Advancing preclinical research on *Mycobacterium abscessus*: Development of Biofilm and Animal Models

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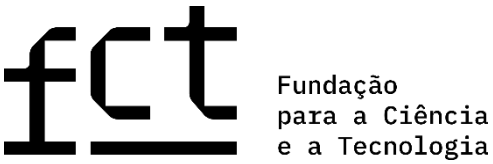
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Resumo

Mycobacterium abscessus (Mab) é um patogénio oportunista que pode causar infeções pulmonares crónicas em pacientes com patologias pulmonares subjacentes, particularmente fibrose cística (CF). Devido à sua resistência a múltiplos antibióticos, a taxa de sucesso dos tratamentos atuais é baixa. Mab também é capaz de formar biofilmes no muco e cavidades pulmonares de pacientes com CF, o que lhe providencia proteção adicional contra antibióticos. Deste modo, é urgente encontrar novos fármacos para combater estas infeções. Porém, para a maioria dos compostos testados contra Mab, a suscetibilidade *in vitro* não se correlaciona com a eficácia clínica. Para prevenir esta discrepância, o desenvolvimento de melhores modelos pré-clínicos é necessário. Assim, o objetivo deste trabalho é estabelecer dois modelos pré-clínicos para *screening* de fármacos contra Mab, usando uma estirpe luminescente e fluorescente. O primeiro modelo é um modelo *in vitro* que consiste num meio sintético de CF (SCFLM), que mimetiza o muco de pacientes com CF, e o segundo modelo é *Galleria mellonella,* um modelo invertebrado *in vivo*. Em relação ao modelo de SCFLM, investigamos o comportamento de Mab em duas versões deste meio que diferem em complexidade – SCFLM1 e SCFLM2. Mab cresceu de modo semelhante em SCFLM2 e 7H9, mas mais lentamente em SCFLM1. Para descobrir o motivo por trás destes resultados, determinamos o efeito do DNA e da mucina no crescimento de Mab. Concluímos que o DNA é parcialmente responsável pelo menor crescimento de Mab em SCFLM1. A mucina não tem impacto no crescimento de Mab, mas promove a formação de biofilmes. Por outro lado, a suscetibilidade de Mab à claritromicina, amicacina, moxifloxacina, imipenem e rifabutina está reduzida em SCFLM1 em comparação ao 7H9. Constatamos também que a mucina aumenta a resistência à amicacina, mas não a outros antibióticos. Surpreendentemente, porém, biofilmes formados em SCFLM1 foram mais suscetíveis a todos estes antibióticos do que biofilmes formados em 7H9. Quanto a *G. mellonella*, verificamos que Mab consegue replicar dentro das larvas, e que a mortalidade das larvas aumenta após infeção com doses altas de bactéria. Além disso, o tratamento com amicacina e meropenem levou a uma redução na carga bacteriana e a uma maior sobrevivência das larvas. Isto indica que este modelo pode ser extremamente útil para avaliar a eficácia de compostos através de uma análise de sobrevivência corroborada por técnicas como contagem de colónias e luminescência.

Palavras-chave: *Mycobacterium abscessus*, Fibrose Cística, biofilmes, mucina, *Galleria mellonella*, desenvolvimento de fármacos

Abstract

Mycobacterium abscessus (Mab) is an emerging opportunistic pathogen that can cause chronic lung infections in patients with underlying lung pathologies, particularly cystic fibrosis (CF). Due to its exquisite multidrug resistance, the treatment success rate is very low; additionally, Mab can form biofilms in the mucus and lung cavities of CF patients, which provide additional protection from antibiotics. Thus, it is imperative to find new drugs for combating these infections. However, for most compounds tested against Mab, *in vitro* susceptibility often does not correlate with clinical efficacy. To shorten this gap, the development of more complex preclinical models is necessary. Therefore, the aim of this work is to improve preclinical models for drug screening against Mab, using a luminescent and fluorescent Mab strain. The first improvement is the use of a synthetic CF lung medium (SCFLM), which mimics the mucus of patients with CF, for the *in vitro* growth of Mab. The second improvement is the use of *Galleria mellonella* larvae to establish an *in vivo* invertebrate infection model. For our SCFLM model, we began by investigating the behaviour of Mab in two variations of this medium – SCFLM1 and SCFLM2, the latter having a more complex composition. Mab grew similarly in SCFLM2 and 7H9 broth, but slower in SCFLM1. To further investigate the reason for these results, we assessed the effect of DNA and mucin on Mab's growth rate. We concluded that DNA is partially responsible for Mab's decreased growth rate in SCFLM1. Mucin had no impact on Mab's growth, but had an important role in promoting biofilm formation. Remarkably, we also found that Mab's susceptibility to clarithromycin, amikacin, moxifloxacin, imipenem and rifabutin was reduced in SCFLM1 compared to 7H9. Interestingly, we also observed that the presence of mucin in 7H9 decreased the effect of amikacin, but not of other antibiotics. Surprisingly, biofilms formed in SCFLM1 were more susceptible to all tested antibiotics than biofilms formed in 7H9. Regarding *G. mellonella*, we observed that Mab can replicate inside the larvae, and higher bacterial load leads to higher larval mortality. Furthermore, treatment with amikacin and meropenem resulted in a reduction of bacterial load and a significant improvement in larval survival. This suggests that this model may be highly valuable in evaluating a compound's effectiveness through survival assessment, corroborated by other techniques such as colony counting and luminescence.

Keywords: *Mycobacterium abscessus*, Cystic Fibrosis, Biofilms, mucin, *Galleria mellonella*, Drug development

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List of Abbreviations

ADC – Albumin-Dextrose-Catalase

AMR – Antimicrobial Resistance

ATP – Adenine triphosphate

c7H9 – Complete 7H9: 7H9 supplemented with 10% ADC, 0.2% glycerol and 0.05% of Tween80

CF – Cystic Fibrosis

CFTR – Cystic Fibrosis Trans-membrane Conductance Regulator

CFUs – Colony-forming Units

DTPA - Diethylenetriaminepentaacetic acid

ECM – Extracellular Matrix

eDNA – Extracellular DNA

EPS – Extracellular Polymeric Substances

GPLs – Glycopeptidolipids

LAM – Lipoarabinomannan

Mab - *Mycobacterium abscessus subsp. abscessus*

MABS – *Mycobacterium abscessus* Complex

MAC – *Mycobacterium avium* Complex

MIC – Minimal Inhibitory Concentration

Mtb – *Mycobacterium tuberculosis*

NTM – Non-tuberculous mycobacteria

OADC – Oleic Acid Albumin-Dextrose-Catalase

PRRs – Pattern Recognition Receptors

RGM – Rapid-growing mycobacteria

SCFLM – Synthetic Cystic Fibrosis Lung Media

SCFLM1* – Synthetic Cystic Fibrosis Lung Media 1 with DNA at 0.6 g/L

SGM – Slow-growing mycobacteria

Introduction

1. Non-tuberculous mycobacteria

Mycobacteria are part of the phylum Actinobacteria, and have a considerable diversity of species, with different lifestyles and a high phenotypical, biochemical and physiological variability (1). The cell wall of this bacterial genus is essentially composed of three layers, peptidoglycan with N-acetylglucosamine and N-acetyl-muramic acid, followed by the highly branched polysaccharide arabinogalactan, which connects peptidoglycan and the layer of mycolic acids. Mycolic acids, the main component of the mycobacterial cell wall, are long-chain fatty acids with 60 to 90 carbons. Together with the glycolipids phosphatidyl inositol mannosides, they confer these bacteria an exquisitely high lipid content, resulting in unique impermeability and hydrophobicity of the cell wall. Additionally, the lipoglycans, lipoarabinomannan (LAM) and lipomannan, are also characteristic of mycobacteria, playing an important role in pathogenicity (2). Additional important components of the mycobacterial cell wall are glycopeptidolipids, which can determine the colony morphotype and condition bacterial virulence (3). A schematic representation of the cell wall is presented in **Figure 1**.

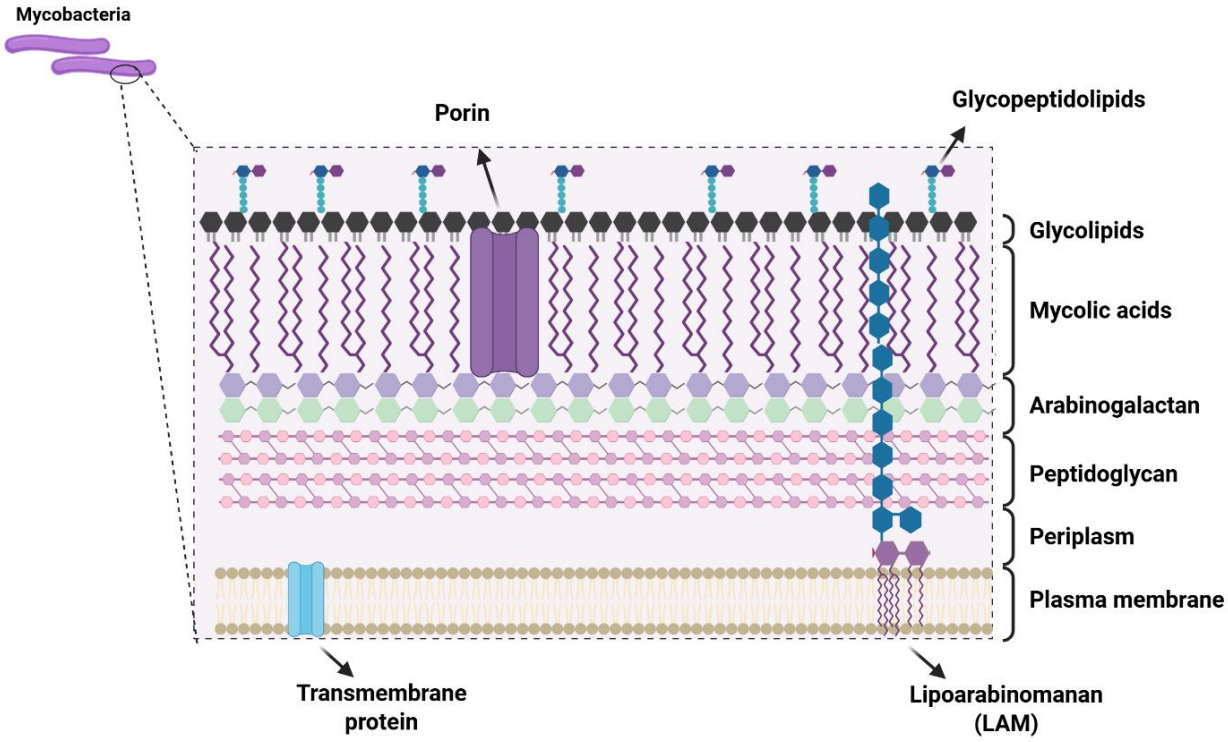
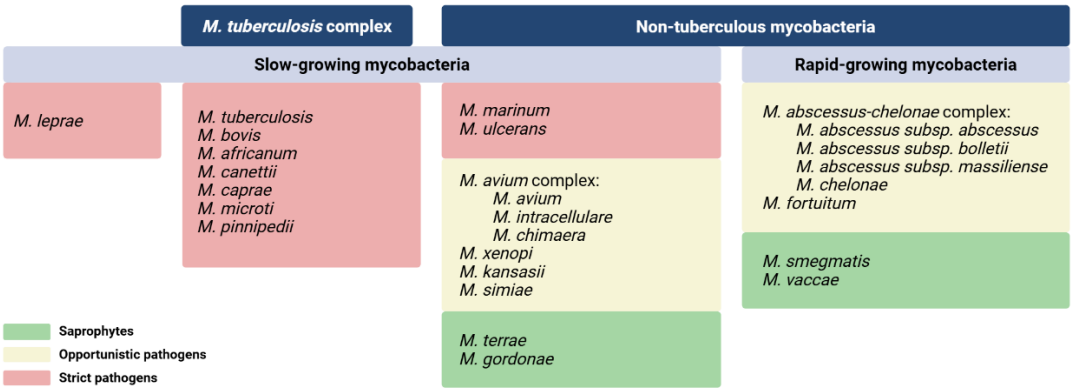


Figure 1 – Cell wall of mycobacteria. Schematic representation of its different components. Created with Biorender.

Mycobacteria species can be divided into three different categories: the *Mycobacterium tuberculosis* complex, *M. leprae* and non-tuberculous mycobacteria (NTM). The first two

groups contain strictly pathogenic mycobacteria, whilst NTM are either saprophytic (i.e., non-pathogenic), or strict or opportunistic pathogens in animals and humans. An overview of the different mycobacterial species and their pathogenicity is presented in **Table 1**. NTM are highly abundant in environmental settings like soil, water sources, residential plumbing systems (4), cattle (5), and even food products (6). They can be classified as rapid-growing mycobacteria (RGM) or slow-growing mycobacteria (SGM), depending on the time they take to form visible colonies in solid agar (3 to 7 days and more than 7 days, respectively).

Table 1 – Mycobacteria and their pathogenicity in animals and humans (adapted from Johansen MD *et al.* (7))



NTM infections are quite diverse and can affect various organs, but lung disease is the most prevalent, with *M. avium* complex (MAC), *M. abscessus* and *M. kansasii* causing the highest number of pulmonary infections among the different NTM species (8). Accordingly, in Portugal, the NTM species most frequently isolated from patients are MAC (40.8%), *M. gordonae* (17.8%), the *M. abscessus-chelonae* complex (9.6%) and *M. fortuitum* (6.3%) (9). Recently, species from the *M. abscessus-chelonae* complex have been gaining increased attention from researchers and healthcare professionals as its prevalence is increasing and it is the most difficult to treat NTM infection.

NTM infections primarily affect individuals with compromised immune systems and those with underlying lung conditions. Recently, however, concerns have been raised as the number of infections caused by these mycobacteria is increasing worldwide (10). This trend is mainly observed in developed countries, where the number of NTM infections may even surpass the number of new tuberculosis infections (11). This could be attributed to various reasons. Firstly, the ubiquitous nature of NTM leads to a greater

number of host-pathogen contacts. Secondly, in developed countries, certain host factors contribute to an increased risk of acquiring NTM infection: the increasing age of the global population (12), the high prevalence of lung conditions such as cystic fibrosis and bronchiectasis (13), immunosuppression (14) and broad-spectrum antibiotic therapy (15). Despite this, it has been reported that NTM can also infect immunocompetent patients with no history of previous health conditions (16). Another factor that can explain the increase in NTM disease prevalence is the improvement of diagnostic methods, and a greater awareness of the community regarding these bacteria.

NTM infections are still poorly understood, mainly due to a lack of investment, and many basic aspects of NTM are still not clarified. NTM disease is also very difficult to diagnose correctly – not only it is prone to be misdiagnosed as tuberculosis, but it is also difficult to say if they are the true cause of the pulmonary disease, or simply a commensal organism. The diagnosis for NTM infection is usually made by evaluating clinical symptoms; in the case of a pulmonary infection, these could be chronic cough, sputum, haemoptysis, fatigue and weight loss; complementary exams such as radiography, high-resolution computed tomography, sputum cultures, lung biopsy samples and histological analysis are required to further confirm NTM infection (17, 18). Despite growing awareness of these infections, misdiagnoses are still fairly common, and lead to an underestimation of the number of global NTM infections.

2. *M. abscessus* – an impending and nightmarish threat

M. abscessus (Mab) is an RGM and belongs to the *M. abscessus* complex (MABS), which includes three subspecies: *M. abscessus subsp. abscessus* (*M. abscessus,* Mab), *M. abscessus subsp. bolletii* (*M. bolletii*) and *M. abscessus subsp. massiliense* (*M. massiliense*). Mab can present itself as two different colony morphotypes – smooth variants (S), which possess surface-associated glycopeptidolipids (GPLs), and rough variants (R), in which GPLs are absent. Mab R colonies are irregular and dry, whilst S colonies are uniform, round and glossy (**Fig. 2**) (7).

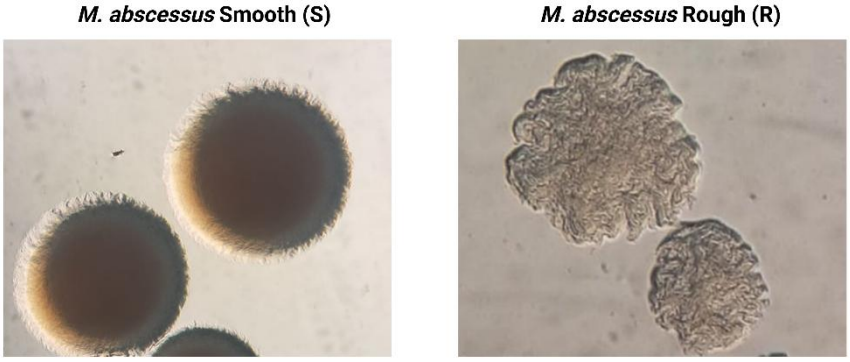


Figure 2 –Different morphotypes of *M. abscessus*. S colonies (left) are round and glossy, whilst R (right) colonies have irregular borders and seem dry. The images above are representative pictures from our laboratory strains.

2.1 The pathophysiology of *M. abscessus*

Mab shares many pathophysiological characteristics with its "cousin" *M. tuberculosis* (Mtb), the pathogen behind tuberculosis. Thus, the infection process has remarkable similarities (7, 19) (**Fig. 3**). The infection cycle begins after contact with the bacteria, which is made through potential environmental sources, such as shower-based aerosols, biofilms and fomites (20, 21). As for person-to-person transmission of Mab, conclusions regarding this possibility are still highly debated (22). Some studies used whole-genome sequencing to track down transmission of Mab between cystic fibrosis (CF) patients and concluded that, since genomic differences between bacteria isolated from different patients were so low and were confined to the same CF centres, it was very likely that they were transmitted from one person to another (23, 24). Additionally, transmission networks in CF patients were also shown to be important for Mab's global spread (25). Despite this, many consider that interhuman transmission of Mab is uncommon, and point to the limitations of using single-nucleotide polymorphisms and base-pairs to suggest this type of transmission, as was performed by the previous studies (26).

After exposure to the bacteria, colonization occurs, and immune cells such as macrophages and neutrophils are recruited to eliminate Mab. This immune response can constrain and destroy most NTM species; however, like other pathogenic mycobacteria, Mab can evade phagosomal defence mechanisms and remain intracellularly. This induces the production of inflammatory cytokines that recruit neighbouring immune cells to the site of infection, culminating in the formation of a granuloma. The granuloma consists of an aggregation of immune cells formed during certain diseases and is a hallmark of mycobacterial infections (7). Similarly to Mtb, Mab can persist in these granulomatous structures and give rise to the formation of caseous lesions (19).

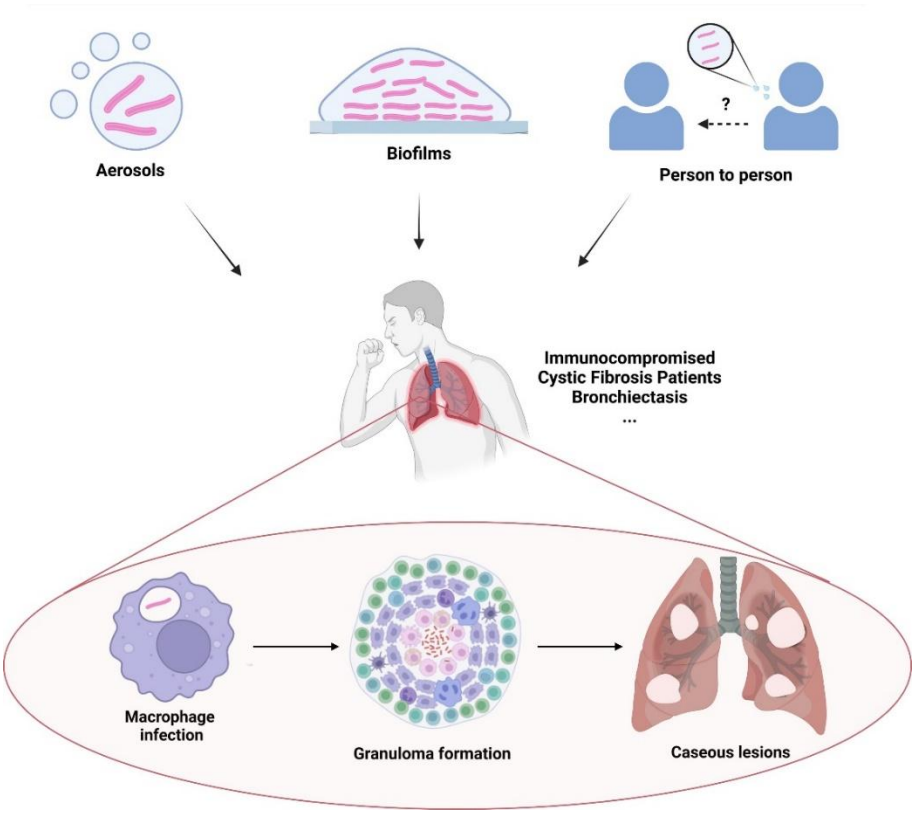


Figure 3 – Infection by *M. abscessus*. The bacteria are represented in pink and macrophages in purple. In the granuloma, macrophages are coloured in purple and pink, lymphocytes in green and light blue, multinucleated giant cells in dark blue, and neutrophils in dark purple. The image was created with Biorender.

During the infection process, the presence of genes involved in pathogenicity and virulence is essential and points to an adaptation of Mab to humans or animals (27). These genes encode for proteins responsible for host cell invasion and colonization, promoting resistance against nitrogen-derivatives and reactive oxygen species produced by macrophages (28, 29). With most RGM being saprophytic, Mab is a notable exception, harbouring the capacity to survive and multiply intracellularly, a characteristic usually observed in SGM. Alarmingly, some researchers believe that Mab is evolving rapidly towards becoming an obligatory lung pathogen due to mutations that enhance intramacrophage survival and acquisition of epigenetic modifiers through horizontal gene transfer (30).

Another peculiarity of Mab resides in the transition from the S to the R morphotype that can occur during infection. This transition is irreversible and occurs through single nucleotide polymorphisms and small insertions/deletions within the *gpl* cluster (27). The stimuli behind the change in morphotypes are still unknown, but the loss of GPLs results in a change of the bacteria's morphology and surface properties, which has several repercussions in pathology and virulence. This is showcased by the fact that the R phenotype is responsible for the most severe forms of disease, causing severe

pulmonary infections (31). Mab R morphotype is also more aggregative and forms cords in solid and liquid medium, unlike its S counterpart. Furthermore, the exposure of GPLs in S variants promotes sliding motility across agar and biofilm formation (7, 32). GPLs influence Mab interactions with the host and the environment, intracellular survival strategies, biofilm formation and sliding motility, which highly impacts clinical outcomes, progression of disease and immune response (7, 33). Interestingly, drug susceptibility and hydrophobic properties can also vary between morphotypes (34, 35).

2.2 Clinical relevance of *M. abscessus*

Besides macrophages, Mab can also use endothelial cells, antigen-presenting cells and fibroblasts as cell hosts (27). Due to this, the type of infections Mab causes is very wide. Mab has been associated with skin and soft tissue infections (36), chronic pulmonary infections (37), nosocomial and otolaryngological infections (38), central nervous system infections (39), urinary tract infections (40) and, in extreme scenarios, disseminated infections (41).

Pulmonary infections with Mab are the most difficult to treat of all NTM infections, with a treatment success rate of less than 50% (42). These lung infections are especially prevalent in patients suffering from CF, an autosomal recessive disease caused by a mutation in the cystic fibrosis trans-membrane conductance regulator (CFTR) gene. CFTR allows for the passage of chloride and water through mucus-producing cells, turning the mucus thin. When this protein is dysfunctional, the mucus becomes thick and sticky, obstructing the airways, and leading to an increased risk of contracting lung infections (43). Common CF pathogens include *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*, but it is becoming increasingly clear that these infections are usually polymicrobial (44). NTM prevalence in CF patients is increasing worldwide (45), with MAC and MABS causing the majority of infections (46). While MAC is most prevalent in the US and Australia, MABS is more commonly reported in Europe and tropical locations (47), and more frequently isolated from younger CF patients and those with more severe lung disease (45, 48).

Both in CF cohorts and in other patients suffering from Mab infection, a common problem emerges – resistance to antibiotics used in current treatments. The most recent guidelines for drug regimens against Mab infection suggest a macrolide-containing multidrug treatment with at least 3 active drugs guided by *in vitro* susceptibility testing (49). The continuation of treatment is recommended until patients are sputum culture-negative for one year, and for an additional 12 months after culture conversion (50).

Besides its long duration, aggressiveness, and low success rate, another problem with this treatment is that, excluding macrolides, the *in vitro* susceptibility of most drugs does not correlate with clinical outcomes (51, 52). As such, no evidence-based treatment exists to treat Mab infection.

Mab is resistant to first-line antituberculosis drugs like isoniazid, rifampicin, ethambutol, and pyrazinamide. It is also resistant to almost all classes of antibiotics, including β-lactams, aminoglycosides, cyclins, sulfonamides, macrolides, tetracyclines and rifamycins (7, 53). For this impressive antimicrobial resistance (AMR), Mab has earned the title of “incurable nightmare”.

Antimicrobial resistance (AMR) can be intrinsic, adaptative or acquired. Intrinsic AMR is referent to the innate ability of certain microorganisms to resist to a drug due to inherent structural or functional traits; adaptative AMR is the ability of a microorganism to rapidly alter gene or protein expression to respond to an antimicrobial; acquired AMR emerges when a sensitive microorganism turns resistant through the acquisition of new genetic material from other microorganisms by horizontal gene transfer or spontaneous mutations (54). For Mab, intrinsic AMR involves the low permeability of the cell wall towards hydrophilic compounds, different nucleotides variants in the *embB* and *gyrA* genes, conferring resistance to ethambutol and fluoroquinolones, antibiotic-modifying enzymes (β-lactamases against β-lactams, ADP-ribosyltransferases against rifampicin, acetyltransferases and phosphotransferases against aminoglycosides) and multidrug efflux pumps (7, 53). Mab’s adaptative AMR can arise upon exposure to macrolides, which induce the expression of *erm41*, a ribosome methylase that methylates the 23S ribosomal RNA (rRNA) and can confer resistance to clarithromycin and azithromycin (55, 56). Examples of acquired resistance are mutations in the *rrl* and *rrs* genes, which encode for 23S rRNA and the 16S rRNA, respectively. These mutations occur after prolonged use of macrolides and aminoglycosides, rendering them ineffective against these Mab strains (57, 58).

All these resistance mechanisms help to explain why Mab is extremely difficult to treat, and why finding a successful treatment regimen is still a challenge. Besides AMR, there are two additional factors that can contribute to the low success rate of current therapies against Mab. The first one is the potential existence of persister cells, defined as a subpopulation of bacteria that can tolerate lethal concentrations of antibiotics despite the lack of heritable drug resistance mechanisms. As a consequence, persisters can transform back into antibiotic-susceptible bacteria and, after the antibiotic pressure is removed, reconstitute a population that displays an antibiotic tolerance identical to that

of the starting culture (59). Persister cells are especially relevant in intracellular infections and in immunocompromised patients who are not able to eliminate all bacteria (60), which reiterates the importance of their study in the context of Mab infection. The second factor is biofilm formation, which will be explored in section 3.

2.3 Drug susceptibility testing against *M. abscessus* – methods and challenges

Mab's emergence as an opportunistic pathogen brings forward a tough clinical challenge, with the need for alternative drugs to combat Mab infection being undeniable. Importantly, the complexity of infection, and aspects that contribute to the persistence of Mab (such as biofilms and persisters) must also be acknowledged when it comes to designing an alternative treatment course.

To find and select new drugs against Mab, there is a need for optimized and improved preclinical models to test and assess the activity of certain antimicrobials. For Mab, the most common *in vitro* model used for drug screening is a 96-well plate with the typical 7H9 broth medium or other laboratory media; for *in vivo* studies, immunocompromised mice are the preferred option. Some studies have also used *Galleria mellonella*, *Drosophila melanogaster* and Zebrafish for drug discovery *in vivo* (61). However, current protocols for drug screening against Mab lack standardization, and often result in inconsistent findings, contributing to the discrepancy between *in vitro* drug susceptibility and clinical efficacy. Additionally, for *in vitro* drug evaluation, most models fail to portray the conditions bacteria face while infecting the host. This is especially relevant for mycobacteria (and, subsequently, Mab) as they present many intrinsic factors that complicate drug development. These factors are their highly hydrophobic and impermeable cell wall, their capacity to remain intracellularly in phagocytic cells, their slow growth, and their ability to enter a dormant state (62). Laboratory-wise, mycobacteria also pose many challenges, as they are prone to adhere and aggregate, and have several colony variations and different strains (62). All these factors have severe repercussions when it comes to drug susceptibility testing.

The gold-standard methods for drug susceptibility testing are dilution methods, either in broth or agar (63). Both these methods are used to discover the minimal inhibitory concentration (MIC) of a compound, i.e., the lowest concentration of an antimicrobial agent that inhibits the growth of bacteria.

Broth dilution involves diluting a certain compound in a liquid growth medium in tubes or 96-well plates. Afterwards, a microbial inoculum with a specific concentration is prepared

and added to each tube or well for further dilution. The MIC will correspond to the concentration at which no turbidity is observed (63). Despite its usefulness, this method can be labour and resource-consuming, and other disadvantages include the risk of errors in the preparation of the antimicrobial solutions, and the intra and interlaboratory variability associated with assessing turbidity with an unaided eye (63). To assess the bactericidal effect of a compound, the time-kill methodology is recommended (64). In this method, the inoculum is multiplied in order to see the rate of bacterial killing in different concentrations, with anti-bacterial activity being defined as a reduction of 3 $\log_{10}$ CFU/mL compared with the initial bacterial concentration.

The agar diffusion method relies on the principle that, when agar plates are inoculated with bacteria and paper discs containing the test compound are placed on the agar surface, the compound diffuses and creates an inhibition zone, where bacteria have their growth inhibited (63). The diameter of this zone can then be measured, which translates into the activity of the compound. However, for slow growing microbes, such as mycobacteria, this methodology is not recommended (64). This is due to the possibility that the inhibition zone may be smaller, as these microorganisms grow poorly, which complicates the interpretation of the results. Additionally, this method cannot determine the MIC value or distinguish between bacteriostatic or bactericidal effects of a compound (63, 64).

Despite their simplicity and accuracy, these methods are significantly time, resource and labour-consuming, and often cannot be applied in settings that translate the complexity of infection. To overcome these limitations, new alternatives have been developed. One of these alternatives is bioluminescence assays. These assays aim to quantify adenosine triphosphate (ATP) produced by living, active bacteria. In the presence of ATP, D-luciferin is converted to oxyluciferin by the enzyme luciferase in a reaction that generates light (63). The quantity of light can then be measured by a luminometer, and correlates linearly with cell viability. This technique has a wide range of applications, from antibacterial drug screening (65) to biofilm studies (66). Bioluminescence can be used in a fast and efficient way in complex infection models, such as macrophages, biofilms and animals, which allows for a more rapid and sophisticated drug screening. Importantly, in *in vivo* settings, it has the particular advantage of avoiding animal sacrifice, especially in the case of mice (67). For mycobacteria, the main advantage of bioluminescent assays is their rapidity. Conventional dilution methods entail long incubation periods, especially for SGM. With luminescence, it is possible to avoid these periods and obtain results in a shorter timeframe (3-4 weeks compared to 3-5 days in the case of Mtb (65)). For Mab, bioluminescent assays have been used for high and medium-throughput drug

screenings (68, 69), assessment of the efficiency of new drugs (70, 71), for *in vivo* drug testing with *Galleria mellonella* (72) as well as measurement of anti-biofilm activity of novel compounds in mice (73). This showcases the wide versatility and applicability of this technique for drug development against Mab.

3 *M. abscessus* and biofilms

Biofilms are defined as biostructures adherent to surfaces and composed of various extracellular polymeric substances (EPS). EPS form an extracellular matrix (ECM), which protects bacteria from environmental stress, and in the context of infections, from antibiotics and the host's immune system (74). These structures are communities of bacteria and consist of an alternative lifestyle that protects them from extreme circumstances, like depletion of nutrients, temperature, pH and antimicrobials (75). The process of biofilm formation can be divided into 5 different stages: (i) reversible attachment of the bacteria to a surface; (ii) irreversible attachment; (iii) EPS production; (iv) biofilm maturation; (v) dispersal/detachment phase (76) (**Fig. 4**). The self-produced EPS in the ECM usually consist of polysaccharides, proteins, glycopeptides, lipids, lipopolysaccharides, and extracellular DNA (eDNA) (77). Biofilms are often associated with chronic infections, not only because they protect bacteria from antibiotics, but also because they harbour persister cells that lead to a relapse of infection after treatment is completed (78). As such, biofilms have emerged as appealing therapeutic targets, with various studies covering antibiofilm agents such as antimicrobial peptides, bacteriophages, enzymes, phytochemicals, synthetic molecules and nanoparticles (74).

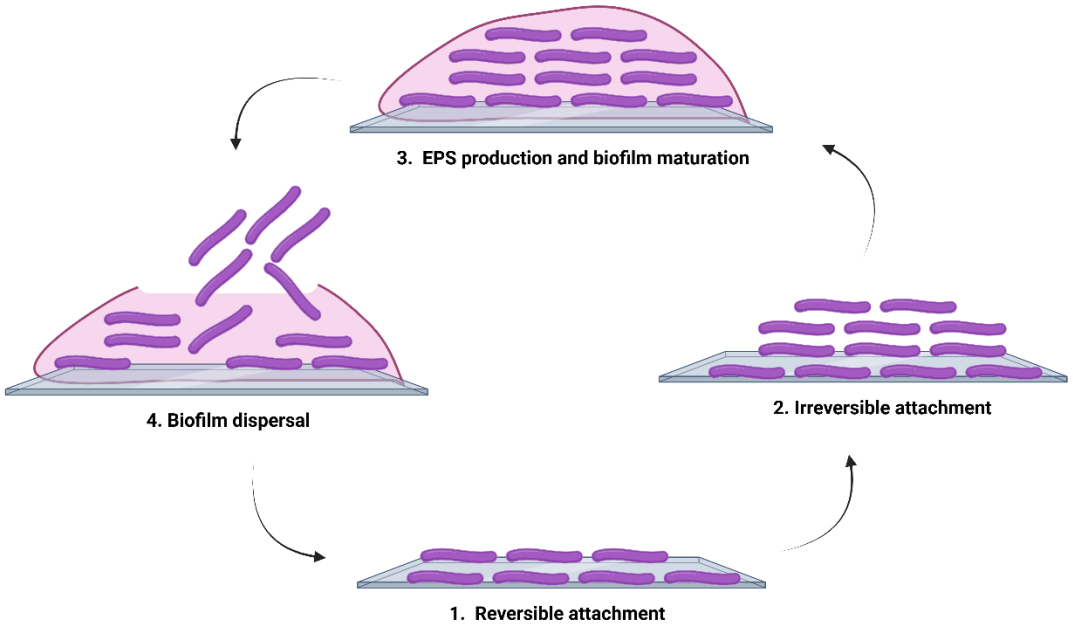


Figure 4 – Biofilm formation cycle. In the first stage, bacteria adhere to a surface in a reversible way. After irreversible

attachment, they produce several EPS and form the ECM, culminating in biofilm maturation. The biofilm can then disrupt, resulting in the release of bacteria that can attach to another surface, recommencing the cycle. The image was created with Biorender.

Many species of the *Mycobacterium* genus are capable of forming biofilms. These include Mtb, Mab, *M. avium*, *M. fortuitum*, *M. smegmatis*, *M. kansasii*, *M. marinum* and *M. ulcerans* (79). NTM are often found residing inside biofilms in their usual environmental settings, and these reservoirs could be potential sources of infection in humans and animals (80). Recently, mycobacterial biofilms have gained increased attention as a research topic, which is attributed to some studies that proved mycobacteria form biofilms both in *in vitro* and *in vivo* settings. Namely, Qvist *et al.* (2015) reported the presence of Mab biofilms in the thickened alveolar walls of a patient with CF (81); subsequent studies registered the presence of Mab biofilms in the lung cavity (82) and in the mucus (83) of CF patients. In 2017, Basaraba *et al.* (2017) argued that extracellular Mtb grows as biofilms in necrotizing lesions (84); 4 years later, Chakraborty P. *et al.* (2021) detected the presence of Mtb biofilms in the lungs of mice, macaques and humans infected with tuberculosis (85). As such, mycobacterial infections have started to be described as biofilm-related diseases. Given all the advantages they offer bacteria, the presence of biofilms can help explain the chronic nature of NTM infections, as well as their drug recalcitrance and long treatment duration (79). Kolpen M. *et al.* (2020) demonstrated that by disrupting MABS biofilms, bacterial susceptibility to amikacin, clarithromycin, imipenem and other antibiotics was increased (86). Therefore, new strategies to inhibit biofilm formation are essential to improve and shorten current therapeutic regimens.

The ECM of Mab biofilms is composed of eDNA, mannose- and glucose-containing glycans, cellulose, phospholipids and proteins (85, 87). Similarly to other NTM, the development of Mab biofilms is tightly regulated by various genes and cell components, such as GPLs (88).

Despite their potential clinical relevance, mycobacterial biofilms are still severely understudied, and much is still to be understood. Additionally, it is undeniable that preclinical testing against biofilms could help in a more accurate selection of drugs against Mab. Mab biofilms can be grown in liquid cultures, in settings such as 96-well plates, test tubes, tissue culture flasks and chambered slides or flow cells (89). Biofilms can also be grown in solid medium, through the placement of an inoculated semi-permeable membrane on top of the agar (89). To perform biofilm quantification, several techniques are used, such as CFU determination, crystal violet assay, resazurin assay, confocal laser scanning microscopy, electron microscopy and several others (89).

Such a wide variety of models and techniques used in different studies can render inconsistent findings and complicate study comparisons. Furthermore, differences in experimental conditions such as culture medium, bacterial strain, incubation temperature and agitation can also severely influence results (89). Therefore, there is a need for a standardized method for Mab biofilms characterization and drug activity testing. Regarding quantification methods, another problem emerges: some protocols are not sophisticated enough to discover if a certain drug affects bacterial viability or biofilm structure itself. This is attributed in some degree to a lack of knowledge on the composition of the ECM of Mab biofilms, which results in a lack of specific markers. These markers are not only important to establish the anti-biofilm activity of a compound but can also be used as biomarkers in clinical settings. In this context, Calcofluor White, a stain that binds to cellulose, could be a potential marker for registering the presence of Mab biofilms (85).

Recently, more complex models such as synthetic cystic fibrosis lung media (SCFLM) (87, 90), 3D lung epithelium models (91) and organoids (92) have been developed for growth of Mab biofilms in environments with greater resemblance to the lung niche.

SCFLM is an approximation of the mucus present in the lungs of CF patients and was initially used to study *Pseudomonas aeruginosa* infections (93). The original composition of this medium (SCFLM1) consists of mucin, DNA, diethylenetriaminepentaacetic acid (DTPA), sodium and potassium chloride, egg yolk emulsion and all the 20 amino acids (93). It represents a middle ground between *in vitro* and *in vivo* settings, presenting a composition similar to what is found under physiologic conditions (e.g., mucin and amino acids), but lacking some key biomolecules of the lung environment, and the systemic interplay only obtained with *in vivo* models (94). To solve this issue, another variation of this medium emerged (SCFLM2), which supplements SCFLM1 with components that improve the approximation to the lung environment, such as N-acetylglucosamine, glucose, and biometals like iron and magnesium (94). Interestingly, C.J. Wiersma *et al.* (2020) demonstrated that genetic expression of Mab grown in SCFLM2 closely mimicked the genetic changes of Mab when present in actual CF mucus (95).

This model is of great value in terms of evaluating the activity of antimicrobials against Mab and its biofilms in an environment more similar to what is found in the lungs of patients with CF. The efficacy of certain compounds may differ from their activity in the 7H9 broth, the usual laboratory media used for drug testing against Mab and other NTM. In this context, Hunt-Serracin AC. *et al.* (2019) discovered that in SCFLM1, planktonic Mab cells were much more sensitive to antibiotics than when associated in biofilms,

whilst in a laboratory medium like 7H9, this tendency was not observed (90). Similarly, Belardinelli *et al.* (2021) reported a higher tolerance of Mab biofilms against conventional antimycobacterial antibiotics, when compared to planktonic bacteria in this medium (87). More recently, E. J. Baker *et al.* (2024) demonstrated that there is a loss of susceptibility to certain antimicrobials when Mab is incubated in this medium (96). In line with these observations, A. Leestemaker-Palmer *et al.* (2025) showed that the presence of mucin in 7H9 broth increased Mab's resistance against amikacin (97). Collectively, these studies seem to indicate that the antibiotic susceptibility of Mab is altered upon incubation in the SCFLM. Given that this model presents a greater approximation to the physiological conditions of bacteria during infection, it might better predict drug efficacy in CF patients with Mab infection.

The reasons why Mab appears more resistant to antibiotics in the SCFLM are still unknown. One possibility could be an altered growth rate for Mab in this medium when compared to the 7H9 broth. However, for Mab, conclusions on this topic are somewhat contradictory. While a study claims that Mab grows at a similar rate in both mediums (95), others report a slower growth rate in the SCFLM (90), and that the presence of mucin does indeed alter the progression of Mab's growth (97). Given that so few studies have covered this topic, further investigation is necessary to better understand if an altered growth rate could, in fact, lead to an increase in antimicrobial resistance.

4 *In vivo* models for *M. abscessus*

The last step for preclinical drug screening is testing hits previously identified *in vitro* in adequate animal models. This is referred to as the *in vivo* evaluation of a drug. Given the discrepancy between *in vitro* testing of compounds against Mab and their clinical outcomes, this *in vivo* evaluation is crucial for the process of discovering more effective drugs to be used in the treatment of Mab infections. Current animal models used for drug screening against Mab are *Galleria mellonella* (*G. mellonella*), *Drosophila melanogaster* (*D. melanogaster*), zebrafish (*Danio rerio*) and mice (*Mus musculus*). The advantages and disadvantages of their application are briefly summarized in **Table 2**. In this work, we will focus mainly on the *G. mellonella* model, given its advantages and recent use for the study of Mab infection.

Table 2 – Pros and cons of different *in vivo* models for Mab

Model	Advantages	Disadvantages
G. mellonella	• Convenient manipulation size • Easy acquisition • Low cost • Presence of innate immune system, humoral responses and granulomatous-like lesions after infection • Few ethical restrictions	• Lack of standardized procedures for acquisition, storage, maintenance and infection route of the larvae • Few mutant strains, microarrays and libraries of RNA interference • Lack of an adaptative immune system • Anatomical differences between larvae and humans
D. melanogaster	• Good accessibility • Low cost • Ease of use • Short life cycle • Great genetic tractability • Presence of an innate immune system • Few ethical restrictions	• Lack of an adaptative immune system • Anatomical differences between flies and humans • Incapacity to incubate at 37 ºC
Zebrafish	• Good insights for compound's tissue selectivity, toxicity and bioavailability • Ease of use • Low cost • Fewer ethical restraints compared to rodents • High number of animals • Small size, fast organogenesis rate • Transparency of the embryo	• Difficulty of drug testing in adult fish • Incapacity to distinguish between lack of permeability and lack of toxicity • Difficulty of imaging deeper tissues
Mouse	• Mammalian animal, with very high phylogenetic proximity to humans • Great physiological and pharmacological conservation between mice and humans • Allows for the determination of the best dosage and administration route of a compound • Very well-established	• Costly, difficult manipulation, time-consuming • Many ethical restrictions • Number of animals that can be used and drugs that can be tested is low • Immunocompetent mice resolve Mab infection

4.1 *Galleria mellonella*

G. mellonella, or the greater wax moth/honeycomb moth, is part of the family *Pyralidae* within the Lepidoptera order. It has a white opaque appearance and develops through four stages: egg, larva, pupa, and adult (98). The physiological characteristics of the larva phase make this organism a great choice for the study of fungi, particularly those with a dimorphic nature (99). However, *G. mellonella* has also emerged as a model to study bacterial pathogens and to perform antimicrobial screenings (100).

The several advantages this model offers (**Table 2**), in particular the fact that there are no requirements of specialized facilities and that larvae can be kept at 37ºC, makes *G. mellonella* a more attractive choice for the study of infectious diseases when compared to other invertebrates such as *D. melanogaster* and *Caenorhabditis elegans* (101).

Furthermore, the innate immune system of the larvae has a good resemblance to that of man, comprising various cellular and humoral components. Haemocytes, which are analogous to mammalian white blood cells, are responsible for the cellular response, performing functions such as phagocytosis, nodulation and encapsulation (102). Humoral responses occur when a pathogen's molecules are recognized by host pattern recognition receptors (PRRs), leading to activation of several signalling cascades and secretion of reactive oxygen/nitrogen species or hydrogen peroxide into the haemolymph (analogous to blood) (101). In the context of humoral responses, the recognition of particular components of the bacterial cell wall by the PRRs can result in the activation of the prophenoloxidase cascade, which leads to a deposition of melanin in damaged tissue and around the foreign body, in order to prevent dissemination of the infection. The occurrence of this process can be easily identified by the presence of grey or black spots in the larvae (**Fig. 5B**), and the amount of melanisation can be useful to monitor disease progression (**Fig. 5A**) (103). When a disseminated dark pigmentation is observed throughout the larvae's body, it indicates that the larvae are very close to death (**Fig. 5A**). Additionally, the expression of immune and antimicrobial peptides, as well as the formation of nodules was observed in *G. mellonella*, reinforcing the similitude to human's immune system (104, 105).

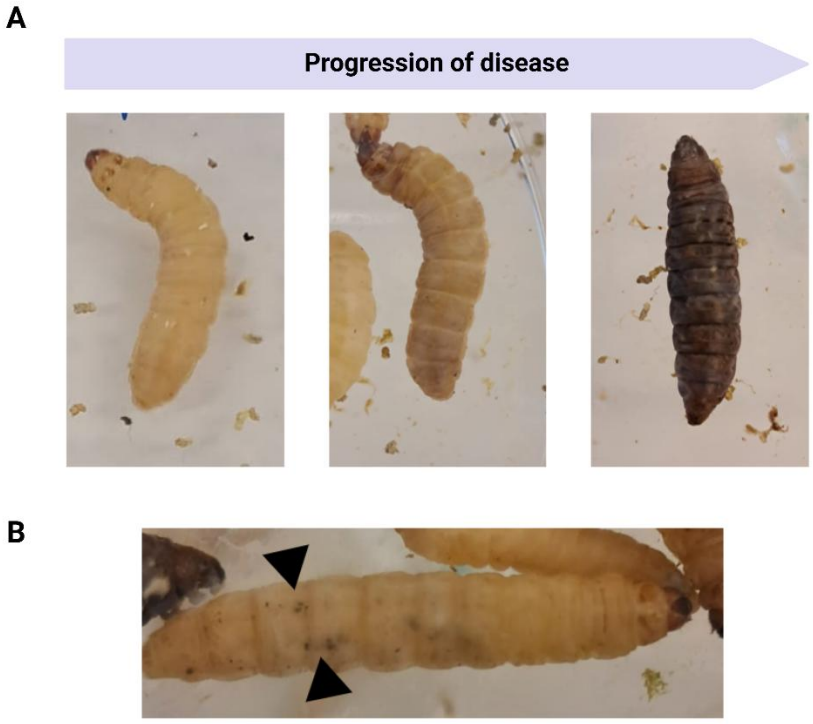


Figure 5 – *G. mellonella* melanisation as an immune response. (A) A higher content of melanin gives the larvae a darker colour; as melanisation is correspondent to an immune response, the darkening of *G. mellonella* allows for monitorization of the disease. (B) The presence of grey or dark spots (marked by the arrow) in larvae is indicative of foci of bacteria

and/or damaged tissue. The image was created with Biorender, and the pictures were taken from the larvae used in our studies.

The high costs, ethical restrictions and difficult manipulation associated with animal models used for drug discovery hinder the progression of many preclinical studies. The fast, easy use, low cost and availability of *G. mellonella* make this organism a potential candidate for a pre-screen of drugs with good *in vitro* activity. This is especially relevant for Mab infections, since current treatments are long, inefficient and there's a gap between *in vitro* susceptibility of compounds meant to target Mab and their clinical efficiency. Furthermore, testing in *G. mellonella* allows for the identification of non-toxic compounds at an earlier stage, resulting in a shortening of the drug development process, and reduction of mammals in research (101).

G. mellonella has been recently used to address both pathogenesis of mycobacterial infections, and drug efficacy against some mycobacteria, including Mtb (106) and Mab (72, 103). In this type of study, larvae are injected with bacteria and the infection can be monitored with different techniques, such as luminescence, CFU counting and histopathology. To assess drug efficacy, larvae mortality, CFU quantification and the bacterial luminescence signal are analysed (72, 107). In this context, Meir M. *et al.* (2018) (72) tested several antibiotics in larvae infected with Mab, namely meropenem, amikacin, tigecycline, azithromycin, levofloxacin, linezolid and piperacillin. Similar to what is observed clinically, treatments with meropenem and amikacin were the most effective. In a different study, Meir M. *et al.* (2018) (108) also used *G. mellonella* larvae to explore the effect of a joint therapy with avibactam and piperacillin against Mab. More recently, *G. mellonella* was used to validate the enzyme mycothione reductase as a potential drug target, where the knockout of the gene responsible for the expression of this enzyme inhibited Mab proliferation in the larvae (109).

Upon infection of *G. mellonella* with Mab, foci of bacteria can be found in the fat body, gut and haemolymph (103). In the latter, most of Mab bacilli are phagocytosed, whilst some remain extracellularly. However, the infection is not reduced, and instead an internal redistribution of bacteria occurs, probably due to Mab's slow growth rate (103). In the fat body of larvae infected with Mab, the presence of granuloma-like structures was registered by Meir M. *et al.* (2018) (72). Interestingly, this type of structure was also detected in *G. mellonella* infected with Mtb, as well as the internalization of Mtb bacilli by haemocytes (110), which indicates that the similarities between Mab and Mtb in regards to the process of infection are maintained in this model.

Despite the utility of *G. mellonella* model for the investigation of pathological aspects of Mab infection, as well as for drug screening, this model still has many limitations (**Table**

2). Particularly, the use of this model is relatively recent for bacterial infections, with very few studies regarding their infection with Mab. As such, and especially for this species, there is a severe lack of standardized procedures when it comes to larvae acquisition, storage and maintenance, as well as for the bacterial load necessary for infection. Furthermore, it is also important to note that pulmonary disease cannot be mimicked in the larvae, since they do not have a respiratory system comparable to mammals (103), and that the pharmacodynamic and pharmacokinetic properties of various compounds in *G. mellonella* are unknown and may vary in comparison to humans (101).

Aim

Drug discovery against Mab is consistently hampered by the failure to translate the *in vitro* efficiency of compounds into clinical success. This leads to treatments that are largely ineffective despite their long duration and aggressiveness. To surpass this obstacle, more refined and complex preclinical models are necessary to improve the identification of compounds with relevant activity against Mab and reduce the number of drugs tested in mice in later stages of drug research. The SCFLM has been described to mimic the mucus of patients with CF. Therefore, the use of this medium for drug susceptibility testing, instead of those commonly used to grow mycobacteria could help in the selection of new drugs against Mab, predicting with greater accuracy whether they will be effective *in vivo.* On the other hand, *G. mellonella* has been increasingly used as a low-cost *in vivo* model animal, with the additional advantages of presenting few ethical restraints and being easy to handle and acquire, especially in comparison to mice.

Given all this, this project aims to:

- Establish *in vitro* drug susceptibility assays based on SCFLM
- Establish *G. mellonella* as an *in vivo* model for medium-throughput screening of compounds against Mab

Materials and Methods

Bacteria

For this work, the following *M. abscessus* (Mab) strains were used: *M. abscessus* 19977 Smooth FF_mScarlet (Mab FF-sc) and *M. abscessus* 19977 Smooth operon_mScarlet (Mab op-sc) (107). Pre-cultures of these strains were grown at 37 ºC with agitation (110 rpm) in Middlebrook 7H9 broth (BD Difco™) supplemented with 10% (v/v) ADC (Albumin-Dextrose-Catalase), 0.2% glycerol (VWR) and 0.05% of Tween80 (Sigma Aldrich). When needed, the strains were grown in Middlebrook 7H10 (BD Difco™) agar supplemented with 10% (v/v) OADC (Oleic acid Albumin-Dextrose-Catalase) and 0.5% of glycerol (VWR).

Growth Media

- **7H9 with mucin and DNA**

Complete 7H9 broth (c7H9) was prepared as described in the section above, with 0.05% Tween80, unless stated otherwise. Afterwards, mucin from Porcine stomach Type II (Sigma-Aldrich or Merck Life Science) or DNA from salmon sperm (Merck Life Science) was added to the medium to achieve a concentration of 0.5 g/L or 5 g/L in the case of mucin, and 4 g/L in the case of DNA. When the mucin and DNA were fully dissolved, the medium was autoclaved at 121 ºC for 10 minutes and supplemented with 10% ADC.

- **Synthetic Cystic Fibrosis Lung Medium (SCFLM)**

The composition of SCFLM1 is represented in **Table 3**. SCFLM1 was prepared in house as described previously (93) by adding mucin, DNA, DTPA, NaCl, KCl, Tris Base, and Casamino Acids. When all components were thoroughly dissolved, pH was adjusted to 7.0 by adding HCl or NaOH 1 M. The medium was then sterilized in an autoclave at 110 ºC for 15 minutes. After cooling, 5 mL of egg yolk emulsion was added, and the medium was stored at 4 ºC for short-term use and at -20 ºC for long-term use.

The composition of SCFLM2 is represented in **Table 4**. SCFLM2 was prepared in house as described previously (111) by firstly dissolving mucin and DNA. Afterwards, this solution was autoclaved at 121 ºC for 10 minutes and added to a filtered buffered base solution supplemented with all 20 amino acids, NaH_2PO_4, Na_2HPO_4, KNO_3, K_2SO_4, NH_4Cl, KCl, NaCl and MOPS. Next, stock solutions of Dextrose, L-lactic acid, $CaCl_2*2H_2O$, $MgCl_2*6H_2O$, $FeSO_4*7H_2O$, N-acetylglucosamine and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were added to the medium**.** Lastly, the pH was adjusted to 6.8 by adding HCl or NaOH 1 M, and the medium was stored at 4 ºC for short-term use and at -20 ºC for long-term use.

Table 3 – Composition of SCFLM1

Reagent	Concentration (g/L)	Brand
Mucin from Porcine stomach Type II	5.0	Merck Life Science
DNA from salmon sperm	4.0	Merck Life Science
DTPA	0.0059	Merck Life Science
NaCl	5.0	Sigma Aldrich
KCl	2.2	Sigma Aldrich
Tris Base	1.8	ThermoFisher Scientific™
Casamino Acids	5.0	Gibco™ Difco™
Egg yolk emulsion	----	ThermoFisher Scientific™

Table 4 – Composition of SCFLM2

Reagent	Concentration (g/L)	Brand
Mucin from Porcine stomach Type II	5.0	Sigma-Aldrich
DNA from salmon sperm	0.60	Sigma-Aldrich
Tyrosine	0.15	Sigma-Aldrich
Asparagine	0.11	Sigma-Aldrich
Tryptophan	0.0026	Merck
Serine	0.15	Fluka
Glutamic acid * HCl	0.28	Merck
Proline	0.19	Fluka
Glycine	0.090	Sigma-Aldrich
Alanine	0.16	Fluka
Valine	0.13	Sigma-Aldrich
Methionine	0.094	Sigma-Aldrich
Isoleucine	0.15	Sigma-Aldrich
Leucine	0.21	Fluka
Ornithine * HCl	0.11	Sigma-Aldrich
Lysine * HCl	0.31	Fluka
Arginine * HCl	0.053	Sigma-Aldrich
Threonine	0.13	Sigma-Aldrich
Cysteine * HCl	0.025	Sigma-Aldrich
Phenylalanine	0.088	Merck
Histidine * HCl * H_2O	0.081	Sigma-Aldrich
NaH_2PO_4	0.18	Sigma-Aldrich
Na_2HPO_4	0.18	Sigma-Aldrich
KNO_3	0.035	Sigma-Aldrich
K_2SO_4	0.047	Merck
NH_4Cl	0.12	Sigma-Aldrich
KCl	1.1	EMSURE
NaCl	3.0	Fluka
MOPS	2.1	MP Biomedicals
Dextrose	0.54	Sigma-Aldrich

L-lactic acid	0.84	Sigma-Aldrich
$CaCl_2 * 2H_2O$	0.26	Merck
$MgCl_2 * 6H_2O$	0.12	Merck
$FeSO_4 * 7H_2O$	0.0010	Merck
N-acetylglucosamine	0.066	Sigma-Aldrich
1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)	1.0	Sigma-Aldrich

Growth Curve in planktonic conditions

Mab FF-sc was grown in c7H9, c7H9 with mucin (0.5 g/L or 5 g/L), c7H9 with salmon sperm DNA (4 g/L), SCFLM1 and SCFLM2, at 37 ºC, with agitation (110 rpm). At 24, 48, 72 and 96 hours, serial dilutions of the content of the cultures were performed in sterilized water with Tween80 and plated in 7H10 agar plates supplemented with 10% OADC and 0.5% glycerol. The plates were incubated at 37 ºC for 2 to 3 days for colony counting and colony-forming units (CFUs) estimation.

MIC assay

Mab op-sc was grown in c7H9 and the optical density at 600 nm (OD_{600}) was measured after 2 days of incubation. When OD_{600} was between 0.3 and 0.5 (exponential phase), bacteria were diluted in different media (depending on the experiment) to a concentration of $5x10^5$ CFU/mL, using a calibration curve. Afterwards, solutions of clarithromycin (Sigma-Aldrich), rifabutin (EDQM), amikacin (Merck), moxifloxacin (Sigma-Aldrich) and imipenem (Merck) with a concentration of 16 µg/mL were prepared in different media as well and 100 µL of each solution were added to a white flat bottom 96 well-plate (ThermoFischer Scientific™). The antibiotics were then diluted by 2-fold in the 96-well plate and 100 µL of the previously prepared bacterial suspension were added on top of each antibiotic solution. For non-treated controls, bacteria were added on top of media without any antibiotics. The plate was then sealed with parafilm and incubated at 37ºC in a humid environment without agitation. After 2 days of incubation, bacterial viability was assessed through luminescence measuring in a Synergy 2 plate reader (BioTek).

Biofilm formation

To form biofilms, it is necessary to remove any detergent in the growth media, and grow the bacteria for a longer incubation period, without any agitation. Thus, the protocols for growing of Mab biofilms were as follows:

- **96 well-plates**

Mab op-sc was grown in c7H9 until the exponential phase, and the OD_{600} was measured (between 0.2 to 0.7). Using a calibration curve, a new bacterial suspension with a concentration of $1x10^7$ CFU/mL was prepared either in c7H9 without Tween80 or SCFLM1. 100 µL was added to each well of a flat bottom white polystyrene 96-well plate. The plate was then sealed with parafilm and incubated at 37 ºC for 7 days in a humid environment without agitation.

After 7 days, the biofilms are fully formed, and treatments can be added. 100 µL of SCFLM1 or c7H9 without Tween80 with or without different antibiotics or Triton X-100 (Sigma-Aldrich) were added on top of the biofilms. Since the final volume was 200 µL, all solutions were prepared at double the desired concentration for each condition. The plates were sealed again with parafilm and incubated for 2 days at 37 °C. The antibiotics tested in the biofilms were the same antibiotics used in the MIC assays. Biofilm viability was assessed through luminescence measuring in a Synergy 2 plate reader.

- **24 well-plates**

Mab FF-sc and Mab op-sc were grown until the exponential phase, and a suspension of $1x10^7$ CFU/mL was prepared as described previously in different media: c7H9 without Tween80, SCFLM1, SCFLM2 and c7H9 with mucin (0.5 g/L and 5 g/L) without Tween80. Afterwards, 1 mL of the bacterial suspensions was added to each well of a transparent, polystyrene 24-well plate (SARSTEDT® or Greiner Bio One). The plate was sealed with parafilm and incubated at 37 ºC for 7 days in a humid environment without agitation.

Galleria mellonella

G. mellonella larvae were purchased from Terramania® (https://www.terramania.nl/) and kept at 4 ºC. For infection, Mab FF-sc was grown until the exponential phase, and the OD_{600} was measured to prepare bacterial suspensions with a calibration curve. Mab was washed with and diluted in PBS 1x, and 10 µL of each bacterial dilution was injected into the last left proleg of the larvae with a 31G needle Hamilton syringe. The larvae were then placed in a petri dish and left in an incubator at 37 ºC for a maximum of 12 days.

For treatment, larvae were injected with 10 µL of previously prepared solutions of amikacin (Sigma-Aldrich) (0.33, 3.3 and 33 mg/kg) and meropenem (TargetMol) (2, 20 and 200 mg/kg) in PBS 1x. The solutions were injected in the last right proleg of the larvae after 1 day of infection.

Luminescence measuring was performed by injecting 10 µL of a solution of D-luciferin (AAT Bioquest) at 0.05 µg/g, into the last right proleg of the larvae. Imaging of the larvae was conducted in IVIS® SPECTRUM (Calliper LifeSciences). For CFU counting, larvae were euthanized by freezing at -80 ºC for 30 minutes and placed in 5 mL tubes (Greiner Bio One) containing 1 mL PBS 1x. Afterwards, the larvae were crushed with a TissueRuptor (QIAGEN) and the resulting liquid was serially diluted in sterilized water with Tween80. Each dilution was then plated in 7H10 agar plates with 10% ADC, 0.5% glycerol, 2 µg/mL vancomycin (Sigma-Aldrich) and 8 µg/mL ceftazidime (EDQM), and incubated at 37 ºC for 2 to 3 days.

Statistical Analysis

In the MIC assays and biofilm experiments, the IC_{50} and IC_{99} (antibiotic concentration that inhibits by 50 and 99% the mycobacterial viability, respectively) values were interpolated by fitting the luminescent experimental data through a 4PL nonlinear sigmoidal curve using the software Prism 10® (GraphPad Software Inc.). Statistical analysis for the survival curves of *Galleria mellonella* was performed with the Logrank (Mantel-Cox) test with the software mentioned previously.

Results

1. Use of Synthetic Cystic Fibrosis Lung Medium for planktonic and biofilm drug testing against *M. abscessus*

Throughout this work, two types of Synthetic Cystic Fibrosis Lung Medium (SCFLM) were used – SCFLM1 and SCFLM2. The composition of each medium compared to c7H9 is summarized in **Figure 6.** SCFLM2 contains the same compositional backbone as SCFLM1, with mucin, DNA, NaCl, KCl and amino acids. However, it includes a wide range of additional components that increase its similitude to the lung environment and make it a more complex preclinical model (**Fig. 6**).

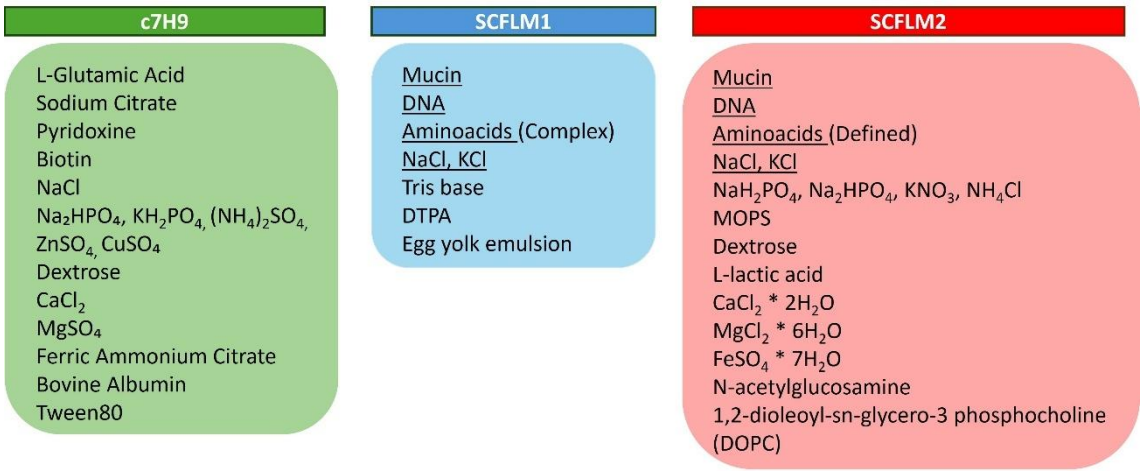


Figure 6 – Different components of c7H9, SCFLM1 and SCFLM2.

1.1. Characterization of SCFLM1 and SCFLM2

To understand if SCFLM1 and SCFLM2 can be adapted for drug screening against Mab, its behaviour upon incubation in these media in comparison to 7H9 broth (the standard laboratory medium for mycobacteria) was thoroughly studied.

Firstly, the planktonic growth rate of Mab in SCFLM1 and SCFLM2 was compared to that of c7H9. The growth curves of Mab FF-sc in these media are represented in **Figure 7**. There is a clear reduction in Mab's capacity to grow when incubated in SCFLM1 compared to c7H9 (**Fig. 7A**). Particularly, within the first 48 hours of incubation, in c7H9, we observe an increase of ~ 2 log, whereas in SCFLM1, this increase is less than 1 log. Interestingly, however, Mab FF-sc has a very similar growth rate both in c7H9 and in SCFLM2 (**Fig. 7A**).

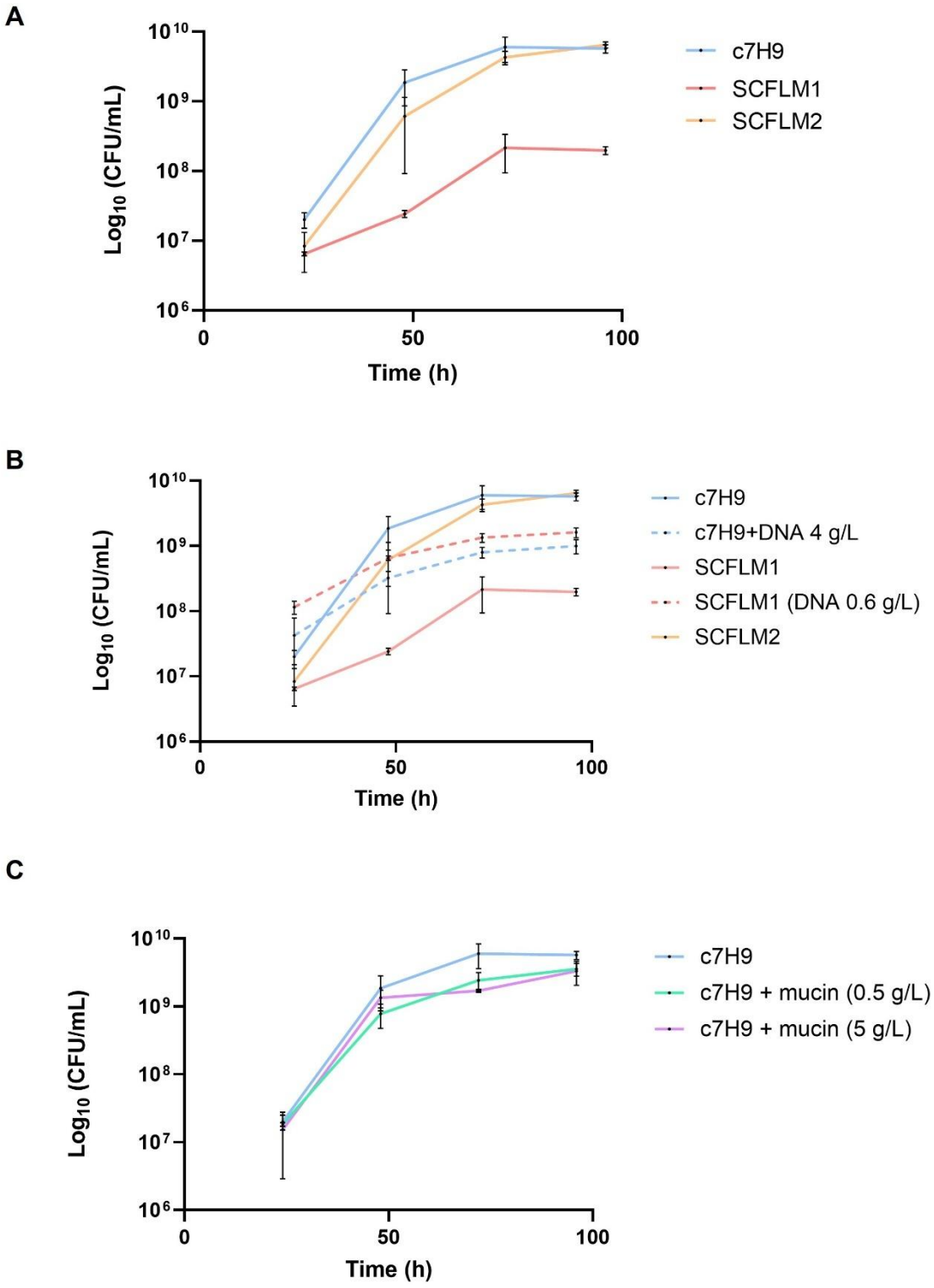


Figure 7 – *M. abscessus* growth is influenced by the composition of the medium. (**A**) Growth curves of Mab FF-sc in c7H9 (blue line), SCFLM1 (red line) and SCFLM2 (orange line). (**B**) Growth curves of Mab FF-sc in c7H9 (blue line), c7H9 supplemented with DNA at 4 g/L (dark blue line), SCFLM1 with a concentration of DNA of 0.6 g/L (dark red line), normal SCFLM1 (red line) and SCFLM2 (orange line). (**C**) Growth curves of Mab FF-sc in c7H9 (blue line), c7H9 supplemented with mucin at 0.5 g/L (green line) and 5 g/L (purple line). The liquid cultures were grown at 37ºC, 110 rpm, for 4 days. At each time point, samples were taken for CFU counting. The symbols represent the average ± standard deviation of 3 independent experiments.

One of the major differences between SCFLM1 and SCFLM2 is the concentration of DNA (4 g/L and 0.6 g/L, respectively) (**Table 3** and **Table 4**). To investigate the influence of DNA in Mab's growth, c7H9 was supplemented with DNA at 4 g/L. Surprisingly, the supplementation of c7H9 with DNA at this concentration resulted in a delayed growth compared to normal c7H9 (**Fig. 7B**). To further confirm if DNA could be influencing Mab's growth in SCFLM1, we reduced its concentration in this medium to 0.6 g/L and plotted the growth curve of Mab in this variation of SCFLM1. Strikingly, an increase in Mab's growth was observed, especially compared to normal SCFLM1, confirming that indeed, a high concentration of DNA hinders Mab's growth both in c7H9 and in SCFLM1.

Even though the concentration of mucin is the same in both SCFLM1 and SCFLM2, since it is the most abundant component in both media, its potential effect on Mab's growth was also assessed. To do so, c7H9 was supplemented with mucin at a concentration of 0.5 g/L and 5 g/L, the latter equal to the concentration of mucin in SCFLM. Remarkably, there was no significant difference between Mab's growth in c7H9 and c7H9 with both concentrations of mucin, proving that indeed this biomolecule does not affect the growth rate of Mab (**Fig. 7C**).

After determining the growth rate of Mab FF-sc in SCFLM1 in planktonic conditions, the biofilm formation capacity of this bacterium in SCFLM1 and SCFLM2 was assessed. To simultaneously determine the influence of mucin and DNA in biofilm formation, Mab biofilms were grown in 24-well plates in c7H9 without Tween80, c7H9 without Tween80 supplemented with mucin at 0.5 and 5 g/L, SCFLM1 and SCFLM1 with DNA at 0.6 g/L (**Figure 8A**). Surprisingly, biofilms appeared to be less developed in SCFLM1 – instead of a pellicle, only significant cording of the bacteria is observed. In SCFLM1 with DNA at 0.6 g/L, pointy aggregates of bacteria are observed, with no visible pellicle either. In c7H9, Mab also appears to form little biofilm. However, when supplemented with mucin at 0.5 g/L, we start to observe a faint pellicle, and in c7H9 with mucin at 5 g/L, this pellicle is quite developed. This suggests that mucin plays an important role in promoting the formation of Mab biofilms, especially at higher concentrations typical of CF patients. In this context, the absence of a mature biofilm in SCFLM1 can be attributed to Mab's decreased growth rate in this medium when compared to c7H9, and not to the interference of mucin or DNA.

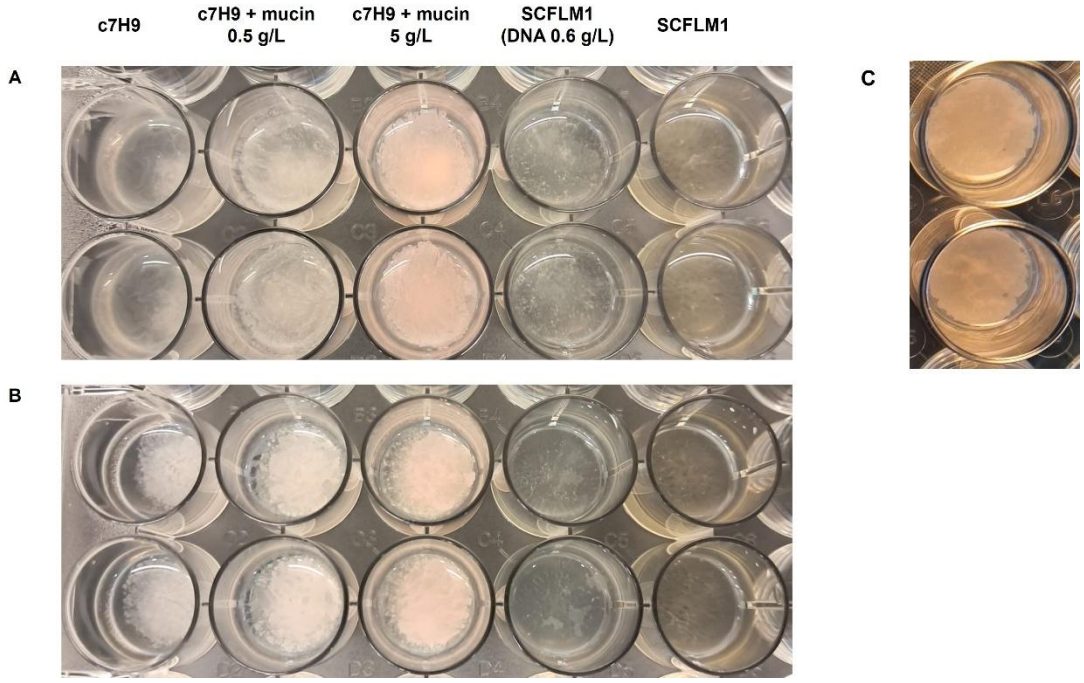


Figure 8 – *M. abscessus* capacity to form biofilms is influenced by different media. (**A**) Mab FF-sc and (**B**) Mab op-sc biofilms in different media after 6 days of incubation. (**C**) Mab FF-sc biofilms in SCFLM2 after 6 days of incubation.

In the context of drug screening, our strain Mab op-sc offers the advantage of being auto-luminescent, not needing the addition of the D-Luciferin substrate, in contrast to Mab FF-sc. Previously, it was established that the growth rates of Mab WT, Mab FF-sc and Mab op-sc in c7H9 medium and planktonic conditions are similar to each other (107). However, in terms of biofilm formation, the comparison between strains was not done. Thus, to perform that comparison, we plated Mab op-sc biofilms in 24-well plates and in all the media mentioned previously (**Figure 8B**). Mab op-sc biofilms were relatively similar to the ones formed by Mab FF-sc in all media. However, in this experiment, in SCFLM1 with DNA at 0.6 g/L, small fragments of a pellicle could be observed. Similarly to the Mab FF-sc results, mucin promoted biofilm formation, observed by the increasing size of the pellicles formed in the different types of c7H9. Interestingly, the pellicle formed in c7H9 and SCFLM1 is remarkably different. The SCFLM1 pellicle is more transparent and thinner, whilst in c7H9, the biofilm seems thicker, more developed, and less cohesive around the edges. This suggests that Mab produces biofilms with different phenotypes depending on the media it encounters. Given their differences in morphology, it is plausible to presume that the drug susceptibility profile of biofilms in SCFLM1 will also vary when compared to biofilms grown in c7H9. This could be of great interest for drug

research, given that biofilms formed in SCFLM1 are presumed to be more similar to Mab biofilms in the lungs of CF patients.

Besides the growth rate in planktonic conditions, Mab's biofilm formation capacity also differs significantly between SCFLM1 and SCFLM2. In this medium, at 6 days post-incubation, a full pellicle is already observed (**Fig. 8C**), in great contrast to Mab FF-sc biofilms in SCFLM1, where at this timepoint, no pellicle was observed (**Fig. 8A**). Indeed, it appears that the increase in complexity of this medium, and the presence of various components that are absent in SCFLM1 increases the capacity of Mab to form biofilms. Similarly to the conclusions drawn for biofilms in c7H9 and SCFLM1, it is possible that the drug susceptibility of these biofilms in SCFLM2 will differ significantly from the biofilms in SCFLM1.

1.2. Suitability of SCFLM1 for drug screening

Given the greater biofilm formation capacity and increased growth rate of Mab in SCFLM1 with DNA at a concentration of 0.6 g/L, for all further experiments, this variation of the medium was used and will henceforth be designated by SCFLM1*.

To verify if SCFLM1* is suitable to perform drug screening against Mab, a MIC assay in 96-well plates was performed with five conventional antimycobacterial antibiotics – clarithromycin, rifabutin, amikacin, moxifloxacin and imipenem. For comparison, a MIC assay in c7H9 was also performed using Mab op-sc. The results are represented in **Figure 9A,** and the IC_{50} and IC_{90} values for each antibiotic in each medium are represented in **Table 5**. Strikingly, Mab appeared to be much more resistant to all five antibiotics in SCFLM1* when compared to c7H9, given the clear increase of both IC_{50} and IC_{90} values in all antibiotics, and the dose-response curves plotted for each medium.

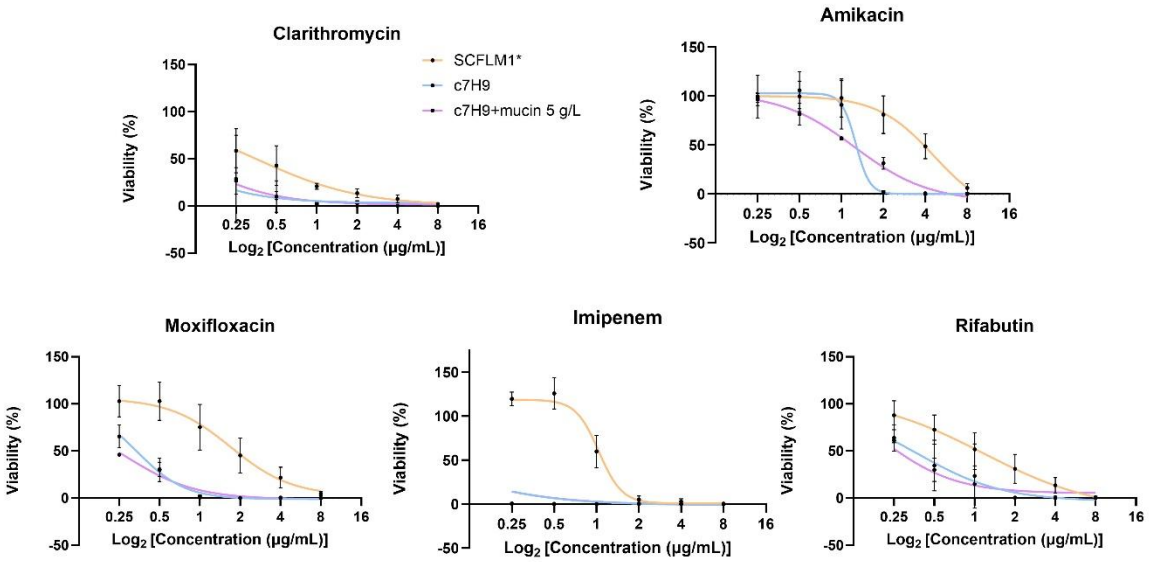


Figure 9 – Different media alter *M. abscessus* antibiotic susceptibility. Mab op-sc was incubated with increasing concentrations of clarithromycin, amikacin, moxifloxacin, imipenem, and rifabutin for 2 days, in either c7H9 (blue line), SCFLM1* (orange line) and c7H9 + mucin 5 g/L (purple line). The symbols represent the average ± standard deviations of three and two (in the case of c7H9 + mucin 5 g/L) independent experiments. Viability in percentage was calculated through luminescence quantification, normalized to the non-treated wells. The lines represent the nonlinear regression (4PL) obtained in GraphPad Prism 10 for each condition.

Table 5 – IC_{50} and IC_{90} values of Mab op-sc in c7H9, SCFLM1* and c7H9 supplemented with mucin 5 g/L

	c7H9		SCFLM1*		c7H9 + mucin 5 g/L	
	IC_{50} (µg/mL)	IC_{90} (µg/mL)	IC_{50} (µg/mL)	IC_{90} (µg/mL)	IC_{50} (µg/mL)	IC_{90} (µg/mL)
Clarithromycin	0.077	0.42	0.35	2.5	0.11	0.54
Rifabutin	0.35	1.5	1.1	4.7	0.26	1.4
Amikacin	1.3	1.6	3.9	7.4	1.2	3.4
Moxifloxacin	0.34	0.80	1.9	6.4	0.24	0.90
Imipenem	0.038	0.35	1.1	1.7	0.037	0.36

These results are extremely promising since they not only validate the use of this medium for drug screening against Mab in an environment more similar to the lung of CF patients but also point to a severe alteration of Mab's susceptibility to conventional antibiotics when the bacteria are growing in more clinically relevant settings. This data also helps shed some light on the great discrepancy observed between compounds' *in vitro* activity and their clinical efficacy against Mab infections.

One of the potential reasons for Mab's increased drug resistance in this medium could be the presence of a negatively charged molecule such as mucin, which may bind to some positively charged antibiotics, such as aminoglycosides, and decrease their efficiency. To explore this hypothesis, we performed another MIC assay with c7H9 supplemented with mucin at a concentration of 5 g/L. Overall, the antibiotic susceptibility profile of Mab in c7H9 and c7H9 with mucin is quite similar. There is a clear juxtaposition

of the graphs for both conditions, and the IC_{50} and IC_{90} values do not differ significantly from the ones Mab showcases in c7H9 (**Fig. 9** and **Table 5**). Nonetheless, a 3-fold increase is still observed in the IC_{90} value of amikacin. However, it is nowhere near the 7-fold increase observed in SCFLM1*, which strongly indicates that the presence of this biomolecule does not entirely explain the increase in Mab's drug resistance in this medium.

After observing this decrease in susceptibility of Mab in SCFLM1*, and to increase our insight on the conditions that modulate Mab's susceptibility to antibiotics, they were tested against Mab's biofilms in c7H9 and SCFLM1*. To test all the five antibiotics mentioned previously, Mab op-sc biofilms were grown in 96 well-plates. Treatment with clarithromycin, amikacin, rifabutin, moxifloxacin and imipenem was performed after 7 days of incubation, and the viability of the biofilms assessed after 2 days of treatment by measuring their luminescence signal. As a control for biofilm disruption, the detergent Triton X-100 at a concentration of 0.1% was used. The results of each treatment are represented in **Figure 10A,** and the IC_{50} and IC_{90} values for biofilm inhibition are listed in **Table 6**.

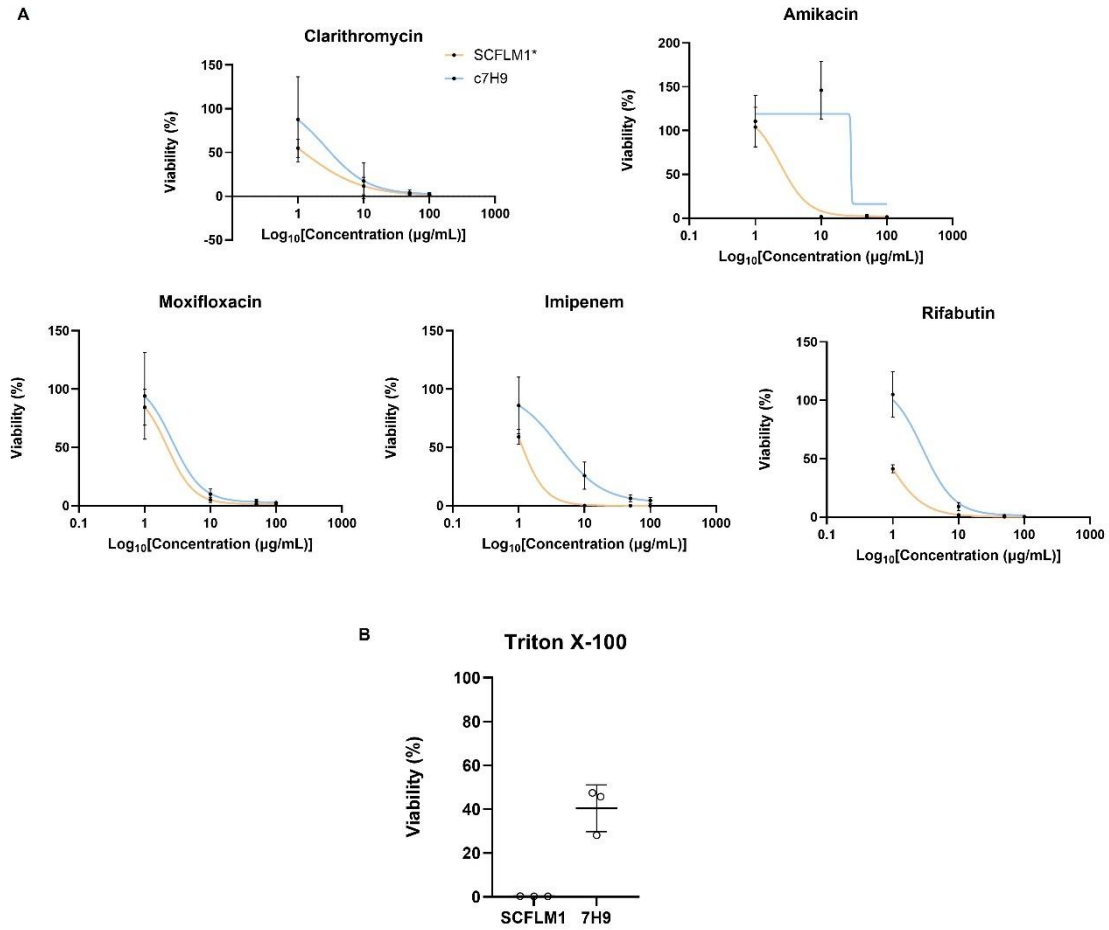


Figure 10 – Different media alter *M. abscessus* biofilms' antibiotic susceptibility. Mab op-sc biofilms were incubated with (**A**) increasing concentrations of clarithromycin, amikacin, moxifloxacin, imipenem, rifabutin, and (**B**) 0.1% Triton X-100, for 2 days, in either c7H9 without Tween80 (blue line) or SCFLM1* (orange line). Viability in percentage was calculated through luminescence quantification, normalized to the non-treated wells. The symbols represent the average ± standard deviations of three independent experiments. The lines represent the nonlinear regression (4PL) obtained in GraphPad Prism 10 for each condition.

Table 6 – IC_{50} and IC_{90} values of Mab op-sc biofilms in c7H9 without Tween80 and SCFLM1*

	c7H9		SCFLM1*	
	IC_{50} (µg/mL)	**IC_{90} (µg/mL)**	**IC_{50} (µg/mL)**	**IC_{90} (µg/mL)**
Clarithromycin	3.1	17	1.2	12
Rifabutin	3.4	11	0.8	3.2
Amikacin	29	<50	2.9	8.8
Moxifloxacin	2.9	10	2.2	6.6
Imipenem	4.3	28	1.2	3.0

Contrary to what was expected given our MIC determinations in planktonic cultures, biofilms grown in SCFLM1* were more susceptible to all the tested antibiotics (**Fig. 10A** and **Table 6**). One possible explanation for these results resides in an apparent decreased matrix production by Mab when incubated in SCFLM1*. In the 24 well-plates, it was clear that Mab biofilms produced at day 6 in SCFLM1* were less mature than the biofilms present in c7H9 (**Fig. 8B**). This can have severe repercussions in terms of drug susceptibility, since the matrix is known for protecting bacteria from antibiotics. A less mature biofilm would also explain why Triton X-100 provoked greater biofilm reduction in SCFLM1* than in c7H9 (**Fig. 10B**), and why IC_{50} and IC_{90} values of our biofilm experiments in this medium are not that different from the values obtained in the MIC assays, except for clarithromycin (**Table 5** and **Table 6**). However, it must be noted that these conclusions regarding matrix production are solely based on visual observations. By staining the matrix of the biofilms in each medium with a dye like Calcofluor White, which stains cellulose, a main component of the mycobacterial biofilm matrix (85), it would be possible to get an idea of how mature the biofilm produced truly is. In a separate experiment, we stained biofilms formed in SCFLM1* and c7H9 in 24-well plates with Calcofluor White and observed a signal in fluorescence microscopy for c7H9, but not for SCFLM1* (data not shown). This somewhat supports the explanation provided earlier and suggests that a longer incubation period may be necessary for a more mature biofilm to form in SCFLM1*. In this context and given the fact that Mab forms more mature biofilms in SCFLM2 (**Fig. 8C**), it would be more interesting to perform drug susceptibility testing against Mab biofilms in this medium than SCFLM1* or SCFLM1.

2. Use of the *Galleria mellonella* infection model to test anti-*M. abscessus* drugs

For the first step of optimization of the *G. mellonella in vivo* model, we decided to find the bacterial concentration that resulted in significant larval mortality. To do so, the larvae were infected with increasing concentrations of Mab FF-sc, incubated at 37ºC, and mortality was assessed daily during incubation. The larvae were considered dead when they showcased increased pigmentation (**Fig. 5**) and did not move upon stimulus. The status of the larvae and the resulting survival curve are represented in **Figure 11A**.

It is clear that a concentration of 10^7 CFU/larva results in significant death of the larvae upon a week of incubation. In the group infected with 10^6 CFU/larva, mortality was significantly less than in the aforementioned concentration, but still considerable. Surprisingly, when larvae were infected with 10^5 CFU/larva, the probability of survival was similar to the control group (PBS 1x), with no statistical significance between the two conditions. Additionally, these larvae exhibited a healthier complexion in comparison to the other infection groups (**Fig. 11A**). This could indicate that the immune system of *G. mellonella* is capable of dealing with relatively low concentrations of Mab, eliminating almost all or at least a significant part of the bacteria that enter upon infection. The enhanced mortality rate observed in the 10^7 CFU/larva group could, therefore, be explained by the inability of the immune system to eliminate such a high load of bacteria. This could potentially lead to a dysregulated immune response which results in greater larval death.

Given the balance between mortality and infection progression observed in the 10^6 CFU/larva group, we decided to infect the larvae with this concentration and delineate the progression of infection by CFU counting on days 2 and 5 post-infection. Interestingly, we noted an increase of about 1 log of bacteria between these two timepoints, meaning that Mab is indeed capable of replicating within *G. mellonella*, ensuring progression of infection during incubation (**Fig. 11B**).

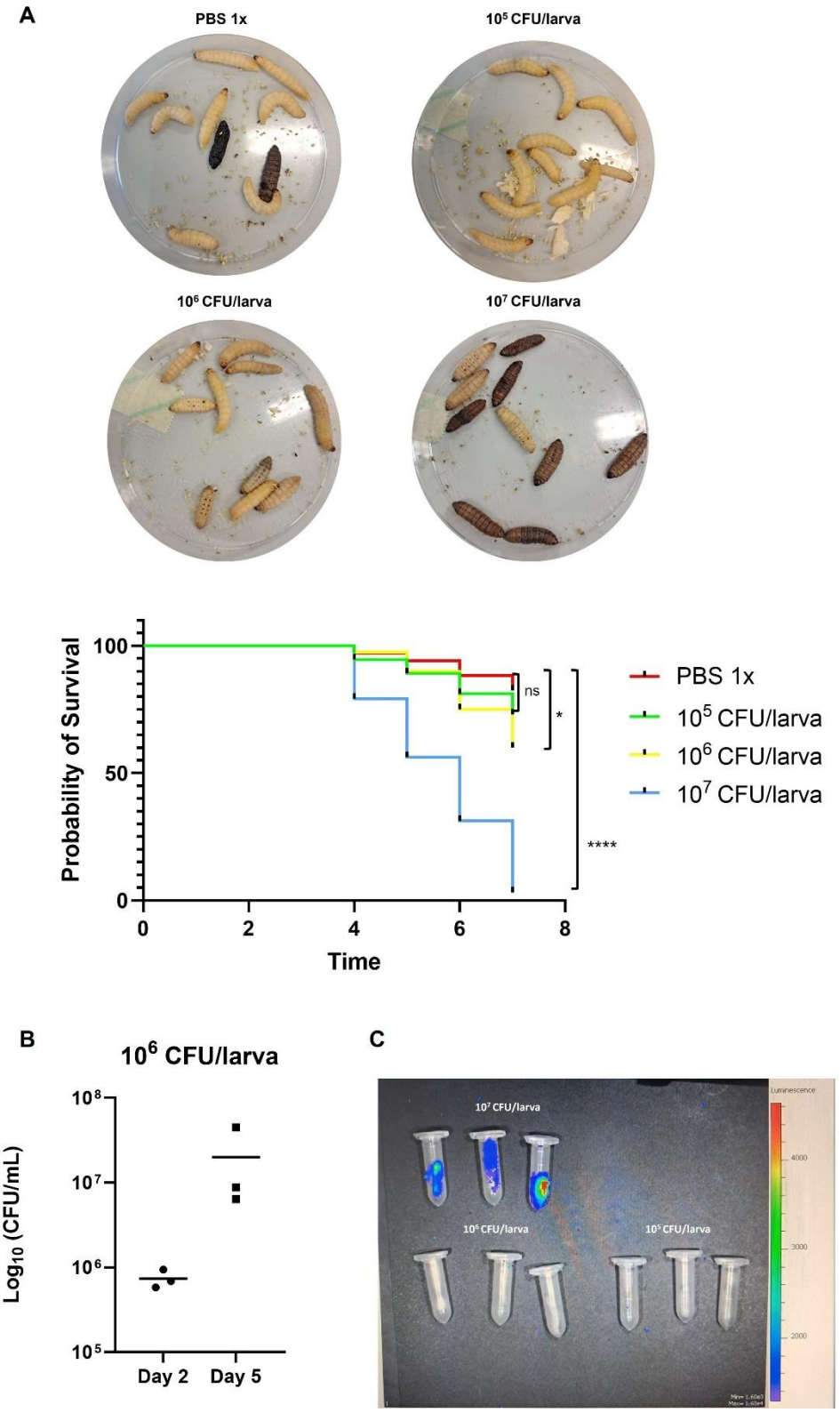


Figure 11 – Characterization of *G. mellonella* upon infection with *M. abscessus*. (**A**) Images of *G. mellonella* injected with the control (PBS 1x) and infected with Mab FF-sc at 10^5, 10^6 and 10^7 CFU/larva, at 6 days post-infection, and the corresponding Kaplan-Meier survival curve (n=30). Statistical analysis was performed with the Logrank (Mantel-Cox) test with GraphPad Prism 10: ns – not significant; * $p<0.05$; **** $p<0.0001$ (**B**) Mab FF-sc CFUs at day 2 and day 5 post-

infection (n=3). (**C**) Luminescence emitted by the larvae infected with 10^7, 10^6 and 10^5 CFU/larva, after 2 days of incubation, with the respective scale bar for luminescent signal intensity.

When performing CFU counting, the larvae are euthanized. A simpler and faster way to assess bacterial load and witness the progression of infection without sacrificing the larvae is through luminescence measuring. To measure the luminescence signal within the infected larvae, they were injected with D-Luciferin and imaged using an *in vivo* imaging system (IVIS, Revvity) (**Fig. 11C**). Disappointingly, a signal was observed in larvae infected with 10^7 CFU, but not in lower concentrations.

Finally, to see if this *in vivo* model can be applied to drug susceptibility testing, the larvae were infected with 10^6 CFU/larva and treated at 1 day post-infection with two conventional antimycobacterial antibiotics – amikacin and meropenem (**Fig. 12**). The highest dose of each antibiotic was chosen based on other *G. mellonella* studies (72) and is an approximation of two daily doses in humans. The efficacy of the different concentrations of these antibiotics was assessed by CFU counting at 3 days post-treatment. For both antibiotics, a dose-dependent response is observed (**Fig. 12A**). Interestingly, it appears that amikacin showcases a more powerful effect than meropenem, provoking a somewhat similar log reduction with a concentration approximately 6 times lower than that of meropenem.

Since only some of the larvae were sacrificed for CFU counting, the remaining ones were incubated until 5 days post-treatment for survival assessment (**Fig. 12B**). At this timepoint, the treated larvae showed enhanced survival when compared to the larvae that received no treatment at all. It is also worth noting that the survival rate correlated well with our CFU counting results and even allowed to better distinguish the differences between different concentrations of both antibiotics, particularly in the case of amikacin. These results indicate that compound efficiency can be easily determined through a simple survival assessment of treated and non-treated larvae after some days of incubation.

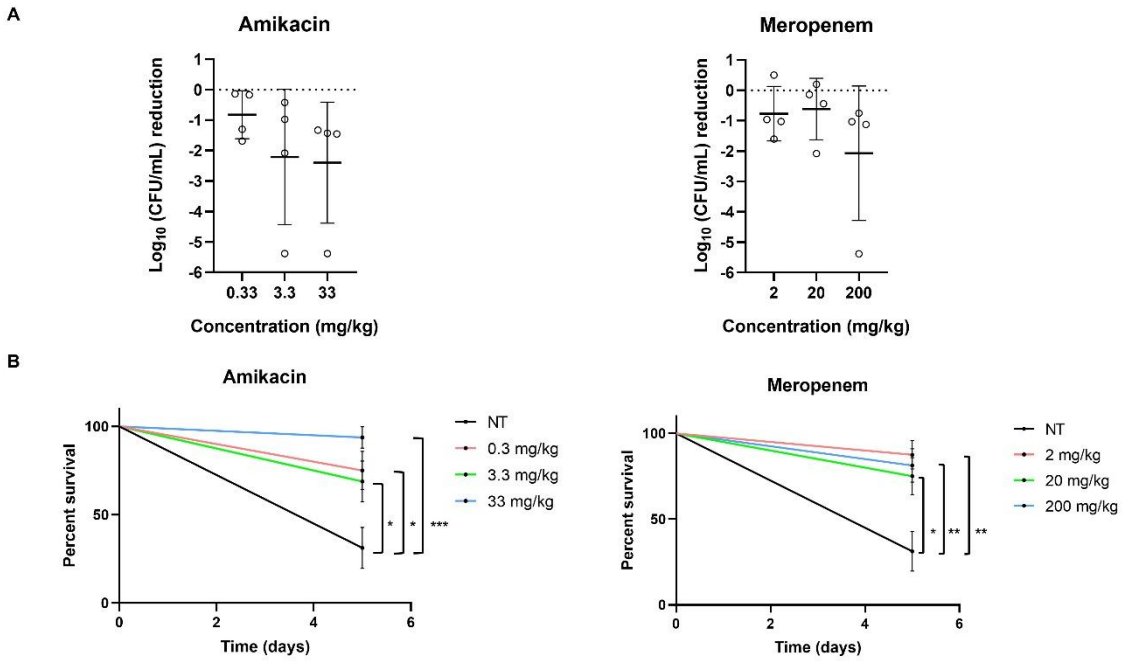


Figure 12 – Drug susceptibility testing on *G. mellonella.* (**A**) The larvae were treated with amikacin (0.33, 3.3 and 33 mg/kg) and meropenem (2, 20 and 200 mg/kg) for 3 days (n=4). The highest dose was chosen based on other *G. mellonella* studies and is an approximation of two daily doses in humans. (**B**) Kaplan-Meier survival curve for larvae treated with amikacin and meropenem in comparison to non-treated larvae (NT) (n=12). Statistical analysis was performed with the Logrank (Mantel-Cox) test with GraphPad Prism 10: * p<0.05; ** p<0.01; *** p<0.001.

Discussion

Mab's emergence as an opportunistic pathogen brings forward a tough clinical challenge, only comparable to the treatment of multidrug-resistant Mtb (112). The exquisite multidrug resistance of this bacteria leads to a drug regimen based solely on medical empirical evidence (51, 53), with an extremely long duration (50), high costs for healthcare services (113) and a low success rate (42); in some cases, the only way to solve infection is through surgical intervention (114). Alarmingly, some researchers believe that Mab is evolving rapidly towards becoming an obligatory lung pathogen (30). As such, the urgency to find alternative drugs to combat Mab infection is undeniable.

Given the wide gap between a drug's activity *in vitro* and its clinical efficacy that consistently haunts Mab infections, there is an urgent need for optimized, improved and more complex preclinical models to solve this issue. Additionally, studies that involve drug discovery against Mab also suffer from a severe lack of standardization, which renders findings inconsistent and makes it impossible to extrapolate them into clinical practice. This reiterates once again the importance of establishing and optimizing novel preclinical models for Mab infection, particularly the ones that have more variability between studies, such as biofilms (89) and recent animal models, such as *G. mellonella*.

In this work, we planned to establish two preclinical models for drug screening against Mab: an *in vitro* model – the biofilm formation in SCFLM, which, due to its composition, mimics the mucus of patients with CF, and an *in vivo* model – *G. mellonella* systemic infection. These two models represent important steps forward in terms of complexity, especially when compared to standard laboratory *in vitro* models (planktonic growth in 7H9 medium).

The SCFLM is a greater approximation to the conditions Mab faces during the infection of CF patients. In the context of drug discovery, it could be a replacement for the normal 7H9 broth, or an intermediate *in vitro* model for a more profound study of the activity of some molecules previously tested in 7H9, for combating Mab infections in patients with CF. The behaviour of Mab in this medium is severely understudied, so this work began with the characterization of the bacteria in both variations of SCFLM – SCFLM1 and SCFLM2.

Interestingly, we observed that Mab's growth differed significantly between SCFLM1 and c7H9, but not between SCFLM2 and c7H9 (**Fig. 7A**). This is in accordance with two previous studies, one which claimed that Mab grew slower in SCFLM1 than in c7H9 (90) and another that reported similar growth rates in c7H9 and SCFLM2 (95). However, in this work, we established that the difference between concentrations of DNA in the two media is partially responsible for Mab's delayed growth in SCFLM1 in comparison to

SCFLM2 (**Fig. 7B**). In contrast, a high concentration of mucin has no influence on Mab's growth rate (**Fig. 7C**).

A higher concentration of DNA can result in pH alterations overtime and, due to its negative charge, may result in the chelation of ions essential for mycobacterial growth, making them less accessible to bacteria. In the future, it may be interesting to measure the pH of SCFLM1 and SCFLM1* at different growth stages and investigate potential alterations that could hinder bacterial growth. Nonetheless, it is important to reiterate that these differences in DNA concentration do not fully explain the delayed growth of Mab in SCFLM1. The higher nutrient richness associated with the increasing complexity of SCFLM2 (**Fig. 6**) also ensures greater bacterial growth, particularly due to the presence of molecules such as glucose, L-lactic acid, N-acetylglucosamine and the supplementation of this medium with ions such as magnesium, calcium and iron, which are also present in c7H9 (**Fig. 6**). Thus, it could be interesting to supplement SCFLM1 with the elements mentioned previously and delineate Mab's growth curves once more, to see if faster replication is observed.

As for biofilm formation, the appearance of Mab biofilms in SCFLM1 was remarkably similar to the ones described by Hunt-Serracin *et al.* (2019) (90) (**Fig. 8A** and **8B**). In SCFLM2, biofilms appeared more mature and developed than the ones formed in SCFLM1 – instead of cording and various aggregates, a full pellicle is observed (**Fig. 8C**). Once again, reasons for this difference very likely reside in the distinct composition of SCFLM1 and SCFLM2, especially in regards to magnesium, an element that is essential for Mab biofilm formation in a CF lung-like environment (87, 115). The induction of biofilm formation can also reside in the myriad of other components present in SCFLM2 and absent in SCFLM1, as well as Mab's increased growth rate in SCFLM2. On the other hand, the formation of biofilms by Mab in SCFLM1 was not affected by the reduction of DNA in SCFLM1*, meaning this molecule appears to have little influence in conditioning biofilm formation (**Fig. 8A** and **8B**). However, the same cannot be said about mucin, as the supplementation of c7H9 with mucin at a concentration of 0.5 g/L and 5 g/L improved biofilm formation significantly (**Fig. 8A** and **8B**). It has been reported that exposure to mucin can change Mab biofilms' morphology, as well as biomass and matrix composition (97). For other bacteria, the role of mucin in biofilm formation has been more thoroughly studied; namely, for *P. aeruginosa*, the presence of mucin has been shown to promote the formation of biofilms and biofilm-like structures (116, 117). Given that *P. aeruginosa* is also a well-known CF pathogen and very often co-infects the lungs of CF patients with Mab, the fact that mucin can induce biofilm formation in both bacteria is extremely interesting. It is important to note, however, that these conclusions are based

solely on visual observations. To more accurately describe the role of mucin in biofilm formation, additional experiments are necessary, such as staining biofilms in different media with matrix-binding dyes and observing them in a fluorescence microscope or quantifying the signal. One of these potential dyes is Calcofluor White, which binds to cellulose, but others are possible – Nile red, which binds lipids, Texas Red, which binds to carbohydrates, SYPRO Ruby, which binds to proteins, Crystal Violet which stains all negatively charged molecules in the biofilm matrix, among others.

After the characterization of Mab's behaviour in SCFLM, we decided to see if this medium could be adapted for high-throughput drug screening. Given the monetary and logistical hurdles associated with SCFLM2, this part of the study was solely performed for SCFLM1*. To see if the latter could indeed be used for performing MIC assays with our auto-luminescent Mab strain, Mab op-sc, we tested five clinically used antibiotics to validate our model: clarithromycin, rifabutin, amikacin, imipenem and moxifloxacin. Strikingly, not only were we able to obtain good linear regressions for all antibiotics in SCFLM1* (**Fig. 9**), but we also concluded that Mab showcases increased antibiotic resistance in this medium when compared to c7H9 (**Table 5**). This is in accordance with a study by E.J. Baker *et al.* (2024), which also reported an increase in MIC values of clarithromycin, amikacin and moxifloxacin when Mab was incubated in SCFLM1 instead of c7H9 (96).

To further investigate the reason for Mab's decreased antibiotic susceptibility in SCFLM1*, we performed a MIC assay in c7H9 supplemented with mucin at 5 g/L, given that this biomolecule has been reported to interfere with the susceptibility of Mab to amikacin and induce loss of antibiotic efficacy in *P. aeruginosa* (97, 117, 118). We concluded that Mab's antibiotic susceptibility profile was quite similar in c7H9 and c7H9 with mucin at 5 g/L, except in the cases of amikacin where a 3-fold increase in the IC_{90} value was observed (**Fig. 9** and **Table 5**). Since amikacin is a positively charged molecule at physiological pH, the decrease in its activity can be explained by its binding to mucin, which is a negatively charged molecule. This would hinder the access of amikacin to the bacteria, and decrease its efficiency. However, it is worth noting that this increase is not in the same degree as the one observed in SCFLM1*. Mab's resistance to amikacin and the other antibiotics is much more pronounced in the latter, which indicates that the presence of mucin alone cannot explain such a decrease in Mab's susceptibility to antibiotics in SCFLM1*. Thus, it is plausible to assume that Mab undergoes profound metabolic, genetic and physiological alterations in SCFLM1* that result in a different antibiotic susceptibility profile, presumably more similar to the one it showcases in clinical settings. These alterations can be linked to its altered growth rate

in SCFLM1* and/or changes in cell physiology and metabolism that only happen in this medium and not in any variation of c7H9. Namely, Hunt-Serracin *et al.* (2019) reported that, in SCFLM1, Mab possesses translucent sheaths that cover each bacterium and hinder penetration of stains like NADA and FM4-64 (90). It is plausible to presume that this sheath also protects the bacteria from antibiotics by hampering their penetration. Regarding the growth rate, most antibiotics have mechanisms of action that enable them to preferentially target metabolically active and replicating bacteria. Thus, the slower growth rate observed in SCFLM1 and even SCFLM1* when compared to c7H9 can indeed lead to a greater antibiotic resistance.

To increase the complexity of our preclinical model, the next step was to perform drug susceptibility testing against Mab biofilms in SCFLM1*. Disappointingly, however, the biofilms formed in SCFLM1* were more susceptible to all the tested antibiotics, which is not in accordance with our MIC assay results, obtained in planktonic cultures (**Fig. 10A** and **Table 6**). Nonetheless, it is important to note that matrix production by Mab in SCFLM1 is not as abundant as in c7H9. Since the matrix functions as a barrier that hinders antibiotic penetration, a more developed biofilm is more resistant to antibiotics. Besides less matrix production, another potential explanation for these results lies in the possibility that, in SCFLM1*, a greater percentage of bacteria residing in biofilms enter a dormant/persister state upon contact with antibiotics. Luminescence cannot distinguish between dead or dormant bacteria, only detecting metabolically active bacteria. In this context, it is important to have a complementary method for biofilm assessment, such as the ones mentioned above for matrix detection. Previously, some of our laboratory results established that crystal violet, a method for matrix detection, correlated well with our luminescence data for biofilms grown in c7H9 (unpublished data). However, given that the crystal violet protocol requires several washing steps, which cannot be performed in biofilms grown in SCFLM as the biofilm forms as a pellicle in the media itself and not attached to the wells' walls, other matrix quantification strategies should be used. Additionally, in the future, drug susceptibility assays against planktonic bacteria and biofilms in SCFLM2 should be performed, to confirm if the increase in complexity leads to alterations in Mab's antibiotic susceptibility profile, and the formation of biofilms more resistant to antibiotics than the ones formed in c7H9 and even SCFLM1*. SCFLM2 is also more complex and more similar to the physiological conditions the bacteria face in the lungs of CF patients, making this medium even more advantageous for drug susceptibility testing in conditions more similar to clinical settings. Furthermore, given our previous results regarding mucin and biofilm formation, it would also be of great

interest to perform drug susceptibility testing against biofilms formed in c7H9 supplemented with mucin at 5 g/L.

Although this model represents a significant advancement in terms of complexity, particularly in comparison to the c7H9 laboratory medium, it is not entirely representative of the lung environment as a whole. Namely, it lacks the presence of cells such as epithelial cells and macrophages, which undoubtedly play an important role in Mab's infection. Furthermore, in this model, Mab is treated as an isolated pathogen, which does not accurately reflect the polymicrobial nature of the lungs in CF patients. In the future, it would be interesting to test antibiotics against Mab in the presence of other bacteria that co-populate the lungs of CF patients, such as *P. aeruginosa* and *S. aureus*. Another limitation of our work also resides in the fact that we did not test any combinations of antibiotics, which are the regimens usually employed clinically. As such, determining the efficacy of these combinatorial regimens against Mab when incubated in SCFLM would be of great interest and importance to greater approximate our conclusions to what is clinically relevant.

After developing this *in vitro* model, we moved to *G. mellonella* as an *in vivo* option, given its numerous advantages and the severe lack of studies with Mab in this particular model (**Table 2**). First, it was important to establish the infection conditions. Survival curves upon infection with increasing bacterial concentrations showed a quite good correlation between a higher bacterial load and greater larval mortality (**Fig. 11A**). This was also observed in the literature (103), although with some differences. In our study, a dose of 10^5 CFU/larva was not able to cause significant larval death after 7 days, whilst in a study by Admella *et al.* (2023), such a concentration was capable of inducing considerable death at 6 days post-infection (103). A possible reason for this difference could be the weight of the larvae – whilst in our work the larvae weighed around 400 mg, in the study mentioned previously, the larvae weighed only 200 mg.

We also proved that Mab was capable of replicating inside the larvae, with an increase of about 1 log between day 2 and day 5 of infection (**Fig. 11B**). This proves that Mab is indeed capable of infecting the larvae, not only because it induces significant death after infection, but also because it can actively replicate inside the larvae. To assess bacterial load, luminescence measuring can also be used. Unfortunately, our Mab op-sc strain has a very low signal, so for luminescence visualization in the larvae, Mab FF-sc was the most appropriate choice. Disappointingly, however, we were only able to obtain a signal for larvae infected with high bacterial load, which results in faster larval death, unfitting for studies of drug susceptibility. The lack of signal in the larvae could be partially

explained by the progressive loss of signal intensity during the time it takes to inject all the larvae and image them. It was previously established that the strain Mab FF-sc produces a luminescent signal up to 30 minutes after the addition of D-Luciferin (107). However, it is a possibility that the low volume of D-Luciferin injected (10 µL) alongside a smaller bacterial load results in a weaker signal that fades faster and/or is not able to be detected by the lack of sensitivity of the IVIS imager. It is also possible that the concentration of D-Luciferin and acquisition time used in this experiment are not adequate for signal detection, meaning that further optimization of luminescence measuring in larvae infected with Mab FF-sc is still necessary.

To validate this model for drug screening, the larvae were treated with amikacin and meropenem in increasing concentrations and the efficiency of the antibiotics was verified by CFU counting at 3 days post-treatment and survival assessment at 5 days post-treatment. Despite great variability, our CFU counting results appear to indicate that reduction in bacterial load is dose-dependent (**Fig. 12A**). As for survival, it is quite clear that the larvae which received no treatment at all survived less than larvae which received the antibiotics (**Fig. 12B**). Surprisingly, the dose dependent effect was even clearer through survival than through CFU counting. These results are extremely promising as they indicate that the efficacy of compounds against Mab can be addressed simply through survival assessment of the larvae. Despite this, to further investigate the activity of compounds against Mab, we consider it necessary to have a complementary method for assessing bacterial load, given the fact that the survival of the larvae can be influenced by other factors besides infection, namely their physiological status and the stress associated with injection. Luminescence is the ideal choice for this method, given its advantage of not requiring the sacrifice of the larvae, allowing for simultaneous survival analysis. In the work developed by Meir *et al.* (2018) (72), a luminescent Mab strain was used to infect the larvae, and the effect of amikacin and meropenem was determined by luminescence measurements and survival analysis. Strikingly, larvae treated with antibiotics exhibited delayed mortality and a reduction in their luminescent signal. Given that the luminescent signal of the Mab strain used in their studies correlates with CFUs, these conclusions correlate quite well with our own, and reiterate the importance of optimizing the luminescence measuring protocol for Mab FF-sc.

All things considered, the *G. mellonella* model shows potential for evaluating novel compounds against Mab in an *in vivo* setting that allows for testing a greater number of compounds than mice. The fact that our findings correlate well with the few existing studies brings hope for standardizing the *G. mellonella* model for drug testing against Mab. However, it is important to note that this model does not offer a complete picture of

Mab's infection, since the larvae do not have lungs and do not have an adaptative immune system, meaning that complementary tests for drug activity should be performed in other settings besides *G. mellonella*.

Conclusion

Throughout this work, we developed two preclinical models that, due to their increased complexity, will yield more sophisticated and accurate conclusions regarding compound selection against Mab than those obtained with c7H9. Remarkably, the use of both these models may help close the gap between *in vitro* activity and clinical efficacy of compounds currently used in treatments against Mab infections, as well as aid in the selection of novel compounds that may be used in future treatment alternatives. Given Mab's extreme resistance to various antibiotics, the fact that these models can help refine current treatments and/or find new ones is of extreme importance. Additionally, a more accurate selection of novel compounds results in fewer drugs to be tested in mice, which promotes research that is not only more precise, but also in conformity with the 3 R's policy.

It is undeniable that there is still a long way to go in finding new strategies to combat Mab and its resistance. Nonetheless, eliminating crossroads through the improvement of preclinical research can shorten the time it takes to reach our destination and increase the odds of success. The optimization of several models for drug screening gains particular significance in a world struggling with the problem of antimicrobial resistance, and in a time when more than ever, bacteria remind us that what does not (truly) kill them makes them stronger.

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