In Vivo

In vivo analysis of lung inflammation remains essential for understanding the pathophysiology of chronic respiratory diseases and for developing new therapeutic

strategies. Traditionally, inflammation in murine models is induced by invasive lung challenge using bacterial cells or pro-inflammatory bacterial products such as LPS, BALF collection post-mortem to assess immune cell infiltration and cytokine profiles [171–173]. This method, while validated and widely used, relies on ex vivo sampling, which requires the sacrifice of animals and becomes particularly costly when using genetically modified or transgenic mice.

In CF transgenic mouse models, such approaches may lack full reproducibility due to the inherent heterogeneity of airway inflammation in CF. Moreover, the inability to perform longitudinal analysis on the same animals represents a major limitation in assessing dynamic disease progression or therapeutic response over time.

To address these issues, both in vitro and in vivo imaging models have been developed to study the molecular mechanisms underlying chronic inflammation and to evaluate the anti-inflammatory effects of candidate therapeutics [174,175].

In recent years, a range of non-invasive imaging modalities has been adopted for longitudinal monitoring of lung inflammation in animal models. Techniques such as micro-computed tomography (micro-CT), magnetic resonance imaging (MRI), and optical imaging allow for serial, real-time assessment of airway remodeling and inflammatory responses without the need to euthanize animals. These technologies, initially designed for clinical use (MRI, CT, PET), have been adapted for small animal imaging and serve as crucial preclinical tools bridging the gap between laboratory research and clinical translation [176–178].

Optical imaging systems, including bioluminescence and fluorescence imaging, have also demonstrated potential in tracking inflammation in live animals, particularly in intra-operative or targeted molecular imaging settings [179,180].

Notably, a murine model was developed with transient expression of a luciferase reporter gene driven by a bovine IL-8 promoter. Although mice lack a true IL-8 homolog, the model exploits conserved inflammatory signaling pathways capable of activating this exogenous promoter, thereby enabling real-time visualization of

inflammation in vivo [181]. More recent developments have improved such reporter systems to enhance sensitivity, specificity, and temporal resolution.

6. Zebrafish embryo as model organism for CF infections

Advantages of Zebrafish as animal model

Zebrafish (*Danio rerio*) have been utilized as an animal model for research for several decades, providing significant insights into a wide range of human diseases. Their small size, transparent embryos, and rapid development make them an invaluable tool for studying various diseases, including cancers, cardiovascular conditions, muscular disorders, and neurological diseases [182–185]. Zebrafish have been particularly instrumental in understanding the genetic and molecular mechanisms underlying these disorders, offering researchers a platform to investigate disease pathways.

A major advantage of the zebrafish model is its strong potential for genomic and large-scale mutant analysis. The zebrafish genome is fully sequenced and well-annotated (ZF version 9; http://www.ensembl.org/Danio_rerio/Info/Index), with over 26,000 protein-coding genes identified. This genome shows a high degree of conservation with human orthologs, particularly in genes related to both the innate and adaptive immune systems[186–188].

Their relatively low cost, ease of large-scale breeding, and transparency of embryos make them particularly advantageous for high-throughput studies, such as drug screening and genetic research [189–192]. Furthermore, the ability to monitor disease progression and treatment effects in real-time through live imaging adds an extra dimension to their utility, making zebrafish an indispensable tool in modern biomedical research.

In addition to their utility in studying human diseases, zebrafish have been widely employed to examine both animal and human infectious diseases [193]. This is due to the fact that the zebrafish immune system closely resembles that of mammals, making it an excellent model for immunological studies [194–196]. The immune system of adult zebrafish encompasses many of the same lymphoid organs and immune

cell types found in humans, including macrophages, neutrophils, and various T and B lymphocytes [194,195].

During the early stages of development, up to approximately two weeks post-fertilization, zebrafish rely solely on innate immunity, with macrophages and neutrophils playing a crucial role in the defence against infections [182,197,198]. This makes them a particularly useful model for studying the initial immune responses to pathogens and understanding the genetic factors that regulate immune development.

The use of zebrafish in infectious disease research has revolutionized our ability to study host-pathogen interactions in vivo, thanks to their small size and remarkable transparency, particularly during the first week after fertilization. This unique feature allows for real-time observation of disease processes using advanced imaging techniques, providing an unprecedented level of detail. Key imaging modalities include differential interference contrast (DIC) and fluorescence microscopy, which enable the visualization of cellular dynamics and pathogen behaviour within living organisms [199–202].

One of the major advantages of using zebrafish in infectious disease studies is the ability to utilize fluorescently labelled pathogens and transgenic zebrafish lines that express fluorescent markers for immune cells, immune response genes, or cytokines/chemokines. These tools allow researchers to track the interactions between the host and pathogen in real-time, offering a detailed view of immune responses at both the cellular and molecular levels. Through these advanced techniques, it is possible to visualize the activation of immune cells, the migration of leukocytes to infection sites, and the dynamics of pathogen colonization [203–207].

Additionally, zebrafish are increasingly considered a viable alternative to traditional animal models due to several key advantages, including high fecundity, small size, ease of maintenance, rapid development, and fewer regulatory and ethical concerns. Since fish embryos in the early developmental stages are not believed to experience pain, suffering, or distress, they are often regarded as an ethical choice for scientific research[208].

Studies have employed zebrafish larvae to examine how the immune system responds to a range of pathogens, including *Mycobacterium marinum* [201,209], *Streptococcus* species [210,211], *Salmonella typhimurium* [212], *S. aureus* [213,214] , *P. aeruginosa* [215–217] and *B. cenocepacia* [218,219]. In each of these cases, zebrafish have provided critical insights into the early immune response, such as the activation of macrophages and neutrophils and pathogens' host immune system evasion mechanisms.

The combination of real-time imaging, genetically engineered zebrafish, and a wide array of fluorescent markers makes this model system a powerful tool for understanding the complex interactions between pathogens and the immune system. These capabilities not only contribute to our knowledge of basic immunology but also offer potential for developing novel therapeutic strategies and evaluating the efficacy of anti-infective drugs.

Zebrafish embryo model

Zebrafish embryos have become an invaluable model for studying the innate immune response to bacterial infections [208,220]. Genetic tools allow for precise manipulation of host genes, either through transient knockdown via translation-blocking antisense oligonucleotides injected at the one-cell stage (creating morphants) or through permanent gene knockdowns by generating mutated fish lines [220].

At the embryonic stage, only the innate immune system is functional, making it an ideal model for investigating early immune responses. Macrophages begin to appear at 22 hours post-fertilization (hpf), and both macrophages and neutrophils are fully functional by 30 hpf. The transparency of zebrafish embryos allows direct, real-time observation of the infection process without the need for animal sacrifice. This non-invasive imaging technique, combined with the use of transgenic fluorescent zebrafish lines, allows for high-resolution monitoring of cellular dynamics and host-pathogen interactions. The ability to visualize pathogens, immune cells, and immune molecules in the three-dimensional context of tissues

provides crucial insights into tissue- and organ-specific immune responses. Additionally, this method allows for detailed imaging of immune cell activities, such as phagocytosis, and the interactions between host cells and invading microbes.

Another advantage of this model is the ability to chemically or genetically manipulate embryos to selectively deplete macrophages or neutrophils, helping to better understand their specific roles during infection.

The large number of zebrafish larvae available for experimentation also enables high-throughput screenings, facilitating large-scale evaluations of pathogen virulence and immune responses [184,202].

Pathogens studied in zebrafish model

Recent studies have provided significant new insights into the mechanisms underlying human infectious diseases through the use of infection models in zebrafish. One prominent example is the use of *M. marinum*, a pathogen closely related to the human pathogen *Mycobacterium tuberculosis* [201]. In zebrafish, *M. marinum* induces granuloma formation, which is a hallmark histopathological feature of human tuberculosis. This makes the *M. marinum* zebrafish infection model an essential tool for studying tuberculosis. Key findings from this model have highlighted several important factors in the host response. For instance, the stabilization of the transcription factor hypoxia-inducible factor (HIF-) has been shown to favour the host's defence against *M. marinum*. Additionally, the CXCR3/CXCL11 signalling axis plays a crucial role in the recruitment of macrophages and the dissemination of mycobacterial infection. Moreover, Dram-1-mediated autophagy has been identified as an important host defense mechanism to counteract *M. marinum* infection [201,204,205]. These findings not only advance our understanding of tuberculosis pathogenesis but also present potential targets for therapeutic intervention against the disease [201,221].

Salmonella typhimurium infections in zebrafish larvae have provided valuable insights into the role of mitochondrial-associated enzymes in immune responses.

One such enzyme, immunoresponsive gene 1 (IRG1), plays a pivotal role in the intracellular degradation of phagocytosed bacteria. IRG1, expressed by macrophages, directs the mitochondrial catabolism of fatty acids, leading to the production of mitochondrial reactive oxygen species (ROS). These ROS contribute to the clearance of intracellular bacteria, emphasizing the critical link between cellular metabolism and immune defense mechanisms. This discovery positions IRG1 as a novel therapeutic target for treating intracellular bacterial infections [206] .

Another important zebrafish infection model involves *B. cenocepacia*, an opportunistic pathogen that poses a significant risk to individuals with cystic fibrosis or those who are immunocompromised. Studies in zebrafish larvae have revealed that *B. cenocepacia* can survive and replicate within macrophages, before disseminating to cause fatal bacteraemia. Furthermore, it was shown that a functional CepIR quorum-sensing system is required for the pathogen's intracellular replication and subsequent dissemination throughout the host [218]. This model underscores the complexity of host-pathogen interactions and highlights potential avenues for therapeutic strategies to combat opportunistic infections.

Different studies have been conducted on *P. aeruginosa*, a well-known pathogen in CF research, utilizing the unique characteristics of the zebrafish embryo model. These studies have provided valuable insights into the interaction between the pathogen and the host immune system [222,223], highlighted the role of bacterial factors in virulence [224], and identified host factors critical for protection against *P. aeruginosa*. Furthermore, zebrafish models have been employed to validate novel antibacterial strategies [225,226]. These findings demonstrate the model's feasibility and effectiveness for advancing our understanding of *P. aeruginosa* pathogenesis and treatment options.

By leveraging the unique advantages of zebrafish, such as their transparency and the ability to observe host-pathogen interactions in real time, these models provide valuable insights into the complex dynamics of infectious diseases. Importantly, zebrafish embryos have proven to be a suitable model for studying several opportunistic bacterial pathogens found in pwCF, such as *S. aureus*, *B. cenocepacia*

[218,219], *M. marinum*[201,209] and *P. aeruginosa* [213,214,222]. This makes zebrafish an excellent tool for investigating a range of similar bacterial infections, such as *Achromobater* spp, and advancing our understanding of host-pathogen interactions.

Summary of the work included in this thesis

A bibliographic research of the existing literature was conducted, focusing on *Achromobacter* spp. prevalence and adaptation in cystic fibrosis lung infection, and culminated in a review examining the current literature regarding *Achromobacter* spp. role in CF, focusing on taxonomy, prevalence in CF lung infections, genomic characteristics, and adaptation strategies including modifications of metabolism and virulence, acquisition of antibiotic resistance, exchange of mobile genetic elements and development of hypermutation.

My research evolved into several studies on different aspects of *Achromobacter* spp. respiratory infection in CF: virulence and antimicrobial resistance, host immune response, and species identification.

The first study provided a comprehensive comparison of the phenotypic characteristics of *Achromobacter* spp. CF clinical isolates from chronic and occasional infections. This investigation revealed critical differences in aspects such as virulence (assessed in *Galleria mellonella* larvae), biofilm formation, cytotoxicity, and antimicrobial susceptibility. This study demonstrated that different *Achromobacter* species exhibit varying virulence and antibiotic resistance profiles, some of which may be linked to persistence in CF lungs and could serve as potential predictive markers of persistence [227]. These results not only improved our understanding of the behavior of these bacteria in CF individuals but also lay the groundwork for more personalized treatment strategies to manage these infections.

The second study investigated the host inflammatory response in CFTR-knockout (KO) and WT mice induced by *Achromobacter* spp. CF clinical isolates selected based on the pathogenic characteristics assessed in the previously described study[227] . The results indicated that virulent isolates caused higher lung inflammation and mice mortality, especially in KO mice. Moreover, both virulent and cytotoxic isolates exhibited greater persistence in the lungs of mice, while biofilm formation was not linked to lung inflammation, mouse mortality, or bacterial persistence. Furthermore, a positive correlation between virulence and lung inflammation was observed [228]. These findings suggest that some pathogenic

traits of *Achromobacter* spp., including virulence and cytotoxicity, may be associated with clinically significant outcomes, highlighting the necessity of understanding better their underlying mechanisms.

Following these studies, we extended our research to an *in vivo* zebrafish larvae model to further investigate the interaction between these selected *Achromobacter* spp. CF clinical isolates and the host innate immune system in a novel model organism. We established a zebrafish embryo infection model. The evaluation of these isolates revealed distinct infection outcomes The evaluation of these isolates revealed distinct infection outcomes: virulent strains were identified caused significant host mortality, while less virulent strains were identified by low host mortality and reduced bacterial burden. One strain exhibited elevated and sustained expression of the pro-inflammatory cytokines *il1b* and *cxcl8*, while the less virulent strain showed a more transient expression of these cytokines, suggesting different mechanisms of immune modulation. Additionally, macrophages played a crucial role in controlling bacterial growth and reducing host damage, as their depletion led to increased bacterial burden and mortality in embryos infected with the less virulent strain.

This result highlights the critical role of macrophages in controlling bacterial growth and limiting infection severity in *Achromobacter* spp. infection. Altogether our results show that zebrafish embryos are a useful and interesting model to further explore interactions of these opportunistic bacteria with innate immune cells in vivo, identify host and bacterial determinants required for virulence, and contribute to the identification of novel antimicrobial strategies.

Finally, regarding the issue of identification of the different *Achromobacter* species, with recent studies, including our previously described first study[227], suggesting that certain strains with higher virulence or resistance are more likely to establish chronic infections in individuals with CF, our research also aimed to develop an accurate qPCR protocol for species-level identification of *Achromobacter* spp., which could be easily implemented in clinical diagnostic laboratories. Through whole genome sequencing of clinical isolates, we identified species-specific single nucleotide polymorphisms (SNPs) within two 16S rRNA gene regions. Based on these variations, we designed two sets of primers and corresponding qPCR probes,

creating a unique identification code for each species. We tested six probes capable of identifying three species: *A. xylosoxidans*, *A. dolens*, and *A. insuavis;* also, we designed two probes capable of identifying two new genotypes that we have found in our collection and do not match phylogenetically with any publicly available sequence. The LOD ranged from 0.005 pg/µL to 1 pg/µL. Combining and matching the probes together for the species identification, sensitivity reached 100% for all combinations, while specificity varied between 97,95% and 100%. These tests suggest a potential and easy application in clinical diagnostic laboratories.

The work described in this thesis has been a multi-step process that has seen the publication of a review on the existing literature about *Achromobacter* spp. and the first two projects and the fourth as peer-reviewed papers on international journals; the other study will be presented in the following sub-sections as original manuscripts not yet published:

1. Veschetti L., Boaretti M., Saitta G.M., Passarelli Mantovani R., Lleò M.M., Sandri A., Malerba G. *Achromobacter Spp. Prevalence And Adaptation In Cystic Fibrosis Lung Infection.* Microbiological Research 263, 2022. DOI: 10.1016/j.micres.2022.127140.

2. Sandri A., Veschetti L., Saitta G.M., Passarelli Mantovani R., Carelli M., Burlacchini G., Preato S., Sorio C., Melotti P., Montemari A.L., Fiscarelli E.V., Signoretto C., Boaretti M., Lleò M.M., Malerba G. *Achromobacter spp. Adaptation in Cystic Fibrosis Infection and Candidate Biomarkers of Antimicrobial Resistance.* International Journal of Molecular Sciences 2022, 23, 9265. DOI: 10.3390/ijms23169265.

3. Sandri A., Saitta G.M., Veschetti L., Boschi F., Passarelli Mantovani R., Carelli M., Melotti P., Signoretto C., Boaretti M., Malerba G., Lleò M. M. *In Vivo Inflammation Caused by Achromobacter spp. Cystic Fibrosis Clinical Isolates Exhibiting Different Pathogenic Characteristics.* International Journal of Molecular Sciences 2023, 24, 7432. DOI: 10.3390/ijms24087432.

4. Saitta G. M., Boyer J., Ma Q., Sandri A., Boaretti M., Lleò M.M., Signoretto C., Vergunst A.C. *Development of an Infection Model to Study Virulence of the Opportunistic Pathogen Achromobacter Spp in Zebrafish Larvae.* Not yet published.

5. Saitta G.M., Veschetti L., Feletti R., Sandri A., Boaretti M., Melotti P., Carelli M., Lleò M.M., Malerba G., Signoretto C. *Development of a Simple and Accurate Molecular Protocol Using 16-SrRNA for Species-Specific Identification f Achromobacter Spp*. Pathogens 2025, 14, 271. DOI: 10.3390/pathogens14030271.

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Achromobacter spp. prevalence and adaptation in cystic fibrosis lung infection

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ABSTRACT

Bacteria belonging to the genus *Achromobacter* are widely distributed in natural environments and have been recognized as emerging pathogens for their contribution to a wide range of human infections. In particular, patients with cystic fibrosis (CF) are the subjects most frequently colonized by *Achromobacter* spp., which can cause persistent infections in their respiratory tract. Although many clinical aspects and pathogenic mechanisms still remain to be elucidated, *Achromobacter* spp. have been a source of expanding interest in recent years. This review examines the current literature regarding *Achromobacter* spp. role in CF, focusing on taxonomy, prevalence in CF lung infections, genomic characteristics, and adaptation strategies including modifications of metabolism and virulence, acquisition of antibiotic resistance, exchange of mobile genetic elements and development of hypermutation.

1. Introduction

Achromobacter spp. are non-lactose fermenting, catalase and oxidase positive Gram-negative bacilli widely distributed in the environment, mainly in moist soil and water sources but also in plants (Edwards et al., 2017). These motile opportunistic pathogens are mainly found in wet environments and are increasingly isolated also in nosocomial settings. Nosocomial outbreaks are often caused by contamined disinfectant solutions, dialysis fluids, saline solutions and deionized water (Gomila et al., 2014). *Achromobacter* spp. strains are usually resistant to a variety of antibiotics and disinfectants, due to both innate and adaptive antibiotic resistance (Edwards et al., 2017). *Achromobacter* spp. colonization events have been associated with a variety of infections such as bacteraemia, meningitis, pneumonia, peritonitis and urinary tract infections (Amoureux et al., 2016a; Neidhöfer et al., 2022). In addition, other conditions such as renal disease, cancer, diabetes, and endocarditis increase the risk of *Achromobacter* infection. These infections usually occur in subjects with underlying immunodeficiency, in subjects with impaired airway clearance due to chronic lung disease and in subjects that underwent surgical procedures. In particular, *Achromobacter* strains are primarily isolated from the respiratory tract of cystic fibrosis (CF) patients (Edwards et al., 2017) where these microorganisms can persist for a long time in both lower and upper airways (Hansen et al., 2010).

CF is a monogenic autosomal recessive disorder that is strictly linked with chronic bacterial respiratory infections: persistent airways infections and the ensuing prolonged lung inflammation lead to lung insufficiency, which accounts for the majority of CF morbidity and mortality (Quon and Rowe, 2016). Many opportunistic pathogens can cause lung infections in people with CF, among them *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia*, *Achromobacter* spp., non-typeable *Haemophilus influenzae*, *Aspergillus* and nontuberculous mycobacteria (Gibson et al., 2003). Although some retrospective studies have found that *Achromobacter* spp. infection in people with CF has no statistically significant impact on lung function (Edwards et al., 2017; Raso et al., 2008; Lambiase et al., 2011; De Baets et al., 2007; Tan et al., 2002), this is controversial since it has been reported that infection with the type

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species *Achromobacter xylosoxidans* results in a heightened host inflammatory response (Hansen et al., 2010). Moreover, chronic infections with *A. xylosoxidans* lead to a greater number of pulmonary exacerbation events (Edwards et al., 2017) and annual hospitalizations (Firmida et al., 2016; Rønne Hansen et al., 2006), thus highlighting the growing role of this opportunistic pathogen in CF.

In the last ten years the number of publications regarding *Achromobacter* spp. has more than tripled in comparison to the preceding decade (Fig. 1), underlining both the increasing research interest for these microorganisms as well as their improved recognition and emergence in the clinical setting, especially in CF. This review examines the current literature regarding *Achromobacter* spp. role in CF, focusing on taxonomy, prevalence in CF lung infections, genomic characteristics and adaptation strategies including modifications of metabolism and virulence, acquisition of antibiotic resistance, exchange of mobile genetic elements and development of hypermutation.

2. Research interest

Although primarily isolated from the airways of people with CF, *Achromobacter* spp. can cause a broad range of infections in hosts with other underlying conditions. Not only are these bacteria able to establish chronic infections associated with lung inflammation in people with CF (Hansen et al., 2010; Lambiase et al., 2011), they also produce biofilm, resist common disinfectants (Gomila et al., 2014; Günther et al., 2016), readily acquire antibiotic resistance (Trancassini et al., 2014) and outcompete resident microbiota (Talbot and Flight, 2016; Jeukens et al., 2017). This could be some of the reasons why there has been an increase in research interest regarding *Achromobacter* spp. in the last 20 years (Fig. 1). In particular, thanks to the advent of next-generation sequencing technologies, the rise in number of publications regarding this CF emerging pathogen has been followed by a steep increase in whole genome sequencing (WGS) data availability (Vincent et al., 2017; Land et al., 2015).

3. Taxonomy

Achromobacter spp. are classified as members of the β-proteobacteria and belong to the order of *Burkholderiales*, which also includes the *Burkholderia* genus. The family name of *Achromobacter* is *Alcaligenaceae,* which is the same family *Bordetella* and *Alcaligenes* belong to (Gomila et al., 2014). Phylogenetically, *Achromobacter* has been found to be closely related to the genus *Bordetella*, most members of which are human pathogens involved in respiratory infections, and a common origin of these microorganisms has been suggested (Li et al., 2013; Gross et al., 2008; Melvin et al., 2014).

Membership within the *Achromobacter* genus is continuously evolving, with recently named novel species being described since 2016 (Vandamme et al., 2016a; Kuncharoen et al., 2017; Singh et al., 2017; Green and Jones, 2018). Indeed, recent studies have resulted in the reclassification of previously described species: *A. spiritinus* has been reclassified as *A. marplatensis,* and *A. sediminum* has been reassigned to the novel genus *Verticia* (Vandamme et al., 2015, 2016b).

Current approaches for identifying members of the *Achromobacter* genus include biochemical testing (e.g. VITEK2), *nrdA* or 16 S rRNA gene sequencing, multi-locus sequence typing (MLST), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). In routine clinical microbiology laboratories, where time-sensitive results are necessary, bacterial identification is often carried out by biochemical testing even though it allows a less accurate species determination and at times results in misidentification due to *Achromobacter* spp. biochemical similarities with other Gram-negative bacilli (Fernández-Olmos et al., 2012; Alby et al., 2013; Isler et al., 2020).

Another identification method is MALDI-TOF MS which allows an accurate identification at genus level (Fernández-Olmos et al., 2012; Alby et al., 2013; Degand et al., 2008). Nonetheless, accurate identification at species level is still hindered by the limited number of species included in MALDI-TOF databases (Isler et al., 2020). Attempts to improve MALDI-TOF accuracy by increasing the number of species

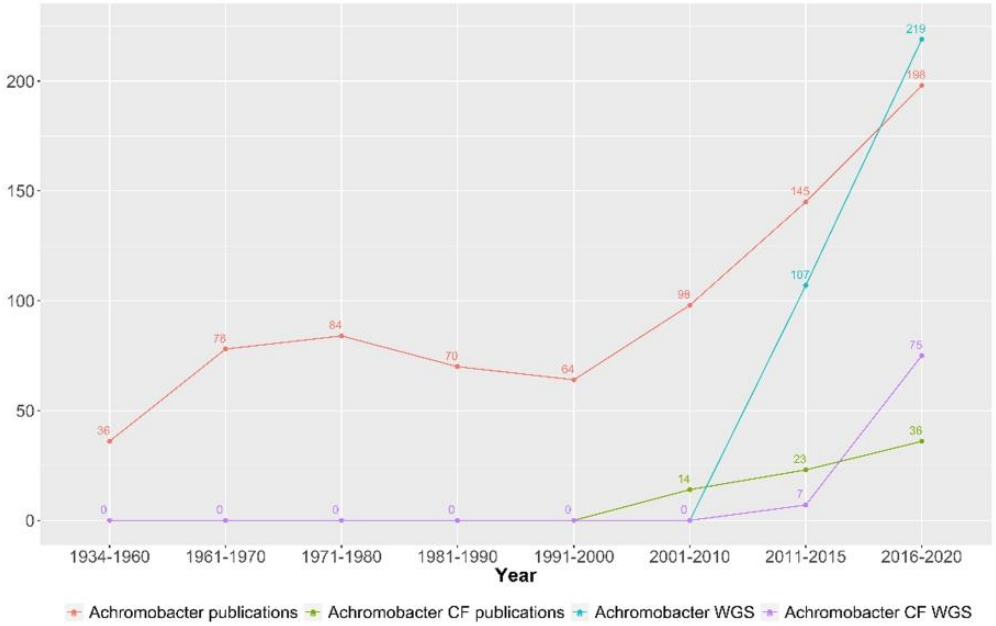

Fig. 1. *Achromobacter* research interest as of February 03, 2021. The number of publications is based on a literature search using PubMed (https://pubmed.ncbi.nlm.nih.gov) with Title=*Achromobacter* and Title=*Achromobacter* AND cystic fibrosis, while the number of whole genome sequencing (WGS) data is based on the content of NCBI (https://www.ncbi.nlm.nih.gov/Traces/wgs/) with Term=*Achromobacter* and Project type=WGS.

included in the databases presented promising results (Garrigos et al., 2021; Papalia et al., 2020a) and their incorporation into commercial databases will allow a more accurate identification at species level.

As regards sequence-based identification, *nrdA* gene sequencing or MLST (MLST scheme comprises 7 genes, namely *nusA, rpoB, eno, gltB, lepA, nuoL,* and *nrdA* - available at PubMLST (Jolley et al., 2018)) offer a more accurate species recognition (Spilker et al., 2012, 2013), whereas 16 S rRNA gene sequencing has been shown to not being able to ensure a definitive species identification due to the conserved nature of the gene (Gomila et al., 2014). With the advancement of sequencing techniques, WGS has become an affordable method for accurately identifying bacteria while obtaining a great amount of data enclosing a richness of information. The increasing use of WGS will enable correct identification of *Achromobacter* to the species level, including the reassignment of taxa that have historically been incorrectly speciated.

To date, the genus *Achromobacter* comprises 22 named species and multiple genogroups (Parte et al., 2020); WGS data is available for all 22 species but complete reference genomes are available only for 6 species, namely *Achromobacter deleyi, Achromobacter denitrificans, Achromobacter insolitus, Achromobacter pestifer, Achromobacter spanius,* and *Achromobacter xylosoxidans*. Detailed information regarding *Achromobacter* child taxa with a validly published name is reported in Table 1.

4. Genome and pan-genome

The first *Achromobacter* spp. complete genome sequence published was that of an environmental strain isolated from soil (*A. xylosoxidans* A8, RefSeq accession: GCF_000165835.1) (Stmad et al., 2022b) whereas the first complete genome assembly of a clinical isolate from a CF patient (*A. xylosoxidans* NH44784–1996, accession: GCF_000967095.2) was published two years later, in 2013 (Jakobsen et al., 2013). Complete reference genomes are available for 6 *Achromobacter* species (out of 22; Table 1).

The *Achromobacter* spp. genomes (Stmad et al., 2022b; Jakobsen et al., 2013; Badalamenti and Hunter, 2015; Li et al., 2018, 2017, 2020; Méndez et al., 2018; Reis et al., 2017; Wass et al., 2019) consists of a single chromosome comprising an average of 6.5 Mbp (range=5,876, 039–7,013,095 bp), presents a relatively high GC content (mean=65.5%, range=63.8–67.7%), and a mean of 5978 (range=5328–6459) open reading frames (ORFs) have been predicted with a coding density of ~90% (information obtained from the annotation files of *Achromobacter* spp. complete genome sequences available on NCBI - n = 41 - accessed December 2021). These genomic features resemble those of major CF pathogens such as *P. aeruginosa* (median total length: 6.6 Mbp; median ORFs count: 6097; median GC: 66.2% - source: NCBI) and *Burkholderia* spp. (median total length: 7.7 Mbp; median ORFs count: 6916; median GC: 66.7% - source: NCBI). Of note, a mean of 19% (range=10–29%) of ORFs still remain classified as having hypothetical function in *Achromobacter* spp., suggesting that some aspects of metabolism, pathogenic potential or adaptation mechanisms might still need to be elucidated and characterized with further studies.

Pan-genome analysis (Jeukens et al., 2017; Li et al., 2013) revealed that *Achromobacter* spp. has an open pan-genome and its conserved core genome consists of ~30% of the genes carried in an average genome of this genus. This means that a great part of the pan-genome is categorized as accessory genome, which comprises genes that are not conserved among isolates. Typically, these features coupled with such a large genome size characterize species living in a community with frequent lateral gene transfer and high adaptability to diverse environmental conditions (Tettelin et al., 2008; Rouli et al., 2015).

5. Contribution to CF infections: prevalence and species variation

Prevalence data from the latest European CF patients annual registry (2020) (ECFS Patient Registry, 2020) showed a high number of

Table 1
Achromobacter child taxa with a validly published name (information retrieved on LPSN (Parte et al., 2020) on 23–06–2022). The RefSeq accession numbers of taxa with reference genomes having "complete genome" as assembly level on NCBI are reported. NA = not available.

Child taxa	Reference (ref)	Current taxonomic status	Ref Seq assembly accession (number of available strains)
Achromobacter aegrifaciens	Vandamme, 2013 (Vandamme et al., 2013a)	Correct name	NA
Achromobacter agilis	Vandamme, 2016 (Vandamme et al., 2016a)	Correct name	NA
Achromobacter aloeverae	Kuncharoen, 2017 (Kuncharoen et al., 2017)	Correct name	NA
Achromobacter animicus	Vandamme, 2013 (Vandamme et al., 2013b)	Correct name	NA
Achromobacter anxifer	Vandamme, 2013 (Vandamme et al., 2013a)	Correct name	NA
Achromobacter deleyi	Vandamme, 2016 (Vandamme et al., 2016a)	Correct name	GCF_016127315.1 GCF_013116765.2 GCF_021432025.1 Amoureux et al. (2016a)
Achromobacter denitrificans	Coenye, 2003 (Coenye et al., 2003a)	Correct name	GCF_013267375.1 GCF_013267395.1 GCF_003812265.1 GCF_002205315.1 GCF_013343095.1 GCF_001514355.1 Quon and Rowe (2016)
Achromobacter dolens	Vandamme, 2013 (Vandamme et al., 2013a)	Correct name	NA
Achromobacter insolitus	Coenye, 2003 (Coenye et al., 2003b)	Correct name	GCF_008245125.1 GCF_001971645.1 GCF_900637265.1 GCF_000783435.2 Neidhöfer et al. (2022)
Achromobacter insuavis	Vandamme, 2013 (Vandamme et al., 2013a)	Correct name	NA
Achromobacter kerstersii	Vandamme, 2016 (Vandamme et al., 2016a)	Correct name	NA
Achromobacter marplatensis	Gomila, 2011 (Gomila et al., 2011)	Correct name	NA
Achromobacter mucicolens	Vandamme, 2013 (Vandamme et al., 2013b)	Correct name	NA
Achromobacter pestifer	Vandamme, 2016 (Vandamme et al., 2016a)	Correct name	GCF_013267355.1 Edwards et al. (2017)
Achromobacter piechaudii	Yabuuchi, 1998 (Yabuuchi et al., 1998)	Correct name	NA
Achromobacter pulmonis	Vandamme, 2013 (Vandamme et al., 2013b)	Correct name	NA
Achromobacter ruhlandii		Correct name	NA

(*continued on next page*)

Table 1 (*continued*)

Child taxa	Reference (ref)	Current taxonomic status	Ref Seq assembly accession (number of available strains)
	Yabuuchi, 1998 (Yabuuchi et al., 1998)		
Achromobacter sediminum	Zhang, 2014 (Zhang et al., 2014)	Synonym (*Verticia* spp.)	NA
Achromobacter spanius	Coenye, 2003 (Coenye et al., 2003b)	Correct name	GCF_900636675.1 GCF_002812705.1 GCF_003994415.1 GCF_002966795.1 Neidhöfer et al. (2022)
Achromobacter spiritinus	Vandamme, 2013 (Vandamme et al., 2013b)	Synonym (*A. marplatensis*)	NA
Achromobacter veterisilvae	Dumolin, 2020 (Dumolin et al., 2020)	Correct name	NA
Achromobacter xylosoxidans	Yabuuchi, 1981 (Yabuuchi and Yan, 1971, 2022a)	Correct name	GCF_008432465.1 GCF_022870085.1 GCF_001457475.1 GCF_013343135.1 GCF_016728825.1 GCF_001558755.2 GCF_016027035.1 GCF_014490035.1 GCF_900475575.1 GCF_900010105.1 GCF_900009125.1 GCF_013282255.1 GCF_001558915.1 GCF_900009115.1 GCF_013282235.1 GCF_001559195.1 GCF_009363015.1 GCF_001051055.1 GCF_000165835.1 Land et al. (2015)

Achromobacter infections in Denmark, Belgium and Portugal (prevalence: 13.58%, 10.31% and 9.80% respectively), while other countries presented a prevalence around or lower than 10%. Moreover, a higher percentage of adults with *Achromobacter* infection has been reported when compared with data regarding pediatric patients.

Even though the European CF patients annual registry has only reported *Achromobacter* spp. infection data starting from 2018, there are national CF registries that have been reporting *Achromobacter* spp. data for a long time. One of them is the French CF registry (Menetrey et al., 2021), which reports an increase in patients colonized over 20 years (6, 7% of the patients in 2018 versus 3.1% in 1999).

Similarly to European data, the latest US annual registry (2020) showed that the prevalence of *Achromobacter* spp. varies by age group, with an increase of infections in adult patients (Patient Registry Annual Data Report, 2020). While a general trend analysis of comprehensive European data is not possible (*Achromobacter* spp. infection data are available starting from 2018), the reported prevalence for *Achromobacter* spp. appears to be stable at around 7%. Concordantly, prevalence data from the US annual registry reported that *Achromobacter* infections rose from 1.9% in 2005 to around 7% in 2011 and have since remained stable (Green and Jones, 2018). Overall, the prevalence of *Achromobacter* species in people with CF is less than 10% at the majority of centers worldwide. Although data about *Achromobacter* spp. prevalence remain rare and are not available for all countries, the distribution of *Achromobacter* species in people with CF appears to be different among countries with available data (Table 2 and Fig. 2): the type species *A. xylosoxidans* is the most often isolated *Achromobacter* species among people with CF (Spilker et al., 2013; Papalia et al., 2020b; Gade et al., 2017; Gabrielaite et al., 2021; Amoureux et al., 2016b; Coward et al., 2016; Veschetti et al., 2021a) in all countries, while *A. marplatensis* and *A. pulmonis* show the lowest prevalence. *A. insuavis* infections are reported with a similar frequency in Denmark and France (20–24% and 19%, respectively), and at a lower rate in UK (12%), Italy (8%) and Argentina (5%). *A. dolens* is most prevalent in US (17%) followed by Argentina (10%), while it has a prevalence $< 10\%$ in UK, Italy and Denmark (8%, 3% and 0–2%). Furthermore, *A. ruhlandii* prevalence is 17–25% in Argentina, US and in Denmark, where an outbreak was reported at two CF centers (Rønne Hansen et al., 2006; Ridderberg et al., 2011); *A. insolitus* seems to have a higher prevalence in Italy (12%) than in France and US (both 4%), and *A. aegrifaciens* has a prevalence of 12–15% in Italy and France while in Denmark is $< 5\%$. Although prevalence data is available in the literature, the limited number of people with CF included in some of the studies and the still suboptimal species-level identification techniques (Fernández-Olmos et al., 2012; Alby et al., 2013; Isler et al., 2020; Saiman et al., 2001; Kidd et al., 2009) coupled with the changing nomenclature hinder an accurate prevalence estimation.

The high prevalence of *A. xylosoxidans*, *A. ruhlandii*, *A. dolens* and *A. insuavis* among clinical isolates, coupled with the phylogenetic clustering of these species and their ability to develop chronic infections (Amoureux et al., 2016b; Barrado et al., 2013; Dupont et al., 2015),

Table 2
Achromobacter CF prevalence in different countries. The number of *Achromobacter* infected people with CF included in each study is reported in the last row.

Achromobacter species	Country						
	Argentina[a] (%)	Denmark - Aarhus (%)	Denmark – Copenhagen (%)	France – Dijon (%)	Italy - Verona (%)	United Kingdom[b] (%)	United States of America[c] (%)
A. aegrifaciens	–	5	2	15	12	–	–
A. dolens	10	2	–	–	3	8	17
A. insolitus	–	–	–	4	12	–	4
A. insuavis	5	24	20	19	8	12	–
A. marplatensis	2	2	–	–	–	2	–
A. pulmonis	2	–	–	–	–	–	–
A. ruhlandii	17	19	25	–	–	3	24
A. xylosoxidans	63	36	52	57	65	61	43
N. patients included in the study	41	43	51	47	26	96	341
Identification method	*nrdA* and bla_{OXA} sequencing, MLST	*nrdA* seq, MLST	WGS	*nrdA* seq, MLST	WGS	*nrdA* and bla_{OXA} seq, MLST, WGS	*nrdA* seq, MLST
Study reference	(Papalia et al., 2020b)	(Gade et al., 2017)	(Gabrielaite et al., 2021)	(Amoureux et al., 2016b)	(Veschetti et al., 2021a)	(Coward et al., 2016)	(Spilker et al., 2013)

[a] Six healthcare centers in Argentina were involved in the study.
[b] Study by the UK national reference laboratory.
[c] Eighty-six CF treatment centers in the US were involved in the study.

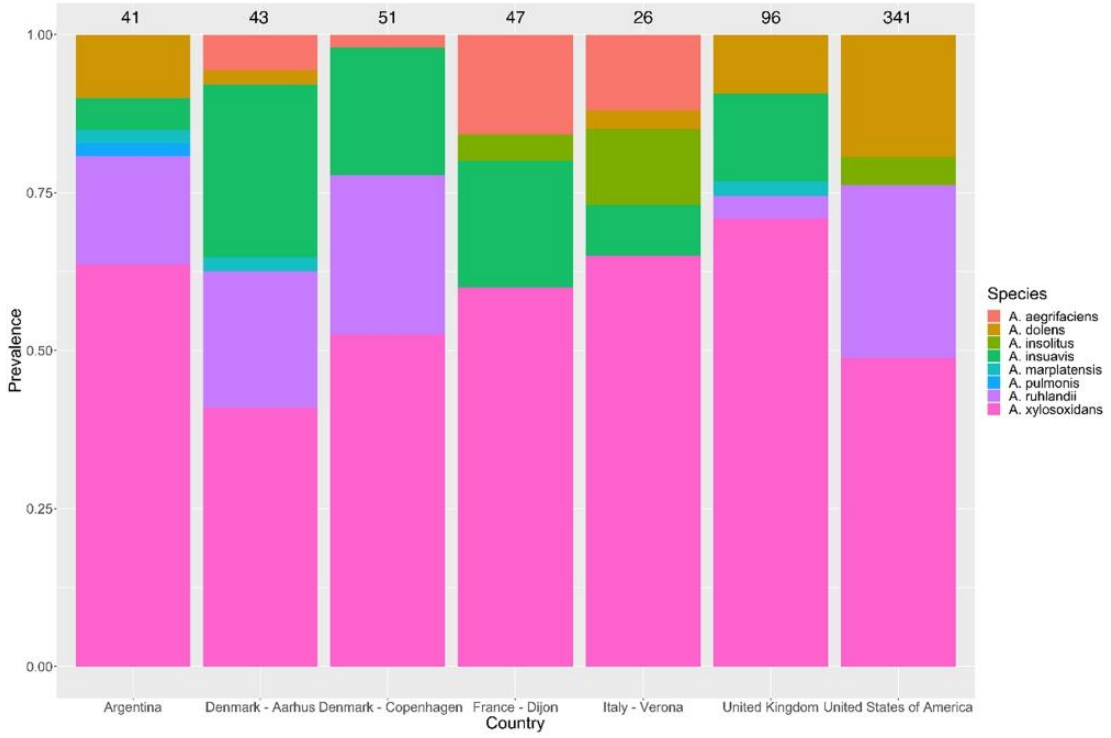

Fig. 2. *Achromobacter* CF prevalence in different countries. The number of *Achromobacter* infected people with CF included in each study is reported over each bar.

might indicate that they could be better adapted to cause opportunistic chronic infections (Jeukens et al., 2017). Even though more than half of people with CF with airway colonization by *A. xylosoxidans* develop chronic infections, usually associated with decline in respiratory function and lung inflammation (Hansen et al., 2010; Lambiase et al., 2011; Firmida et al., 2016; Gade et al., 2017; Amoureux et al., 2016b; Pereira et al., 2011), the clinical impact of different *Achromobacter* species is still not well characterized.

According to the available longitudinal studies (Gabrielaite et al., 2021; Ridderberg et al., 2011; Amoureux et al., 2013), the majority of people with CF with *Achromobacter* spp. chronic lung infection seem to harbor a unique strain or clone type (identified by WGS or PFGE). Interestingly, two of these studies (Gabrielaite et al., 2021; Amoureux et al., 2013) observed that 20–24% of patients were infected with more than one *Achromobacter* species and/or clone types over the sampling period, suggesting that not all *Achromobacter* spp. strains lead to chronic lung infections.

Cases of cross-infection among patients have been reported, even by indirect person-to-person transmission (Rønne Hansen et al., 2006; Hansen et al., 2013). For example, the *A. ruhlandii* Danish epidemic strain has been identified in multiple patients attending the same CF center and, more recently, patient-to-patient transmission was verified also for *A. xylosoxidans* and *A. insuavis* strains. In some cases, clear epidemiological connections (e.g. sibling pairs, visit-based) were found, but in other instances no epidemiological connection to support cross-infection could be identified (Green and Jones, 2018; Gabrielaite et al., 2021). In all the reported cases, WGS proved to be essential for *Achromobacter* species typing and identification of patient-to-patient transmission.

6. Adaptation strategies

The increase in the number of sequenced *Achromobacter* spp. genomes enabled researchers to focus at first on the genomic differences among clinical and environmental isolates and afterwards on the genomic determinants of pathogenicity and adaptation during persistent infections, allowing to identify a variety of adaptation mechanisms in the CF lung environment. A phylogenetic study aimed at evaluating differences among environmental and clinical strains showed that in the latter 35 genes involved in metabolism (COG functional categories: Amino acid transport and metabolism, Carbohydrate transport and metabolism, Cell wall/membrane/envelope biogenesis, Coenzyme transport and metabolism, Energy production and conversion, Inorganic ion transport and metabolism, Secondary metabolites biosynthesis, transport and catabolism, Lipid transport and metabolism), regulation, and efflux pumps were positively selected, and that this group of isolates carried a greater number of antibiotic resistance genes, namely for resistance against aminoglycosides, β-lactams, chloramphenicol and sulfonamides (Jeukens et al., 2017). Interestingly, it was shown that the most frequently mutated genes were involved in general metabolism (COG functional categories: Energy production and conversion, Amino Acid metabolism and transport, Carbohydrate metabolism and transport, Lipid metabolism, Inorganic ion transport and metabolism), which is the key to adapt to host conditions and outcompete the resident microbiota as well as other opportunistic pathogens (Sandri et al., 2021; Menetrey et al., 2020; Olive and Sassetti, 2016). Indeed, during CF lung colonization, bacteria survive under the selective pressure imposed by the host immune system and antibiotic therapies by increasing the efficiency in nutrient acquisition, developing the ability to avoid toxic compounds and to evade immune response, and promoting the colonization of new areas (Houry et al., 2012). An overview of *Achromobacter* spp. main adaptation strategies reported to date is represented in Fig. 3.

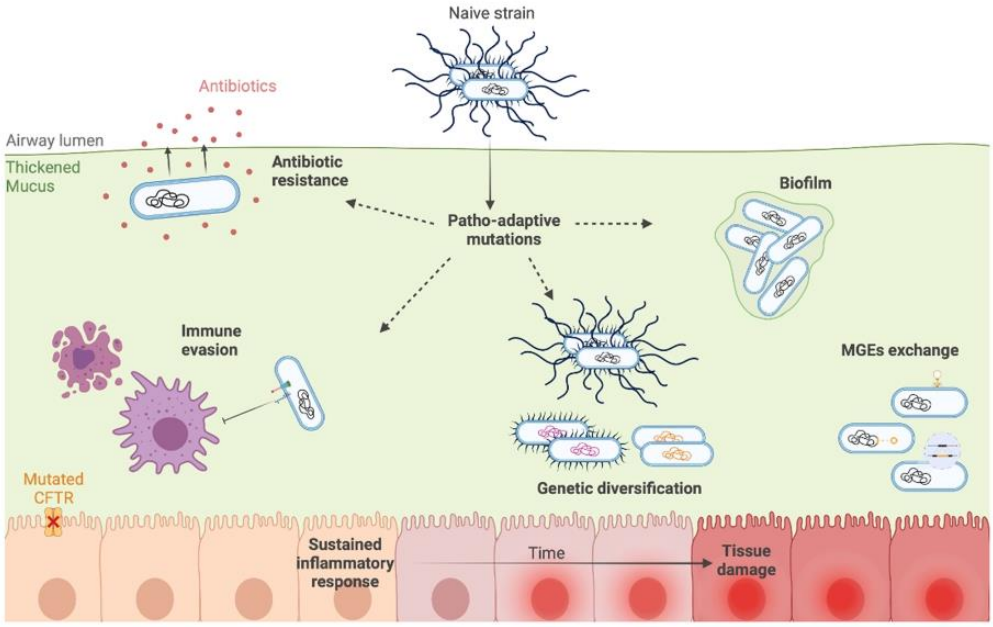

Fig. 3. *Achromobacter* spp. adaptation strategies reported in the literature to date: antibiotic resistance, immune evasion, genetic diversification, biofilm production and exchange of mobile genetic elements. MGEs = mobile genetic elements, CFTR = cystic fibrosis conductance transmembrane regulator. This figure was created on BioRender.com.

6.1. Metabolism

Studies on within-host evolution of *Achromobacter* spp. in people with CF have found that the most mutated genes during adaptation are mainly involved in general metabolism, leading to attenuation of functions not essential for survival in the CF lung environment, such as amino acid synthesis (since amino acid concentrations are elevated in CF sputum and bacteria have sufficient supply) (Ridderberg et al., 2015). In particular, the ability to survive with limited oxygen has been identified as one of the possible adaptive mechanisms favouring persistence of *Achromobacter* in the CF airways environment (Jeukens et al., 2017). Indeed, hypoaerobic/anaerobic growth ability confers to microorganisms the possibility to locate deeper within the mucous layer and within biofilm structures or in more hypoxic regions of the lung, where some antibiotics and penetration default can be dramatically less effective due to anaerobic conditions (Borriello et al., 2004). The use of denitrification for energy production in oxygen depleted environments, such as CF mucus, has been demonstrated for *P. aeruginosa*, another CF pathogen (Schobert, 2010), and there is evidence that molybdenum uptake, upon which denitrification depends, is essential for anaerobic proliferation and influences virulence in this pathogen (Pederick et al., 2014; Périnet, 2016). Results suggesting that *Achromobacter* and *P. aeruginosa* may share this adaptive mechanism have been reported in a study regarding CF clinical isolates (Jeukens et al., 2017), and anaerobic growth ability has also been suggested as a key difference between occasional and chronic infection isolates (Veschetti et al., 2021a).

6.2. Virulence

The *Achromobacter* genus has been found to be phylogenetically related to *Bordetella* and this strong link highlights the potentially pathogenic nature of this CF emerging pathogen from the phylogenetic perspective (Li et al., 2013; Gross et al., 2008; Melvin et al., 2014). The mechanisms underlying *Achromobacter* spp. ability to colonize the respiratory tract as well as other sites of the human body are still not fully clear, but a number of virulence factors have been described that likely support their invasiveness and survival in hostile environments. Similar to better-studied pathogens, biofilm production, motility, lower oxygen tolerance, and virulence factor secretion have been shown to play important roles in *Achromobacter* (mainly *A. xylosoxidans*) infection, adaptation, and persistence.

Biofilms consist of bacterial microcolonies encased in a matrix of polysaccharides, DNA and proteins and they can have variable morphology, growing attached to a surface or as unattached aggregates in mucus or in sputum (Mantovani et al., 2012; Høiby et al., 2017). Biofilm formation is a growth phenotype that many bacteria causing CF infections use for survival and proliferation in hostile environment; this bacterial ability allows to shield pathogens against environmental stress and increase tolerance towards antibiotics and host defences (Høiby et al., 2017; Nielsen et al., 2016). The presence of genes linked to biofilm formation in other bacteria has also been reported in *Achromobacter* spp. genomes. Among them, the *flgB* gene, which encodes a flagellar basal body rod protein implicated in *Bordetella bronchiseptica* and *P. aeruginosa* biofilm formation (Nicholson et al., 2012; de la Fuente-Núñez et al., 2012), and the pgaABCD operon, which encodes the polysaccharide β-1, 6-GlcNAc involved in both cell-cell adherence and cell-surface adherence in other CF pathogens (Jakobsen et al., 2013). Members of *Achromobacter* genus also present peritrichous flagella that enable swimming motility, contributing to biofilm formation and host cell invasion (Swenson and Sadikot, 2015). A study of 52 *A. xylosoxidans* strains isolated during an outbreak at a CF Centre in Rome (Italy) found that the great majority of strains were motile and biofilm producers; moreover, a significant prevalence of strong biofilm-producing strains was found in patients with severely impaired lung function (Trancassini et al., 2014). These results were explained with an enhanced adaptation of *A. xylosoxidans* to the CF nosocomial environment. In particular, biofilm production seems to play an important role in bacterial persistence as it has been reported that gene expression profiles and antimicrobial susceptibility at biofilm stage differ from planktonic cells. Indeed, in biofilm stage, *Achromobacter* spp. genes associated with

anaerobic respiration were found to be upregulated, suggesting the adaptation to the microaerobic and anaerobic conditions prevalent in the late stage of CF chronic infections (Nielsen et al., 2017). Interestingly, a reduced biofilm formation has been observed in chronic strains over time of infection, which may result from within-host adaptation to the CF lung during chronic colonization (Nielsen et al., 2016). *Achromobacter* spp. has also been reported to form mixed biofilms with *P. aeruginosa* in vitro, where it can affect *P. aeruginosa* biofilm formation (Sandri et al., 2021; Menetrey et al., 2020).

Similarly to other Gram-negative pathogens, *Achromobacter* spp. express membrane-bound virulence factors. Among them, the Vi capsular polysaccharide, which enables surface adhesion and protection from environmental toxins; the O-antigen, involved in eliciting the host immune response (Li et al., 2013); and lipopolysaccharide (LPS), which induces the production of key inflammatory cytokines such as IL-6, IL-8 and TNF-α (Mantovani et al., 2012). The accumulation of mutations in genes involved in LPS production has been suggested to be involved in *Achromobacter* spp. persistence in CF lungs, probably leading to a reduced recognition by the host defense system (Veschetti et al., 2021a). Indeed, a reduction in the number of LPS lipid A acyl chains by other bacteria was shown to modulate the recognition of LPS by toll-like receptors (Qureshi et al., 1991).

Among cell membrane components, secretion systems also have an important function in bacterial pathogenicity since they are involved in the release of toxins, proteases and other virulence factors. Numerous genes encoding different types of secretion systems have been identified in *Achromobacter* genome, including: type II secretion system (T2SS), which is widely conserved among γ-proteobacteria and is involved in the release of extracellular toxins and proteases; type III secretion system (T3SS) that delivers virulence factors directly into the host cell; type VI secretion system (T6SS) that mediates the transport by direct contact with the target cells; and type VII secretion system (T7SS) which includes sigma-fimbriae encoding genes (Green and Mecsas, 2016). In particular, T3SS is known to enhance the bacterial ability to infect host cells with effector proteins and to contribute in immune evasion (Li et al., 2013; Jakobsen et al., 2013; Swenson and Sadikot, 2015). Recently, a phospholipase A2 (PLA2) encoded by the majority of *A. xylosoxidans* genomes, termed AxoU, was identified as a T3SS substrate that induces cytotoxicity in macrophages suggestive of a pathogenic or inflammatory role in the CF lung (Pickrum et al., 2020). The presence of T3SS has also been considered as a key discriminant among clinical and environmental *Achromobacter* strains. Comparative genomics analyses showed that virulence genes related to T3SS are more common in *Achromobacter* CF isolates rather than in environmental strains (Jeukens et al., 2017; Li et al., 2013), linking this feature with the infection ability of these microorganisms. Furthermore, the presence of functional T3SS genes seems to be associated with the establishment of chronic infections in the CF lung, while occasional infection isolates show a lack of functional T3SS genes (Veschetti et al., 2021a).

As concerns secreted virulence factors, genomic studies reported the presence of genes encoding colicin V, a cytotoxic protein that likely gives *Achromobacter* spp. environmental advantages by eliminating competing flora and enabling tissue invasion, and AepA, which facilitates the production of cellulases and proteases enabling mucosal invasion (Jakobsen et al., 2013). The presence of secreted proteases was also recently assessed (Veschetti et al., 2020). In addition, production of phospholipase C was observed, which allows hydrolysis of phospholipids of the alveolar surfactants and tissue disruption (Pederick et al., 2014), and a heat-stable cytotoxic factor has been identified and associated with increase of pro-inflammatory cytokines in vitro (Mantovani et al., 2012).

Interestingly, it was also found that some *Achromobacter* spp. strains are able to inactivate *P. aeruginosa* quinolone signal (PQS), participating in the Quorum Sensing (QS) mechanisms used for coordination of gene expression when bacterial cells reach a critical cell density (Papenfort and Bassler, 2016). The QS creates a global regulatory network and is believed to regulate the expression of up to 12% of the *P. aeruginosa* genome (Lin et al., 2018). Therefore, the ability to disrupt the QS could give *Achromobacter* spp. a competitive advantage over *P. aeruginosa* and maybe over other CF pathogens like *S. maltophilia* during co-habitation in the same lung environment, e.g., by affecting their growth, motility and /or biofilm formation (Sandri et al., 2021; Menetrey et al., 2020; Soh et al., 2015).

6.3. Antibiotic resistance

An important factor for the survival of *Achromobacter* spp. within the host is its resistance to antibiotics, mediated by naturally occurring and acquired systems of defense, rendering infections particularly hard to eradicate. A variety of mechanisms contribute to the resistance patterns of bacteria such as production of degrading enzymes, efflux pump system or changes in the antibiotic target. Isler et al (Isler et al., 2020). recently wrote a comprehensive overview of *Achromobacter* antibiotic resistance mechanisms, so we shall report here a brief summary regarding this matter.

Achromobacter spp. show an innate resistance to many classes of antibiotics, especially to aminoglycosides, some monobactams (aztreonam), tetracyclines, some penicillins (penicillin G, ticarcillin) and cephalosporins, which include antibiotics relevant to CF lung infection treatment (Trancassini et al., 2014; Swenson and Sadikot, 2015; Almuzara et al., 2010). In particular, trimethoprim-sulfamethoxazole, ceftazidime, piperacillin, and carbapenems are the most active agents against *Achromobacter* spp. isolates. Among carbapenems, several studies showed imipenem to be more active than meropenem against *Achromobacter* isolates (Amoureux et al., 2019; Caverly et al., 2019; Díez-Aguilar et al., 2019).

The most conserved genes conferring antibiotic resistance among *Achromobacter* spp. can be classified in 5 groups: class B β-lactamase, group B chloramphenicol acetyltransferase, rRNA methylases, class A β-lactamase and resistance-nodulation-cell division (RND) efflux pump (Hu et al., 2015). Particularly, many members belonging to the RND efflux pump group are associated with intrinsic resistance. Among them, AxyABM, which is able to extrude most cephalosporins, fluoroquinolones, aztreonam and chloramphenicol, and AxyXY-Opr, which extrudes aminoglycosides but can also accommodate cefepime, tetracyclines and carbapenems (Swenson and Sadikot, 2015; Bador et al., 2013, 2011).

Another important resistance mechanism is the production of β-lactamases. Interestingly, bla_{OXA} genes follow a species-specific distribution: while the specificity of $bla_{OXA-114}$, $bla_{OXA-243}$, $bla_{OXA-364}$ for *A. xylosoxidans*, *A. insuavis* and *A. dolens* respectively was already reported in literature (Bador et al., 2011), no bla_{OXA} genes were recently reported for *A. aegrifaciens* and some *A. insolitus* isolates, whereas bla_{OXA-2} was identified in *A. insolitus* strains (Menetrey et al., 2021). OXA-114-like enzymes show great activity in vitro against penicillin G, early cephalosporins, piperacillin, and ticarcillin (Isler et al., 2020). However, phenotypic piperacillin susceptibility results common among OXA-114-positive *A. xylosoxidans* isolates (Isler et al., 2020). Moreover, extended-spectrum β-lactamase, AmpC type β-lactamase, and metallo-β-lactamase have been observed in *A. xylosoxidans* CF isolates and appear to contribute to resistance to β-lactams including carbapenems (Traglia et al., 2012; Filipic et al., 2017; Vali et al., 2014; Neuwirth et al., 2006; Shibata et al., 2003; Riccio et al., 2001; Sofianou et al., 2005; Shin et al., 2005).

Many studies have focused on the role of antibiotic resistance in *Achromobacter* CF infections. Among them, Jeukens et al (Jeukens et al., 2017). performed a pan-genomic analysis of publicly available *Achromobacter* spp. and reported that clinical strains carry more resistance genes than other stains, namely for resistance against aminoglycosides (six additional genes), β-lactams (six additional genes), chloramphenicol (three additional genes) and sulfonamides (two additional genes). These additional genes presumably contribute to acquired resistance, but no

genomic information about their origin (e.g. mobile genetic elements, recombination) is reported. In addition, clinical strains showed positive selection of three genes encoding efflux pump components: *emrA*, *macA* and *mexW* (Jeukens et al., 2017). The protein products of these genes are elements of a major facilitator superfamily multidrug export complex, an ABC efflux pump that exports macrolides and an RND-type efflux pump, respectively. Positive selection of these genes suggest that efflux pumps represent another key mechanisms for adaptation to a pathogenic lifestyle as they are implicated in bacterial virulence (Alcalde-Rico et al., 2016) and show a tendency to favor loss of specificity, which translates into multi-drug resistance (Lewis, 1994; Vargiu et al., 2016).

Analysis of 54 *Achromobacter* genomes from people with CF presenting chronic and occasional infections found that there is no significant difference in resistance genes between chronic and occasional isolates. Moreover, 54% of isolates presenting deleterious variants in antibiotic resistance genes carried mutations in at least one *bla* gene (Veschetti et al., 2021a). Another recent study of 101 *Achromobacter* clinical isolates showed that development of antibiotic resistance is associated with chronic infections; in particular, late isolates were statistically significantly less susceptible than early and single isolates (Gabrielaite et al., 2021). Moreover, nearly all isolates were resistant or intermediate resistant to aztreonam, ceftriaxone, cefuroxime, ciprofloxacin, moxifloxacin, penicillin, rifampicin, tobramycin and trimethoprim. Interestingly, it was also reported that isolates belonging to the Danish epidemic strain (*A. ruhlandii*) were resistant or intermediate resistant to a median of 20 antibiotics, while the median was 14 for other *Achromobacter* isolates (*A. xylosoxidans* and *A. insuavis*), which could be one of the reasons this strain has become so widespread among people with CF in Denmark.

Taken together, acquired antimicrobial resistance in chronic *Achromobacter* CF infections, either by chromosomal mutation or horizonal gene transfer, is a growing concern, especially as it can be shared among genetically similar pathogens like *P. aeruginosa*, *Ralstonia* spp. and *Burkholderia* spp. Current knowledge deficits could be addressed by greater antimicrobial susceptibility testing of *Achromobacter* clinical isolates to enable better diagnosis, monitoring and treatment of antimicrobial resistance emergence and persistence in people with CF.

6.4. Mobilome

Horizontal gene transfer is the transfer of genetic elements among microorganisms by means other than vertical transmission and is a well-described mechanism that has been increasingly studied due to its role in the rapid dissemination of genetic elements among bacteria (Soucy et al., 2015). In particular, the acquisition of MGEs harboring genes related to virulence and antibiotic resistance can enable their microbial host to synthesize products that affect the fitness of resident microbiota and co-infecting pathogens or confer antibiotic resistance (Botelho et al., 2020). MGEs have been detected in the great majority of prokaryotic organisms and a rich variety of MGEs carrying resistance genes have been identified in *A. xylosoxidans* clinical isolates, such as plasmids, IS26, IS440, and class I and class II integrons (Hu et al., 2015; Traglia et al., 2012). Nevertheless, literature about the scale and importance of mobilome is still scarce for *Achromobacter* species.

A bioinformatic study found through pan-genome analysis that the most likely candidates to be involved in horizontal transfer with *Achromobacter* spp. were *Sinorhizobium* sp. as well as *Ralstonia*, *Pseudomonas* and *Burkholderia* sp (Jeukens et al., 2017)., which share a similar GC content with *Achromobacter* and are soil microorganisms that are also responsible for CF opportunistic infections (Mahmood et al., 2016; LiPuma, 2015). These results have been confirmed in a recent study that identified MGEs - phages, insertion sequence (IS) elements, integrative and conjugable elements (ICEs), and integrative and mobilizable elements – through genome analysis of 54 *Achromobacter* spp. clinical isolates from occasional and chronic CF lung infection (Veschetti et al., 2021b).

Among MGEs, phages (viruses which infect bacteria) are drivers of bacterial evolution (Clokie et al., 2011). Interestingly, most of the conserved phages in all *Achromobacter* species were previously described in other pathogens and carried genes related to MGE stability, biofilm formation and stress responses, highlighting the importance of MGE in *Achromobacter* pathogenicity. Moreover, type II toxin-antitoxin systems, which have been reported to occur more often in pathogenic bacteria and have been evaluated as antimicrobial targets (Williams and Hergenrother, 2012), were identified in *Achromobacter* isolates (Veschetti et al., 2021b). Additionally, an ancestral uptake of the phage Bcep176 from *Burkholderia* has been proposed for *A. xylosoxidans* (Veschetti et al., 2021b).

As another type of MGEs, different classes of ISs, which are frequently associated to antibiotic resistance genes and to class I and II integrons (Hu et al., 2015; Traglia et al., 2012), were also detected in *Achromobacter* spp., either inside of or in proximity to pathogenicity islands. In particular, ISs from a wide variety of microorganisms have been identified, especially from species of clinical interest including *B. cepacia* complex, *P. aeruginosa*, and *S. maltophilia* (Veschetti et al., 2021b).

Also, a great number of ICEs carrying genes related to a variety of functions such as secretion, motility, quorum sensing, metabolism, mismatch repair, and resistance to different classes of antimicrobial molecules have also been found in *Achromobacter* genomes (Veschetti et al., 2021b). In particular, the most represented antibiotic resistance genes were the sulfonamide resistance gene *sul1* and the *aac(6')* family aminoglycoside acetyltransferase, which are frequently found within MGEs such as integrons, plasmids and transposons carried by other Gram-negative opportunistic pathogens (Domingues et al., 2012). Additionally, while phages and ISs have shown high consistency in longitudinal isolates, variations in the presence and pathogenic content of ICEs over time were observed, indicating a frequent exchange of MGEs within the CF lungs (Veschetti et al., 2021b).

Little is still known about *Achromobacter* spp. plasmid content, especially regarding strains isolated from people with CF. Indeed, the majority of the available literature concerns environmental strains from aquacultures and soil. In particular, plasmids that have been reported in *Achromobacter* spp. are the wide host range IncP plasmids (Traglia et al., 2012), a 27 kbp plasmid coding for nitrite reductase and nitrous oxide reductase (Kathiravan and Krishnani, 2014), the 70 kbp 2,4-dichlorophenoxyacetic acid-degradative pEST4011 plasmid (Vedler et al., 2000), and the 98 kbp plasmid pA81 harboring genes encoding heavy metal resistance determinants (Jencova et al., 2008).

Overall, these findings underline MGEs contribution to the genomic plasticity of *Achromobacter* isolates and support that MGEs might play an important role in pathogenesis and adaptation during chronic infections, highlighting the need for further studies.

6.5. Hypermutation and clonal diversification

Among the variety of adaptation mechanisms identified in the CF lung environment, another important aspect is the high-rate accumulation of pathoadaptive mutations leading to hypermutation (Marvig et al., 2013). Typically, short-term adjustments are believed to be the result of regulatory alterations in gene expression whereas long-term adaptation is the result of the accumulation of pathoadaptive mutations (Ridderberg et al., 2015). Interestingly, the generation rate of mutations can be accelerated due to defects in DNA repair or error avoidance systems in hypermutable strains, thus giving rise to clonal diversification within the host (Oliver et al., 2000). Some of the genes involved in this phenomenon, also referred to as mutator genes, are *mutL*, *mutS*, *pfpI*, *superoxide dismutase*, *radA*, *radC*, *rad50*, *uvrA*, *uvrB*, *uvrC*, and *uvrD* (Oliver, 2010). The occurrence of hypermutation has been demonstrated for various CF pathogens, such as *Pseudomonas aeruginosa* (Oliver et al., 2000; Ciofu et al., 2010; Hogardt et al., 2007; Mena et al., 2008) and

Burkholderia cepacia complex (Martina et al., 2014), and more recently for *Achromobacter (*Veschetti et al., 2020; Oliver, 2010). In particular, *Achromobacter* spp. hypermutation events appear to be observed frequently (60–78%, (Veschetti et al., 2021a)) in strains isolated from the lungs of chronically infected patients while no occasional infection isolate showed hypermutator characteristics to date (Gabrielaite et al., 2021; Veschetti et al., 2021a, 2020; Oliver, 2010), thus suggesting that hypermutation might constitute an advantageous adaptive mechanism in the lung environment. Interestingly, a recent study reported the presence of two copies of *mutS* gene in *A. dolens* genome and a variable copy number of this gene in *A. insuavis (*Veschetti et al., 2021a*)*. Both species were isolated from chronically infected CF patients, but these findings were validated by analyzing publicly available reference genomes. Moreover, in the same study, *A. dolens* hypermutator isolates carrying mutations in a single *mutS* gene have been identified, thus suggesting that both genes are needed for effective mismatch repair in this species.

Hypermutation is a key feature for within-host evolution of clonal lineages leading to clonal diversification, which results from co-evolution of several subpopulations from an original infecting isolate (Winstanley et al., 2016). During this process, mutation of genes involved mainly in the general metabolism, but also in virulence and antimicrobial resistance, was observed in CF chronic infections (Ridderberg et al., 2015). Interestingly, genes required for initiation of acute infection were found to be selected against, e.g. genes of the type I and type III secretion systems and genes related to pilus and flagellum formation or function, while mutations of antimicrobial resistance genes or their regulatory genes were found, that likely caused increased resistance to meropenem. In particular, *A. insuavis* has been reported to show higher diversification compared to other species (Dupont et al., 2015). In addition to hypermutation, other mechanisms might also contribute to clonal diversification, such as IS-related genomic rearrangements (Dupont et al., 2015).

7. Concluding remarks: challenges and areas of further research

Achromobacter spp. are a subject of increasing interest for their pathogenic characteristics and their growing prevalence in people with CF; nonetheless, many clinical aspects and pathogenic mechanisms remain to be elucidated. This genus still suffers from difficulties in diagnosis due to misidentification caused by its biochemical similarity to other Gram-negative bacilli and continuously evolving taxonomy. Some advances have already been made regarding the identification of *Achromobacter* species by the introduction of *nrdA* gene analysis, MLST (Spilker et al., 2012, 2013) and the creation of databases for MALDI-TOF-MS (Fernández-Olmos et al., 2012; Alby et al., 2013), which is the most employed technique in routine clinical microbiology laboratories for this aim. The growing clinical concern and research interest for *Achromobacter* spp. is determining and will continue to determine an increase in the number of collected isolates, both in CF centers and across other diseases. This could lead to the creation of new or updated MALDI-TOF databases that will allow a more accurate identification at species level if incorporated into commercial databases or made publicly available. Moreover, with the advancement of sequencing techniques, WGS could become feasible even in routine clinical microbiology laboratories and the growth of sequences in public repositories (e.g. NCBI, RefSeq) will allow a comprehensive analysis in terms of phylogeny, virulome, resistome and mobilome. In such circumstances, *Achromobacter spp.* genomic analysis could offer a plethora of information which might assist clinicians in choosing the best course of action. Additionally, we still have a limited understanding of *Achromobacter* adaptation to the CF lungs environment attributable to the restricted number of comparative studies, especially involving isolates from occasionally infected patients. In fact, the comparison between chronic and occasional infection isolates may allow the identification of genetic markers of persistence, while studies of less clinically characterized *Achromobacter* species could help in understanding whether some species are more likely to establish a chronic colonization of the CF airways. Studies focusing on *Achromobacter* interactions with other opportunistic pathogens or with lung microbiota also lack, limiting our understanding of the interplays occurring in the airway environment and of their participation in adaptation and persistance.

Another problem contributing to the difficult understanding of *Achromobacter* spp. adaptation is the large number of ORFs classified as having hypothetical function. Bioinformatic and functional studies regarding the product of these genes could give further insights on their role and contribution to *Achromobacter* metabolism, pathogenic potential and adaptation mechanisms.

Noteworthily, the variety of MGEs identified in *Achromobacter* genomes and their diverse virulence and antibiotic resistance profiles have confirmed *Achromobacter* spp. as a reservoir of MGEs. Not only they do contribute to genomic plasticity, but some of these elements can also even become a constitutive part of the bacterial genome (Veschetti et al., 2021a), thus highlighting the need for further studies to better elucidate MGEs clinical impact and their potential to become antimicrobial targets in treatment regimens. For example, the exploitation of type II toxin-antitoxin systems as an antibacterial strategy via artificial activation of the toxin has been proposed (Williams and Hergenrother, 2012).

Overall, the increasing number of studies focusing on this emerging pathogen and the continuous refinement of research techniques will likely allow a deeper knowledge of *Achromobacter* species that could successfully be translated into the clinical setting to the benefit of the patients.

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Author Contributions

L.V. performed literature search and wrote the original draft. M.B., G.M.S. and A.S. contributed to the text of the article. R.P.M., G.M., A.S. and M.M.L. supervised and reviewed the content of the article. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

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Achromobacter spp. Adaptation in Cystic Fibrosis Infection and Candidate Biomarkers of Antimicrobial Resistance

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Abstract: *Achromobacter* spp. can establish occasional or chronic lung infections in patients with cystic fibrosis (CF). Chronic colonization has been associated with worse prognosis highlighting the need to identify markers of bacterial persistence. To this purpose, we analyzed phenotypic features of 95 *Achromobacter* spp. isolates from 38 patients presenting chronic or occasional infection. Virulence was tested in *Galleria mellonella* larvae, cytotoxicity was tested in human bronchial epithelial cells, biofilm production in static conditions was measured by crystal violet staining and susceptibility to selected antibiotics was tested by the disk diffusion method. The presence of genetic loci associated to the analyzed phenotypic features was evaluated by a genome-wide association study. Isolates from occasional infection induced significantly higher mortality of *G. mellonella* larvae and showed a trend for lower cytotoxicity than chronic infection isolates. No significant difference was observed in biofilm production among the two groups. Additionally, antibiotic susceptibility testing showed that isolates from chronically-infected patients were significantly more resistant to sulfonamides and meropenem than occasional isolates. Candidate genetic biomarkers associated with antibiotic resistance or sensitivity were identified. *Achromobacter* spp. strains isolated from people with chronic and occasional lung infection exhibit different virulence and antibiotic susceptibility features, which could be linked to persistence in CF lungs. This underlines the possibility of identifying predictive biomarkers of persistence that could be useful for clinical purposes.

Keywords: *Achromobacter*; cystic fibrosis; drug resistance; virulence; biomarkers; adaptation

1. Introduction

Achromobacter spp. are opportunistic pathogens that can colonize the lungs of patients with cystic fibrosis (CF), causing chronic or occasional infections. In particular, chronic colonization has been associated with a decline in respiratory function, increased frequency of exacerbations and lung inflammation [1]. Although virulence factors supporting invasiveness and survival have been described (e.g., swimming motility, biofilm formation, lipopolysaccharide, type III secretion system, phospholipase C, proteases) [2–13], virulence

features related to the ability of *Achromobacter* spp. to colonize the lungs of CF patients chronically or occasionally are still not fully clear.

Additionally, multidrug resistance strongly contributes to *Achromobacter* spp. persistence in CF patients. These bacteria show an innate resistance to many classes of antibiotics, especially to those relevant to CF lung infection treatment such as aminoglycosides, aztreonam, tetracyclines, penicillins and cephalosporins [2,14,15]. Moreover, clinical isolates exhibit acquired resistance, especially to β-lactams. The most active agents against *Achromobacter* spp. are, among others, trimethoprim–sulfamethoxazole, ceftazidime, piperacillin and carbapenems [16]; however, mutations of genes related to antibiotic resistance may occur, causing resistance to these antibiotics [13]. Indeed, isolates from long-term chronic infection tend to be resistant to more antibiotics than earlier or occasional isolates [16].

The variation in virulence factors and antibiotic resistance among *Achromobacter* spp. isolates highlights the necessity to better understand the involvement of these features in the pathogenic potential and mechanisms of colonization of these microorganisms. To this purpose, we evaluated virulence, cytotoxicity, biofilm formation and antibiotic susceptibility of clinical isolates causing occasional and chronic CF infections.

2. Results

Ninety-five *Achromobacter* spp. isolates were analyzed in this study. Seventy-nine isolates (range = 1–11 successively collected isolates, mean = 3.3 isolates/patient) were recovered from 24 chronically-infected CF patients with a mean time delay of 469 days (range = 21–1825 days). One isolate was recovered from each occasionally-infected CF patient (n = 14), except for two patients, P06 and P12, from whom we recovered 2 isolates with a time delay of 112 days and 155 days, respectively. We compared phenotypic features such as virulence, biofilm formation, cytotoxicity and antibiotic susceptibility between chronic and occasional and between early and late chronic isolates.

2.1. Virulence

Virulence is an important feature for bacterial pathogenicity, invasion and interactions with the host. Virulence testing (Figures1 and S1) in a *G. mellonella* larvae model showed that isolates from occasionally-infected patients induced significantly higher mortality of larvae than chronic infection isolates (Kaplan–Meier survival estimate p-value = 0.02; Cox hazard ratio = 1.32; 95% CI = 1.04–1.66). When comparing early against late chronic isolates, no significant difference was observed.

Biofilm formation plays an important role in the persistence of bacteria in CF chronic lung infections, protecting pathogens against environmental stress and increasing tolerance towards antibiotics and host defenses. No significant difference was observed in biofilm production among chronic and occasional isolates and between early and late chronic isolates (Figure 2A,B).

The ability to cause cytotoxicity could play an important role in tissue inflammation and degeneration. Cytotoxicity was assessed on both WT and CF bronchial epithelial cells. Although no statistically significant difference was found, we observed that chronic infection isolates showed a trend for greater cytotoxicity than occasional isolates in both cell types (Figure 2C,E). Moreover, when comparing early and late chronic isolates we observed an increase (Wilcoxon Mann–Whitney test p-value = 0.05 after 10,000 permutations) of cytotoxicity from early to late isolates in WT cells (Figure 2D).

Virulence, cytotoxicity and biofilm formation results per isolate are shown in Figure S2.

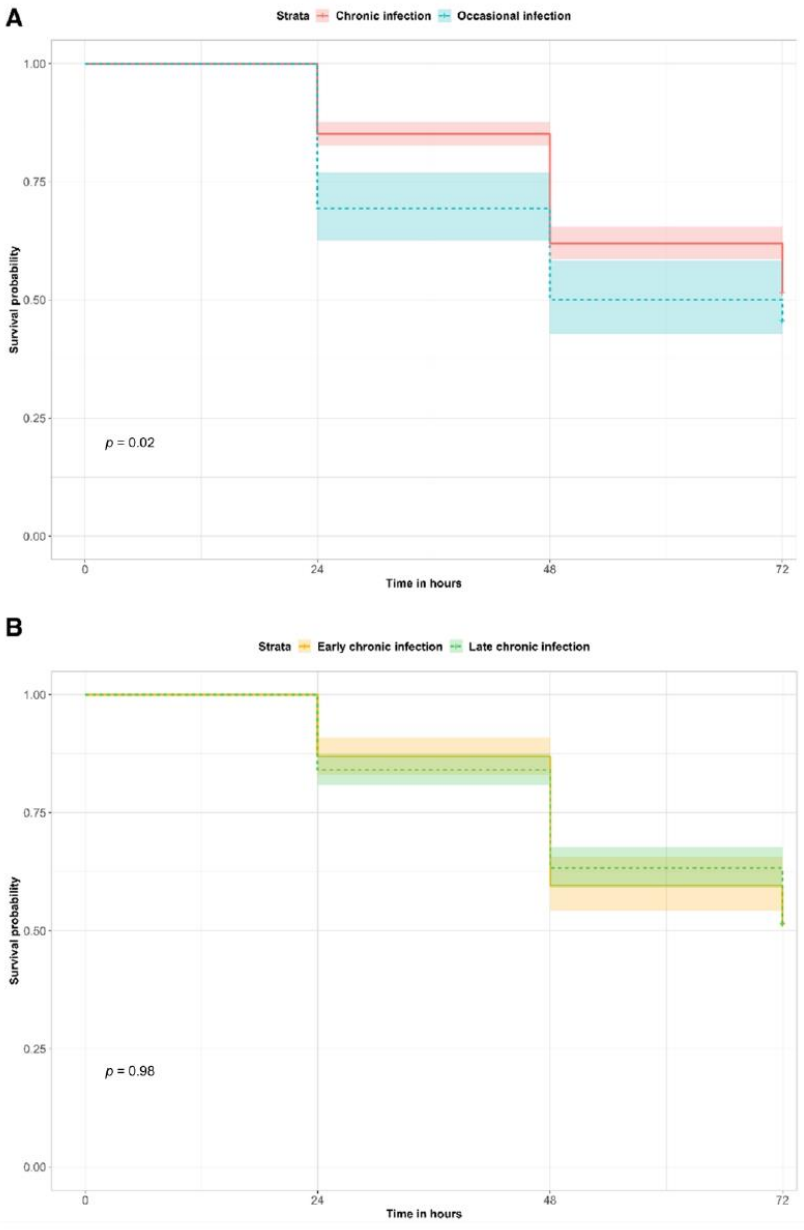

Figure 1. *G. mellonella* survival curve for virulence testing. The survival probabilities of *G. mellonella* larvae infected with chronic or occasional infection isolates (**A**) and with early or late chronic infection isolates (**B**) are reported at each time point. The *p*-values of the survival curve comparisons are indicated with *p*.

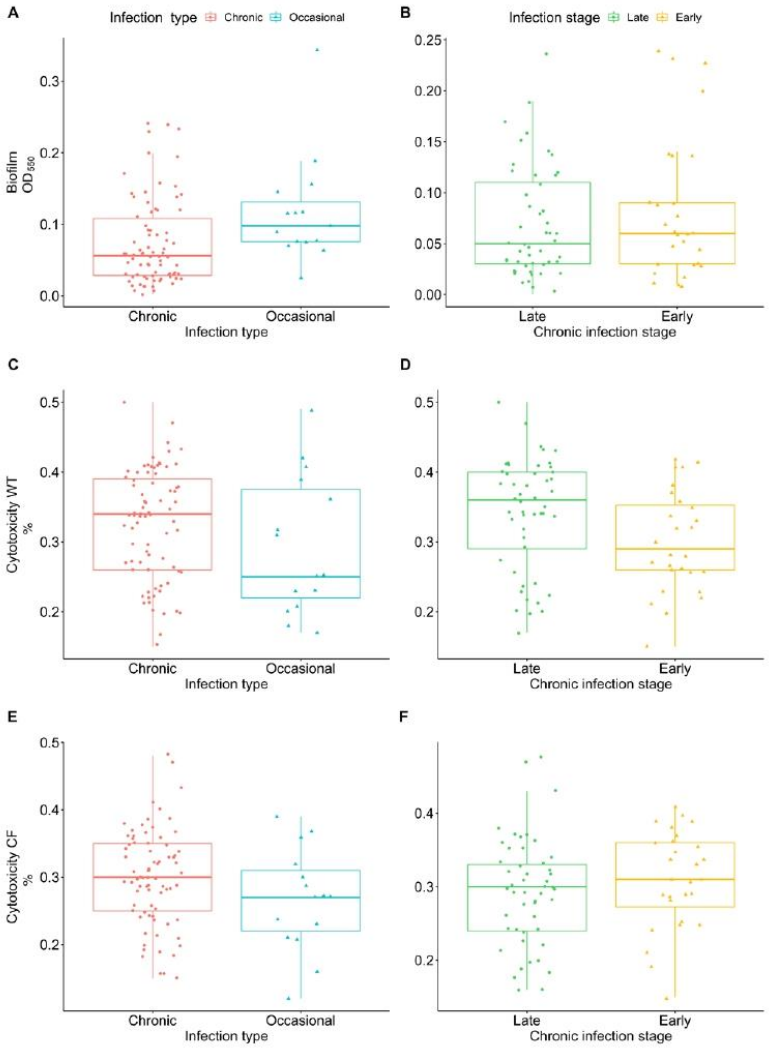

Figure 2. *Achromobacter* spp. biofilm formation and cytotoxicity on WT and CF bronchial epithelial cells. Biofilm formation in occasional and chronic isolates (**A**) and in early and late chronic isolates (**B**) measured by crystal violet staining (OD_{550}). Cytotoxicity of occasional and chronic isolates and of early and late chronic isolates on WT cells (**C**,**D**) and CF cells (**E**,**F**) expressed as percentage of LDH release compared to the maximum value (positive control).

2.2. Antimicrobial Susceptibility

An important factor for the survival of infectious bacteria is their resistance to administered antibiotics, making infections hard to eradicate. We evaluated six antibiotics that have been reported to show variable susceptibility in chronic strains [16]: SXT, TGC, SSS, IPM, TZP and MEM.

The majority of strains were resistant to SXT and TGC and sensitive to IPM and TZP, while different susceptibility between occasional and chronic isolates was observed for SSS and MEM (Figure 3): when compared to occasional isolates, strains from chronically-infected patients were significantly more resistant to SSS (Fisher's exact test p-value = 0.04

after 10,000 permutations; CI = 0.042–0.62; odds ratio = 0.17) and MEM (Fisher's exact test p-value = 0.01 after 10,000 permutations; CI = 0–0.34; odds ratio = 0). No significant difference in resistance was observed when comparing early and late chronic isolates (Figure 4). Antimicrobial susceptibility results per isolate are shown in Figure S3.

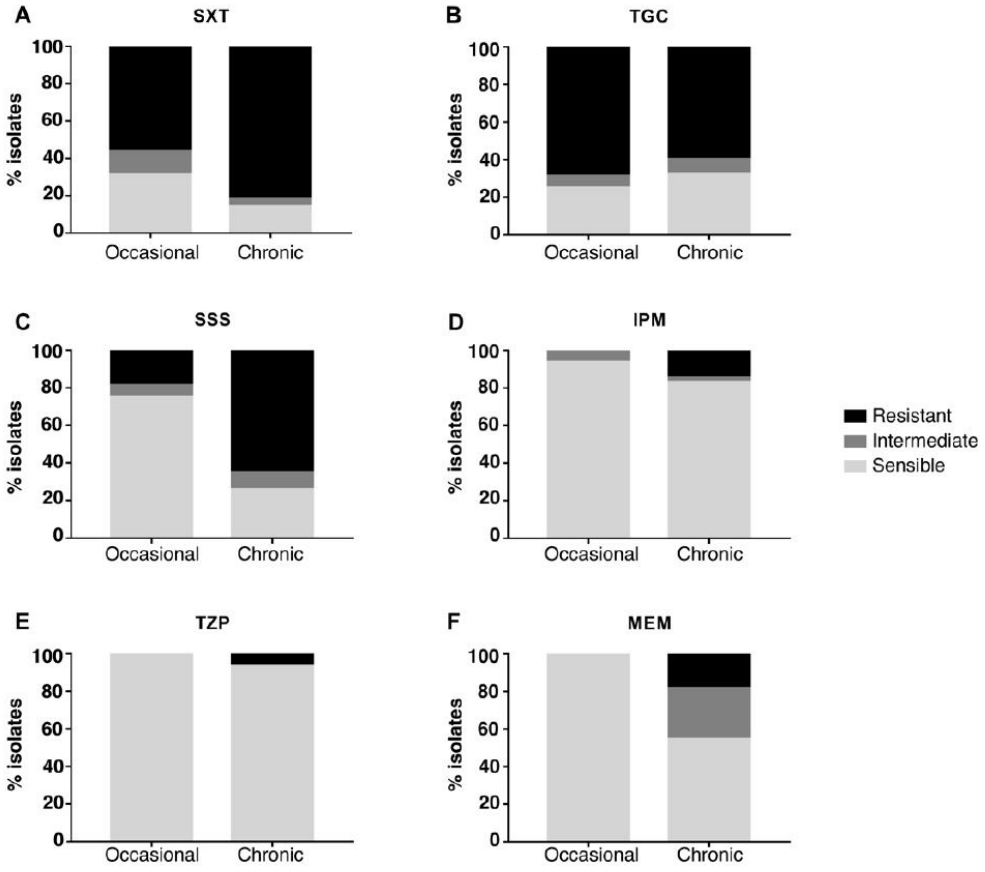

Figure 3. Antimicrobial susceptibility testing of chronic and occasional isolates. The percentage of resistant, intermediate and sensible isolates from chronic and occasional infection are represented for each antibiotic tested. SXT = trimethoprim–sulfamethoxazole (**A**), TGC = tigecycline (**B**), SSS = sulfonamides (**C**), IPM = imipenem (**D**), TZP = piperacillin-tazobactam (**E**), MEM = meropenem (**F**).

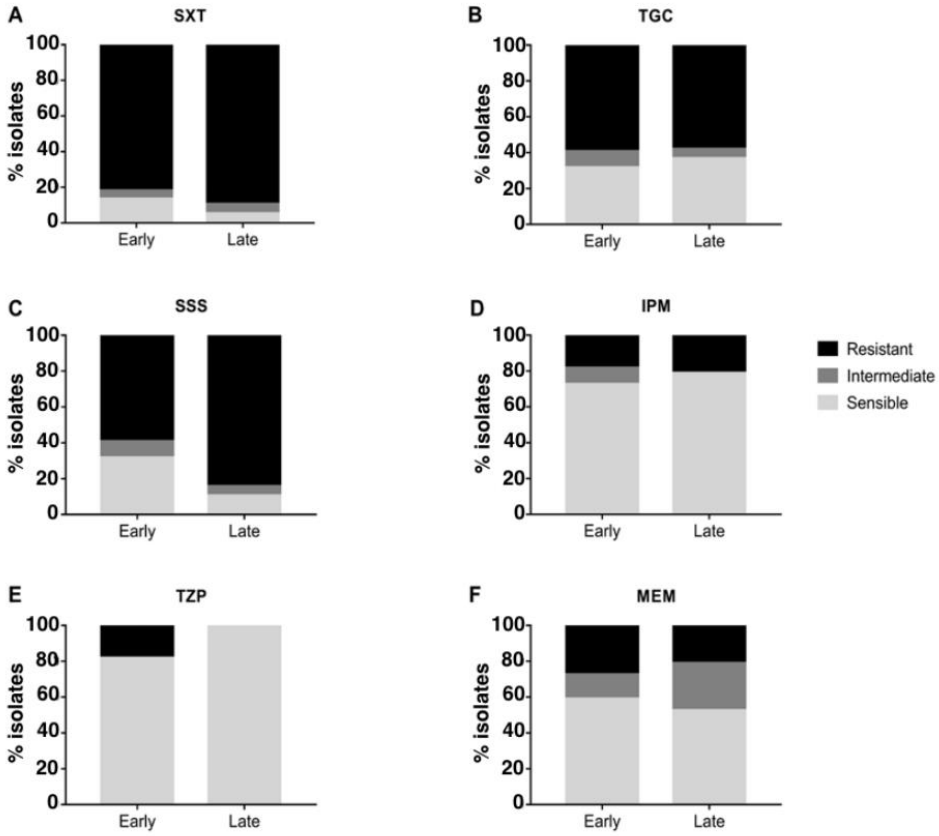

Figure 4. Antimicrobial susceptibility testing of early and late chronic isolates. The percentage of resistant, intermediate and sensible isolates from chronic and occasional infection are represented for each antibiotic tested. SXT = trimethoprim–sulfamethoxazole (**A**), TGC = tigecycline (**B**), SSS = sulfonamides (**C**), IPM = imipenem (**D**), TZP = piperacillin-tazobactam (**E**), MEM = meropenem (**F**).

2.3. Biomarkers of Antimicrobial Resistance

Genome analysis of the 54 *Achromobacter* spp. isolates from the Verona collection was performed to identify genetic components significantly associated with virulence, biofilm, cytotoxicity and antimicrobial resistance. As regards the former three features, no genetic loci were significantly associated to the phenotypic traits. Within the tested antibiotics, we found various candidate biomarkers of resistance or sensitivity linked to transmembrane transporters and efflux pumps (e.g., secretory components, ABC transporters), transcriptional regulators (e.g., AraC) and metabolic enzymes (Tables 1 and S1 for details). In addition, some hypothetical proteins were associated with sensitivity to SSS and resistance to MEM. Some node sequences showed statistically significant correlation (q-value ≤ 0.05) with both IPM and MEM, and with both SSS and SXT (Table S1).

Table 1. Genetic loci significantly associated with antimicrobial resistance. The most significant results of association analysis are reported. IPM = imipenem, SXT = trimethoprim–sulfamethoxazole, SSS = sulfonamides (sulphadiazine, sulphathiazole), MEM = meropenem, *q*-value = *p*-value adjusted for the False Discovery Rate.

Antimicrobial	Associated with	Node ID	Sensitivity (%)	Specificity (%)	*q*-Value	Annotation
IPM	Resistance	n1316743	100.0	100.0	8.4×10^{-23}	Arginine-tRNA ligase
IPM	Resistance	n284357	100.0	100.0	8.4×10^{-23}	Diguanylate cyclase (GGDEF domain) with GAF sensor
IPM	Resistance	n1001723	100.0	100.0	8.4×10^{-23}	ABC transporter
IPM	Resistance	n1228398	100.0	100.0	8.4×10^{-23}	Type II secretory pathway component GspD
IPM	Resistance	n1320154	100.0	100.0	8.4×10^{-23}	AraC family transcriptional regulator
IPM	Resistance	n119911	100.0	100.0	8.4×10^{-23}	NA
IPM	Resistance	n1360642	100.0	100.0	8.4×10^{-23}	General secretion pathway protein GspN
IPM	Resistance	n1607259	100.0	100.0	8.4×10^{-23}	Fe^{2+}-dicitrate sensor, membrane component, FecR
IPM	Resistance	n1496359	100.0	100.0	8.4×10^{-23}	Glutathione S-transferase family protein
MEM	Resistance	n382985	88.9	97.1	1.3×10^{-2}	ABC transporter ATP-binding protein
MEM	Resistance	n776344	88.9	97.1	1.3×10^{-2}	Hypothetical protein
MEM	Resistance	n1454097	88.9	97.1	1.3×10^{-2}	ABC transporter ATP-binding protein
SSS	Susceptibility	n477893	81.3	86.1	2.8×10^{-3}	Hypothetical protein
SSS	Susceptibility	n1248808	81.3	86.1	2.8×10^{-3}	ABC transporter ATP-binding protein
SSS	Susceptibility	n888310	81.3	86.1	2.8×10^{-3}	Aminomethyl-transferring glycine dehydrogenase
SXT	Susceptibility	n1221225	85.7	100.0	7.6×10^{-9}	NA
SXT	Susceptibility	n1593088	85.7	100.0	7.6×10^{-9}	16S rRNA (uracil(1498)-N(3))-methyltransferase
SXT	Susceptibility	n222900	85.7	100.0	7.6×10^{-9}	NA
SXT	Susceptibility	n979447	85.7	100.0	7.6×10^{-9}	DNA mismatch repair endonuclease MutL
SXT	Susceptibility	n120539	85.7	100.0	7.6×10^{-9}	Efflux RND transporter periplasmic adaptor subunit
SXT	Susceptibility	n335885	85.7	100.0	7.6×10^{-9}	Efflux RND transporter periplasmic adaptor subunit
SXT	Susceptibility	n71145	85.7	100.0	7.6×10^{-9}	M61 family metallopeptidase
SXT	Susceptibility	n346933	85.7	100.0	7.6×10^{-9}	Acyl-CoA synthetase
SXT	Susceptibility	n1069885	85.7	100.0	7.6×10^{-9}	Helix-turn-helix domain-containing protein
SXT	Susceptibility	n1157928	85.7	100.0	7.6×10^{-9}	Helix-turn-helix domain-containing protein
SXT	Susceptibility	n527920	85.7	100.0	7.6×10^{-9}	Patatin-like phospholipase family protein

3. Discussion

To identify phenotypic features related to the ability of *Achromobacter* spp. to establish chronic or occasional colonization in CF airways, we evaluated virulence, biofilm formation, cytotoxicity, and antimicrobial susceptibility of 95 clinical isolates and compared results between chronic and occasional isolates and between early and late chronic ones.

Isolates were collected at the CF Center of Verona (Italy) and Bambino Gesù Hospital in Rome (Italy). While the Verona collection included a larger number of occasional isolates, the Rome one comprised many longitudinally-isolated chronic strains encompassing a long period of time. The two collections showed similar phenotypic characteristics (Figures S2 and S3); so, their combination allowed the analysis of a more homogeneous group of strains than using them separately; however, the final collection still included a lower number of occasional (n = 16) than chronic isolates (n = 79, 30 early and 49 late chronic isolates).

To evaluate the virulence of *Achromobacter* spp. isolates, we used the well-characterized *G. mellonella* larvae model [17,18]. Occasional infection isolates caused higher mortality than strains from chronic infection, indicating that *Achromobacter* spp. exhibit higher virulence during occasional infection. This observation suggests that virulence attenuation could be a key factor during the establishment of chronic infection. Differences in virulence between early and late chronic strains of another CF pathogen, *Pseudomonas aeruginosa*, were also previously highlighted [18], leading to the hypothesis that multiple mutations could be responsible for virulence attenuation during late infection. Although we did not observe a significant difference in virulence between early and late chronic isolates of *Achromobacter* spp., a similar mechanism of adaptation could be proposed for occasional and chronic isolates, where selection of strains with an increasing ability to persist may occur.

As for biofilm formation, the majority of our isolates showed a low-moderate production of biofilm, confirming the poor adhesion ability of *Achromobacter* spp. on surfaces [7,9]. Although no significant difference in biofilm production was observed between chronic and occasional isolates nor between early and late chronic isolates, the great majority of strains unable to form biofilm were isolated from chronic infection. This could suggest a mechanism of within-host adaptation in the CF lung; e.g., acquisition of mutations in genes with a role in surface adhesion could lead to decreased biofilm production or to formation of unattached aggregates [10,13,19].

To investigate whether a reduced virulence in the chronic isolates coincided with lower cytotoxicity, we compared the cytotoxic potential of chronic and occasional strains in WT and F508del human bronchial epithelial cultured cells, but no significant difference was found. Although no statistically significant difference was found, we observed that chronic infection isolates induced slightly greater cytotoxicity than occasional isolates—an opposite trend compared to the results of biofilm and virulence testing. We observed an increased cytotoxicity from early to late isolates in cells expressing WT CFTR. No significant difference was observed in CF cells; this could indicate an underlying adaptation of late chronic strains to the CF lung environment leading to a more indolent colonization.

Biomarkers analysis of virulence traits (biofilm, cytotoxicity and virulence in *G. mellonella* larvae) showed no associated genetic loci. This is probably due to the fact these aspects are known to be mainly regulated through RNA modulation [20,21] rather than through the accumulation of genomic mutations.

Achromobacter spp. are reported to increasingly develop resistance to various antibiotics. We tested susceptibility of all isolates to 6 antibiotics that have been reported to show variable susceptibility in chronic strains [16]. *Achromobacter* spp. strains generally displayed resistance to TGC and susceptibility to TZP and IPM, confirming their previously-reported innate resistance to tetracyclines [2] and susceptibility to beta-lactams and carbapenems [16]. Interestingly, an increased resistance to MEM was observed in chronic isolates. Moreover, even though IPM and MEM belong to the same class of antimicrobials, we observed both imipenem resistant but meropenem susceptible (IRMS) and meropenem resistant but imipenem susceptible (MRIS) phenotypes. These phenotypes were previously reported for *P. aeruginosa* and various members of the *Enterobacteriaceae* family [22] as well as for *Achromobacter* spp. clinical isolates [22,23]. In addition, we observed significantly higher resistance to SSS in chronic infection isolates than in occasional ones, in concordance with previous studies [23,24]. Resistance of chronic infection isolates to SSS could be associated with the use of SXT to eradicate CF pathogens. The majority of both occasional and chronic isolates was resistant also to SXT, in contrast with previous investigations; e.g., a recent one found that the majority of *Achromobacter* spp. strains from Danish CF patients were sensitive to SXT [16], considered as one of the most active agents against *Achromobacter* spp. infections [25]. This suggests that the acquisition of resistance to SXT might have occurred among isolates in our collections, e.g., through the spread of mobile genetic elements carrying resistance genes [26] or due to the different antibiotic treatment regimens used.

Candidate biomarkers were identified for SXT, IPM, MEM and SSS by analyzing sequenced isolates. Some of them are known to be involved in antibiotic resistance, such as ABC transporters [27], while the role of other candidates—associated with antibiotic resistance or with sensitivity—should be further investigated. Of particular interest are hypothetical proteins, which could provide additional information on *Achromobacter* spp. resistance mechanisms upon further characterization. These candidate biomarkers could help in the identification of strains that are becoming persistent and support their eradication before chronic infection is fully developed. Finally, in order to assess whether biomarkers presence could be linked to an adaptation mechanism such as clonal expansion or horizontal gene transfer, we further performed genome analysis but neither of these hypotheses was confirmed. In conclusion, our results show that *Achromobacter* spp. isolates from chronic and occasional lung infection exhibit different virulence and antibiotic resistance characteristics, some of which might be linked to persistence in CF lungs. We identified potential predictive markers of persistence such as decreased virulence, higher cytotoxicity, resistance to antibiotics, as well as genetic biomarkers [3], that could be translated into the clinical setting either to help preventing the development of chronic infections or to support therapeutic treatments aimed at eradicating *Achromobacter* spp.

4. Materials and Methods

4.1. Samples Collection

Ninety-five *Achromobacter* spp. isolates were collected from the sputum samples of 38 patients followed at the CF Center of Verona and Bambino Gesù Hospital in Rome (Italy): 54 isolates were recovered from 26 patients in Verona [3,26], while 41 isolates were recovered from 12 patients in Rome. Patients were classified as occasionally- and chronically-infected with *Achromobacter* spp. according to the European Consensus Criteria or Leeds criteria. In the Verona collection, 43 longitudinal isolates were collected from 17 patients with chronic infections while 11 strains were collected from 9 patients with occasional infection. The Rome collection comprised 36 strains collected over time from 7 patients with chronic infection and 5 strains collected from 5 patients with occasional infections. Isolates from chronically-infected patients were further classified as early (<1 year from 1st colonization event) and late isolates (>1 year from 1st colonization). Relatedness of strains was verified to confirm that subsequent isolates from one patient actually represented a single strain. An overview of the isolates included in each collection is reported in Table 2. Informed consent was obtained according to projects CRCFC-CEPPO026 and CRCFC-CEPPO031, approved by the Ethical Committee. All the isolates included in this study were identified as *Achromobacter* spp. by MALDI-TOF-MS (bioMerieux Marcy-l'Étoile, France). Strains were stored in Microbank (Pro-Lab Diagnostics, Neston, UK) at −80 °C. Detailed information regarding the collections is reported in Table S2.

Table 2. *Achromobacter* spp. collections summary. The number of isolates included in each collection is reported in the table; the number of patients from which the strains were collected is indicated in parenthesis. E = early chronic infection isolates; L = late chronic infection isolates.

	Rome-*n* (Patients)		**Verona-*n* (Patients)**	
Chronic infection isolates	36 (7)	E: 6 (4) L: 30 (7)	43 (17)	E: 24 (10) L: 19 (10)
Occasional infection isolates	5 (5)		11 (9)	

4.2. Virulence Testing

Virulence was assessed in *Galleria mellonella* larvae. Ten larvae were inoculated with a 1×10^6 CFU bacterial suspension of each clinical isolate through the last proleg into the haemocoel using a 0.3 mL syringe and incubated in Petri dishes, on filter paper, at 37 °C, in the dark. In the control group, larvae were injected with sterile saline solution. Larvae were

monitored daily up to 72 h and death was assessed by lack of movement after stimulation and blackening.

4.3. Biofilm Formation Assay

Bacterial strains were plated onto LB agar and grown at 37 °C for 24–48 h. A single colony was inoculated in BHI medium and grown for 16 h at 37 °C with shaking. OD_{600} was measured, cultures were diluted to 0.1 OD/mL and 200 µL/well were incubated in a 96-well plate for 24 h at 37 °C. Wells were washed with saline solution and stained with 0.1% crystal violet solution for 15 min, then rinsed, washed with water and air dried. After 30 min of incubation with 30% acetic acid at 37 °C, absorbance at 550 nm was measured.

4.4. Cytotoxicity Testing

Human CF bronchial epithelial cell lines CFBE14o- 4.7 WT-CFTR (WT cells) and DeltaF508-CFTR (CF cells) (Merck, Darmstadt, Germany), overexpressing WT and F508del CFTR cDNA, respectively, were cultured in 200 µL EMEM supplemented with 1% Fetal Bovine Serum, 0.5–2 µg/mL Puromycin and 2 mM L-Glutamine into Fibronectin/Collagen/BSA-coated 96-well plates incubated at 37 °C and 5% CO_2. At 80% confluency, 50 µL/well of 2 OD_{600}/mL bacterial suspension were added to cell cultures and incubated at 37 °C for 4 h. CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) measuring LDH release was used according to manufacturer's instructions. Briefly, 50 µL of cells suspension were added with 50 µL of CytoTox 96 Reagent and incubated for 30 min in the dark. After adding 50 µL of Stop Solution, absorbance at 450 nm was recorded. Cytotoxicity was calculated by dividing for the absorbance of the positive control (treated with Lysis Solution).

4.5. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was determined by disk diffusion assay. Bacterial suspension at 0.5 McFarland was streaked onto Mueller–Hinton agar plates, antibiotic-containing disks (Oxoid) were placed onto the agar surface, plates were incubated at 37 °C for 48 h and the diameter of the zone-of-inhibition was measured. Disks contained 1.25/23.75 µg trimethoprim–sulfamethoxazole (SXT), 15 µg tigecycline (TGC), 300 µg sulfonamides (sulphadiazine, sulphathiazole) (SSS), 10 µg imipenem (IPM), 100/10 µg piperacillin-tazobactam (TZP) or 10 µg meropenem (MEM). Since no EUCAST or CLSI breakpoint standard is available for *Achromobacter* spp., susceptibility profiles were interpreted as resistant (R), intermediately resistant (I) or sensible (S) based on breakpoints proposed in previous literature [15,16,28].

4.6. Statistical Analysis

Statistical analysis was carried out to compare chronic and occasional isolates and early and late chronic isolates. Virulence results were tested by the Kaplan–Meier method using the log-rank test to compare the overall survival of larvae over an observation period of 72 h. Hazard ratios were computed with a Cox regression model. Cytotoxicity and biofilm formation results were tested using a Wilcoxon Mann–Whitney test. Outlier values were observed in biofilm formation results (n = 2, isolates 7-3 and 12-2) and were excluded from statistical analysis. Fisher's exact test was used to ascertain the significance of antibiotic susceptibility results. Since observations per isolate were not independent due to a longitudinal collection strategy, for each test, p-values were adjusted performing 10,000 permutations of the infection type (chronic, occasional) or infection stage (early chronic, late chronic) stratified by collection (Verona, Rome). R version 4.0.4 (R Foundation for Statistical Computing, Vienna, Austria) [29] was used for statistical analysis and for results visualization. Boxplots were generated using ggpubr v0.4.0, survival curves using survminer v0.4.9, and heatmaps using pheatmap v1.0.8 R libraries.

4.7. Identification of Genetic Loci Associated with Virulence, Biofilm, Cytotoxicity and Antimicrobial Resistance

The DBGWAS 0.5.4 software (bioMerieux, Lyon, France) [30] was used to identify genetic components significantly associated with virulence, biofilm, cytotoxicity and antimicrobial resistance. In previous work [3,26] we sequenced the whole genome of the 54 *Achromobacter* spp. isolates from the Verona collection. A phylogenetic analysis and a comparison of virulence and resistance genes, genetic variants and mutations, and hypermutability mechanisms between chronic and occasional isolates was also performed. The de novo assembled contigs of these genomes were used as input for the association analysis. The following phenotypic cut-offs were used as parameters when running the analysis: biofilm production of 0.115 (absorbance at 550 nm), cytotoxicity of 0.36 (% cytotoxicity vs. positive control), virulence causing 5 dead larvae in 48 h; while the cut-offs for antimicrobial resistance were defined as described in Section 4.5. In particular, DBGWAS accepts continuous phenotypes, so we translated the categorical variables "resistant (R)", "intermediately resistant (I)" and "sensible (S)" to a dummy variable such that S = 0, I = 1 and R = 2. All available annotations of *Achromobacter* genes from the UniProt database (www.uniprot.org, ref. 320,589 genes; accessed on 1 March 2022) were used in the annotation step of the virulence, biofilm and cytotoxicity association analysis, whereas all known bacterial resistance genes from the UniProt database (www.uniprot.org, ref. 36,658 genes; accessed on 1 March 2022) were used for annotation in the antimicrobial resistance association analysis.

Sensitivity and specificity of candidate components having the same order of magnitude of the lowest *q*-value—i.e., *p*-value adjusted for the False Discovery Rate—of each analysis were calculated using the following formulas: for nodes positively associated to the phenotype sensitivity = Pheno1Count/Pheno1TotalCount and specificity = (Pheno0TotCount-Pheno0Count)/Pheno0TotCount; for nodes negatively associated to the phenotype sensitivity = Pheno0Count/Pheno0TotCount and specificity = (Pheno1TotCount-Pheno1Count)/Pheno1TotCount. In particular, Pheno1Count = isolates displaying the phenotype and carrying the allele, Pheno1TotalCount = isolates displaying the phenotype, Pheno0Count = isolates not displaying the phenotype and carrying the allele, Pheno0TotCount = isolates not displaying the phenotype. All candidate components having the highest sensitivity and specificity, down to a threshold of 80%, and lowest *q*-value were further analyzed (tests were considered to be statistically significant if q-value < 0.05). Every k-mer present in only one isolate was discarded.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23169265/s1.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Azienda Ospedaliera Universitaria di Verona (protocol code CRCFC-CEPPO026 and CRCFC-CEPPO031).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Sequences of the 54 *Achromobacter* spp. isolates from the Verona collection have been deposited at the NCBI SRA database under project n. PRJEB40979.

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In Vivo Inflammation Caused by *Achromobacter* spp. Cystic Fibrosis Clinical Isolates Exhibiting Different Pathogenic Characteristics

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Abstract: *Achromobacter* spp. lung infection in cystic fibrosis has been associated with inflammation, increased frequency of exacerbations, and decline of respiratory function. We aimed to evaluate in vivo the inflammatory effects of clinical isolates exhibiting different pathogenic characteristics. Eight clinical isolates were selected based on different pathogenic characteristics previously assessed: virulence in *Galleria mellonella* larvae, cytotoxicity in human bronchial epithelial cells, and biofilm formation. Acute lung infection was established by intratracheal instillation with 10.5×10^8 bacterial cells in wild-type and CFTR-knockout (KO) mice expressing a luciferase gene under control of interleukin-8 promoter. Lung inflammation was monitored by in vivo bioluminescence imaging up to 48 h after infection, and mortality was recorded up to 96 h. Lung bacterial load was evaluated by CFU count. Virulent isolates caused higher lung inflammation and mice mortality, especially in KO animals. Isolates both virulent and cytotoxic showed higher persistence in mice lungs, while biofilm formation was not associated with lung inflammation, mice mortality, or bacterial persistence. A positive correlation between virulence and lung inflammation was observed. These results indicate that *Achromobacter* spp. pathogenic characteristics such as virulence and cytotoxicity may be associated with clinically relevant effects and highlight the importance of elucidating their mechanisms.

Keywords: *Achromobacter*; virulence; cytotoxicity; biofilm; acute respiratory infection; lung inflammation

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1. Introduction

Cystic Fibrosis (CF) is a genetic disorder caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene, encoding for CFTR protein, an important chloride channel of exocrine glands [1]. Although CF is a multisystemic disease, 85% of the mortality results from lung impairment [2]. Lack of functional CFTR expression in the airways results in excessive thickening of the mucus layer, which favors the development of infections by a large number of opportunistic pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia*, and *Achromobacter* spp. [3]. These bacteria are able to colonize persistently the lungs of people with CF, influencing the course of the disease, usually with decline of lung function and increased risk of mortality. Among them, in the last decade, *Achromobacter* spp. have generated growing interest as emerging opportunistic pathogens in CF that can cause severe chronic infections leading to lung inflammation, increased frequency of exacerbation, and decline of the respiratory function [4].

The pathogenic mechanisms of many CF pathogens are nowadays well known; in particular, *P. aeruginosa* has been extensively studied, due to its high incidence in CF patients and difficult eradication. Conversely, little is known about the pathogenicity of *Achromobacter* spp. Similar to other CF-related pathogens such as *P. aeruginosa*, a number of virulence factors have already been described in *Achromobacter* spp., such as production of secreted factors (e.g., colicin V and proteases), expression of membrane-bound factors (e.g., Vi capsular polysaccharide and O-antigen), secretion systems (e.g., type II and III secretion systems), and biofilm formation, which are important for survival and proliferation in hostile environments [5–8]. Moreover, these bacteria show innate and acquired resistance to many classes of antibiotics, especially to aminoglycosides, aztreonam, tetracyclines, penicillins, cephalosporins, and β-lactams [9]. Nonetheless, the pathogenic mechanisms and virulence features connected to *Achromobacter* spp. capability of colonizing CF lungs chronically or occasionally are still unclear.

In a previous study, we observed that *Achromobacter* spp. clinical isolates can exhibit different pathogenic characteristics (e.g., virulence, cytotoxicity, biofilm formation) that could be associated with different clinical outcomes [10]. Lung inflammation has already been reported to be influenced by bacterial virulence for another CF pathogen, *P. aeruginosa* [11]. We hypothesized that a similar situation could exist and be relevant for *Achromobacter* spp. too, and evaluated in vivo the inflammatory effects of *Achromobacter* spp. clinical isolates presenting different pathogenic characteristics.

2. Results

Wild-type (WT) and CFTR-knockout (KO) mice were intratracheally challenged with 8 *Achromobacter* spp. clinical isolates expressing different pathogenic characteristics: virulence in *G. mellonella* larvae, cytotoxicity in bronchial epithelial cells, and biofilm formation previously assessed [10], as shown in Table 1 and defined in Tables S1–S3. Mice were grouped and analyzed based on the pathogenic characteristics of the isolates. For each characteristic, strains were divided into 2 groups: high–medium and low–no activity.

Table 1. Type of infection of the clinical isolates and levels of activity for each pathogenic characteristic.

Isolate	Species	Virulence	Cytotoxicity	Biofilm
2-4	*A. xylosoxidans*	No	Medium	Low
7-2	*A. xylosoxidans*	High	Medium	Low
8-2	*A. xylosoxidans*	High	Medium	High
12-2	*A. agrifaciens*	No	Low	High
16-1	na	No	Low	Low
17-1	*A. xylosoxidans*	Medium	No	High
20-1	*A. insolitus*	No	Medium	Medium
23-1	*A. xylosoxidans*	High	Low	Low

na = not available. Average nucleotide identity < 95% against all available genomic assemblies used for phylogenetic analysis [12].

2.1. Lung Inflammation Induced by Isolates Exhibiting Different Pathogenic Characteristics

Transgenic WT and KO mice expressing a luciferase gene under control of bovine interleukin-8 (IL-8) promoter were intratracheally challenged with *Achromobacter* spp. clinical isolates. IL-8-dependent lung inflammation was monitored by in vivo bioluminescence imaging after 4, 24, and 48 h. Isolates showing high–medium virulence in larvae induced significantly higher IL-8-dependent bioluminescence emission in both WT ($p = 0.026$) and KO ($p = 0.038$) mice in comparison to isolates with low–no virulence (Figure 1). When analyzing the isolates individually, three out of four strains showing high–medium virulence activity induced a significant increase of IL-8-dependent bioluminescence emission in both WT and KO mice (isolates 8-2, 17-1), or in KO mice only (isolate 7-2), in comparison to control mice (Figure 2).

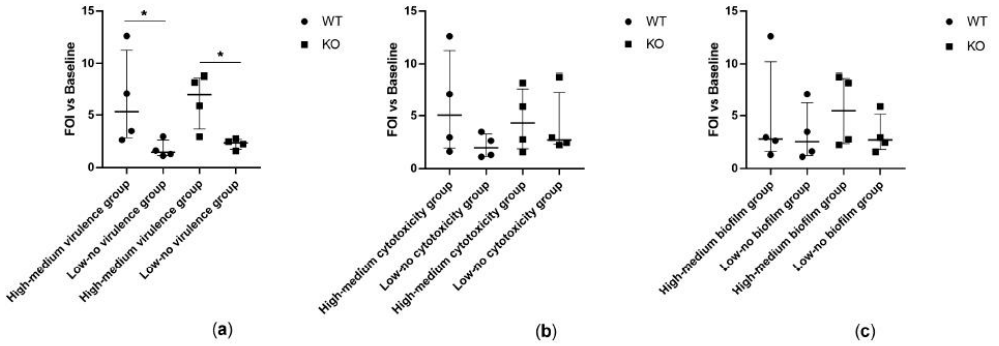


Figure 1. IL-8-dependent bioluminescence emitted by WT and KO mice at 24 h after intratracheal challenge with clinical isolates showing (**a**) high–medium vs. high-medium virulence in *G. mellonella* larvae, (**b**) high–medium vs. low–no cytotoxicity in human bronchial epithelial cells, and (**c**) high–medium vs. low–no biofilm formation. Photon emission is expressed as Folds of Induction (FOI) vs. baseline (before lung challenge). Each value represents the mean of four animals challenged with each isolate. The median ± interquartile range of 16 mice per group is shown. Statistical analysis was performed by Kruskal–Wallis test; * $p < 0.05$.

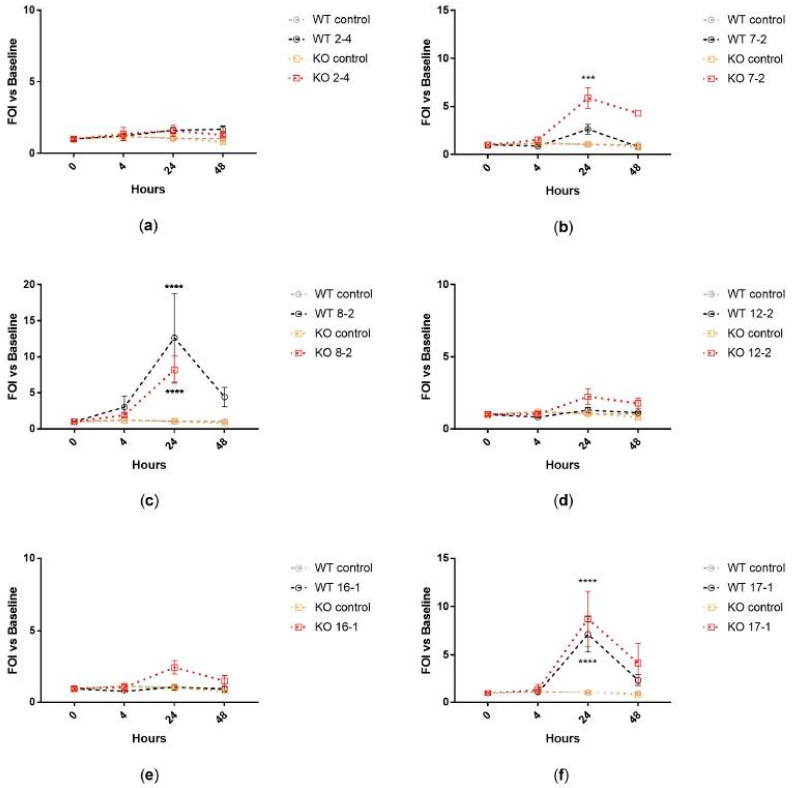
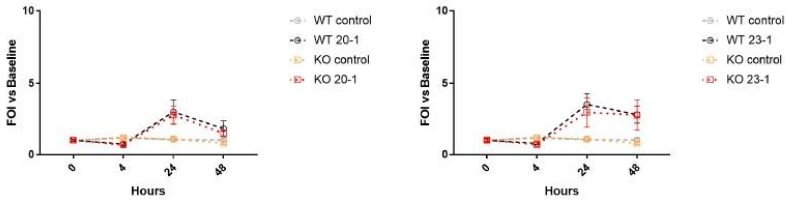


Figure 2. *Cont.*

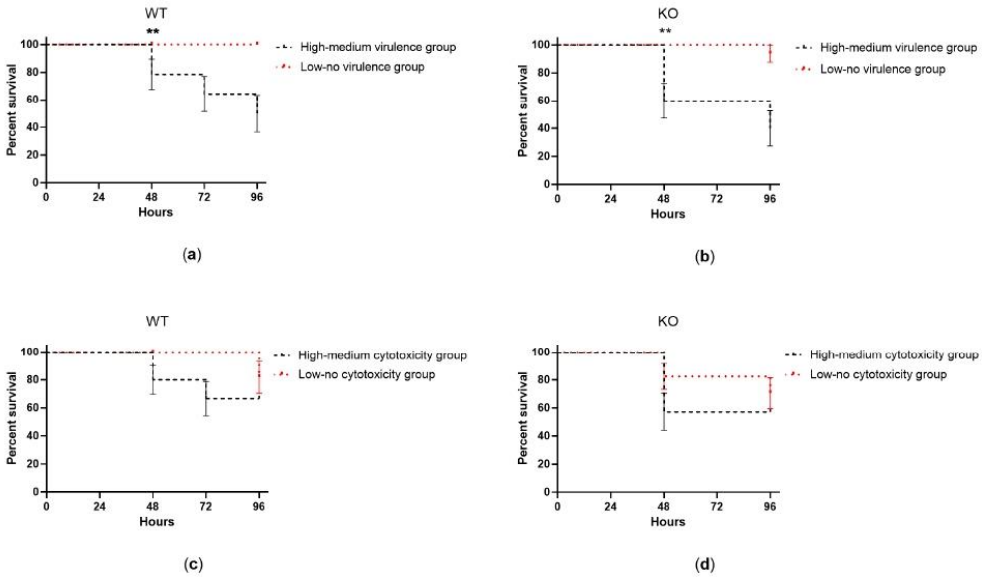


(**g**) (**h**)

Figure 2. IL-8-dependent bioluminescence emitted by WT and KO mice after intratracheal challenge with each clinical isolate: 2-4 (**a**), 7-2 (**b**), 8-2 (**c**), 12-2 (**d**), 16-1 (**e**), 17-1 (**f**), 20-1 (**g**), 23-1 (**h**). Imaging was performed before lung challenge (0 h) and after 4, 24, and 48 h. FOI = Folds of Induction vs. baseline (0 h). Each value represents the mean ± SEM of four animals. Statistical analysis of treated vs. control mice was performed by repeated measures 2-way ANOVA followed by Tukey's multiple comparison test; *** $p < 0.001$, **** $p < 0.0001$.

2.2. Mice Survival during Acute Infection with Isolates Exhibiting Different Pathogenic Characteristics

Mice survival was monitored up to 96 h from the intratracheal challenge with the clinical isolates. Isolates showing high–medium virulence in larvae induced significantly higher mortality in both WT ($p = 0.0035$) and KO ($p = 0.0012$) mice in comparison to isolates with low–no virulence (Figure 3). When analyzing the isolates individually, two strains showing high–medium virulence activity caused over 65% mortality within 72 h from lung challenge in both WT and KO mice (isolates 7-2, 8-2) (Figure 4). One of them (isolate 8-2) induced significantly faster mortality in KO mice (24 h) than in WT animals (48 h) (Figure 4c).



(**a**) (**b**)

(**c**) (**d**)

Figure 3. *Cont.*

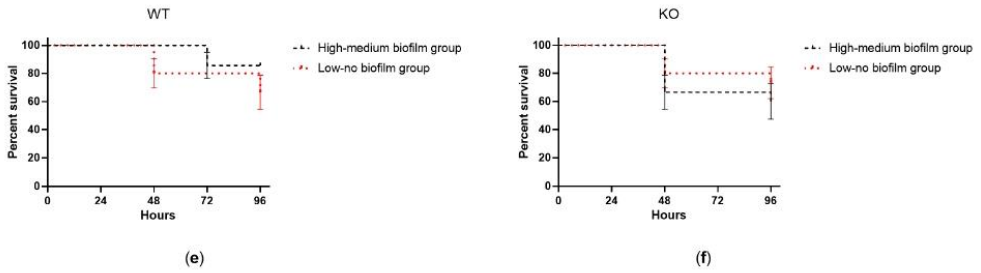


Figure 3. Percent survival of WT and KO mice up to 96 h after intratracheal challenge with clinical isolates showing (**a**,**b**) high–medium vs. low–no virulence in *G. mellonella* larvae, (**c**,**d**) high–medium vs. low–no cytotoxicity in human bronchial epithelial cells, and (**e**,**f**) high–medium vs. low–no biofilm formation. The mean ± SEM of 16 mice per group ($n = 4$ treated with each isolate) is shown. Statistical analysis was performed by log-rank test; ** $p < 0.01$.

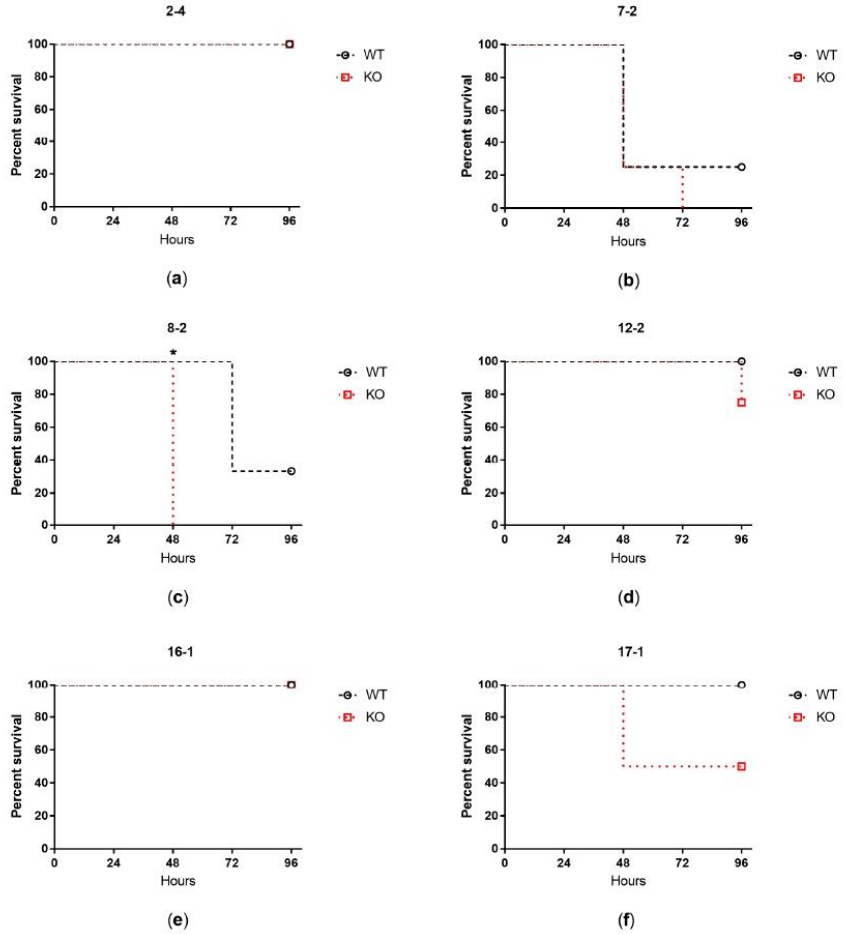


Figure 4. *Cont.*

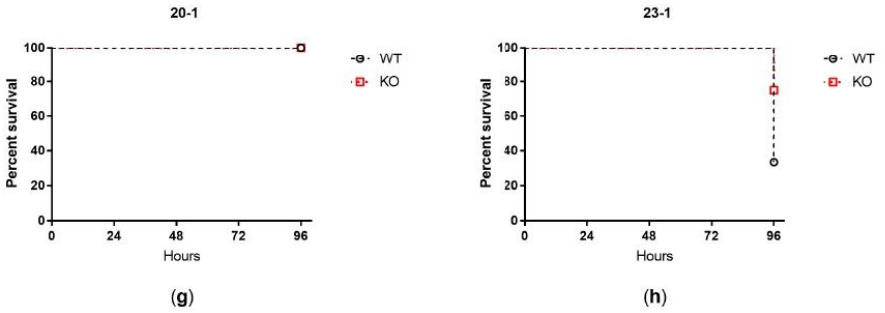


Figure 4. Percent survival of WT and KO mice up to 96 h after intratracheal challenge with each clinical isolate: 2-4 (**a**), 7-2 (**b**), 8-2 (**c**), 12-2 (**d**), 16-1 (**e**), 17-1 (**f**), 20-1 (**g**), 23-1 (**h**). The mean of four mice per group is shown. Statistical analysis was performed by log-rank test; * $p < 0.05$.

2.3. Bacterial Load during Acute Infection with Isolates Exhibiting Different Pathogenic Characteristics

Bacterial load in the lungs was evaluated after 24 h from the intratracheal challenge with the clinical isolates. For all the three pathogenic characteristics analyzed, no significant difference was observed between isolates exhibiting high–medium and low–no activity (Figure 5). Nonetheless, when analyzing the isolates individually, after 24 h from the lung challenge, two isolates with both high–medium virulence and cytotoxicity (7-2, 8-2) showed a high bacterial load in the mice lungs comparable to the challenging dosage, indicating that no clearance occurred, while the bacterial load of all the other isolates was significantly reduced in comparison to the challenging dosage (Figure 6).

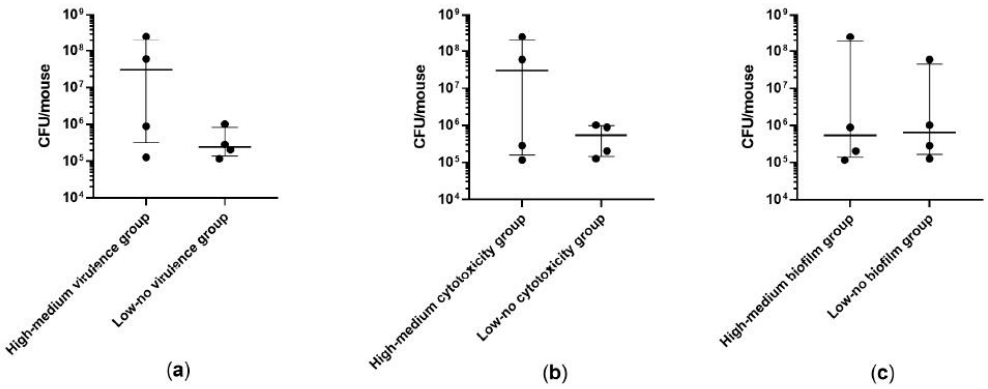


Figure 5. CFU recovered from WT mice lungs at 24 h after intratracheal challenge with clinical isolates showing (**a**) high–medium vs. low–no virulence in *G. mellonella* larvae, (**b**) high–medium vs. low–no cytotoxicity in human bronchial epithelial cells, (**c**) high–medium vs. low–no biofilm formation. Each value represents the mean of three animals challenged with each isolate. The median ± interquartile range of 12 mice per group is shown.

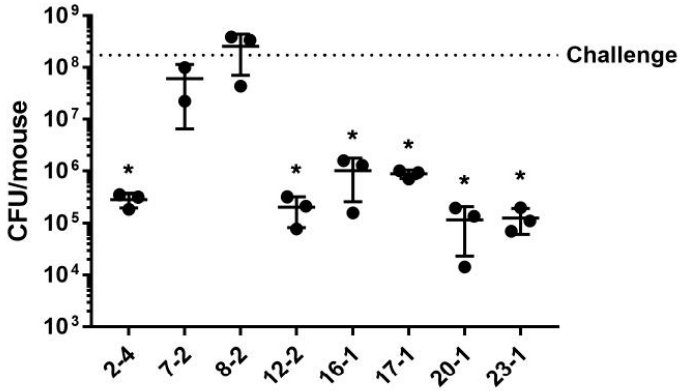


Figure 6. CFU recovered from WT mice lungs at 24 h after intratracheal challenge with eight clinical isolates. The bacterial load used for the challenge is indicated (dotted line). The mean ± SD of three mice per group is shown. Statistical analysis of each isolate vs. challenge was performed by 1way ANOVA test; * $p < 0.05$.

2.4. Lung Inflammation in Mice Correlates with Virulence in G. mellonella Larvae

Based on results from IL-8-dependent bioluminescence emission, mice survival, and lung bacterial load, we identified virulence as the most interesting pathogenic characteristic among those analyzed. We found a positive correlation between virulence of the isolates in *G. mellonella* larvae and IL-8-dependent bioluminescence emission at 24 h after intratracheal challenge in both WT and KO mice ($p = 0.027$ in WT mice, $p = 0.032$ in KO mice; Spearman r = 0.78 in WT mice, r = 0.77 in KO mice) (Figure 7).

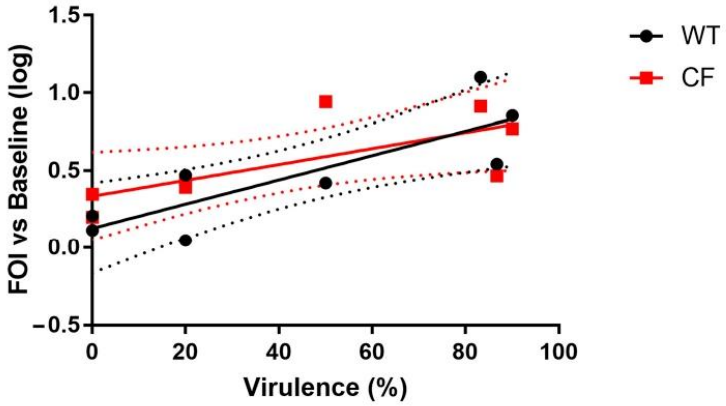


Figure 7. Correlation between IL-8-dependent bioluminescence emission in WT and KO mice at 24 h after intratracheal challenge with the clinical isolates and virulence in *G. mellonella* larvae infected with the same isolates. FOI = Fold of Induction vs. baseline (before lung challenge). Each point represents the mean ± SEM of four mice challenged with each isolate. Linear regression ± SEM (dotted lines) is shown.

3. Discussion

Achromobacter spp. exhibit different pathogenic characteristics that might be linked to clinical outcomes in CF lung infection. We previously evaluated the pathogenicity of *Achromobacter* spp. in terms of virulence in *G. mellonella* larvae, cytotoxicity in human bronchial epithelial cells, and biofilm formation, and observed wide differences for each of these three characteristics among clinical isolates [10]. Based on these results, in the present

study, we selected eight isolates showing different pathogenic characteristics and used them to induce acute lung infection in WT and KO mice to observe eventual differences in lung inflammation, mice survival, and bacterial load that could reveal the importance of these characteristics for *Achromobacter* spp. pathogenicity in CF lung infection.

We found a significant positive correlation between *Achromobacter* spp. virulence in *G. mellonella* larvae and in mice (both WT and KO). In particular, virulent isolates inducing high larvae mortality also induced high lung inflammation and mortality in mice. A correlation between mortality in mice and larvae had been reported for *P. aeruginosa* PA14 mutants [13], indicating that insect model systems can provide a tool for the characterization of microbial virulence involved in causing diseases in mammals. Our results further confirm that *G. mellonella* can be a useful infection model to study virulence of CF respiratory pathogens, also related to virulence-induced inflammation.

There are a number of virulence factors that could be involved in pathogenicity in both larvae and mice, from extracellular proteases to exotoxins to lipopolysaccharide, which were reported to exhibit pathogenicity in both models when treated with *P. aeruginosa* [11,13–16]. Our previous genomic profiling of the isolates used in this study shows that three (out of four) of the less virulent isolates (i.e., 16-1, 12-2, 20-1) also lack several genes associated with the ability to infect cells [12], chemotactic movement, type 3 secretion system, and proteases. This could explain both their low virulence in the *G. mellonella* model, and the low inflammation and mortality observed in mice.

Interestingly, only two isolates expressing both cytotoxicity and virulence showed a higher bacterial load in the mouse lungs after 24 h from the infection procedure, suggesting a higher persistence ability, while all the other strains lacked the combination of both characteristics (virulence and cytotoxicity) and resulted in a bacterial load significantly lower than the challenging dosage. This suggests that both cytotoxicity and virulence could be necessary for successful colonization of the lung environment by *Achromobacter* spp.

Conversely, biofilm formation was not relevant for the induction of lung inflammation and mice mortality, nor for the maintenance of a high bacterial load. The latter might seem surprising at first, since biofilm has been documented as an important mechanism of infection for many pathogens in several diseases (e.g., infective endocarditis, otitis media, urinary tract infections, etc.) including CF [17,18]. However, *Achromobacter* spp.'s poor adhesion ability is already known [5,6], suggesting that these bacteria rely on other mechanisms for colonization. For instance, various studies highlighted *Achromobacter* spp.'s ability to form unattached or loosely attached aggregates held together by polysaccharides and characterized by the scattering and dispersal of planktonic cells [19,20].

Few differences associated with the only two isolates expressing both cytotoxicity and virulence were observed between WT and KO animals in our study in terms of higher IL-8-dependent bioluminescence emission and faster mortality in KO than in WT animals. Therefore, the KO mice seem to be more sensitive to *Achromobacter* spp. pathogenicity than the congenic WT animals; this is as expected, considering that the C57BL/6J Cftr^{tm1UNC} strain was reported to consistently develop various aspects of spontaneous and progressive lung disease [21]. Many studies have proved the suitability of this animal model to study induced CF lung infection and inflammation with various microorganisms [22–29]; our study confirms its suitability to study *Achromobacter* spp. lung infection.

Our study is limited to a small number of clinical isolates that could be used in vivo. Using a larger number of strains, each exhibiting only one specific pathogenic characteristic among the three analyzed (virulence, cytotoxicity, and biofilm formation), would have been preferable. However, very few strains with this characteristic were available in our collection. As such, we decided to group the eight strains used in the study based on each pathogenic characteristic, obtaining an equal number of strains (n = 4) in each group and ensuring that the same isolates were never grouped together for more than one characteristic (virulence, cytotoxicity, biofilm), as shown in Table 1. This strategy allowed us to evaluate all three characteristics, even using a low number of isolates. Further studies are encouraged to elucidate the extent of the correlations that we have first observed.

In conclusion, our results indicate that *Achromobacter* spp. Pathogenicity may be associated with increased lung inflammation, mortality, and bacterial persistence in vivo. In particular, virulence in terms of *G. mellonella* larvae mortality correlates with pro-inflammatory effects and, coupled with cytotoxicity, could support *Achromobacter* spp.'s persistence. This highlights the importance of elucidating the mechanisms underlying *Achromobacter* spp. pathogenicity and virulence and suggests that the treatment of virulent isolates at an early stage of the infection could help prevent the worsening of the lung disease.

4. Materials and Methods

4.1. Clinical Isolates

Eight *Achromobacter* spp. clinical isolates were collected from the sputum samples of eight patients at the CF Center of Verona, Italy. All the isolates were identified by whole genome sequencing and phylogenetic analysis, as previously reported [12]. Strains were stored in Microbank (Pro-Lab Diagnostics, Neston, UK) at −80 °C.

For data analysis, the eight clinical isolates were divided in groups (n = 4 per group) based on each of the three pathogenic characteristics previously assessed in vitro: virulence in *G. mellonella* larvae, cytotoxicity on human bronchial epithelial cells, and biofilm formation [10]. Briefly, virulence was tested through inoculation of bacteria in *G. mellonella* larvae; in vitro cytotoxicity was tested by quantitative measurement of lactate dehydrogenase in human bronchial epithelial cells; biofilm production was measured by crystal violet staining of surface-attached bacteria cultured in static conditions [30]. Cut-off values were defined, and each strain was assigned a level of activity (high, medium, low, none) for virulence, cytotoxicity, and biofilm formation, as shown in Tables S1–S3, respectively. For each characteristic, strains were divided in two groups: high–medium and low–no activity.

4.2. Experimental Animals

Female 12-weeks old congenic C57BL/6NCrlBR (WT) and C57BL/6NCrlBR Cftr^{tm1UNC}TgN(FABPCFTR) [21,31] (KO) mice were provided by the Cystic Fibrosis Animal Core facility (San Raffaele Hospital, Milan, Italy) and bred by Charles River (Calco, Lecco, Italy). Animals were maintained under conventional housing conditions. Prior to use, animals were acclimatized for at least 7 days to the local vivarium conditions, having free access to standard rodent chow and tap water. Animal experiments were conducted in compliance with national (Legislative Decree 26/2014, authorization no. 265/2020-PR) and international laws and policies (Guide for the Care and Use of Laboratory Animals).

4.3. Reporter Construct

Experimental animals were transfected with the bovine interleukin-8 promoter/luciferase (bIL-8-Luc) construct, containing a luciferase gene under the control of the bovine IL-8 promoter (kindly provided by Professor Gaetano Donofrio, University of Parma, Italy) [32]. Competent *Escherichia coli* DH5α cells were transformed by heat shock and the plasmid was purified by Qiagen Plasmid Plus Mega Kit (Qiagen, cat. no. 12981; Qiagen, Valencia, CA, USA). Plasmid concentration and purity were evaluated using NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Fremont, CA, USA).

4.4. In Vivo Gene Delivery

In vivo JetPEI (Polyplus Transfection, Illkirch-Graffenstaden, France) was used as carrier for delivering bIL-8-Luc construct to lung tissue. As previously described [32], DNA and JetPEI were mixed with a final nitrogen/phosphate (N/P) ratio of 7–7.5 following the manufacturer's instructions. Briefly, 38–42 μg DNA and 50.3–60.3 μL JetPEI were separately diluted in 200 μL 5% glucose, mixed, and incubated at room temperature for 15 min; 400 μL of the mixture was intravenously injected through the tail vein after warming the animals for 5 min under a heating lamp. Expression and inactivation of the reporter were monitored by in vivo bioluminescence imaging after 24 h and 7 days, respectively.

4.5. Bacterial Preparation for Lung Challenge

Achromobacter spp. strains were plated onto Luria–Bertani (LB) agar (Oxoid, Basingstoke, UK) and grown at 37 °C for 24–48 h. A single colony was inoculated in LB medium and grown for 16 h at 37 °C shaking. Bacteria were washed twice and resuspended in saline solution. Absorbance at 600 nm was measured, and bacterial cells were diluted to the appropriate load for lung challenge.

4.6. Intratracheal Instillation

BIL-8-Luc transgenic mice were intratracheally challenged with 1.5×10^8 CFU of *Achromobacter* spp. cells for the development of acute lung infection [33]. As previously described [32], mice were anesthetized with 2.5% isoflurane and placed on an intubation platform, hanging by their incisor teeth. After visualization of the opening of the trachea using a laryngoscope, 50 µL of bacterial suspension was instilled by an intubation tube connected to a pressure control system. After 4, 24, and 48 h, reporter activation was monitored by in vivo imaging. Control non-infected mice were intratracheally instilled with saline solution. All mice were also imaged before the intratracheal instillation (baseline).

4.7. In Vivo Bioluminescence Imaging

Bioluminescence imaging of experimental animals was performed, as previously described [32], using IVIS Spectrum imaging system (Xenogen, Alameda, CA, USA). Ten minutes prior to bioluminescence recording, mice were anesthetized with 2.5% isoflurane and intraperitoneally injected with 150 mg/kg D-Luciferin (PerkinElmer, cat. n. 122,799). After 5 min-long luminescence recording, the photons flux emitted from the chest region was quantified using Living Image 4.7 software (PerkinElmer, Milan, Italy).

4.8. Lung Recovery and CFU Count

Mice were euthanized by cervical dislocation and lungs were excised and homogenized using the gentleMACS™ Dissociator (Miltenyi Biotec, Bologna, Italy) according to the manufacturer's instructions. Briefly, lungs were placed in 2 mL sterile saline solution within a M tube (Miltenyi Biotec, Bologna, Italy) and processed twice using the RNA_01 program. Homogenate was plated on LB agar. Plates were incubated at 37 °C for 24–48 h and CFU were counted.

4.9. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 7.0 software. Mice bioluminescence emission was analyzed by repeated measures 2-way ANOVA followed by Tukey's multiple comparison test. Mice bioluminescence between the different groups was analyzed by Kruskal–Wallis test. Mice survival was analyzed by log-rank test. CFU between different groups were analyzed by Mann–Whitney test. CFU among clinical isolates were analyzed by 1-way ANOVA. Linear regression and Spearman correlation of mice bioluminescence vs. larvae mortality induced by the clinical isolates were calculated.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24087432/s1.

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Data Availability Statement: Data are available upon reasonable request to the corresponding author.

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Development of an infection model to study virulence of the opportunistic pathogen *Achromobacter* spp in zebrafish embryos

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Abstract

Achromobacter spp. are emerging opportunistic pathogens of concern in cystic fibrosis (CF) and nosocomial infections. These bacteria are known for their antibiotic resistance and immune evasion. To investigate their pathogenicity and interaction with the innate immune system, we established a zebrafish embryo infection model using seven clinical isolates with varying pathogenic traits.

The evaluation of these isolates revealed distinct infection outcomes: virulent strains were identified caused significant host mortality, while less virulent strains were identified by low host mortality and reduced bacterial burden. One strain exhibited elevated and sustained expression of the pro-inflammatory cytokines *il1b* and *cxcl8*, while the less virulent strain showed a more transient increase in expression of these cytokines. Interestingly, macrophages played an important role in controlling bacterial growth and reducing host damage, as their depletion led to increased bacterial burden and mortality in embryos infected, with a strain that otherwise persists without excessive host damage.

This study highlights the critical role of macrophages in controlling bacterial growth and limiting infection severity in *Achromobacte*r spp. infection. Moreover, it underscores zebrafish embryos as a valuable model for studying *Achromobacter*-host interactions, particularly the role of immune cells. The model provides insights into host-pathogen dynamics and supports the development of targeted therapeutic

strategies against *Achromobacter* infections in vulnerable populations, complementing murine studies and offering real-time insights into host-pathogen interactions.

Introduction

Bacteria from the genus *Achromobacter* are commonly found in various natural environments and they take part in a variety of human infections. Indeed, they are known to cause numerous nosocomial infections, including pneumonia, bacteremia, and urinary tract infections, particularly in immunocompromised individuals[1,2]. Notably, people with CF are often the most affected, therefore *Achromobacter* spp. have garnered growing attention as emerging opportunistic pathogens in CF, where these bacteria can cause severe chronic infections that lead to lung inflammation, increased frequency of exacerbations, and a decline in respiratory function[3–5]. Several virulence factors have been identified in *Achromobacter* spp., including the production of secreted substances like colicin V and proteases, the expression of membrane-associated factors such as the Vi capsular polysaccharide and O-antigen, secretion systems like type II and III, and biofilm formation. These factors play a crucial role in the bacterium's ability to survive and multiply in challenging environments [6,7]. Additionally, the ability of *Achromobacter* to establish persistent infections is attributed to its capability to evade host immune defenses, while its intrinsic antibiotic resistance further complicates treatment [8,9].

Cystic fibrosis is characterized by a chronic inflammatory state in the lungs, where immune cells such as neutrophils, macrophages, and lymphocytes play a central role in both defense and tissue damage. While neutrophils are considered the main immune players, macrophages have also been shown to have an important role in modulating the immune response, initiating phagocytosis of pathogens and producing pro-inflammatory cytokines like IL-1β and TNF-α. Infections by *Achromobacter* spp. further exacerbate this inflammatory environment, as these opportunistic pathogens evade immune system and even exploit immune cells for replication. Pickrum et al. demonstrated a process of internalization and initial replication in vacuoles within these innate immune cells [10]. Additionally, Turton et

al. found that certain *Achromobacter* species can trigger macrophage pyroptosis via type III secretion system mechanisms *in vitro*, leading to the release of pro-inflammatory mediators such as IL-8, which normally results in recruitment of neutrophils and perpetuates inflammation [11]. IL-8-mediated inflammatory response to *Achromobacter* spp. infection was also recently observed in vivo in CFTR-knockout mice[12]. This interplay between *Achromobacter* infection and immune cell dynamics underscores the importance of investigating their interactions to understand pathogenicity and develop effective interventions.

Zebrafish embryos have emerged as a powerful model system for studying host-pathogen interactions due to their external development, optical transparency, and conserved vertebrate biology. This model system has been effectively used to study various diseases, including tuberculosis, septicemia, and viral infections, offering insights into disease mechanisms and host immune responses [13–16]. The optical transparency of zebrafish embryos allows for high-resolution noninvasive live imaging, making it possible to observe the real-time interactions between pathogens and host cells. Additionally, zebrafish embryos develop rapidly and share significant genetic and physiological similarities with humans, making them a relevant and efficient model for biomedical research. Leveraging these unique advantages, researchers have developed a variety of infection models to investigate the pathogenicity of diverse microbial species, including CF pathogens like *Pseudomonas aeruginosa, Staphylococcus aureus* and *Burkholderia cenocepacia* [17–22].

The zebrafish model could therefore be suitable for the study of *Achromobacter* spp. infections, and especially of their interplay with the innate immune system. In fact, zebrafish embryos have a functioning innate immune system with macrophages and neutrophils that can phagocytose and kill microbes from 28 hours post-fertilization. This enables studying innate immune responses without the interference of adaptive immunity, which can be more complex to parse in murine models [23–25]. While the latter provide critical insights into adaptive immunity and lung-specific inflammation, zebrafish models excel in studying initial host-pathogen interactions, innate immune dynamics, and macrophage-mediated

responses [26–28]. Therefore, they can serve as a complementary system to murine studies, allowing for cross-validation of findings.

Building on a previous study where we observed that *Achromobacter* spp. diverse pathogenic characteristics (i.e., virulence, cytotoxicity) can influence lung inflammation in infected CFTR-knockout mice [12], in the present study we have further investigated the interplay with the innate immune system using a zebrafish embryo model. Here we established a zebrafish embryo infection model to assess its suitability as an effective model for studying the pathogenicity of *Achromobacter*, explore the interaction of *Achromobacter* spp. isolates with the host innate immune system, and to better determine the role of macrophages in infection.

Material and Methods

Zebrafish embryos

Zebrafish (*Danio rerio*) were maintained and managed according to the European Union guidelines for laboratory animal handling. The research conducted at VBIC received approval from the Direction Départementale de la Protection des Populations (DDPP) du Gard (ID 30-189-4). Infection experiments were concluded at 5 days post-fertilization and were not classified as animal experiments under the 2010/63/EU Directive. Zebrafish lines used in this study are listed in Table 1.

Table 1. Zebrafish lines used in this study.

Name	Description	Reference
AB	Wildtype	-
Tg(mpx:eGFP^{i114})	Neutrophil marker-GFP, referred to as mpx:GFP	[27]
Tg(mpeg1:eGFP^{caax})	Macrophage marker-GFP, referred to as mpeg:eGFP	[29]

Bacterial strains and plasmids

Seven *Achromobacter* spp. clinical isolates were collected from the sputum samples of eight patients at the CF Center of Verona, Italy. All the isolates were identified by whole genome sequencing and phylogenetic analysis, as previously reported[6,30]. Strains were stored in Microbank (Pro-Lab Diagnostics, Neston, UK) at −80 ◦C.

Bacterial cells were electroporated with a plasmid expressing the fluorescent protein DsRed (pIN29) using conditions described earlier [21,31]. Briefly, electrocompetent cells (40 µl) were mixed with 1-2 µl of the DNA solution and incubated on ice for 1 minute. The mixture was then transferred to a chilled electroporation cuvette (0.2 cm), which was placed into the chamber electrodes. The electroporation apparatus (MicroPulserTM) was activated, delivering a pulse of 3.8 ms with 2.5 kV. *Achromobacter* spp. strains containing DsRed or eGFP plasmids were cultured overnight in 5 ml of LB medium with 100 µg/ml chloramphenicol at 37°C. Fluorescence was confirmed using a Nikon AZ100 microscope equipped for both bright-field and fluorescence imaging.

Zebrafish embryo infection

Microinjection of bacterial cells in zebrafish embryos was carried out as follows [31–33]. Bacteria expressing DSRed were cultured overnight in LB broth supplemented with suitable antibiotics. The bacteria were then harvested by centrifugation at 3,000 × g for 2 minutes and resuspended in phosphate-buffered saline (PBS) (Gibco). Bacterial dilutions (typically 200 bacteria/nl) were made in PBS with 0.05% phenol red added for visualization during microinjection. Embryos were manually dechorionated 2 hours before injection. Before starting injection, embryos were anesthetized in E3 medium with 0.02% buffered MS222 (tricaine; ethyl-3-aminobenzoate methanesulfonate salt; Sigma). Using a Femtojet microinjector (Eppendorf) and a micromanipulator with pulled microcapillary pipettes, bacteria were injected intravebously (iv) into embryos staged at 30 hours post-fertilization (hpf) under a stereo light microscope (Leica MS5). During experiments we determined the initial inoculum by injecting directly onto agar plates (typically 3 plates per strain).

Injection was carried out using 1-2 nL of bacterial suspension (about 200-400 CFU) [21,22,25]. Embryos in each group (strain infection) were assigned at random to either CFU count (n = 5 per experiment per stated time point) or survival monitoring up to 4 days post-injection.

CFU count

Following injection of bacterial cells in zebrafish embryos, the bacterial load was determined by plating individual lysed embryos and measuring the colony forming units (CFU) [21,25]. Briefly, after injection, embryos were rinsed in E3 medium, anesthetized in tricaine, and individually transferred to 1.5 ml Eppendorf tubes containing 45µl of distilled water. Embryos were disrupted using a pestle, which then was rinsed with 50ul of distilled water and finally let to incubate for 10 minutes at room temperature to favour cells lysis. Then, the total lysate and serial dilutions were plated on LB-agar plates with 100µg.ml-1 chloramphenicol. Plates were incubated at 37˚C overnight. The number of colonies was counted the following day.

Analysis of gene expression by qRT-PCR

At specific time points, embryos were collected for RNA isolation, cDNA synthesis, and qPCR analysis. The peptidylprolyl isomerase A-like (*ppial*) gene was used as the house keeping gene for the analysis. For each condition, 15 to 20 embryos were transferred to 500µl of TRIzol, homogenized, and stored at -80˚C as previously described [21,31]. RNA extraction for each pool followed the method described [34] and was purified using the RNeasy MinElute Cleanup kit (Bio-Rad). Reverse transcription of each sample (500 ng total RNA) was carried out using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's instructions. Quantitative PCR was conducted using the LightCycler 480 SYBR Green I Master mix (Roche) on a LightCycler 480. The cycling parameters were: 95˚C for 10 minutes to activate the polymerase, followed by 45 cycles of 95˚C for 15 seconds and 60˚C for 40 seconds. Fluorescence measurements were taken at the end of each cycle. A melting curve analysis was performed to confirm the absence of primer dimers and non-specific products. The stability of the housekeeping gene *ppial* was checked for each experiment. Results were analyzed using the ΔΔCt method.

Neutrophil quantification

Embryos were transferred to glass-bottom dishes (MatTek Corp., Ashland, MA) in E3 containing 0.02% MS222. A Nikon AZ100 equipped for bright-field and

fluorescence imaging, coupled with Coolsnap HQ2 (Roper Scientific) using MetaVue software was used to record full size embryos, and images were processed using Imaris software. We selected a magnification allowing the imaging of entire individual embryo (40x). The embryo was manipulated in order to place it laterally, so that the entire trunk was in the same focal plane as the tail region[29]. This allowed the majority of fluorescent cells to be in focus. Imaris (Oxford Instrument) was used to quantify the number of neutrophils per image using using the "spots" option.

Macrophages ablation: Pu.1 knockdown embryos

Embryos were treated with 1 nL of tMO_pu.1 (5'-CCTCCATTCTGTACGGATGCAGCAT-3', 0,1 mM) and sMO_E4I5_pu.1 (5'-GGTCTTTCTCCTTACCATGCTCTCC-3', 0,38 mM), mixed together [21,35]The indicated combination of sMO/tMO allows the specific ablation of macrophages, without affecting neutrophils. The procedure followed was described by Su et al [35].

Microscopy

For analysis of intracellular bacteria, the confocal laser scanning microscope Fv10i (Olympus Life Science) and Fluoview software (Olympus Life Science) was used. Images were further treated in Imaris (Oxford Instrument), by using surface structure (Figure 3, right).

Statistical analysis

Statistical analysis was performed using Prism 6 (GraphPad). Survival assays results are shown as Kaplan-Meier graphs and analysed with a Log rank (Mantel-Cox) test. In experiments determining CFU, significance between multiple selected groups was determined using one-way Anova, with Kruskal-Wallis's test. CFU counts were log transformed and are presented in dot graphs showing the geometric mean per time point, each dot representing a single embryo. Significance of neutrophil counts was determined using one-way Anova, with Sidak's Multiple comparisons test. QRT-PCR data were log_2-transformed, and significance was analysed using one-way Anova, with Tukey's Multiple Comparison Test at each time point by comparing treatments with each other and the PBS control, for each time point.

Results

In our study, we infected zebrafish embryos through intravenous injections of a panel of seven clinical *Achromobacter* spp. isolates with varying pathogenic characteristics: virulence in *G. mellonella* larvae, cytotoxicity in bronchial epithelial cells and biofilm formation (Table 1)[12,36].

***Table 2*. Levels of activity for each pathogenic characteristic (Sandri et al, 2022).**

Isolate	Species	Virulence	Cytotoxicity	Biofilm
7-2	*A. xylosoxidans*	High	Medium	Low
8-2	*A. xylosoxidans*	High	Medium	High
12-2	*A. agrifaciens*	No	Low	High
16-1	*na*	No	Low	Low
17-1	*A. xylosoxidans*	Medium	No	High
20-1	*A. insolitus*	No	Medium	Medium
23-1	*A. xylosoxidans*	High	Low	Low

Zebrafish embryos survival after blood circulation infection with *Achromobacter* spp. strains

AB (wild type) Zebrafish embryos were intravenously injected with 7 clinical isolates of *Achromobacter* spp., and their survival was monitored up to 4 days post-injection (dpi). Isolate 7-2, that was highly virulent for *G. mellonella* larvae and caused cell cytotoxicity, induced acute fatal infections also in zebrafish embryos, killing most of the larvae within 4 days (87,5 % of dead larvae). Three other virulent

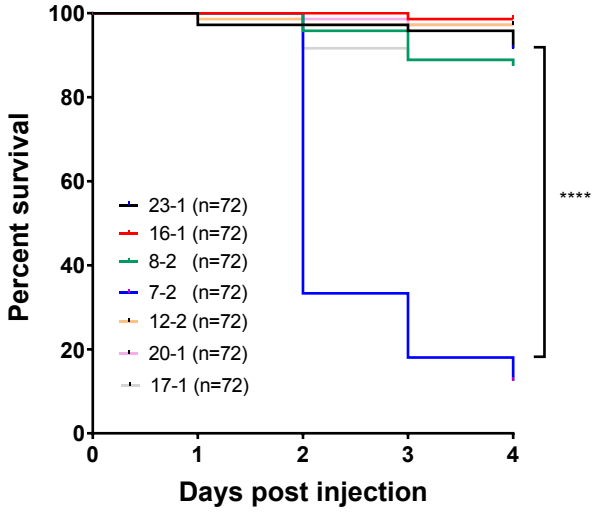

Figure 1. Percent survival of Zebrafish embryos injected iv with *Achromobacter* clinical isolates. In the graph three biological replicates are shown (24 embryo for each strain); Log rank (Mantel-Cox) test; significance is indicated with: **** = $p \leq 0.0001$.

isolates (8-2, 17-1, 23-1) caused 8,4-12,5% mortality. The remaining three strains, all previously classified as not virulent, induced significantly lower mortality (<3%) in the embryos. The results of these experiments are shown in Figure 1.

Bacterial burden in zebrafish embryos after injection in the blood circulation with *Achromobacter* spp. strains

The highly pathogenic isolate 7-2 showed a significant increase in bacterial load at 1dpi, in agreement with the high mortality rate shown in Figure 1. Due to the substantial death of the AB embryos at this early time point, CFU counts at 2 and 3 dpi were performed only on larvae that were still alive. In contrast, the less virulent strains (23-1, 16-1, 12-2, and 20-1) showed a significant reduction in bacterial load at 1dpi, indicating a decrease in bacterial proliferation. Finally, the two moderately virulent strains, 8-2 and 17-1, exhibited a smaller reduction in bacterial load compared to the less virulent strains, with the strain 17-1 showing the least reduction.

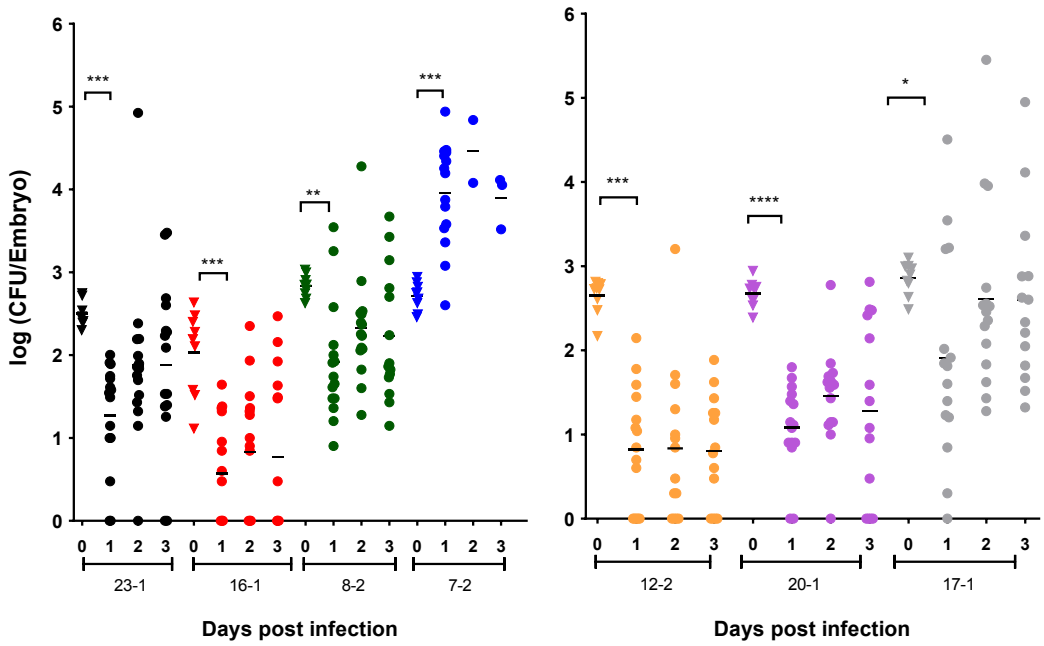

Figure 2 Zebrafish embryos survival and bacterial burden during blood circulation infection with *Achromobacter* spp. Strains. Geometric means with each data point representing an individual embryo. Dead embryo are not represented in the graphs. One-way Anova, with Bartlett's test , * = $p \leq 0.05$; ** = ≤ 0.01; *** = $p \leq 0.001$.

Interactions with innate immune system cells

In order to investigate the interaction between host immune cells and *Achromobacter* spp. during infection, we injected *mpeg:eGFP* zebrafish embryos expressing GFP in macrophages, with strain 7-2, expressing DSRed using confocal imaging. These images, taken at 150 minutes post-infection (mpi), bacteria internalized by macrophages (Figure 3, left).

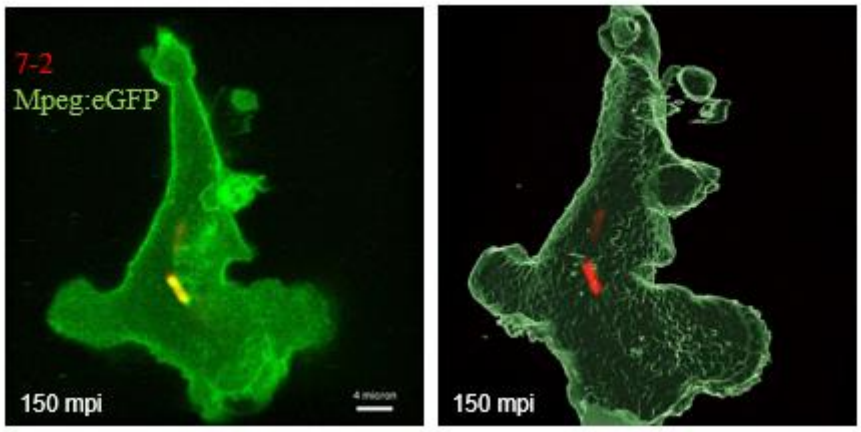

Figure 3 Confocal microscopic images of zebrafish embryos 150 mpi with the most virulent *Achromobacter* spp. strain 7-2. The images show macrophages internalized by the bacteria. These observations highlight the early immune response in the zebrafish infection model.

Analysis of gene expression by qRT-PCR

Next, we focused on two specific strains, 7-2 and 16-1, representing the most and least virulent strain in zebrafish embryos, respectively. We conducted a gene expression analysis of *il1b* and *cxcl8*, two key pro-inflammatory cytokine genes.

At 3 hours post-infection (hpi), *il1b* and *cxcl8* expression was higher in infected embryos compared to the PBS-injected control group, with no significant difference between the two strains for either gene (Figure 4). At 24 hpi, however *il1b* and *cxcl8* expression increased in embryos infected with strain 7-2 compared to both the control and strain 16-1-infected embryos. In contrast, *il1b* and *cxcl8* levels in strain 16-1-infected embryos were reduced, showing no significant difference from the PBS control.

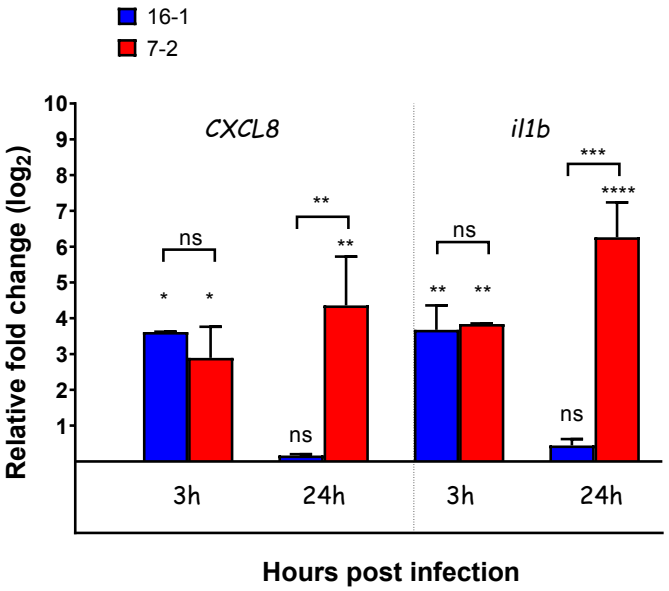

Figure 4. Transcriptional analysis of il1b and cxcl8 gense. Mean relative *il1b* and *cxcl8* gene expression levels (qRT-PCR) in embryos injected with strain 7-2 (red bars) and strain 16-1 (blue bars), normalized to a PBS-injected control group at each time point. Error bars represent mean with SEM of two biological replicates. Asterisks above each bar indicate significance compared to the PBS control at each time point, significance between groups per timepoint is indicated with a horizontal line. Significance is indicated with: ns = non-significant, * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$

Neutrophil count in Zebrafish embryos during blood circulation infection with *Achromobacter* spp. Strains

Mpx:GFP embryos (neutrophils marked with GFP) were injected with strain 7-2 and strain 16-1, while non-infected controls were injected with PBS; neutrophils

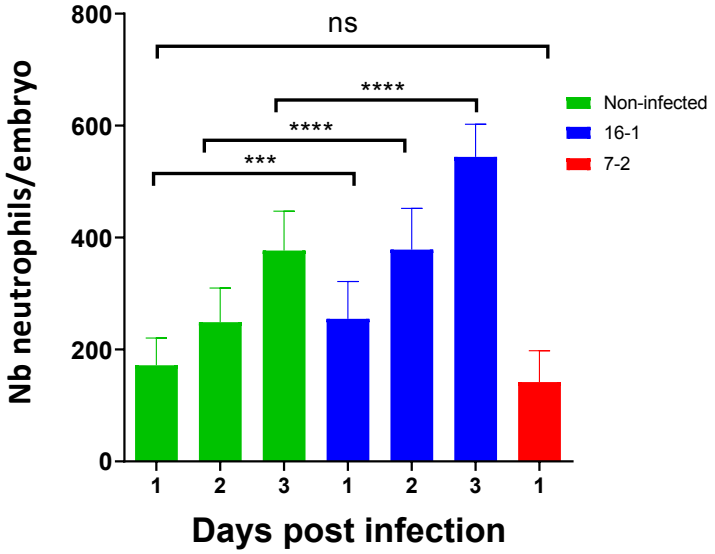

Figure 5. Neutrophils count in zebrafish embryos during blood circulation infection withn *Achromobacter* spp strains. Subsequent comparisons were not feasible due to the highe virulence of strain 7-2, which killed most of the larvae within a few days. Two biological replicates are shown. One-way Anova, with Sidak ultiple comparisons test have been done, ns= non-significant, ***=$p \leq 0.001$, ****= $p \leq 0.0001$

were quantified up to 3 days post infection (Figure 3). There was no difference between the number of neutrophils in embryos infected with strain 7-2 and the non-infected control at 1 day post infection; subsequent comparisons were not feasible due to the high virulence of strain 7-2, which killed most of the larvae in the following days.

Conversely, in embryos infected with strain 16-1 we observed a significant increase of neutrophil numbers from the first day post infection, as well as in the second and third day.

Macrophage-depleted zebrafish embryos infected with a non-virulent strain

We further analysed the role of macrophages during infection by using the non-virulent isolate, 16-1. Zebrafish Pu.1 knockdown embryos and control (not treated) embryos were intravenously injected with clinical isolates 16-1.

Their survival was monitored up to 2 dpi. These results show a significant difference ($p \leq 0.0001$.) between the macrophage-depleted and non-depleted embryos after infection with strain 16-1 (Figure 6A): 12% of macrophage-depleted

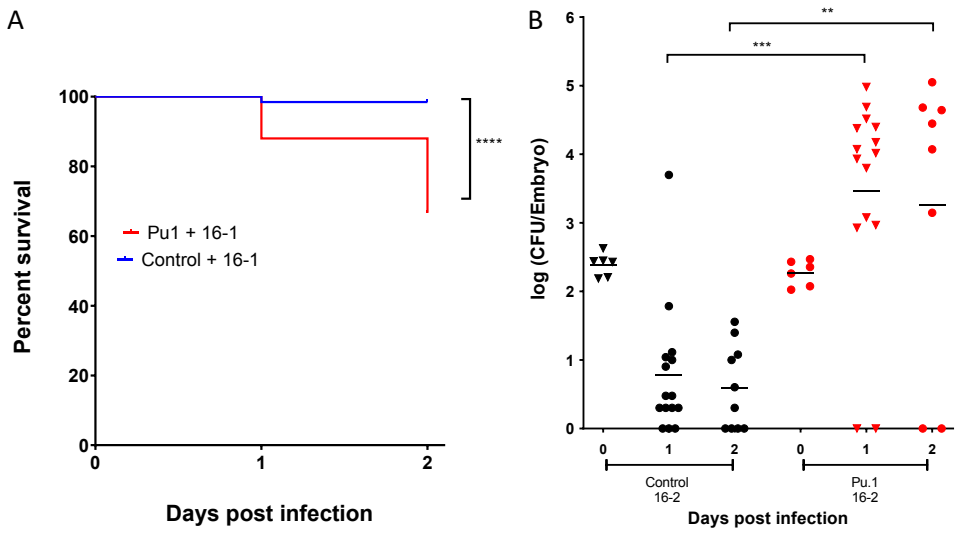

Figure 6. Survival over time of control and Pu.1 knockdown embryos injected iv with less virulent strain. Embryo were treated with MO for ablation of macrophages, by knocking down pu.1, a transcription factor involved in early myeloid progenitor formation, without affecting neutrophils. For survival analysis (A), a Log rank (Mantel-Cox) test was performed; **** = $p \leq 0.0001$. For CFU counts (B), Geometric means with each data point representing an individual embryo. One-way Anova, with Bartlett's test, ** = $p \leq 0.01$; *** = $p \leq 0.001$.

embryos died at 1 dpi and 33% at 2 dpi, while negligible mortality (<2%) was observed in non-depleted embryos.

Bacterial burden in Pu.1 knockdown embryos and control embryos infected with isolate 16-1 was evaluated up to 3 days post-infection (Figure 6B). At 1 and 2 dpi, the bacterial load in ablated than in non-ablated (control) embryos.

Discussion

Achromobacter spp. exhibits various pathogenic characteristics that may influence the outcome of infections in which it is involved. For this study, we used clinical strains that had been previously phenotypically characterized for pathogenic traits such as virulence in *G. mellonella* larvae, cytotoxicity in human bronchial cells, and biofilm formation. The strains considered showed different levels of these three characteristics. In a previous study, we used this panel of strains to infect wild-type (WT) and CFTR-knockout (KO) mice to assess differences in pulmonary inflammation, survival, and bacterial load, which could reveal the significance of these traits for the pathogenicity of *Achromobacter* during infections, particularly in the context of pulmonary infections in cystic fibrosis. Results supported that two pathogenic traits, virulence and cytotoxicity, are particularly important for *Achromobacter* spp. in CF infection.

In the current study we confirmed these findings and explored further the involvement of immune cells in the host response to *Achromobacter* spp in a zebrafish embryo infection model, demonstrating the utility of this model for investigating the pathogenicity of these microorganisms and their interplay with the host immune system. Our findings provide novel insights into the virulence-dependent variability in the immune responses *Achromobacter* spp. elicit, which align with and extend observations from our previous studies and existing literature.

The zebrafish embryo model revealed distinct infection outcomes among *Achromobacter* strains, ranging from lethal infections caused by virulent isolates to low-burden infections caused by more indolent strains. These differences correlate with pathogenic features previously described in murine and in vitro models. For

example, a strain with high virulence (i.e., strain 7-2) exhibited elevated mortality and bacterial burden, consistent with our previous observations in a murine model [12] and to other reports of *Achromobacter*-induced lung infections, where *Achromobacter* spp mice lungs' infection were linked to acute inflammation and rapid host death [11].

The strong induction of *il1b* and *cxcl8* cytokine expression by strain 7-2 further aligns with its acute infection profile and high mortality. These findings parallel reports of heightened pro-inflammatory cytokine production in severe *Achromobacter* infections in murine models where excessive inflammation contributes to tissue damage and mortality[37,38]. The high, durable expression of both *il-1b* and *cxcl8* also suggests that the production of a cytokine storm may contribute to the rapid death of the embryos. The robust induction of pro-inflammatory cytokines il1b and cxcl8 in response to strain 7-2 further aligns with similar findings in zebrafish embryos infected with other CF pathogens. Indeed, a similar correlation has been observed with *P. aeruginosa* infection, where a rapid cytokine response contributes to infection severity[17]. Likewise, *B. cenocepacia* induces a swift and robust increase in pro-inflammatory cytokine expression, leading to acute infections[21]. Similarly, in *S. aureus* infections, virulent strains trigger a rapid cytokine response, which can result in excessive inflammation and tissue damage, ultimately causing higher mortality in embryos [39–41].

Conversely, the lower cytokines response elicited by a strain with low virulence (i.e., strain 16-1) is indicative of a host immune system managing the infection without excessive host damage (Edwards et al., 2018), probably going towards clearance. Interestingly, neutrophil recruitment as well as transient induction of *il1b* and *cxcl8* was significant in embryos infected with strain 16-1, suggesting that another immune population, i.e. macrophages, may contribute to controlling the infection and resolving inflammation.

A key finding from our study is the critical role of macrophages in controlling bacterial growth and limiting infection severity. In macrophage-depleted zebrafish embryos, infection with the low virulent strain 16-1 led to significantly higher

bacterial burden and increased host mortality, underscoring the essential role of these immune cells in defense against *Achromobacter* spp. Also, during microscope observation, we noticed bacteria inside macrophages; These observations are consistent with in vitro studies showing that *Achromobacter* spp. can survive and replicate within macrophages, suggesting that these cells may act both as a defense mechanism and a potential niche for bacterial persistence[10,11]. We can hypothesize that, similar to other CF pathogens like *Pseudomonas aeruginosa* and *Staphylococcus aureus*, *Achromobacter* spp. may have evolved mechanisms to evade complete clearance by macrophages, possibly by surviving within vacuoles or utilizing other strategies [42]. The results from macrophage depletion in our study highlight that macrophages play a critical and fundamental role in controlling *Achromobacter* spp. infections for the host. This contrasts with pathogens such as *Burkholderia cenocepacia*, where macrophages are primarily essential for supporting bacterial growth and replication. Notably, in *Burkholderia* infections, the depletion of macrophages results in a significant reduction in bacterial burden[21]. These observations suggest that different pathogens employ distinct strategies to evade immune defenses, with *Achromobacter* spp. potentially relying on mechanisms beyond macrophage interaction to establish and sustain infections. Furthermore, the increased host damage observed with macrophage depletion underscores the potential vulnerability in individuals with compromised macrophage function, such as those with cystic fibrosis.

Compared to murine models, zebrafish embryos offer unique advantages for studying *Achromobacter* infections, including real-time imaging of host-pathogen interactions and the ability to manipulate immune cell populations genetically. While murine models provide insights into lung-specific adaptive immune responses, our results highlight the zebrafish model's strength in elucidating innate immune mechanisms, particularly the role of macrophages and neutrophils in controlling bacterial growth and modulating inflammation.

While our study highlights the utility of zebrafish embryos as a model for investigating *Achromobacter spp.* pathogenicity and host immune responses, it is not without limitations. Zebrafish embryos lack an adaptive immune system at the

stages used, which precludes analysis of adaptive immune mechanisms that play a crucial role in chronic infections. Additionally, while zebrafish embryos provide a robust system for studying innate immunity, their immune responses may not fully replicate those of mammals, particularly regarding CF-specific lung inflammation. The absence of CF airway conditions may also affect bacterial persistence and immune interactions. Finally, while strain-specific differences in virulence were observed, the molecular mechanisms behind these differences require further investigation. Future studies combining zebrafish with other models are needed to validate our findings and provide a more comprehensive understanding of *Achromobacter*-host interactions in CF.

In conclusion, our study aligns with and expands on the existing literature by demonstrating that zebrafish embryos are a powerful and complementary model for studying *Achromobacter spp.*'s pathogenicity and immune evasion strategies. Future research should focus on integrating findings from zebrafish and murine models to develop a comprehensive understanding of *Achromobacter*-host interactions, with the ultimate goal of identifying novel therapeutic strategies to mitigate infections in vulnerable populations, such as cystic fibrosis patients.

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Brief Report

Development of a Simple and Accurate Molecular Protocol Using 16SrRNA for Species-Specific Identification of *Achromobacter* spp.

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Abstract: The *Achromobacter* genus comprises 22 species and various genogroups. Some species with higher virulence or antibiotic resistance are more likely to cause chronic infections in people with cystic fibrosis (CF). Current identification methods often fail to accurately distinguish between the species or result in misidentifications due to biochemical similarities. This study aims to develop an accurate qPCR protocol for species-level identification that is applicable in clinical diagnostic laboratories. Whole-genome sequencing of clinical isolates from different *Achromobacter* species identified species-specific single-nucleotide polymorphisms (SNPs) in two 16S gene regions. Based on these SNPs, two sets of primers and qPCR probes were designed to generate unique identification profiles. Thermal profiles were optimized, and qPCR was performed on serial bacterial DNA dilutions to determine the detection limit (LOD). Four probes successfully identified three species: *A. xylosoxidans*, *A. dolens*, and *A. insuavis*. Two additional probes were designed for novel genotypes unrelated to publicly available sequences. The LOD ranged from 0.005 pg/µL to 1 pg/µL. Combined probes achieved 100% sensitivity, with specificity ranging from 97.95% to 100%. This qPCR protocol enables accurate species identification, overcoming the limitations of current methods, and represents a reliable tool for clinical diagnostics.

Keywords: *Achromobacter*; identification; cystic fibrosis; qPCR

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1. Introduction

Achromobacter spp. are Gram-negative bacilli broadly present in the environment, especially in moist soil, water sources, and plants [1]. *Achromobacter* spp. infections have been associated with various clinical conditions, including bacteriemia, meningitis,

pneumonia, peritonitis and urinary tract infections [2,3]. In addition, *Achromobacter* spp. exhibit resistance to multiple classes of antibiotics [1,4,5].

Recently, *Achromobacter* spp. have gained attention as emerging opportunistic pathogens in cystic fibrosis (CF), a genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [6]. These mutations result in a defective chloride transport in exocrine glands, leading to a thickened airway mucus, which fosters bacterial colonization [6]. While CF affects multiple organ systems, its primary cause of mortality is progressive lung disease, exacerbated by persistent infections with opportunistic pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Burkholderia cepacia complex*, *Stenotrophomonas maltophilia*, and *Achromobacter* spp. [7,8]. These infections contribute to lung inflammation, disease progression, and pulmonary impairment, ultimately increasing the risk of mortality [9,10].

Among these pathogens, *Achromobacter* spp. have been reported to cause chronic infections that exacerbate lung damage and respiratory decline [1,9–12], highlighting the critical need for improved ways of the identification and treatment of these opportunistic pathogens. The *Achromobacter* genus comprises 22 species and various genogroups. Among these, *Achromobacter xylosoxidans* is the most prevalent in CF, followed by *Achromobacter insuavis* and *Achromobacter dolens*. Other species associated with CF lung infections include *Achromobacter ruhlandii*, *Achromobacter dentifricans*, *Achromobacter insolitus*, and *Achromobacter aegrifacens* [13,14].

Achromobacter species exhibit regional variability in prevalence among CF patients. In Europe, *A. xylosoxidans* is the most frequently isolated species (36–65%, depending on the country), followed by *A. insuavis*, which has higher infection rates in Denmark (20–24%) and France (19%), and *A. dolens*, with lower rates compared to *A. insuavis* [2,15–20]. *A. xylosoxidans*, along with *A. ruhlandii*, *A. dolens*, and *A. insuavis*, is particularly adept at causing chronic infections in CF patients. These four species possess genes and mechanisms that facilitate long-term airway colonization, which can result in significant lung damage and functional decline. Indeed, up to half of CF patients with *A. xylosoxidans* colonization develop chronic infections, often accompanied by lung inflammation and respiratory deterioration [9,11,19,21–24], and a greater number of pulmonary exacerbation events and annual hospitalizations [1,12].

Recently, species-specific virulence and antibiotic resistance genetic profiles were reported, showing that some *Achromobacter* spp. are intrinsically resistant to several antibiotics, especially aminoglycosides, monobactams, tetracyclines, some penicillins, and cephalosporins [23–25]. Resistance to the most frequently used antimicrobial agents (e.g., trimethoprim–sulfamethoxazole, ceftazidime, piperacillin, and carbapenems) is on the rise, and there are no standard treatment protocols, requiring a case-by-case approach for treatment [23,26]. Considering this indication, accurate species identification could support clinical decisions. For example, *A. insuavis* is more sensitive to tigecycline (49%) compared to *A. xylosoxidans* (23%), while both species exhibit moderate sensitivity to ceftazidime–avibactam [27]. *A. rhulandii* shows low susceptibility to tigecycline, meropenem, and pieracillin–tazobactam, while *A. aegrifaciens* responds better to meropenem [27]. Without precise species identification, clinicians might select suboptimal antibiotics, potentially leading to treatment failure. Currently, methods for identifying *Achromobacter* species include biochemical testing (e.g., VITEK2), gene sequencing (such as *nrdA* gene encoding for ribonucleoside-diphosphate reductase 1 subunit alpha or 16S rRNA a ribosomal component found in all bacteria and archaea), multi-locus sequence typing (MLST), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Biochemical testing remains the most commonly used method of routine bacterial identification in clinical laboratories due to its speed and reliability [28]. However, for

Achromobacter spp., biochemical testing can sometimes result in inaccurate species identification due to similarities and, thus, difficult discrimination with other Gram-negative bacilli [23,28–31]. MALDI-TOF MS provides accurate genus-level identification but still faces challenges with *Achromobacter* species-level resolution due to the limited representation of species in databases [23]. Efforts to improve the accuracy of MALDI-TOF MS by expanding species databases have shown promise, but the results are still not satisfactory [31,32]. The limitations of these methods have already been highlighted both in clinical practice and in the literature: as shown by several studies, they can lead to false-positive and false-negative errors for *Achromobacter* spp. identification or to a limited species-level determination [33–35]. Sequence-based methods such as *nrdA* gene sequencing or MLST (which analyzes seven genes—*nusA*, *rpoB*, *eno*, *gltB*, *lepA*, *nuoL*, and *nrdA*—and is available via PubMLST) provide more accurate species identification [16,36]. However, nrdA gene sequencing requires bioinformatics tools for sequence analysis, while MLST, though simpler and faster than whole-genome sequencing (WGS), remains more costly and time-consuming than other molecular methods, such as qPCR. Although several methods and guidelines have been proposed to improve accurate identification, as previously described, differentiation of various species remains particularly challenging. This study aims to develop a reliable and accessible qPCR protocol for species-level identification of *Achromobacter* species using specific TaqMan probes (Eurofins Genomics, Ebersberg, Germany). This protocol could be widely applied in clinical diagnostic laboratories to support more informed decision-making in the management of CF patients.

2. Materials and Methods

2.1. Clinical Isolates

Fifty-two clinical isolates of *Achromobacter* spp. were collected from the sputum samples of patients at the CF Center of Verona, Italy. Informed consent was obtained according to projects CRCFC-CEPPO026 and CRCFC-CEPPO031, approved by the local Ethics Committee. The clinical isolates were recovered from twenty-six patients occasionally and chronically infected with *Achromobacter* spp. (according to the European Consensus Criteria or Leeds criteria); forty-one longitudinal isolates were collected from seventeen patients with chronic infections, while eleven strains were collected from nine patients with occasional infection. All strains isolated from each chronic patient were clonally related. Only one isolate was recovered from nine occasionally infected patients, while from two of these patients, we recovered two isolates; one of them harbored clonal isolates, while different clones were identified in the other one. A unique clonetype is considered to be the first isolate collected from a group of clonally related longitudinal isolates within the same patient, while subsequent isolates are considered to be clonally related variants of the original strain. The indications regarding the sampling timeframe are provided in Table S2. All the isolates were identified at the species level by whole-genome sequencing (WGS), followed by genome de novo assembly and phylogenetic analysis, as reported in our previous studies [14,37]. Strains were stored in Microbank (Pro-Lab Diagnostics, Neston, UK) at −80 °C.

2.2. Primers and Probes Design

Nucleotide sequences of 35 complete 16S ribosomal rRNA genes were retrieved from the de novo assemblies of the collection isolates, belonging to different species (*A. xylosoxidans*, *A. aegrifaciens*, *A. dolens*, *A. insolitus*, *and A. insuavis)* and two new genotypes (not phylogenetically related to any publicly available sequence) that we found in our collection. The 16S gene sequence from the *A. ruhlandii* reference genome (RefSeq accession: GCA_001051055.1) was also included in the dataset given the clinical rele-

vance of this species [14,37]. Multiple-sequence alignment was performed using Clustalw 2.1 [38], and probes and primers were designed for the identification of *A. xylosoxidans*, *A. dolens*, *A. insuavis*, and the two new genogroups (*Achromobacter* NG). Probes and primers were designed using the software Primer3Plus 3.3.0 with default parameters (available at https://www.primer3plus.com/index.html, accessed on 8 October 2022). Candidate probes and primers were manually examined and selected to optimize the number of reactions needed to obtain the identification results. The specific probes were chosen with a Tm 5–10 °C higher than the Tm of the primers and a length of fewer than 30 nucleotides. Two sets of primers covering two distinct regions of the 16S gene harboring single-nucleotide polymorphisms (SNPs) and six probes were selected for experimental validation (Table 1). All the probes were designed as non-extendible hybridization probes, with FAM as reporter at the 5′ end and TAMRA as quencher at the 3′ end. The results obtained by the different qPCR must be associated, creating an identification profile specific for each *Achromobacter* species analyzed (Table 2).

Table 1. Primers, probes, and species identification. NG: new genogroup.

Primers	Tm [°C]	GC Content [%]	Probes	Tm [°C]	GC Content [%]	Species
F1 TTGTAAAGCACTTTTGGCAG	53.2	40	Probe_A AGAAACGTCG(Y)GGGTTAATAC	58	45.2	*A. xylosoxidans* *A. dolens*
R1 CCAGTAATTCCGATTAACGC	55.3	45	Probe_B AGAAACGTCATGGGCTAATAC	58	43	*Achromobacter* NG
			Probe_C AGAAACGTCATGGGTTAATAC	58	38	*A. insuavis*
F2 CGGTGGATGATGTGGATTAA	55.3	45	Probe_1 AATGCCGAAGAGATTTGGCAGT	64	41	*A. xylosoxidans*
R2 GGACTTAACCCAACATCTCA	55.3	45	Probe_2 AATGCCGAAGAGATTTGGTAGT	60	41	*A. insuavis* *A. dolens*
			Probe_3 AATTCCGAAGAGATTTGGAAGT	60	36	*Achromobacter* NG

Table 2. *Achromobacter* species identification profiles.

Species		Identification	
A. xylosoxidans	Probe_A	Probe_1	
A. insuavis	Probe_C	Probe_2	
A. dolens	Probe_A	Probe_2	Probe_1 *
Achromobacter NG	Probe_B	Probe_3	

* Probe 1 is specific for *A. xylosoxidans*. A negative result is needed to confirm *A. dolens* identification.

2.3. DNA Extraction

Achromobacter spp. strains were isolated on Luria–Bertani (LB) agar (Merck, Darmstadt, Germany) and incubated at 37 °C for 48 h. We inoculated 1–2 of the obtained colonies in 5 mL of Brain Heart Infusion (BHI) culture medium (Merck, Darmstadt, Germany) and incubated them at 37 °C on a shaker for 16–18 h. A total of 0.5–1 mL of the bacterial suspension was centrifuged for 10 min at 5000× *g*. Genomic DNA was extracted within 1 h of collection using the QIAamp DNA Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. DNA was eluted in 100 µL of double-distilled water and stored at −20 °C. A NanoDrop 2000 UV/Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to evaluate DNA concentration and quality.

2.4. Real-Time PCR

The protocol used to analyze the different strains included a defined mixture of reagents (Table S1). Real-time PCR was carried out on a 7500 Fast Dx PCR system (Thermo Fisher Scientific, USA) with a first denaturation step at 95 °C for 2 min and 40 cycles of denaturation at 95 °C for 30 s; annealing and extension were performed in a single step at the same temperature at 65 °C for 30 s. All samples were tested in duplicate. The qPCR cut-off was redefined in each session, with manual setting of the threshold value as a function of the positive and negative control curves; in any case, the DeltaRn was between the values of 10^4 and 10^5.

3. Results

Fifty-two *Achromobacter* spp. clinical isolates were identified at the species level through WGS. The species distribution was as follows: 35 *A. xylosoxidans*, 4 *A. insuavis*, 4 *A. dolens*, 3 *A. aegrifacens*, 3 *A. insolitus*, and 3 *Achromobacter* NG [14,37]. Probes were tested on DNA extracted from these strains to assess analytical sensitivity and diagnostic sensitivity and specificity.

3.1. Analytical Sensitivity

For the analytical sensitivity test, the first qPCR was performed using a sequential 10-fold dilution of extracted DNA samples starting from 10^{-2} to 10^{-7} ng/µL for each probe. Furthermore, starting from the last 10-fold diluted concentration that can be detected using the qPCR protocol, we performed another qPCR experiment with 2-fold serial dilutions, from 2^{-1} to 2^{-4} g/µL. This was performed to determine the limit of detection (LOD) for each qPCR protocol, which ranged between 0.05 and 1 pg/µL. The lowest amount of DNA detectable for each probe is shown in Table 3.

Table 3. Limit of detention (LOD) of each probe.

Probes	LOD
Probe_A	0.275 pg/µL
Probe_B	0.1 pg/µL
Probe_C	1 pg/µL
Probe_1	0.05 pg/µL
Probe_2	0.05 pg/µL
Probe_3	0.05 pg/µL

3.2. Diagnostic Sensitivity and Specificity

Diagnostic sensitivity and specificity were assessed by testing each probe combination with all 52 different strains of our collection, and the identifications obtained by qPCR were compared with the actual WGS identification of the isolates (Table S2) [14,37].

The diagnostic sensitivity for each combination of the probes was assessed with the following Equation (1) [39]:

$$\text{Diagnostic sensitivity} = [\text{True positive (TP)}/(\text{TP} + \text{False negative (FN)})] \times 100\%, \quad (1)$$

Sensitivity was 100% for all combinations of the probes (Table 4).

The diagnostic specificity for each probe was assessed with the following Equation (2) [39]:

$$\text{Diagnostic specificity} = [\text{True negative (TN)}/(\text{TN} + \text{False positive (FP)})] \times 100\%, \quad (2)$$

Specificity ranged between 97.95% and 100%. The results obtained for each species are shown in Table 5.

Table 4. Results of diagnostic sensitivity for each combination of probes.

Species	Identification	Diagnostic Sensitivity	TP	FN
A. xylosoxidans	Probe_A + Probe_1	100%	35	0
A. insuavis	Probe_C + Probe_2	100%	4	0
A. dolens	Probe_A + Probe_2 + Probe_1	100%	4	0
Achromobacter NG	Probe_B + Probe_3	100%	3	0

Table 5. Results of diagnostic specificity for each combination of probes.

Species	Identification	Diagnostic Specificity	TN	FP
A. xylosoxidans	Probe_A + Probe_1	100%	17	0
A. insuavis	Probe_C + Probe_2	100%	48	0
A. dolens	Probe_A + Probe_2 + Probe_1	100%	48	0
Achromobacter NG	Probe_B + Probe_3	97.95%	48	1

The sensitivity and specificity calculated on the unique clonetypes (17 *A. xylosoxidans*, 2 *A. aegrifacens*, 1 *A. dolens*, 3 *A. insolitus*, 2 *A. insuavis*, and 2 *Achromobacter* NG) were 100% for all combinations of probes.

4. Discussion

In this study, a new qPCR-based method for species-specific identification of *Achromobacter* spp. was successfully developed, demonstrating 100% sensitivity and 97.95–100% specificity. By targeting SNP regions of the 16S rRNA gene, this approach addresses the persistent challenge of accurately identifying species within the genus—a critical issue given their distinct clinical relevance in people with CF. Compared with the existing literature, our molecular assay reveals advantages over previously described methods, highlighting its diagnostic potential.

Traditional biochemical testing methods, such as VITEK 2, are limited by the significant overlap of biochemical properties among *Achromobacter* species, often leading to misidentification [23,29]. While MALDI-TOF MS has been reported to improve genus-level identification [31,32], species-level resolution remains inconsistent due to limited representation in proteomic reference databases. Efforts to expand these databases have shown promise but remain constrained by the availability of comprehensive protein spectra for all species [20]. 16S rRNA sequencing is also time-consuming and laborious. In contrast, by focusing on species-specific SNPs, our qPCR assay overcomes these limitations and provides precise species differentiation, including identifying novel genotypes that were isolated in our CF center.

Our study leverages whole-genome sequencing (WGS) data for probe design, aligning with the approach taken in multi-locus sequence typing (MLST), as both methods utilize specific genetic markers to differentiate species based on their unique nucleotide sequences [40]. However, MLST is labor-intensive, expensive, and less feasible for routine clinical use, whereas our qPCR assay is simple, rapid, and cost-effective, making it more practical for diagnostic laboratories.

The qPCR method requires approximately one working day, including 3–4 h for bacterial DNA extraction and 40 min for the qPCR run, providing a direct and rapid result. In contrast, sequencing-based methods, while highly accurate, require time-consuming bioinformatic post-analysis. For instance, WGS involves multiple steps, such as sample preparation, library construction, and sequencing, with a total processing time of 3–5 days [41,42]. Similarly, MLST, although simpler and faster than WGS, remains costly and labor-intensive compared to other molecular techniques. On the other hand, the

MALDI-TOF MS system enables extremely fast analysis (15–30 min) [43–45]. However, its ability to discriminate at the species level remains limited. Finally, the Vitek2 system, which involves sample preparation and analysis, takes significantly longer than other methods, requiring between 20 and 30 h [46–48]. Among these techniques, qPCR offers an optimal balance between speed, cost, and accuracy. It provides a direct result within hours, avoiding the lengthy processing times of sequencing methods while ensuring greater precision compared to MALDI-TOF. This makes qPCR a highly efficient choice for *Achromobacter* spp. identification in routine diagnostics (Table S3).

The diagnostic sensitivity of our assay (100%) is comparable to or exceeds that reported in studies using advanced sequence-based techniques. For example, Papalia et al. reported diagnostic inconsistencies using MALDI-TOF MS when identifying less common species like *A. dolens* [32], which our assay successfully differentiated. Furthermore, our ability to identify novel genotypes not phylogenetically related to any publicly available sequences suggests a broader applicability and adaptability to evolving genetic diversity within the genus.

Several studies have noted the difficulty of achieving both high specificity and inclusiveness in diagnostic assays for *Achromobacter* spp. [23,36]. Our qPCR assay demonstrated 97.95% specificity for *Achromobacter* NG; although this does not considerably impact the diagnostic accuracy, further probe refinement may enable the elimination of potential cross-reactivity with closely related species. These findings are in agreement with those of Fernández-Olmos et al. [29], who emphasized the importance of iterative optimization in diagnostic tool development.

Our findings align with recent studies highlighting the clinical importance of distinguishing *Achromobacter* species due to their varying virulence and antibiotic resistance profiles [13,25]. For instance, *A. xylosoxidans*, the most prevalent species in CF patients, has been strongly associated with chronic infections and significant respiratory decline [9,10]. Similarly, *A. dolens* and *A. insuavis* exhibit distinct geographical prevalence and infection patterns, as observed in Denmark and France [16,18,19,49]. By enabling accurate identification, our assay supports personalized treatment strategies tailored to the specific pathogen, aligning with the recommendations of Gabrielaite et al. for individualized CF management and Coward et al. for improved diagnostic precision in CF care [18].

We are aware, however, that this study has some limitations. First, the assay currently targets only a subset of the 22 known *Achromobacter* species, focusing on *A. xylosoxidans*, *A. dolens*, and *A. insuavis*, among the most frequent species, and novel genotypes of interest at our CF center. Expanding the probe set to include additional species will be essential for broader applicability. Additionally, the specificity for *Achromobacter* NG (97.95%) suggests potential cross-reactivity with closely related non-target species, warranting further probe refinement. Another potential limitation is the possible cross-reactivity with closely related species, which could reduce the assay's specificity and potentially cause false-positive results. Despite our assay reaching very high sensitivity, we could only test a limited number of samples of *A. insuavis*, *A. dolens*, and *Achromobacter* NG due to the fact that *A. xylosoxidans* is the most prevalent species in CF.

In our study, we analyzed 52 isolates from 26 different patients. *Achromobacter* is often associated with chronic colonization in CF patients, so we considered it essential to validate the test under conditions where repeated sampling occurs; indeed, it is known that some genetic variation also occurs during chronic infection. In support of this, some previous studies on *Achromobacter* and other CF-related pathogens have included longitudinal isolates to assess the intra-patient variability and reliability of molecular identification methods over time [21,50]. To address potential concerns regarding data evaluation, we have also calculated sensitivity and specificity using only unique clonetypes, confirming

that the results remain consistent. However, we believe that intra-patient variability justifies the inclusion of longitudinal isolates, as it allows for a more comprehensive assessment of the assay's robustness in clinical setting.

Further testing on a higher number of samples belonging to these three species will be necessary for a full validation in clinical environment. Lastly, the assay's reliance on high-quality DNA may pose challenges in a clinical environment where sample quality can vary, potentially impacting sensitivity. To address this, optimizing the clinical DNA extraction process is crucial to enhance sample quality and ensure the reliable application of this protocol.

The innovation of our qPCR-based method, compared to other existing qPCR approaches, such as the duplex-real time PCR assay by Price et al., lies in its ability to distinguish a subset of major *Achromobacter* species, reducing the risk of misclassification—an essential feature for targeted treatment decisions [51]. Our assay enables precise species differentiation, including novel genotypes, which is particularly relevant in CF care, where different *Achromobacter* species exhibit varying pathogenicity and antibiotic resistance profiles.

In contrast, the assay developed by Price et al. offers a more practical solution in some clinical settings, as it allows for the simultaneous detection of *Achromobacter* spp. and *A. xylosoxidans* with a streamlined workflow [51]. Their method benefits from a highly conserved target region (rpoB gene), reducing the likelihood of false negatives and making it applicable even in cases where DNA quality is suboptimal. However, while their approach is well-suited for rapid genus- and species-level screening, it does not achieve full species resolution, which may limit its usefulness in clinical decision-making where precise identification is necessary.

Despite requiring high-quality DNA and facing potential cross-reactivity with closely related species, our method remains a valuable tool for improved clinical management. The ability to accurately identify *Achromobacter* species at a refined level enhances diagnostic precision and supports more tailored therapeutic strategies, making it a significant advancement in the molecular identification of this pathogen. Based on this work, future studies could expand the assay to include additional *Achromobacter* species and validate its performance across larger, geographically diverse datasets. Integrating this method into multiplex diagnostic panels for CF pathogens may further enhance its utility. Additionally, longitudinal studies examining the impact of accurate species identification on treatment outcomes and resistance management are encouraged. In conclusion, our study aims to address a gap in the diagnostic landscape of *Achromobacter* spp., providing a practical, precise, and adaptable tool for clinical use.

5. Conclusions

Accurate species-level identification is crucial for understanding the specific pathogenic potential of different *Achromobacter* spp., particularly given the varying degrees of virulence and antimicrobial resistance among different species. The qPCR protocol developed in this study represents a step forward in addressing the challenges faced by clinicians in diagnosing *Achromobacter* spp. infections quickly and accurately. With its high sensitivity and specificity, this method could be implemented in clinical diagnostic laboratories to improve the speed and accuracy of diagnosis, ultimately leading to better-targeted therapies for critical patients such as those with CF. This is especially important as the incidence of *Achromobacter* infections in CF patients continues to rise, and timely, accurate identification is essential for optimal treatment.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/pathogens14030271/s1, Table S1: Reagents and concentration for the qPCR protocol; Table S2: WGS identification and qPCR identification; Table S3: Comparison of time efficiency between our method and other identification methods.

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Abbreviations

The following abbreviations are used in this manuscript:

CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
SNPs	Single-nucleotide polymorphisms
MLST	Multi-locus sequence typing
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
WGS	Whole-genome sequencing
LOD	Limit of detection
NG	New genogroup
TP	True positive
FN	False negative
TN	True negative
FP	False positive

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Conclusion of the Thesis

This research has provided a comprehensive examination of various aspects of *Achromobacter* spp. respiratory infections in pwCF, focusing on the key areas of virulence, antimicrobial resistance, host immune response, and species identification. By investigating the behaviour of these bacteria through different models and methodologies, the study has not only expanded our understanding of *Achromobacter* spp. in the context of CF, but also contributed to the development of potential diagnostic and therapeutic strategies for managing these infections.

One of the critical aspects that this research highlighted is the demonstration that different pathogenic profiles exhibited by different *Achromobacter* spp. strains may influence the persistence and severity of infections in CF lungs. These findings challenge the traditional view of *Achromobacter* as a single, homogenous group of bacteria, suggesting that tailored approaches to treatment and management may be needed depending on the specific species and strain involved. Furthermore, the correlation between bacterial virulence and the host response in a CF mouse model has highlighted the importance of understanding the interplay between these pathogens and the host's immune defences. Strains with high virulence and cytotoxicity were associated with greater persistence and more severe outcomes in the host, underlining the importance of considering these factors when evaluating the risk of chronic infections.

The use of the zebrafish embryo model has proven to be an innovative and valuable approach in understanding the host-pathogen interactions of *Achromobacter* spp. The ability to observe bacterial persistence, innate immune response, and host mortality in a living organism, combined with the relatively low cost and simplicity of the model, offers a powerful platform for future research. This model not only provided insights into bacterial virulence and immune responses but also revealed the crucial role of macrophages in defending the host against *Achromobacter* spp. infections.

Another key contribution of this research lies in the development of a robust and reliable diagnostic tool for *Achromobacter* spp. identification. Accurate species-

level identification is crucial for understanding the specific pathogenic potential of different *Achromobacter* strains, particularly given the varying degrees of virulence and antimicrobial resistance among different species. The qPCR protocol developed in this study represents a step forward in addressing the challenges faced by clinicians in diagnosing *Achromobacter* spp. infections quickly and accurately. With its high sensitivity and specificity, this method could be implemented in clinical diagnostic laboratories to improve the speed and accuracy of diagnosis, ultimately leading to better-targeted therapies for pwCF. This is especially important as the incidence of *Achromobacter* infections in pwCF continues to rise, and timely, accurate identification is essential for optimal treatment.

Throughout this research, the integration of multiple experimental approaches—ranging from phenotypic characterization to in vivo models and advanced molecular techniques—has been crucial in providing a holistic view of *Achromobacter* spp. infections in CF. The findings suggest that understanding the genetic and phenotypic diversity of *Achromobacter* is vital for developing more effective clinical management strategies. Moreover, the research has underscored the need for personalized medicine in the treatment of chronic respiratory infections, as different strains may require different therapeutic approaches.

In conclusion, this PhD thesis has contributed significantly to the field of CF microbiology by improving our understanding of *Achromobacter* spp. infections. The findings not only enhance our knowledge of the pathogenesis and persistence mechanisms of these bacteria but also pave the way for more precise diagnostic tools and targeted therapies. Future research should focus on further elucidating the molecular mechanisms underlying *Achromobacter* virulence and resistance, as well as expanding the use of innovative model systems to explore potential therapeutic interventions. With these advances, it is hoped that the management of *Achromobacter* infections in pwCF can be improved, ultimately leading to better clinical outcomes and enhanced quality of life for these individuals.

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