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# Universidade Nova de Lisboa

# Instituto de Higiene e Medicina Tropical

## Restoring Drug Resistant Mycobacteria Susceptibility to β-lactam Antibiotics

**Bianca Carolina Ribeiro Gama**

**DISSERTAÇÃO PARA OBTENÇÃO DO**

**GRAU DE MESTRE EM CIÊNCIAS BIOMÉDICAS**

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# Restoring Drug Resistant Mycobacteria Susceptibility to β-lactam Antibiotics

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# Abstract

Tuberculosis (TB) is one of the top 10 causes of death worldwide, and the emergence of multi (MDR) and extensively drug resistant (XDR) *Mycobacterium tuberculosis* strains is a major public health concern. The potential use of antibiotics that are not usually included in tuberculosis treatment is currently being considered and recent studies have highlighted the potential use of β-lactams such as carbapenems to treat MDR-TB. Carbapenems are a subclass of β-lactam antibiotics, which target peptidoglycan biosynthesis, that are particularly resistant to inactivation by the BlaC a β-lactamase, produced by *Mycobacterium tuberculosis*.

Mycobacteria have a characteristic cell envelope, consisting of a long chain mycolic acids layer, a highly branched arabinogalactan polysaccharide and a very cross linked and modified meshwork of peptidoglycan. This barrier contributes to the virulence, persistence and intrinsic resistance of mycobacteria to several drugs, and modulates host-pathogen immune response.

The aim of this thesis was to study how exposure to isoniazid and ethambutol – antibiotics that inhibit the synthesis of mycolic acids and arabinogalactan – lead to increased accessibility of peptidoglycan to antibiotics that target its biosynthesis, the β-lactams.

To address this, minimum inhibitory and bactericidal concentrations (MIC and MBC) of β-lactams (amoxicillin, cefotaxime, meropenem and imipenem), isoniazid and ethambutol were determined for four different mycobacteria species (*Mycobacterium smegmatis, Mycobacterium fortuitum, Mycobacterium bovis* and *Mycobacterium tuberculosis*). All four species were susceptible to at least one of the β-lactams tested, with better efficacy registered for meropenem, and clavulanate – a β-lactamase inhibitor – was essential for enhancement of β-lactams activity. Additionally, we tested if exposure of mycobacteria to isoniazid or ethambutol in two sub-MIC (½ MIC and ¼ MIC) and subsequent and/or simultaneous exposure to β-lactams could improve its efficacy. It was notable that ethambutol had an enhancing effect over the activity of β-lactams, with amoxicillin and meropenem MIC being significantly lower when combined with ethambutol and clavulanate, frequently changing the bacteria classification from resistant to susceptible. Isoniazid was also advantageous, but further work must be done, especially for slow growing mycobacteria. These data suggests that treatment with sub-MICs of

isoniazid and ethambutol halters the proper biosynthesis of outer cell envelope components, leaving peptidoglycan more accessible for β-lactams activity.

In order to confirm the exposure of the mycobacterial peptidoglycan after treatment with isoniazid or ethambutol, co-precipitation assays were done with *Drosophila* receptors that specifically recognize peptidoglycan, peptidoglycan recognition proteins (PGRPs). Fluorescence microscopy corroborated the results from antibiotics synergistic assays, showing that mycobacterial peptidoglycan was only recognized by PGRPs after exposure to sub-MIC of isoniazid and ethambutol.

The results presented here are preliminary and assays are being optimized to be tested in with clinical isolates of *Mycobacterium tuberculosis*, including MDR and XDR-TB strains, in the near future. This work will help to establish a connection between unknown mechanisms of resistance to anti-TB drugs and potential vulnerabilities in the cell envelope of mycobacteria, which could be further exploited for therapeutic purposes.

Keywords: *Mycobacterium tuberculosis*; Multidrug-resistant tuberculosis (MDR-TB); Extensively drug-resistant tuberculosis (XDR-TB); β-lactams; Peptidoglycan.

# Resumo

A tuberculose (TB) é uma das 10 principais causas de morte no mundo, e o surgimento de estirpes de *Mycobacterium tuberculosis* multirresistente (MDR) e extensivamente resistente a medicamentos (XDR) é um grande problema de saúde pública. O uso de antibióticos que normalmente não são incluídos no tratamento da tuberculose tem sido considerado, e estudos recentes destacaram o uso de β-lactâmicos, como os carbapenemos, no tratamento da MDR-TB. Os carbapenemos são uma subclasse dos antibióticos β-lactâmicos, que têm como alvo a biossíntese do peptidoglicano e que são particularmente resistentes à inativação pela β-lactamase BlaC, produzida por *M. tuberculosis*.

As micobactérias têm um envelope celular característico, constituído por uma camada de ácidos micólicos de cadeia longa, um polissacárido de arabinogalactano altamente ramificado e uma malha de peptidoglicano reticulada e modificada. Essa barreira contribui para a virulência, persistência e resistência intrínseca das micobactérias a vários antibióticos e modula a resposta imune do hospedeiro.

O objetivo desta tese foi estudar como a exposição à isoniazida e ao etambutol - antibióticos que inibem a síntese de ácidos micólicos e arabinogalactano - leva ao aumento da acessibilidade do peptidoglicano aos antibióticos que inibem a sua biossíntese, os β-lactâmicos.

Para este fim, foram determinadas concentrações mínimas inibitórias e bactericidas (MIC e MBC) de β-lactâmicos (amoxicilina, cefotaxima, meropenem e imipenem), isoniazida e etambutol para quatro espécies de micobactérias (*Mycobacterium smegmatis, Mycobacterium fortuitum, Mycobacterium bovis* e *Mycobacterium tuberculosis*). Todas as espécies mostraram-se suscetíveis a pelo menos um dos β-lactâmicos testados, com melhor eficácia registada para o meropenem, e o clavulanato – um inibidor de β-lactamases – foi essencial para o aumento da atividade dos β-lactâmicos. Adicionalmente, verificámos se a exposição de micobactérias à isoniazida ou ao etambutol em duas sub-MIC (½ MIC e ¼ MIC) e subsequente e/ou simultânea exposição a β-lactâmicos poderia melhorar a sua eficácia. Notavelmente, o etambutol teve um efeito potencializador da atividade dos β-lactâmicos, com as MICs da amoxicilina e do meropenem significativamente mais baixas quando combinados com etambutol e clavulanato, alterando frequentemente a classificação das bactérias de resistente para suscetível. A

combinação com isoniazida também foi benéfica, mas as condições devem ser otimizadas, especialmente para as micobactérias de crescimento lento. Este resultado sugere que o tratamento com sub-MICs de isoniazida e etambutol interfere na biossíntese correta dos componentes do envelope celular externo, deixando o peptidoglicano mais acessível para a atividade dos β-lactâmicos.

Para quantificar a exposição do peptidoglicano micobacteriano após tratamento com isoniazida ou etambutol, foram realizados ensaios de co-precipitação com recetores de *Drosophila* que reconhecem o peptidoglicano, proteínas de reconhecimento de peptidoglicano (PGRPs). A microscopia de fluorescência corroborou os resultados dos ensaios sinergísticos de antibióticos, mostrando que o peptidoglicano micobacteriano apenas é reconhecido pelas PGRPs após a exposição a sub-MICs de isoniazida e etambutol.

Os resultados apresentados são preliminares e os ensaios estão a ser otimizados para serem realizados com isolados clínicos de *Mycobacterium tuberculosis*, incluindo estirpes MDR e XDR-TB, no futuro próximo. Este trabalho ajudará a estabelecer uma conexão entre mecanismos desconhecidos de resistência a antibióticos anti-TB e possíveis vulnerabilidades no envelope celular de micobactérias, que poderão ser exploradas para fins terapêuticos.

Palavras-chave: *Mycobacterium tuberculosis*; Tuberculose multirresistente (MDR-TB); Tuberculose extensivamente resistente (XDR-TB); β-lactâmicos; Peptidoglicano.

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# Abbreviation list

6-APA – aminopenicillanic acid

7-ACA – aminocephalosporanic acid

AG – Arabinogalactan

Ala – Alanine

AMX – Amoxicillin

Ara*f* – Arabinofuranosyl

BCG – Bacille Calmette-Guérin

BSL – Biosafety level

cfu – Colony forming unit

CLA – Clavaulanate

CTX – Cefotaxime

D-isoGlu – D-iso glutamate

DHP-I – Dehidropeptidase-I

DNA – Deoxiribonucleic acid

DOTS – Directly Observed Treatment Short course

DST – Drug susceptibility testing

EMB – Ethambutol

ESBL – Extended spectrum beta-lactamase

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FAS – Fatty acid synthetase

Gal*f* – Galactofuranosyl

GlcNAc - Glycolylmuramic acid

HF – Hydrofluoridic acid

HIV – Human immunodeficiency virus

IFN-γ – Interferon-γ

IL – Interleukin

Imd - Immune deficiency pathway

IMP – Imipinem

INH – Isoniazid

InhA – enoyl-acyl carrier protein reductase

IPTG – Isopropyl β-D-1-thiogalactopyranoside

LAM – Lipoarabinomannans

LB – Luria-Bertani broth

LDT – L,D-transpeptidases

LM – Lipomannans

m-DAP – meso-diaminopimelate

MA – Mycolic acids

mAGP - Mycolyl-arabinogalactan-peptidoglycan complex

MBC – Minimum bactericidal concentration

MDR-TB – Multi-drug resistant tuberculosis

MEM – Meropenem

MIC – Minimum inhibitory concentration

MRSA – Methicillin resistant *Staphylococcus aureus*

*Mtb – Mycobacterium tuberculosis*

MurGlyc – *N*-glycolylmuramic acid

MurNAc – *N*-acetylmuramic acid

MyD88 – Myeloid differentiation primary response gene 88

NAD – Nicotinamide adenine dinucleotide

NAG – *N*-acetylglucosamine

NAM – *N*-acetylmuramic

NLR – NOD-like receptor

NOD – Nucleotides oligomerization domain

NTM – Non-tuberculous mycobacteria

OADC – Oleic acid dextrose catalase

PAMPs – Pathogen-associated molecular patterns

PBP – Penicillin binding protein

PBS – Phosphate buffer saline

PG – Peptidoglycan

PGRP – Peptidoglycan recognition protein

PIM – Phosphatidylinositol mannosides

RR-TB – Rifampicin-resistant tuberculosis

SDS – Sodium dodecyl sulfate

SDS-PAGE – Sodium dodecyl sulfate-polyacrylamide gel eletrophoresis

TB – Tuberculosis

TLR – Toll-like receptor

TMM – Trehalose monomycolate

TNF-α – Tumor necrosis factor-α

UDP – Uridine diphosphate

WHO – World Health Organization

XDR-TB – Extensively-drug resistant tuberculosis

# Introduction

## 1. Tuberculosis

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), is an ancient infectious disease that remains one of the top causes of death worldwide (World Health Organization, 2018a). *Mtb* is very contagious, transmitted by airborne particles, and usually affects the lungs but can infect other organs (Forbes *et al.*, 2018). TB can remain clinically silent for years in the form of a granuloma at the lung, latent TB, or can cause acute disease, with symptoms like cough with blood, fever, night sweats, weakness and weight loss (Salvatore and Zhang, 2017; Centers for Disease Control and Prevention, 2019). The emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) consists a global health threat, with longer, more expensive and less effective treatments (Green and Garneau-Tsodikova, 2013).

### 1.1. Tuberculosis history overview

TB is an ancient disease, with the *Mtb* common ancestor being probably more than 3 million years old with origin in the African continent, coincident with the location of early hominids (Gutierrez *et al.*, 2005). There is evidence of TB-like deformities in Egyptian mummies from the reign of pharaohs, with more than 4000 years, and throughout history there are written records describing TB as a fatal disease, from India, China, Ancient Greece and in the Roman Empire (Barberis *et al.*, 2017).

Several epidemics were registered between the 17$^{th}$ and 19$^{th}$ century, killing mostly habitants of poor areas and the term ‘tuberculosis’ was introduced in the mid-19$^{th}$ century (Barberis *et al.*, 2017). In the late-19$^{th}$ century, Robert Koch isolated and cultivated the tubercle bacilli in animal serum, and his achievements were awarded by the Nobel prize in 1905 (Gradmann, 2001). Since this discovery, significant advancements were made in the area of drug and vaccine development, with the globalization of the vaccine with Bacille Calmette-Guérin (BCG) (Luca and Mihaescu, 2013).

### 1.2. Epidemiology

Currently, tuberculosis is still a major public health concern, being on the top ten of causes of deaths worldwide. World Health Organization (WHO) last report on TB (World

Health Organization, 2018a) estimates 10 million cases and 1,6 million deaths by TB globally in 2017. Higher incidence is registered in low income countries (Figure 1), such as India, that accounts with 27% of total cases. Also, there is almost 1 million of HIV positive individuals infected with TB, 720000 of which from Africa (World Health Organization, 2018a).

The END TB strategy has the goal to end TB global epidemics, with the target of ending TB in 2035 (World Health Organization, 2018b). Between 2000 and 2017 there was a decrease of 33% of deaths, but by 2020 the milestone is to reach a 35% reduction of deaths compared to 2015. In order to reach the ultimate goal of ending TB, strong efforts must be done to conjugate medical care interventions, political supportive systems and research and innovation (World Health Organization, 2018b).

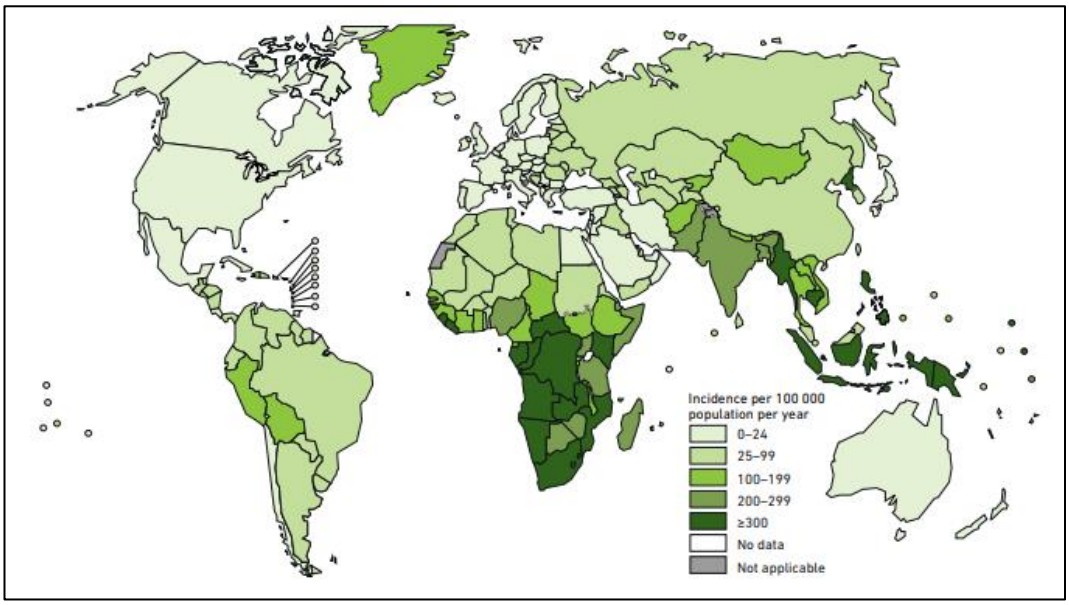


Figure 1 – Incidence rates of TB cases, per 100000 population, 2017. Adapted from WHO, 2018a.

## 1.3.Diagnosis and treatment

A drug treatment for TB was not yet available when the biggest epidemics occurred (Sotgiu *et al.*, 2015). The first treatment for this disease was the concept of sanatorium, introduced by Hermann Brehmer in late-19$^{th}$ century and is noted as the first successful cure to tuberculosis (Barberis *et al.*, 2017). This consisted in facilities where patients were isolated from the community, preventing transmission, and could rest in open air, with constant medical attention and adequate nutrition (Daniel, 2011).

Diagnosis of the disease can be made microscopically by sputum smear staining which is a low sensitive test, or by culturing, which is an accurate test but can take up to

12 weeks to give positive results (World Health Organization, 2018a). In alternative, rapid molecular tests have been developed, and the Xpert® MTB/Rif assay is recommended by WHO because of its high accuracy and quick response, of only two hours. Xpert® MTB/Rif assay provides not only the identification of *Mtb*, but also the prediction of resistance to rifampicin (Caulfield and Wengenack, 2016).

Nowadays, the treatment strategy recommended by WHO consists in the directly observed treatment short course (DOTS), which involves commitment from political, health care and patient parts of the programme. This strategy is recognized as highly efficient and cost-effective. TB 1st-line treatment consists in the combination of different antibiotics – isoniazid, rifampicin, ethambutol and pyrazinamide – for at least six months. Considering the duration of the treatment and unpleasant adverse effects of the drugs, poor patience adherence is very common, and can lead to treatment failure, relapses and drug resistance emergence (Sotgiu *et al.*, 2015).

Rifampicin resistant (RR-TB) and MDR-TB have become a major health emergency on the last two decades (Figure 2). MDR-TB is caused by *Mtb* strains that do not respond to at least isoniazid and rifampicin, the two most powerful 1st-line anti-TB drugs. In the case of MDR-TB, treatment becomes more longstanding, with regimens that can last for up to two years. Additionally, the drugs used on MDR-TB treatment – amikacin, capreomycin, kanamycin, new-generation fluoroquinolones, ethionamide and cycloserine – called 2nd-line anti-TB drugs, are less effective, more expensive and toxic (Sotgiu *et al.*, 2015; World Health Organization, 2018c). Even more resistant strains can arise, and XDR-TB *Mtb* strains have additional resistance to any fluoroquinolone – levofloxacin, moxifloxacin or ofloxacin – and at least one injectable agent. The estimate of RR/MDR-TB cases on 2017 was 558 000 (161 000 reported cases), 8,5% being XDR-TB (World Health Organization, 2018a).

It is evident that the scenario of tuberculosis burden is still far from the ideal, and further investigation should be done to improve prevention, diagnostics and treatment.

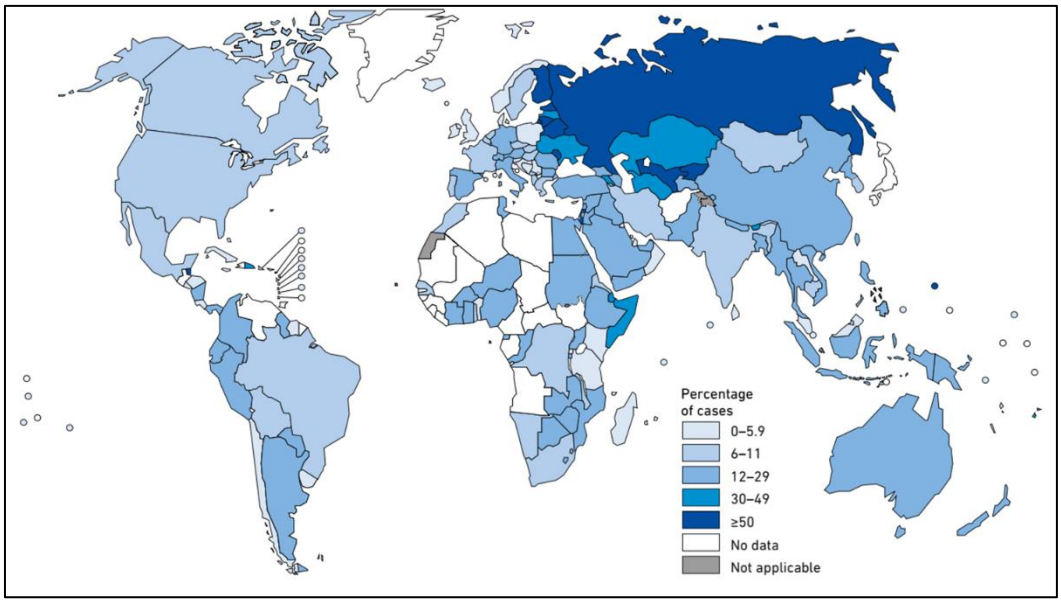


Figure 2 - RR/MDR-TB percentage in previously treated TB cases, in the period between 2005 and 2018. Adapted from WHO, 2018a.

## 2. Host-pathogen interactions in the context of TB infection

*Mtb* is an intracellular pathogen, transmitted by aerosols and a droplet containing 1 to 3 bacilli is enough for transmission (Salvatore and Zhang, 2017). When bacteria reach the lung and are phagocytosed by alveolar macrophages (Figure 3-1), replication occurs until the host cell dies due to necrosis or apoptosis (Figure 3-2). The release of innate immune cytokines recruits phagocytic cells to the site of infection, forming the characteristic TB granuloma (Davis and Ramakrishnan, 2009) (Figure 3-3 and 3-4). After activation of the adaptive immune response and macrophage differentiation to Th1 cells, bacterial growth can be controlled, forming the mature granuloma which leads to a calcified lesion on the lung containing the infection in a latent state (Figure 3-5). This happens to the majority of infections, and a small portion of these can be eventually reactivated in the case of immunosuppression, causing secondary symptomatic TB (Figure 3-6). In some infected individuals, bacteria cannot be retained in the granuloma, leading to symptomatic disease called primary progressive TB (Salvatore and Zhang, 2017). Figure 3 has the representation of *Mtb* infection of human macrophages.

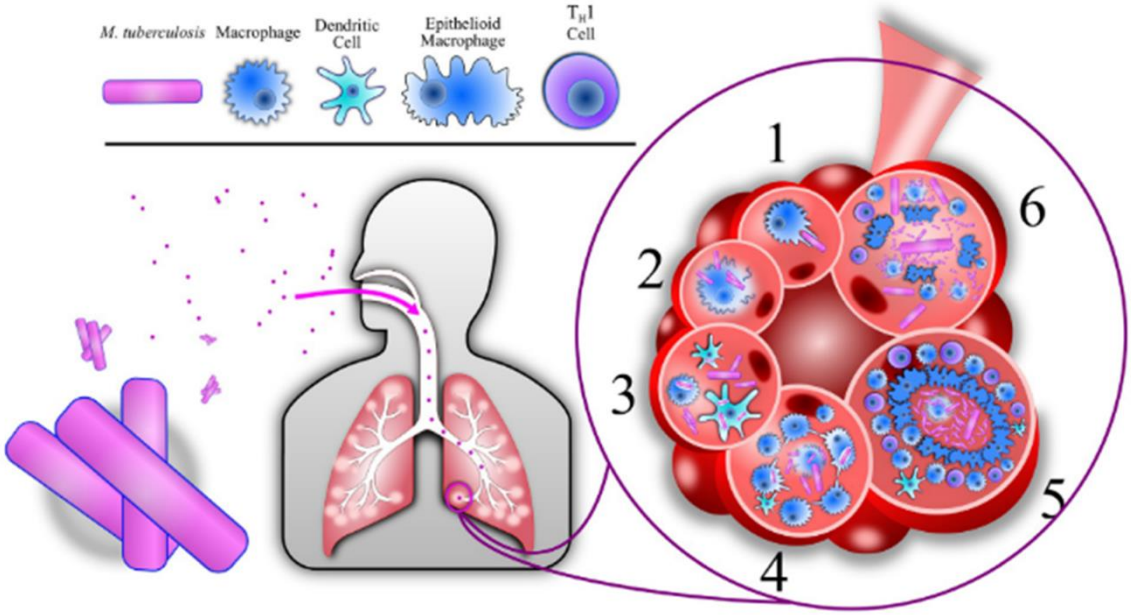


Figure 3 - Representation of the infection by *M. tuberculosis*. Pink rods represent the bacteria, that is transmitted by air droplets, with a diameter up to 5 μm that can go through the airways and deposit in the lung's alveoli. Blue cells are human immune phagocytic cells that are infected by *Mtb* and are responsible for the control of the infection. 1 – The bacilli is phagocytized by an unactivated macrophage. 2 – The bacterium is capable of killing the immune cell by necrosis or apoptosis and escapes the alveolar tissue. 3, 4 – Bacteria start to replicate in the extracellular space and macrophages and dendritic cells are recruited to the site of infection by signalling cytokines, forming the early granuloma. 5 – Mature Th1 cells and epithelioid macrophages are capable of containing the infection by effective killing and surrounding the necrotic area, forming the mature granuloma. 6 – In the case of immune suppression, bacteria can destroy the granuloma, causing progressive disease and further transmission. Adapted from Salvatore and Zhang, 2017.

## 2.1.Macrophage receptors involved in mycobacteria recognition

Mycobacterial cell envelope has a distinct structure (see section 4 – Mycobacterial cell envelope), which plays an important role in pathogenesis and host recognition. Bacterial cell surface lipid derivatives are recognized by C-type lectin mannose receptors expressed by macrophages and dendritic cells, triggering phagocytosis and anti-inflammatory responses, but can also be recognized by Toll-like receptors (TLRs) which activates pro-inflammatory responses (Van Crevel *et al.*, 2011).

TLR2, TLR4 and TLR9 are known to be involved in the recognition of *Mtb* pathogen-associated molecular patterns (PAMPs) (Salvatore and Zhang, 2017). These membrane receptors activate the myeloid differentiation primary response gene 88 (MyD88) signalling pathway, which induces the transcription of pro-inflammatory cytokines like interleukin (IL)-1β, tumor necrosis factor-α (TNF-α) and IL-6, and interferon-γ (IFN-γ) that recruit immune cells to the site of infection (Sasindran and Torrelles, 2011). The cytosolic nucleotide oligomerization domain-like receptors (NLR) NOD-1 and NOD-2 also participate in this process (Mortaz *et al.*, 2015). These receptors recognize

mycobacterial glycolipids, such as phosphatidylinositol mannosides (PIM); lipomannans (LM) and lipoarabinomannans (LAM), and peptidoglycan (PG) motifs (Amaral, Lasunskaia and D'Império-Lima, 2016).

In order to investigate which mycobacterial molecules are more capable of being recognized by macrophages, structural studies must be done, such as recognition of peptidoglycan by specific receptors.

### 2.1.1. Peptidoglycan recognition proteins

PG recognition proteins (PGRPs) are specific receptors of bacterial PG that are essential for the innate immune system activation in insects and vertebrates. In humans, PGRPs are secreted to the blood and have either amidase or bactericidal effects (Dziarski and Gupta, 2006). The fruit fly *Drosophila melanogaster*, often used as a model organism for studies of the innate immune system, is known to express PGRPs that recognize different types of PG activating distinct immune pathways (Dziarski, 2004). PGRP-SA is an extracellular receptor that binds to Lys-type PG from Gram-positive bacteria and activates the Toll pathway, an important immune pathway against Gram-positive bacteria (Leulier *et al.*, 2003). Recently, PGRP-SA was shown to also recognize both Lys and DAP-type PG from Gram-negative bacteria (Vaz *et al.*, 2019). PGRP-SD recognizes DAP-type PG and participates in the activation of the Toll pathway (Leone *et al.*, 2008); PGRP-LC is a transmembrane protein that activates the Immune deficiency pathway (Imd) after recognition of mainly DAP-type PG from Gram-negative or Gram-positive bacteria (Dziarski, 2004); PGRP-LE does not have a transmembrane domain, functioning as a intracellular or extracellular receptor of DAP-type PG and participates in the activation of the Imd pathway (Dziarski, 2004).

## 3. Mycobacteria genus taxonomy

*Mycobacterium* spp. have a particular cell envelope, that among other constituents, contains mycolic acids (MA), which are present in very few other organisms (Jankute *et al.*, 2015; Forbes *et al.*, 2018). These are aerobic, non-motile and non-spore forming bacteria, that can be categorized as 'acid fast bacilli', because of its resistance to acid alcohol decolourization, and as Gram-positive bacteria, although there are some discrepancies on this classification (Abrahams and Besra, 2016). From the DNA-based molecular grouping point of view, mycobacteria are similar to other Gram-positive

bacteria – due to its GC-rich DNA, but also have some resemblance with Gram-negative bacteria (constituted by an inner membrane, a thin layer of PG, a periplasm and an outer membrane) by not always retaining the Gram staining and having porins present on its outer layer (Hett and Rubin, 2008). Mycobacteria species can be categorized in three groups: *Mycobacterium leprae*, *Mtb* complex, and non-tuberculous mycobacteria (NTM). Additionally, mycobacteria can be classified by pigmentation (scotochromogenic, photochromogenic and nonphotochromogenic), pathogenicity (strict pathogens, potential or opportunistic pathogens, nonpathogenic and saprophytic) and growth rate (slow and rapidly growing) (Forbes *et al.*, 2018). All strict pathogens and most opportunistic pathogens are slow growing species, and few rapidly growing species are pathogenic.

## 3.1.*Mycobacterium tuberculosis* complex

*Mtb* complex group comprises several species that can cause tuberculosis on mammals, namely *Mtb*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium bovis*, *Mycobacterium suricattae*, *Mycobacterium mungi*, *Mycobacterium orygis*, *Mycobacterium caprae*, *Mycobacterium pinnipedii*, *and Mycobacterium canettii* (Forbes *et al.*, 2018; Fieweger, Wilburn and VanderVen, 2019).

*Mtb* is the etiologic agent of human TB, and can cause not only pulmonary disease, but also lymphatic, pleural, central nervous system and even disseminated infections (Salvatore and Zhang, 2017). Due to the burden of this disease, investigators around the world are constantly trying to understand *Mtb* resistance mechanisms, improve the development of new diagnostic tools, new drugs, and the discovery of new drug targets. To standardize studies, there is a widely used strain of *Mtb*, H37Rv, that was isolated from a clinical sample in 1905 and is currently used as a deduction of susceptibility of clinical isolates, once it is susceptible to all anti-TB drugs (Heinrichs *et al.*, 2017). *Mtb* is a slow growing mycobacteria with a replication time of approximately 24 hours, can take 3 to 4 weeks to grow on laboratory media and microscopically is recognized by the formation of aggregates or clumps. The avirulent/attenuated version of *Mtb* H37Rv, *Mtb* H37Ra (Figure 4), is also used as a reference strain, with the advantage of not having to be handled in a biosafety level-3 (BSL) laboratory.

*M. bovis* causes TB in animals such as cattle and can be transmitted to primates and humans. In the 20$^{th}$ century about one third of TB cases in Europe were caused by *M. bovis*, but cattle control programs and milk pasteurization process lead to eradication of

this transmission (Forbes *et al.*, 2018). The live attenuated strain – Bacille Calmette-Guérin (BCG) – is widely used for neonatal vaccination, essentially in high-prevalence areas to prevent disseminated disease in children. Despite rarely and only on immunodeficient individuals, complications from vaccination with BCG can happen, and the vaccine is poorly protective for adolescents and adults. Therefore, efforts are being made for novel anti-TB vaccines development (World Health Organization, 2019). BCG takes about 2 -3 weeks to grow on laboratory media, colonies have an irregular and translucid aspect (Figure 4) and should be handled in a BSL-2 laboratory, as *Mtb* H37Ra.

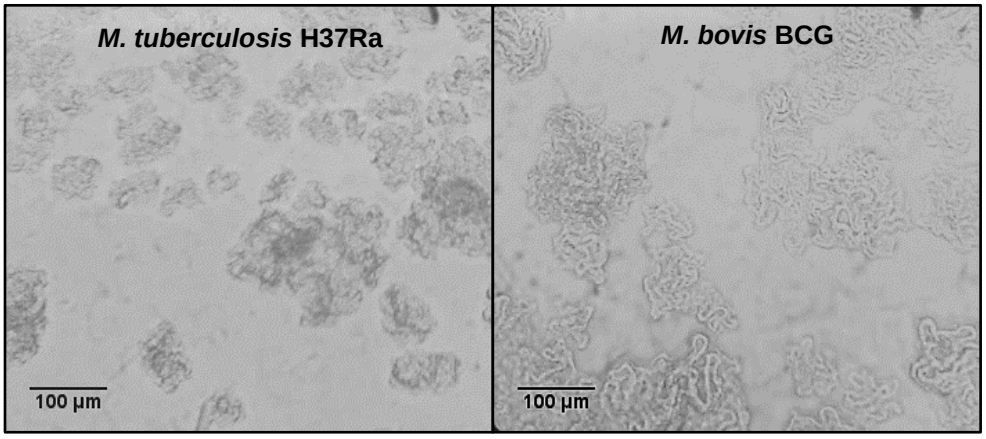


Figure 4 – Light microscopy images of *M.tuberculosis* H37Ra (left) and *M. bovis* BCG (right) colonies. 20x ampliation.

## 3.2.Rapidly growing mycobacteria

Rapidly growing mycobacteria are NTM, usually identified by the capacity of growing into visual colonies within a week (Tran *et al.*, 2019). Most of these species are non-pathogenic and saprophytic, that are present on the environment, mainly in water reservoirs. However, some of this NTM species can cause diseases, especially on immune compromised individuals (Falkinham, 2015).

The *Mycobacterium fortuitum* complex is an example, that includes species as *M. fortuitum*, *Mycobacterium peregrinum*, *Mycobacterium porcinum*, among others. *M. fortuitum* was isolated from a post injection abscess in 1938 (Brown-Elliott and Wallace, 2002), and is known as an opportunistic pathogen that often causes localized post-traumatic wound infections, skin, soft tissue and bone infections after surgical interventions, like plastic or cardiac surgery (Brown-Elliott and Wallace, 2002; Forbes *et al.*, 2018). This species takes 4 to 5 days to grow on laboratory media, presents as light-yellow large colonies with little branches on the microscope (Figure 5) and should be handled in a BSL-2 laboratory.

*M. smegmatis* was isolated on 1885 from genital secretions, and is categorized as non-pathogenic mycobacteria, although there are reports of its isolation from health care-associated infections (Brown-Elliott and Wallace, 2002). The strain $mc^2155$ is frequently applied in genetic studies, because of its high transformability and ease to cultivate mycobacteriophages (Etienne *et al.*, 2005). This species has a fast doubling time, with visible colonies in 2 to 3 days in laboratory media (Figure 5) and can be handled at a level-1 BSL laboratory.

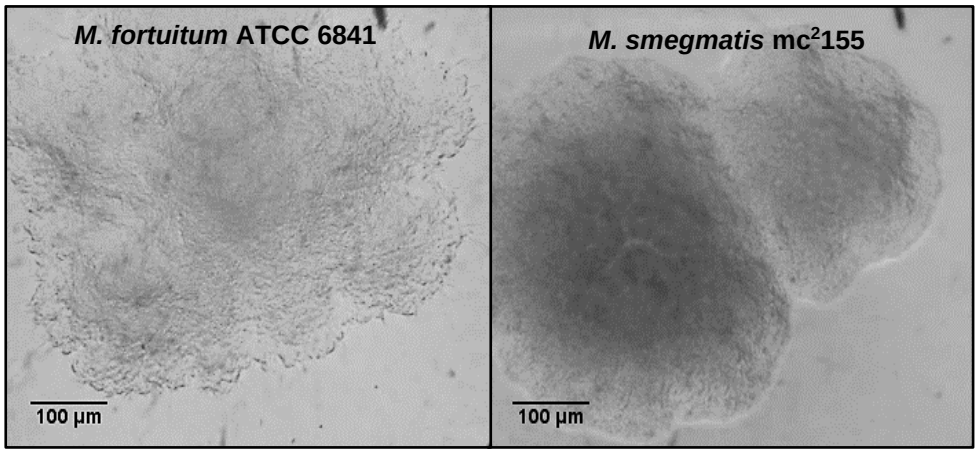


Figure 5 - Light microscopy images of *M. fortuitum* ATCC 6841 (left) and *M. smegmatis* $mc^2155$ (right) colonies. 20x ampliation.

## 4. Mycobacterial cell envelope

As mentioned, *Mycobaterium* spp are known to have a distinct cell envelope (Figure 6), which contributes to its high impermeability, virulence, persistence and intrinsic resistance to several drugs (Jarlier and Nikaido, 1994). This cell envelope is constituted by an outer layer of long-chain MA, a highly branched arabinogalactan (AG) polysaccharide and a cross-linked meshwork of (PG) – the mycolyl-arabinogalactan-peptidoglycan complex (mAGP) complex. Additionally, glycolipids and lipoglycans (PIM, LM and LAM) are intercalated in the MA layer, and a capsule of glycolipids, polysaccharides and proteins surrounding the mAGP complex (Jankute *et al.*, 2015). Due to the uniqueness and importance of the mycobacterial cell envelope, its biosynthesis is the most explored target for antibiotics (Abrahams and Besra, 2016; Catalão, Filipe and Pimentel, 2019).

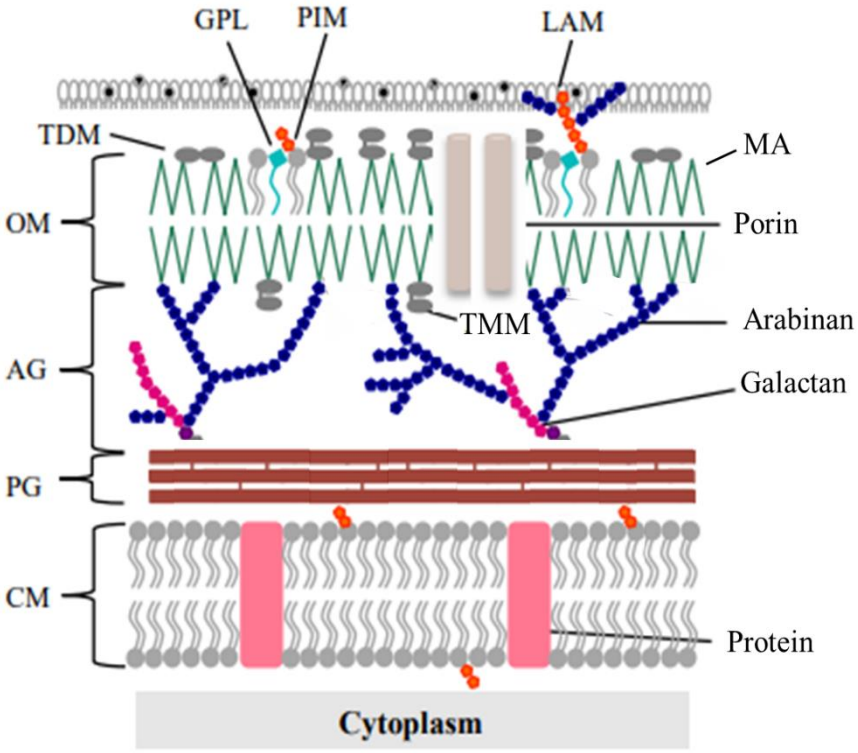


Figure 6 - Representation of the mycobacterial cell envelope. GPL: glycolipids, PIM: phosphatidylinositol mannosides, LAM: lipoarabinomannan, TDM: trehalose dimycolate, MA: mycolic acids, OM: outer membrane, AG: arabinogalactan, PG: peptidoglycan, CM: cell membrane. Adapted from Catalão, Filipe and Pimentel, 2019.

## 4.1.Mycolic acids

MA are long chain fatty acids that are mainly covalently bound to the PG-AG complex but are also present on the outer cell envelope. MA are essential for cell permeability, viability and virulence (Jankute *et al.*, 2015; Abrahams and Besra, 2016).

Biosynthesis of MA occurs on the cytoplasm, by two distinct but dependent pathways: Fatty acid synthetase (FAS) type I and type II. FAS-I system takes place on a single multi-enzyme complex and is responsible for the *de novo* generation of short-chain esters, that can form a saturated α-branch or be extended by FAS-II system, which involves four enzymes that increase the hydrocarbon chain by two carbons by each cycle (Takayama, Wang and Besra, 2005; Hett and Rubin, 2008). After the cytoplasmic synthesis of mycolates, these must be transported to cell envelope and attached to the PG-AG complex (Abrahams and Besra, 2016). This process remains unknown, but the principal hypothesis is that MA are transferred in the form of a trehalose monomycolate (TMM), by a TMM transporter (an efflux pump) and an extracellular mycolyltransferase catalyses the binding to the AG and separation from trehalose (Takayama, Wang and Besra, 2005). Mature mycolates have three subclasses (Figure 7): α-mycolates – *cis*-

cyclopropane configuration; methoxy-mycolates and keto-mycolates – *cis*- or *trans*-configuration (Minnikin *et al.*, 2015). The distribution of these three types of mycolates is similar between strains but differs between species and is related to the virulence of the bacteria. One interesting example is the distinct profile of the strains *M. tuberculosis* H37Rv and H37Ra: the avirulent strain has a deficiency on the mycolate elongation but a better production of α-mycolates than the virulent strain. These differences appear to be a factor to the lack of virulence of the H37Ra strain (Watanabe *et al.*, 2001; Heinrichs *et al.*, 2017).

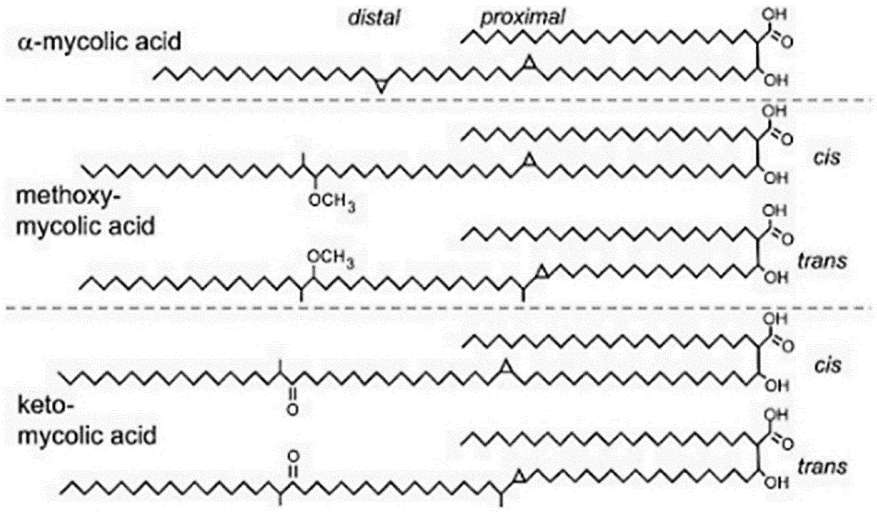


Figure 7 – Chemical structure of MA from *M. tuberculosis*. The three subclasses are represented: α-mycolates, methoxy-mycolates and keto-mycolates. Adapted from Takayama, Wang and Besra, 2005.

Genetic and computational analysis has helped to identify genes encoding proteins involved on MA biosynthesis, which can be candidates for drug targets (Abrahams and Besra, 2016; Rather and Maqbool, 2017). Examples of targets that are explored as targets for therapy is the *inhA* gene, that encodes the enoyl-acyl carrier protein reductase (InhA), an essential enzyme that participates in the last step of the FAS-II system (Marrakchi, Lanéelle and Daffé, 2014). Isoniazid, the main 1$^{st}$-line anti-TB antibiotic, is a pro-drug that needs to be activated by the mycobacterial catalase-peroxidase KatG, which is encoded by the gene *katG*, forming an adduct with nicotinamide adenine dinucleotide (NAD) – Figure 8 (Rawat, Whitty and Tonge, 2003). This adduct inhibits the InhA enzyme, causing oxidative stress, accumulation of intracellular elongated mycolates and eventually cell death (Rawat, Whitty and Tonge, 2003). Isoniazid has a rapid bactericidal activity, therefore is used in the treatment of both active and latent TB. Ethionamide is a

structural analogue of isoniazid, and a similar mode of action (Takayama, Wang and Besra, 2005; Abrahams and Besra, 2016).

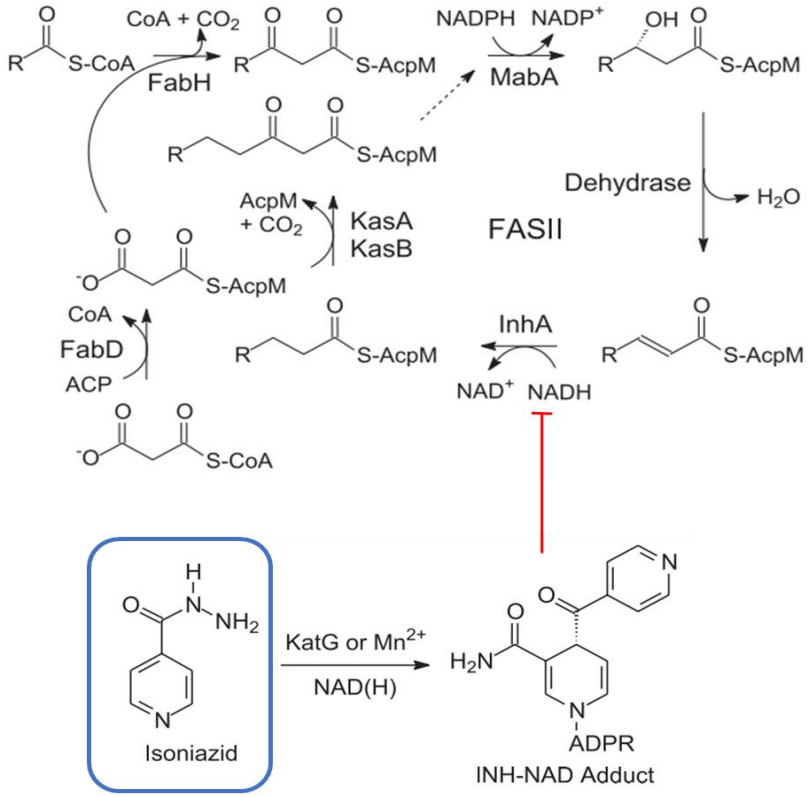


Figure 8 - Chemical structure of the pro drug isoniazid, activation by the catalase-peroxidase KatG, and action on the FASII system, inhibiting the elongation of mycolic acids. Adapted from Rawat, Whitty and Tonge, 2003.

Mycobacteria has developed ways to escape the action of isoniazid, like mutations on the *katG* gene, leading to weak or no activation of the pro-drug and consequent failure of the treatment (Rather and Maqbool, 2017). Alternative mechanisms of resistance are mutations on the *inhA* locus turning the target insensitive to the drug, or increased expression of the gene leading to drug saturation (Green and Garneau-Tsodikova, 2013). To overcome the challenge of drug resistance, it is essential to understand and characterize the mechanisms and mutations involved in resistance in order to design new effective drugs or discover new drug targets in order to bypass it.

## 4.2. Arabinogalactan

AG is the major heteropolysaccharide of the mycobacterial cell envelope and is basically composed of galactose and arabinose residues, in the furanose ring form, which is rare in nature (Alderwick *et al.*, 2015; Abrahams and Besra, 2016). AG is attached to the PG by a single linker unit. This single linker unit consists in a disaccharide of rhamnopyranose (Rha*p*) and *N*-glycolylmuramic acid (GlcNAc) – α-L-Rha*p*-(1→3)-α-D-

GlcNAc-(1→P) – and the ligation is catalysed by a phosphotransferase, Lcp1, encoded by the essential gene *lcp1* (McNeil, Daffe and Brennan, 1990; Harrison *et al.*, 2016).

The galactan domain polymerizes from the single linker unit linearly, alternating roughly 30 β(1→5) and β(1→6) galactofuranosyl (Gal*f*) residues (Jankute *et al.*, 2015). Arabinan chains are specifically linked to the 8$^{th}$, 10$^{th}$ and 12$^{th}$ residues of the galactan domain. The galactan chain biosynthesis occurs on the cytoplasm, and after transport possibly by a flipase, the elongation of the arabinan domain occurs on the extracellular face of the plasma membrane (Abrahams and Besra, 2016). The arabinan domain has around 30 arabinofuranosyl (Ara*f*) residue, and is built on a α(1→5) linked Ara*f* backbone, with the formation of branches on 3,5-α-D-Ara*f* residues and termination with β(1→2) Ara*f* residues (Alderwick *et al.*, 2015). At the reducing end of the AG, the galactan domain is linked to a GlcNAc from the PG, and the end of the arabinan branches anchors MA. Figure 9 represents the structure of mycobacterial AG.

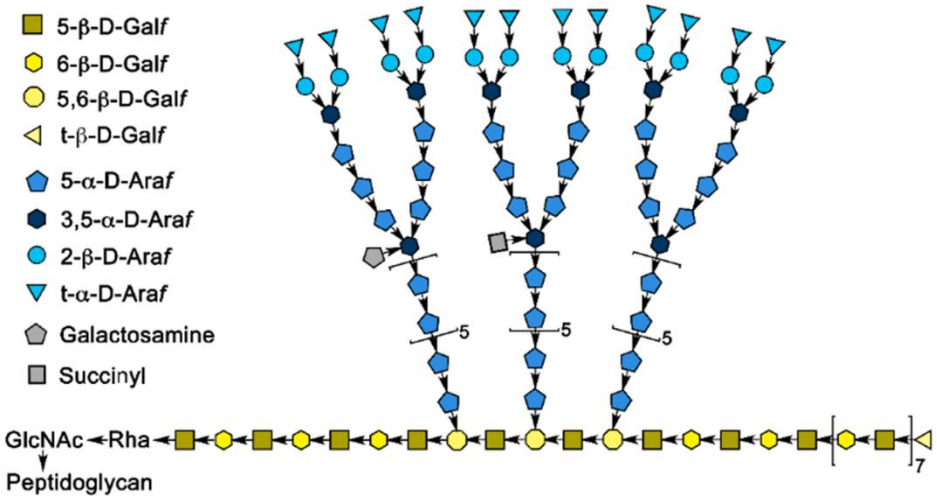


Figure 9 - Representation of the structure of the mycobaterial arabinogalactan. Adapted from Minnikin et al, 2015.

After reorientation of the galactan domain to the outside of the cell, EmbA and EmbB, are two out of four arabinosyl transferases responsible for the α(1→5) Ara*f* polymerization (Abrahams and Besra, 2016). Ethambutol, Figure 10, an important 1$^{st}$-line anti-TB drug, inhibits these two enzymes, restraining the polymerization of the arabinan domain and therefore compromising the cell envelope integrity (Rather and Maqbool, 2017). This is usually a bacteriostatic antibiotic and helps to increase cell permeability to other drugs (Rastogi *et al.*, 1998). Resistance to ethambutol is associated

to mutations in the *embABC* locus, and there is evidence that some mutations on the *embB* gene can lead to the development of *Mtb* resistance to other anti-TB antibiotics (Hazbón *et al.*, 2005).

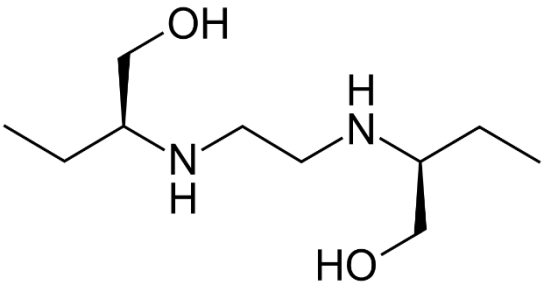


Figure 10 - Chemical structure of the antibiotic ethambutol, that acts by inhibiting arabinosyl transferases, enzymes that are responsible for arabinan polymerization.

Other glycolipids and lipoglycans (PIMs, LM and LAM) are synthetized by some common enzymes involved in the AG biosynthesis (Abrahams and Besra, 2016). These are important components, abundantly present noncovalently linked on the inner and outer cell envelope, with roles on the permeability, cell division and modulation of host-pathogen interaction. Ethambutol can also inhibit the biosynthesis of some of these components (Jankute *et al.*, 2015).

## 4.3.Peptidoglycan

PG is a polymer of glycan and peptides strands, with alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic (MurNAc) residues connected by β(1→4) linkages (Alderwick *et al.*, 2015). It is an important constituent of both Gram-positive and Gram-negative bacteria, once it provides stability and rigidity to the cell envelope and plays an essential role in bacterial growth (Abrahams and Besra, 2016). Being a structure exclusively present on bacterial cells, PG is very explored as a drug target (Hett and Rubin, 2008). In mycobacteria, the PG constitutes the basal layer of the mAGP complex, being surrounded by lipids, carbohydrates, and lipoglycans, which provide a barrier against drugs (Catalão, Filipe and Pimentel, 2019).

Mycobacterial PG has some special features, such as the presence of *N*-glycolylmuramic acid, that is only reported in few other genera than mycobacteria; amidation of the carboxylic acids in the peptide stems; additional glycine and serine residues and a high level of direct peptide cross-links (Mahapatra *et al.*, 2005; Raymond *et al.*, 2005; Catalão, Filipe and Pimentel, 2019). The gene *namH* encodes an uridine diphosphate (UDP)-*N*-acetylmuramic acid hydroxylase, which converts *N*-acetylmuramic acid (MurNAc) into *N*-glycolylmuramic acid (MurNGly) on mycobacteria, is not essential for *in vitro* growth of mycobacteria, but its deletion seems

to increase mycobacteria susceptibility to β-lactams and lysozyme (Raymond *et al.*, 2005).

The biosynthesis of mycobacterial PG starts in the cytoplasm by the generation of a UDP-GlcNAc (Figure 11) (Alderwick *et al.*, 2015). Then, a sequential pathway of muramyl ligases catalyses the formation of the UDP-*N*- UDP-MurAc/Glyc pentapeptide. This pentapeptide (Figure 11) is generated by the incorporation of L-alanine (L-Ala), D-isoglutamate (D-isoGlu), meso-diaminopimelate (m-DAP) and two residues of D-alanyl-D-alanine (D-Ala) (Hett and Rubin, 2008). UDP-MurNAc/Glyc pentapeptide is then anchored to the cell membrane, forming Lipid I, which is then β(1→4) linked to GlcNAc and MurNAc/Glyc, forming the called Lipid II, the monomer of PG construction (Abrahams and Besra, 2016). After "flipping" of the Lipid II to the extracellular side of the membrane, PG is polymerized by the action of penicillin-biding proteins (PBPs) – Figure 11 (Catalão, Filipe and Pimentel, 2019).

PBPs have transglycosylase and transeptidase activities, and catalize the (3→4) cross-linkages between m-DAP and D-Ala of adjacent pentapeptide chains, with cleavage of terminal D-Ala, forming the classical DAP-type peptidoglycan (Alderwick *et al.*, 2015). On *Mtb*, about 80% of the cross-links are (3→3) links between m-DAP residues of two adjacent stems, with the release of the D-Ala on the fourth position, which are catalysed by non-classical L,D-transpeptidases (LDTs) (Catalão, Filipe and Pimentel, 2019).

Most bacterial infections can be treated with β-lactam antibiotics that target the PG biosynthesis, by binding to different PBPs (Figure 11), inhibiting its transpeptidase activity and therefore disrupting PG synthesis (Abrahams and Besra, 2016). Several bacteria produce β-lactamases, proteins that inactivate these drugs by degradation of the β-lactam ring, and mycobacteria produce a broad spectrum β-lactamase, BlaC (Abrahams and Besra, 2016). Conjugation with a β-lactamase inhibitor such as clavulanate is essential to improve β-lactams efficacy. LDTs are resistant to most β-lactams, but susceptible to carbapenems, a class of β-lactams which are a poor substrate to BlaC (Papp-Wallace *et al.*, 2011). Some studies have been exploring this feature, and the combination of carbapenems with β-lactamase inhibitors seems to be effective against MDR and XDR-TB (Hugonnet *et al.*, 2009). Resistance can also arise from mutations in the targets genes, and from efflux pumps that actively transports the drugs out of the cell (Green and Garneau-Tsodikova, 2013).

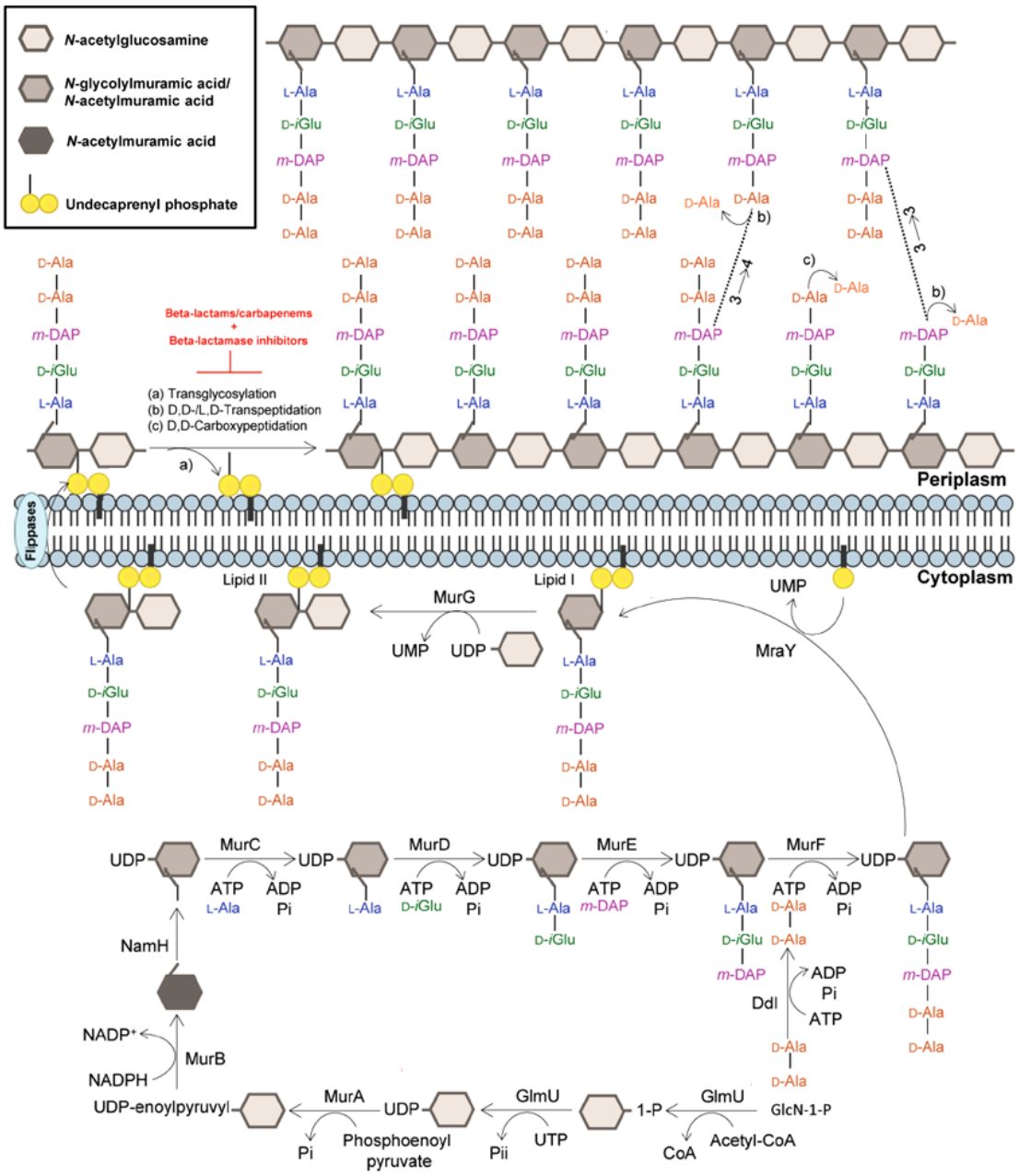


Figure 11 - Mycobacterial peptidoglycan structure and biosynthesis. First steps and precursor formation occurs at the cytoplasm, and polymerization happens at the outer layer of the cytoplasmatic membrane. β-lactams and β-lactamases inhibitors, in red, are inhibitors of peptidoglycan synthesis. Adapted from Catalão, Filipe and Pimentel, 2019.

## 5. β-lactam antibiotics

Since the discovery of penicillin in 1928, β-lactams are one of the most used class of antibiotics against bacterial pathogens (Cho, Uehara and Bernhardt, 2014). However, β-lactams are not usually included in TB treatment regimens, because of the mycobacterial singular and impermeable cell envelope, which provides intrinsic resistance to antibiotics, and the potent β-lactamase BlaC (Solapure *et al.*, 2013). With the emergence of MDR and XDR-TB cases, the need for alternative therapeutics is urgent and the discovery and

development of new drugs is not capable to follow the pace. Recently, the application of β-lactams on MDR and XDR-TB has been revisited, with the intention of repurposing well studied and established drugs on TB treatment (Cohen *et al.*, 2016).

The structure of β-lactams consists in a core β-lactam ring, and variable side groups (Figure 12). The side group defines the subclass of the antibiotic, which can be a penicillin derivative, a cephalosporin, a carbapenem, a monobactam or a β-lactamase inhibitor.

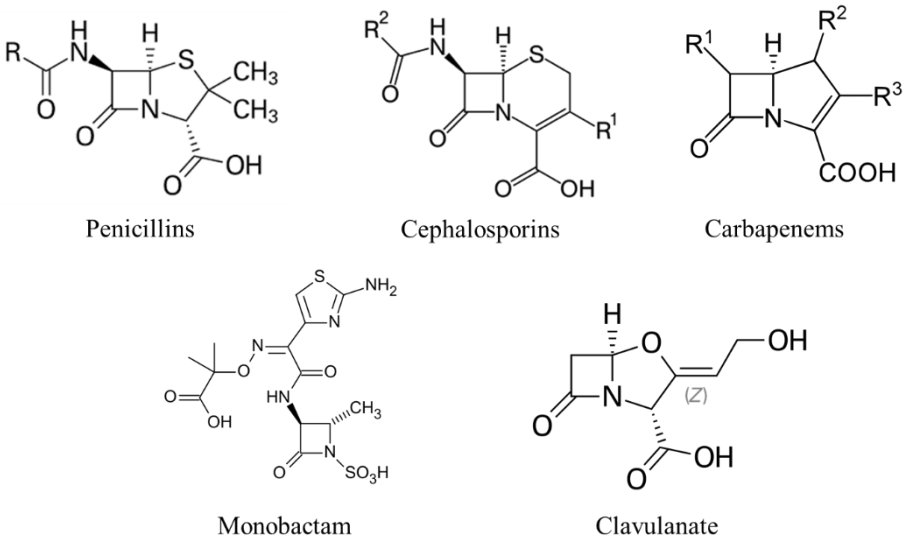


Figure 12 - Subclasses of β-lactam antibiotics. R stands for side groups. Monobactam (Aztreonam) is only effective against aerobic Gram-negative bacteria and will no longer be mentioned on this work. Clavulanate is a β-lactamase inhibitor, despite of having a β-lactam ring on its structure, it is not considered as an antibiotic.

## 5.1.Penicillin derivatives

Penicillin is the oldest antibiotic, which was accidentally discovered by Alexander Fleming and isolated from the fungus *Penicillium* and has become an essential tool in the fight against bacterial infections (Rolinson, 1979). The structure of a penicillin consists in a 6-aminopenicillanic acid (6-APA) with a β-lactam ring and variable side chains. As other β-lactams, penicillin acts indirectly in the inhibition of the crosslinking of PG, by binding to PBPs because of its conformation similar to terminal D-Ala-D-Ala of the pentapeptide (Virudachalam and Rao, 1977; Rolinson, 1979).

Amoxicillin (Figure 13) is an amino-penicillin and is one of the most used antibiotics against both Gram-positive and Gram-negative bacteria. It is a semi-synthetic penicillin, that inhibits D,D-transpeptidases, with a broader activity than the penicillin, and a better oral absorption than ampicillin (the first semi-synthetic penicillin) (Sutherland, Croydon and Rolinson, 1972; Zaffiri, Gardner and Toledo-Pereyra, 2012). Although amoxicillin is a bactericidal antibiotic, bacteria have rapidly found a way to overcome the drug activity, by producing β-lactamases that degrade the antibiotic.

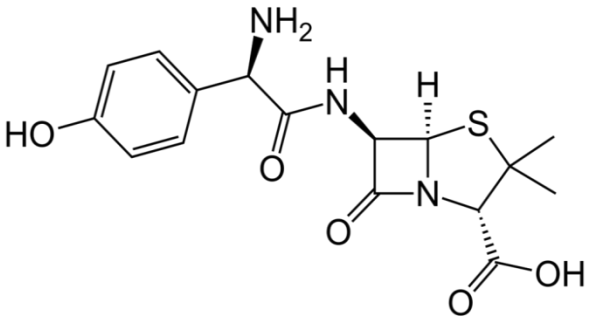


Figure 13 - Chemical structure of the antibiotic amoxicillin, a penicillin derivative applied in the treatment of various bacterial infections.

## 5.2. Cephalosporins

Cephalosporins are derivatives from a substance produced by the fungus *Cephalosporium*, which has a 7-aminocephalosporanic acid (7-ACA) instead of a 6-APA as principal side group, having an action mode similar to penicillins. These antibiotics are usually parenterally administered (intravenous or intramuscular) and are divided in five groups, from first to fifth generation, differing in the antibacterial spectrum, stability, pharmacokinetics, pharmacodynamics and side-effects (Craig and Andes, 2015). First-generation cephalosporins are active against Gram-positive bacteria, while second-generation have a wider spectrum, with activity in some anaerobic Gram-negative bacteria. Third generation cephalosporins are more potent against Gram-negative bacilli and fourth generation cephalosporins have even a broader activity. The fifth-generation group includes cephalosporines active against methicillin resistant *Staphylococcus aureus* (MRSA) but have a similar activity to third generation cephalosporins when treating Gram-negative bacteria. Third, fourth and fifth generation cephalosporins have the important capacity of reaching the central nervous system (Zaffiri, Gardner and Toledo-Pereyra, 2012; Craig and Andes, 2015).

Cefotaxime (Figure 14) is one of the third generation cephalosporins, and is a potent antibiotic applied in the treatment of nosocomial infections, with activity against penicillin-resistant pneumococci and the anaerobial *Bacteroides fragilis* (Klein and Cunha, 1995; Craig and Andes, 2015). Cefotaxime is one of the third generation

cephalosporins with best activity against Gram-positive bacteria (Klein and Cunha, 1995). However, the frequency of organisms with wide spectrum β-lactamases is increasing and can overhang the antibiotics activity.

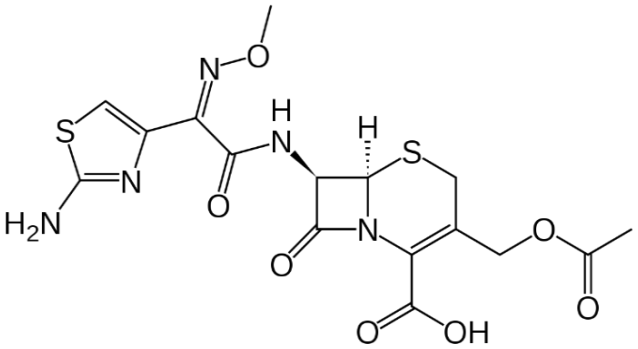

Figure 14 - Chemical structure of the antibiotic cefotaxime, a broad spectrum third-generation cephalosporin used against Gram-positive and penicillin-resistant bacteria infections.

## 5.3. Carbapenems

Carbapenems are, amongst the class of β-lactam antibiotics, the group with greater antimicrobial spectrum and potency (Papp-Wallace *et al.*, 2011). These are inhibitors of L,D-transpeptidases active against both Gram-positive and Gram-negative bacteria, and are especially resistant to β-lactamase action, being slow-substrates or sometimes inhibitors of these proteins. For that reason, carbapenems are mostly used as last line agents for resistant infections (Bradley *et al.*, 1999).

The first carbapenem, thienamycin, was isolated from *Streptomyces catlleya* in the late 1970s and served as parent compound to a wide diversity of more stable and effective antibiotics (Kahan *et al.*, 1979). Imipenem (Figure 15) was the first thienamycin derivative and shows strong potency against serious infections like sepsis, nosocomial pneumonia or polymicrobial diseases (Bradley *et al.*, 1999). This antibiotic is stable to the action of most β-lactamases but can be hydrolysed by extended spectrum β-lactamases (ESBL), produced by some Enterobacteriaceae and transferable by plasmids, and by metallo-β-lactamases, also called carbapenemases (Rodloff, Goldstein and Torres, 2006). Imipenem must be administered in combination with cilastatin, an inhibitor of the human dehydropeptidase-1 (DHP-1), present in the kidney and capable of degradation of the antibiotic (Rodloff, Goldstein and Torres, 2006).

Meropenem (Figure 15) is a parenteral antibiotic, considered to have a slightly higher activity against Gram-negative bacteria but a lower bactericidal effect on slow-growing or static bacteria (Wiseman and Balfour, 1994) – actually, this is expected to happen to any β-lactam, because these antibiotics acts on the active growing phase of the microorganisms. Meropenem appears to be more resistant to β-lactamases compared to imipenem and has the advantage of not being susceptible to DHP-1 (Wiseman and Balfour, 1994; Bradley *et al.*, 1999).

Other mechanisms of resistance to these antibiotics include efflux pumps and mutations that lead to altered function or structure of PBPs or porins – increased impermeability (Papp-Wallace *et al.*, 2011).

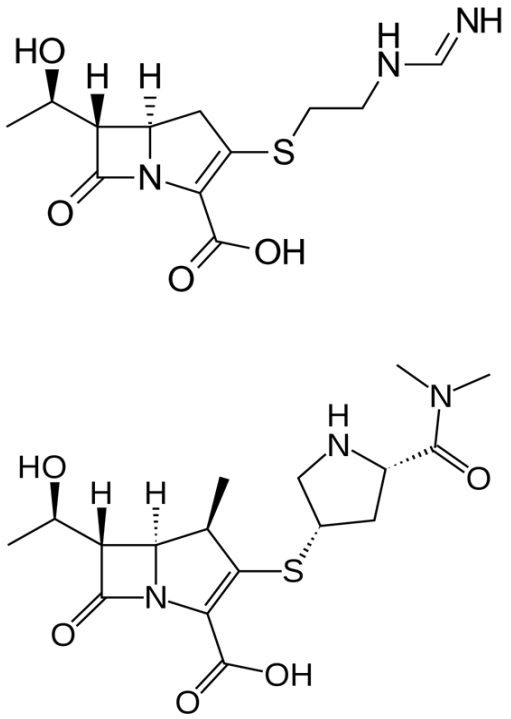


Figure 15 - Chemical structure of carbapenems. Up: imipinem, down: meropenem. These antibiotics are applied in the treatment of serious bacterial infections.

Several more recent carbapenems like biapenem, ertapenem or dorapenem, show even wider spectrum of activity and can be promising tools in the treatment of drug resistant infections, such as ESBL producing bacteria (Bassetti *et al.*, 2009). Carbapenems are used in combination therapies, which can be the way to overcome resistance emergence, when the combined drugs act synergistically (Seaworth and Griffith, 2017).

## 5.4.β-lactamase inhibitors

Although having a β-lactam like structure, β-lactamase inhibitors are not considered antibiotics. These compounds are able to bind covalently to bacterial β-lactamases, hindering its enzymatic activity, preventing the degradation of β-lactam antibiotics and therefore enhancing the activity of the drug (Drawz and Bonomo, 2010). These agents are used in combination with β-lactamase susceptible antibiotics, in order to treat infections caused by β-lactamase producing bacteria (Zaffiri, Gardner and Toledo-Pereyra, 2012). Clavulanate (Figure 12) is used in combination with a wide variety of β-lactam antibiotics, since it inhibits β-lactamases produced by most of penicillin and cephalosporin-resistant bacteria, reverting the resistant profile of the microorganism

(Rolinson, 1979). It is also capable of inhibiting both slow and rapidly-growing mycobacteria β-lactamases (Soroka *et al.*, 2016).

## 6. Evidence of β-lactams efficacy on MDR/XDR-TB treatment

Although this class of antibiotics is not included in the WHO guidelines for TB treatment, the emergence of resistance at fast pace obligates clinicians and investigators to find alternative therapies (Solapure *et al.*, 2013). So far, amoxicillin in combination with clavulanate is held as a last resource on TB treatment because of the lack of proven efficacy data, but recently several studies have revisited the application of antibiotics as meropenem on TB treatment (Gonzalo and Drobniewski, 2013). Cohen and colleagues (2016) studied the effect of meropenem and amoxicillin combined with clavulanate in *Mtb* clinical isolates and in reference strains, observing that MDR and XDR-TB isolates were more susceptible to β-lactams than the reference strains, which were susceptible to 1$^{st}$- and 2$^{nd}$-line anti-TB drugs. Once all the strains belonged to phylogenetic groups with intrinsic amoxicillin resistance, they concluded that this was an acquired susceptibility and noted that the presence of clavulanate was essential for β-lactamase inhibition. Meropenem-clavulanate has been studied by other investigators, with good results (Hugonnet *et al.*, 2009). A retrospective study concluded that the majority of patients with XDR-TB treated with meropenem and clavulanate had successful outcomes, supporting its safety and efficacy (Payen *et al.*, 2018). Imipenem has also shown good outcomes when given to patients with MDR-TB treatment failure (Seaworth and Griffith, 2017) and recent reviews gather information that supports the potential of exploring β-lactams in the mycobacterial context (Sotgiu *et al.*, 2016; Catalão, Filipe and Pimentel, 2019).

Evidence shows that there is a pattern of increased susceptibility to β-lactams on strains that are resistant to other anti-TB drugs. It has been hypothesized that mycobacteria treatment with 1$^{st}$-line anti-TB antibiotics leads to an increased exposure of the inner layer of the cell envelope improving β-lactams access to the PG. Being applied in combination with other anti-TB drugs, β-lactams, specially carbapenems, could be a way to reduce the duration of TB treatment. The biggest challenge so far for the widespread use of this antibiotics are the lack of efficacy and safety data, the high cost and intravenous dosing (Cohen *et al.*, 2016; Catalão, Filipe and Pimentel, 2019).

## 7. Mycobacterial drug susceptibility determination

When a patient has symptoms of TB disease, it is critical to have a quick diagnosis and to know the susceptibility profile of the infection, in order to choose the adequate treatment and for surveillance of TB (Forbes *et al.*, 2018). Since *Mtb* is a slow-growing mycobacteria, microbiological tests such as culture isolation and tests by tube dilution take at least one month, and although these are the gold standard methods, fastest answers are needed specially when the burden of MDR-TB is high (Reller, Weinstein and Woods, 2000). Drug susceptibility testing (DST) is included in the pillars of the END TB strategy and several molecular based tests have been developed to help screen and report RR/MDR/XDR-TB (World Health Organization, 2018a).

The tube dilution test consists in the inoculation of mycobacteria in liquid or solid laboratory media with a known concentration of the antibiotic to be tested. Serial dilutions are made and after incubation, the lowest concentration of antibiotic without visible bacterial growth is annotated as the minimum inhibitory concentration (MIC). When testing a high number of bacterial strains with more than one antibiotic, this method can be very laborious and to overcome this, a micro dilution test is the best alternative. The serial dilutions of antibiotics are made in a microtiter plate with liquid media and after incubation readings can be done visually or by spectrophotometer (Wiegand, Hilpert and Hancock, 2008).

Additionally, it is important to determine the minimum bactericidal concentration (MBC), to assure if the antibiotic is effectively killing or only inhibiting bacterial growth. After MIC determination, the wells from the microtiter plate where no growth was observed must be tested in laboratory media without antibiotic, and after further incubation, if no bacterial growth is observed, the antibiotic tested had a bactericidal effect. On the other hand, if the bacteria recover and grow, the effect was bacteriostatic. (Bettencourt *et al.*, 2010).

### 7.1. Synergistic effect between antibiotics

Combination therapies are usually applied to minimize the probability of resistance emergence, which easily happens in monotherapies (Sotgiu *et al.*, 2015). Prior to clinical treatment it is essential to understand if the antibiotics combined have a synergistic effect and enhance the bactericidal effect of the treatment. Although uncommon, antagonistic

effects may happen, being a very disadvantageous scenario in clinical settings (Acar, 2000).

In the context of the work presented here, an innovative approach was designed to test synergistic effects between antibiotics. We intended to observe the effect of two 1st-line anti-TB drugs that target the biosynthesis of the outer layer of the cell envelope, with β-lactams, that target the PG biosynthesis. To test this, there are three possibilities of combination strategies: i) the antibiotics can be given simultaneously, acting on different targets at the same time; ii) mycobacteria can be treated with the 1st-line anti-TB drugs at sub inhibitory concentrations, being weakened and possibly with exposition of the PG layer for posterior treatment with β-lactams only; iii) the treatment with 1st-line anti-TB drugs can be previous and simultaneous to the assay with the β-lactams. When the MIC value of the antibiotics combined is lower than the antibiotics alone, it is considered to have a synergistic effect. This type of interaction between anti-TB drugs and β-lactams has been studied for *Corynebacterium glutamicum*, and it was shown that the exposure of bacteria to ethambutol causes a depletion of the mAGP layer, exposing PG for an enhanced β-lactam activity (Schubert *et al.*, 2017).

## 8. Objectives

As mentioned, the emergence of MDR and XDR-TB is a major health concern and measures must be taken in order to develop new diagnosis and treatment strategies. The development of new antibiotics can take years from research to clinical use, and the concept of applying old antibiotics for TB treatment has been revisited. The main goals of this project are: i) to determine how mycobacteria susceptibility to β-lactams, an old and well characterized class of antibiotics, can be enhanced after treatment with 1st-line anti-TB drugs that target the cell envelope biosynthesis; ii) to address how mycobacterial PG recognition by specific receptors is enhanced after exposition to ethambutol and isoniazid. To achieve this, the specific objectives are:

- To determine the MIC and MBC of two slow growing mycobacteria (*M. bovis* BCG and *M.tuberculosis* H37Ra), and two rapidly growing mycobacteria (*M. smegmatis* $mc^2155$ and *M. fortuitum* ATCC6841) to four β-lactams (amoxicillin, cefotaxime, meropenem and imipenem) with and without the addition of clavulanate, a β-lactamase inhibitor; and to isoniazid and ethambutol, two 1st-line anti-TB drugs that target MA and AG biosynthesis, respectively;

- Study how MA and AG biosynthesis inhibition by isoniazid and ethambutol, respectively, improves the accessibility of PG to β-lactams, that target its biosynthesis, by treatment of mycobacteria with sub-MIC of the 1$^{st}$-line anti-TB drugs and determination MIC and MBC of amoxicillin and meropenem, with and without clavulanate.
- Confirm the accessibility of mycobacterial PG by binding to PGRP fluorescent derivatives (mCherry) and determine the effect of treatment with sub-MIC of isoniazid and ethambutol on PG recognition.

The data obtained with this work is valuable and will be used as a starting point for the study with clinical isolates of MDR and XDR *Mtb* strains, and later can be a promising therapy option for TB treatment. The use of antibiotics with well-studied tolerability is a fast solution for the treatment of drug resistant TB and can help to lower therapeutics duration.

# Material and Methods

## 1. Mycobacterial strains and culture conditions

Since virulent *M. tuberculosis* strains or clinical isolates must be handled in a BSL-3 laboratory, special procedures, training and authorizations would be required, making work more time-consuming and cumbersome. To overcome this, it was decided that this study would be realized with non-pathogenic and avirulent mycobacteria that serve as models for *Mtb* and can be manipulated in a BSL-2 laboratory. Assays have been optimized for future application on *Mtb* H37Rv reference strain and clinical isolates present in the BSL-3 of the Faculty of Pharmacy of the University of Lisbon.

Four mycobacteria species were used, to explore how differences on the cellular envelope might influence antibiotic effects:

*Mtb* H37Ra is the avirulent/attenuated version of *Mtb* H37Rv, which was isolated in 1935 by William Steeken. Although these two strains have genomic and proteomic differences, studies show that *Mtb* H37Ra is a good surrogate for *Mtb* H37Rv strain and for clinical isolates (Heinrichs *et al.*, 2017).

*Mycobacterium bovis* Bacile Calmette-Guérin (BCG) is the attenuated version of the etiologic agent of zoonotic TB, used globally as a vaccine against TB. The strain BCG Pasteur 1173P2 was isolated at the Pasteur Institute Paris, in France and is widely used by research laboratories (Brosch *et al.*, 2007).

*Mycobacterium smegmatis* is a non-pathogenic NTM and rapidly growing species used as a *Mtb* model in genetic studies. The efficient-plasmid-transformation mutant strain $mc^2155$, that has a high transformability and less clumping properties than most mycobacteria species, was used (Etienne *et al.*, 2005).

*Mycobacterium fortuitum* is a rapidly growing and opportunistic NTM, that usually causes skin, bone and tissue infections on immunocompromised individuals. The strain ATCC 6841 was used (American Type Culture Collection, 2018).

Additionally, two strains of *Staphylococcus aureus* were used for quality control of β-lactam antibiotics activity, once susceptibility/resistance breakpoints for this species are described. The strain ATCC 43300 that is oxacillin and methicillin resistant (MRSA) derived from a clinical isolate; and the strain FF219 that was isolated in the Faculty of Pharmacy of the University of Lisbon and is susceptible to all β-lactam antibiotics, were used. MIC values from the European Committee on Antimicrobial Susceptibility Testing

(EUCAST) were used as reference to assure the quality of β-lactam antibiotics (EUCAST: European Committee on Antimicrobial Susceptibility Testing, 2019).

Aliquots of bacterial suspensions with 20% (v/v) of sterile glycerol (Invitrogen) solution were made and stocks were stored at -80°C until needed.

For mycobacteria growth, media must be enriched, and OADC is a commonly used supplement. OADC contains oleic acid, used on mycobacteria metabolism; albumin, that protects bacteria from toxic agents and helps the recovery on primary isolation; dextrose, which serves as energy source; catalase, that destroys toxic peroxides often present on the media; and sodium chloride (NaCl) to maintain osmotic equilibrium. This supplement was prepared in the laboratory following the formulae from Difco (Systems, 2009): 50 g of bovine albumin Fraction V (NZYtech), 20 g of dextrose (Sigma Aldrich), 8,5 g of NaCl (NZYtech), 0,03 g of catalase (Sigma Aldrich) and 600 μL of oleic acid (AlfaAesar) were dissolved in 1 L of purified water by magnetic agitation for 1 to 2 hours, filtered to a sterile flask overnight (VWR Vacuum Filtration 0,2 μm PES filter) and stored at 4°C for up to 3 months.

For 1 L of 7H10 agar culture media, 19 g of dehydrated 7H10 agar (Difco Middlebrook) culture and 2,5 mL of glycerol were dissolved in 900 mL of distilled water and sterilized by autoclave (15 minutes at 121°C). Sterile media was melted and after adding 100 mL of OADC supplement, distributed in petri plates. After solidification, plates were maintained at 4°C.

For 1 L of 7H9 broth media, 4,5 g of dehydrated 7H9 broth (Difco Middlebrook) and 1 mL of glycerol were dissolved in 900 mL of distilled water and sterilized by autoclave. Media was maintained at 4°C.

Bacterial stocks were inoculated on 7H10 agar plates, incubated at 37°C with 5% $CO_2$ (v/v) and after visible growth a colony was inoculated in 7H9 media supplemented with 10% OADC (v/v) and 0,05% tyloxapol (v/v) (Sigma Aldrich), a detergent used to prevent clump formation. To further eliminate clumps, sub-cultures were made and used when desired optical density at 600 nm ($OD_{600nm}$) was reached. Optical density was spectrophotometrically measured (WPA S1200 visible diode-array spectrophotometer Biochrom). Incubations were made at 37°C with 5% $CO_2$ (v/v) for two to three weeks for slow growing mycobacteria, three to four days for *M. fortuitum* and one to two days for

*M. smegmatis*. Rapidly growing mycobacteria were incubated with agitation (160 rpm maximum).

## 2. Antibiotics stocks

Ethambutol, amoxicillin, cefotaxime, meropenem, and imipenem (Sigma Aldrich) stocks were made by dissolving each one on purified water to a final concentration of 1,28 mg/mL, having in account purity degrees. When needed, solutions were shaken at 37°C for 5 minutes. After complete dissolving, solutions were filtered through a 0,2 μm filter and aliquots stored at -80°C up to 6 months.

Clavulanate (Sigma Aldrich) was dissolved in 0,1 M phosphate buffer pH 6,0 (Sigma Aldrich) to 10 mg/mL and stored as other antibiotics.

Isoniazid (Sigma Aldrich) was available at the laboratory in 10 mg/mL stocks, stored at -20°C. Ten-fold and hundred-fold dilutions of these stocks were made in purified water and stored at 4°C for up to one month.

## 3. Minimum inhibitory and bactericidal concentrations (MIC and MBC) assays

### 3.1. Quality control of β-lactam antibiotics

As quality control, the broth microdilution method (Wiegand, Hilpert and Hancock, 2008) was applied to determine the MIC for two different strains of *Staphylococcus aureus*: FF219, a susceptible strain and ATCC 43300, a resistant strain. The assay was adapted from (Wiegand, Hilpert and Hancock, 2008), using Mueller-Hinton infusion (MHI) (Sigma Aldrich) supplemented with 4% NaCl 1 M (v/v) (NZYtech) and tryptic soy agar (TSA) (Sigma Aldrich) plates for colony forming units (cfu)/mL counting.

Bacteria were inoculated in TSA plates, from -80°C stocks, and incubated at 37°C. After visible growth, one colony of each was inoculated in 3 mL of MHI 4% NaCl (v/v) and incubated at 37°C with agitation (160 rpm) overnight.

96 wells microtiter plates (Sarsted) were prepared by adding 100 μL of MHI 4% NaCl (v/v) per well, except in the first row. On wells where β-lactams were going to be tested conjugated with clavulanate, it was added in the media at 5 μg/mL. Antibiotics solutions were prepared by a ten-fold dilution of stocks in MHI 4% NaCl (v/v), to a concentration of 128 μg/mL, with and without clavulanate at 5 μg/mL. In the first row of

each plate, 200 µL of each antibiotic were added and two-fold consecutive dilutions were made, except in the last two rows that served as controls without antibiotics. After bacterial addition, final concentration of antibiotics on the first well was 64 µg/mL and clavulanate was constant at 2,5 µg/mL.

When bacterial cultures were on the exponential phase, with an $OD_{600nm}$ between 0,8 and 1, bacteria suspensions were prepared by dilution in MHI 4% NaCl (v/v) to have $6\times10^5$ cfu/ml, considering the growth curve of this bacteria (Squiggins *et al.*, 1990), in which an $OD_{600nm}$ of 0,001 corresponds to approximately $3\times10^5$ cfu/mL. The suspensions were then distributed on the respective plates, 100 µL per well, except in the last row that served as negative control, without antibiotic and bacteria. The row with bacteria and no antibiotics was the positive control and cfu's were counted by inoculation of two consecutive ten-fold dilutions ($10^{-2}$ and $10^{-3}$) of this well in TSA plates. This procedure is outlined on Figure 16. Plates were incubated at 37°C and 5% $CO_2$ (v/v) without agitation and results were read after 18 or 24 hours. MIC value was annotated as the first antibiotic concentration without visible bacterial growth. This assay was done whenever new stocks of β-lactams were prepared, and results are presented on Annex 1.

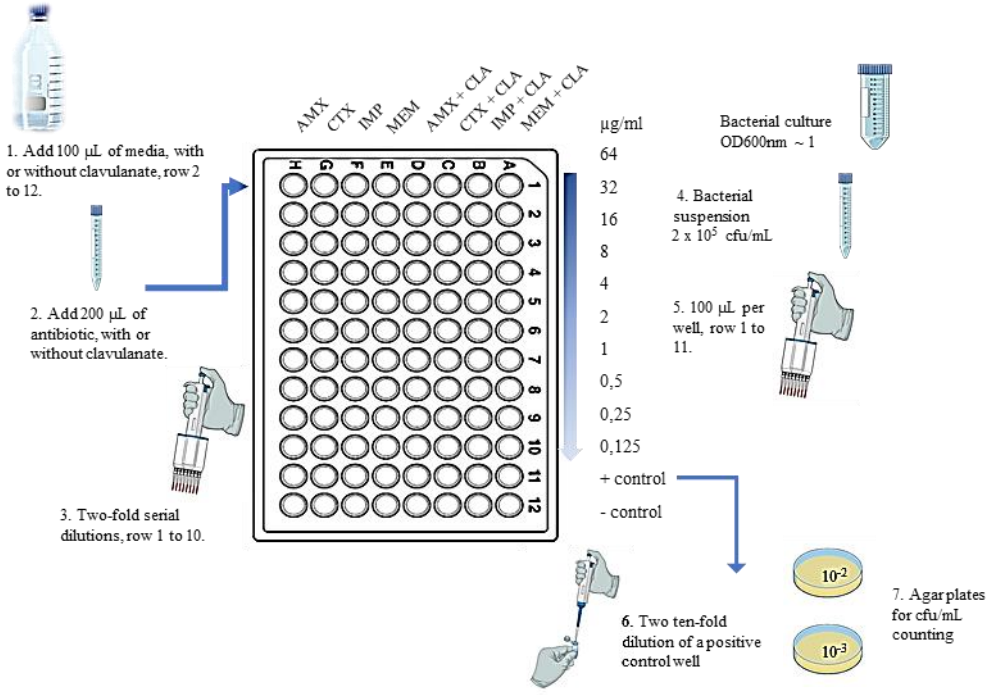


Figure 16 - Representation of the protocol for MIC determination of *S. aureus* resistant (ATCC 43300) and sensitive (FF219) strains, as quality control of β-lactam antibiotics. All plates were incubated at 37°C with 5% $CO_2$ between 18h and 24h.

## 3.2.Mycobacteria MIC determination

The protocol for MIC determination of mycobacteria was adapted from (Wiegand, Hilpert and Hancock, 2008) using 7H9 broth media supplemented with 10% OADC (v/v) and 0,05% (v/v) tyloxapol for bacterial growth, the same media without tyloxapol during the assay, and 7H10 agar with 10% OADC and 0,25% glycerol (v/v) plates for cfu/mL counting.

96 well microtiter plates were prepared by adding 100 μL of 7H9 media with 10% OADC (v/v) per well, with clavulanate at 5 μg/mL where β-lactams were going to be tested. On the first row of the plates, instead of the media, 200 μL of each antibiotic solution was added. Ethambutol and isoniazid initial concentration for all four species was 128 μg/mL, as were β-lactams for slow-growing mycobacteria. For rapidly growing mycobacteria, β-lactams were prepared at an initial concentration of 512 μg/mL. Two-fold serial dilutions of the antibiotics were done, except in the last two rows - that served as controls without antibiotics (Figure 17).

Mycobacterial cultures were done as described before. After bacterial growth to mid to late-log phase, corresponding to an $OD_{600nm}$ between 0,8 and 1, 2 mL of each bacterial culture were centrifuged at 3000×g for 5 minutes, resuspended in 1 mL of 7H9 media without tyloxapol, ultra-sounded for 5 minutes (Bardelin sonorex RK-52) to eliminate clumps, and centrifuged at 500×g for 1 minute to separate remaining cell aggregates. After new $OD_{600nm}$ reading of the supernatant, bacterial suspensions were prepared to have $2\times10^5$ cfu/mL, considering that an $OD_{600nm}$ of 0,001 corresponds to $1\times10^5$ cfu/mL (Peñuelas-Urquides *et al.*, 2013). Bacterial suspensions were added to the plates, 100 μL per well, except in the last row, that was the negative growth control. Two consecutive ten-fold dilutions, $10^{-2}$ and $10^{-3}$, of a growth control well were done and inoculated at 7H10 agar plates. Incubation was done without agitation at 37°C and 5% $CO_2$ (v/v), for 24 hours for *M. smegmatis*, five days for *M. fortuitum*, and seven to ten days for BCG and *Mtb* H37Ra. MIC values were annotated as the lowest concentration of antibiotic without visual bacterial growth. The assay was done with amoxicillin, cefotaxime, imipenem and meropenem (with and without clavulanate), isoniazid and ethambutol at least three times for each species and median value and ranges were calculated.

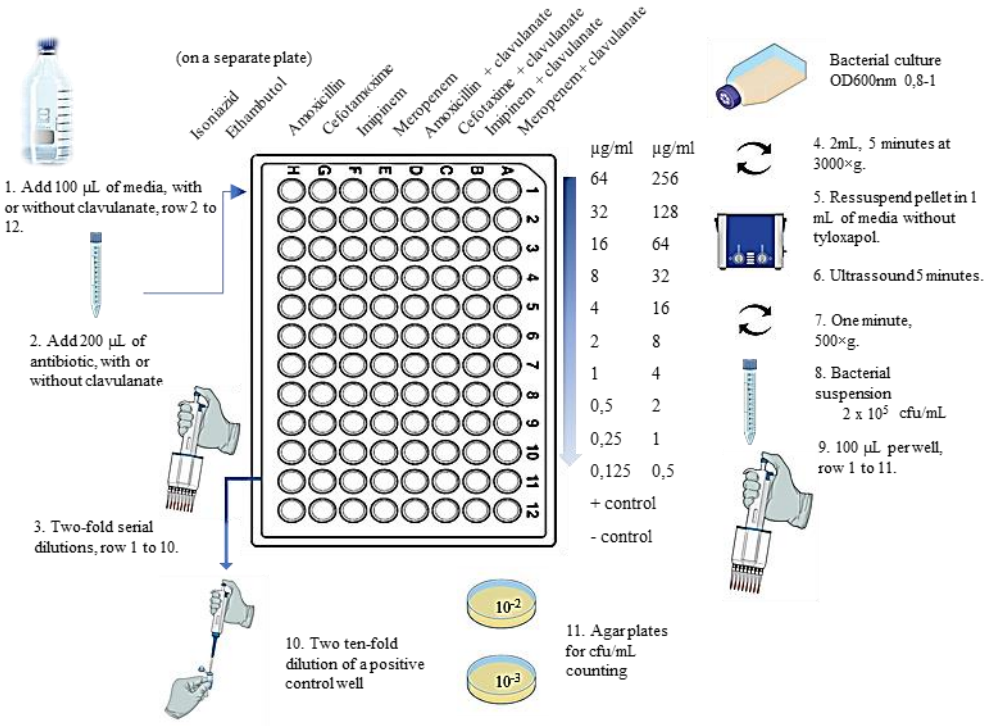


Figure 17 - Representation of the protocol for MIC determination for *M. smegmatis* mc²155, *M. fortuitum* ATCC 6841, *M. tuberculosis* H37Ra and *M. bovis* BCG. Four β-lactams (amoxicillin - AMX, cefotaxime - CTX, meropenem - MEM and imipenem - IMP) were tested alone and conjugated with clavulanate (CLA). Isoniazid (INH) and ethambutol (EMB) were also tested. All plates were incubated without agitation, at 37°C with 5% $CO_2$ for the adequate time for each mycobacteria species.

## 3.3.Mycobacteria MBC determination

MBC values were determined by an adaptation of the micro-colony forming assay described by Bettencourt *et al* (2010). After the adequate time of incubation and MIC determination, a 100 µL sample of the MIC and two wells above (2-fold MIC and 4-fold MIC) were transferred to a new microtiter plate and a 10-fold dilution was done in sterile water (Figure 18). A drop of 5 µL of each sample was applied in a 7H10 agar plate and after drying, incubated at 37°C and 5% $CO_2$ (v/v) until visual growth or for five days for *M. smegmatis* and *M. fortuitum*, and three weeks for *Mtb* H37Ra and BCG. MBC values were considered as the lowest concentration where no visual growth was detected. If no visual growth was detected MBC was considered equal to MIC value, and the antibiotic was bactericidal in the tested conditions. If there was visual growth in all concentrations applied, MBC was considered higher than four times the MIC, and the antibiotic was bacteriostatic. MBC determination assays were performed for each MIC determination assay and median value and ranges were calculated.

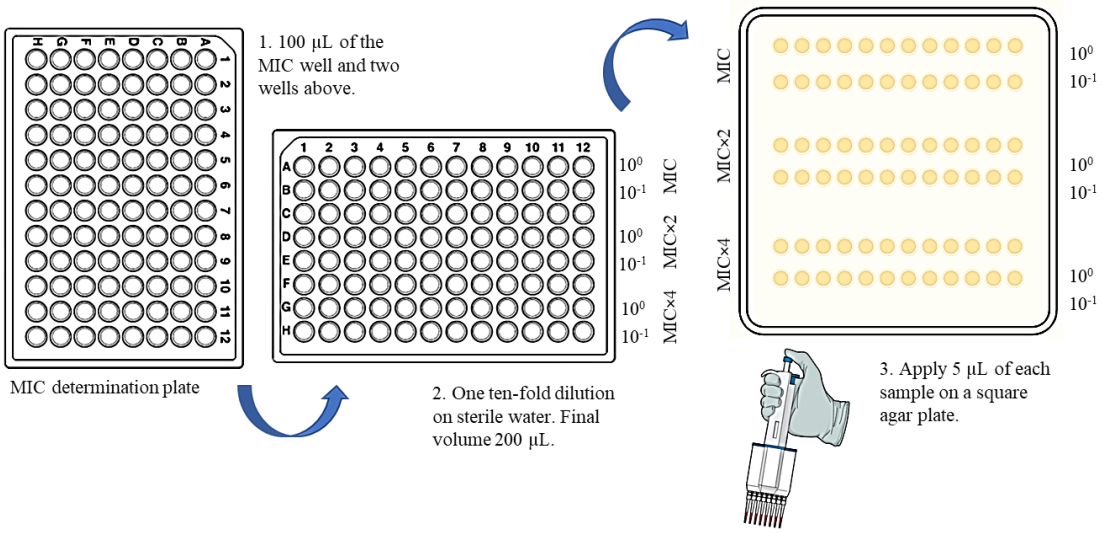


Figure 18 - Representation of protocol for MBC determination. Samples were applied in square 25 $cm^3$ 7H10 agar plates.

Table 1 has the summary of the antibiotic concentrations and incubation periods that were used for each mycobacteria species.

Table 1 - Incubation periods and concentration of the antibiotics used on MIC and MBC assays for each mycobacteria species.

| | ***M. tuberculosis* H37Ra** | ***M. bovis* BCG** | ***M. smegmatis* mc²155** | ***M. fortuitum* ATCC 43300** |
|---|---|---|---|---|
| **Period of culture incubation** | 7-10 days | | 1-2 days | 2-3 days |
| **Ethambutol and Isoniazid concentrations (µg/mL)** | 64-0,125 | | | |
| **β-lactams concentrations range (µg/mL)** | 64-0,125 | | 256-0,5 | |
| **MIC assay incubation** | 7-10 days | | 1-2 days | 5-7 days |
| **MBC assay incubation** | 2-3 weeks | | 2-5 days | 1 week |

## 3.4. MIC determination of β-lactams in combination with isoniazid or ethambutol

To determine if bacteria exposure to isoniazid/ ethambutol can potentiate the efficacy of β-lactams, three different conditions were tested:

i) mycobacteria were pre-exposed to sub-MIC of isoniazid or ethambutol and MIC determination was done for β-lactams only;

ii) exposure of mycobacteria to sub-MIC of isoniazid or ethambutol was done before and during the β-lactams MIC determination assay;

iii) the MIC assay was done with both antibiotics (isoniazid/ethambutol + β-lactam), without pre-exposure.

In order to pre-treat mycobacteria, cultures were grown to an $OD_{600nm}$ between 0,3 and 0,4, corresponding to early-log phase, and divided by tubes containing isoniazid or ethambutol at two sub-MIC (median of MIC determined before divided by 2 and 4) and one without antibiotics as control.

When $OD_{600nm}$ between 0,6 and 1 was reached (mid to late-log phase), or after a week of incubation in the case of slow growing mycobacteria, cultures were centrifuged at 3000×g for 5 minutes, resuspended in 1 mL of 7H9 media without tyloxapol, ultra-sounded for 5 minutes and centrifuged at 500×g for 1 minute for clumps removal. Bacterial suspensions of $2x10^5$ cfu/mL were prepared after new $OD_{600nm}$ reading, in 7H9 media with and without isoniazid or ethambutol. MIC determination assay was done as described before for amoxicillin and meropenem with and without clavulanate. The tested conditions are listed in Table 2 and the procedure is outlined on Figure 19. As control, β-lactams alone were tested with the non-treated culture (a culture that was grown simultaneously but was not exposed to isoniazid or ethambutol).

Table 2 - Conditions applied on MIC assays for amoxicillin and meropenem with and without clavulanate, in different combinations with sub-MIC of isoniazid or ethambutol. In the experimental process, these conditions were distributed in seven microtiter plates and incubation was done at the same conditions as previous assays.

| **Pre-treatment** | | **On plate** |
|---|---|---|
| **INH** | ½ MIC | INH ½ MIC + β-lactams |
| | | Only β-lactams |
| | ¼ MIC | INH ¼ MIC + β-lactams |
| | | Only β-lactams |
| **EMB** | ½ MIC | EMB ½ MIC + β-lactams |
| | | Only β-lactams |
| | ¼ MIC | EMB ¼ MIC + β-lactams |
| | | Only β-lactams |
| **Non treated** | | INH ½ MIC + β-lactams |
| | | INH ¼ MIC + β-lactams |
| | | EMB ½ MIC + β-lactams |
| | | EMB ¼ MIC + β-lactams |
| | | Only β-lactams |

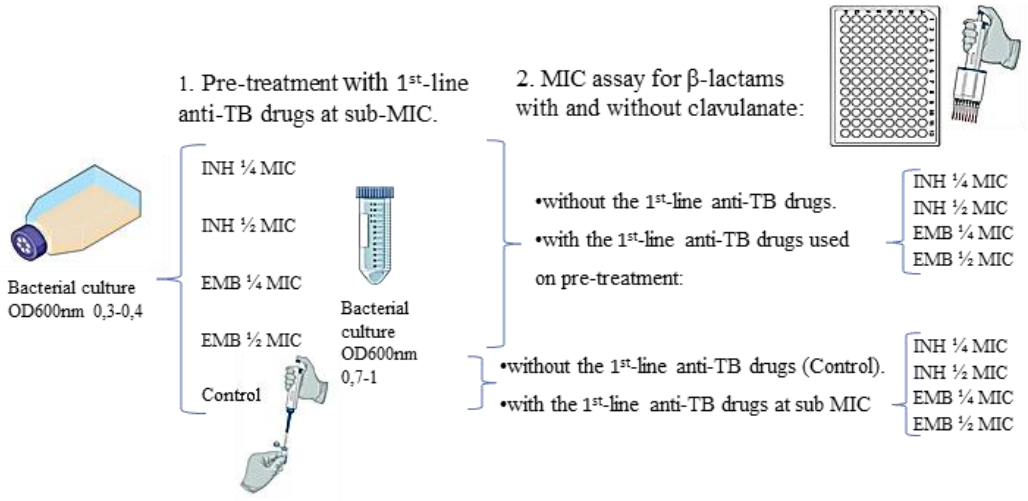


Figure 19 - Representation of the protocol of different combinations between pre-treatment of mycobacteria with sub-MIC of isoniazid or ethambutol, and consequent MIC determination for β-lactams.

The plates were incubated at 37°C and 5% $CO_2$ (v/v) for the adequate period for each species and MBC determination assays were done as before. It was considered that a synergistic effect was present when the MIC and/or MBC values of the combination of antibiotics were significantly lower than β-lactams MIC and MBC values when alone.

## 4. Peptidoglycan binding assays

To address if in mycobacteria treated with sub-MIC of isoniazid or ethambutol the peptidoglycan layer is more exposed, binding of fluorescent derivatives of Drosophila PGRPs was determined by fluorescence microscopy. This part of the project was performed at the Bacterial Cell Surfaces and Pathogenesis Lab, ITQB NOVA.

### 4.1. *Drosophila* PGRP purification

To obtain the four PGRP fluorescent derivatives, the plasmid pET21a carrying the gene for PGRP-SA, PGRP-LC (Atilano *et al.*, 2011), PGRP-SD (Wang *et al.*, 2008), or PGRP-LE (not published) with a hexahistidine tag and a mCherry reporter, was used to transform *E. coli* BL21a. A single colony of each transformant was grown overnight in Luria-Bertani (LB) broth supplemented with 100 μg/mL of ampicillin and a dilution was made to an $OD_{600nm}$ of 0,05. Sub-cultures were incubated at 25°C with agitation (120 rpm) until an $OD_{600nm}$ of 0,5 was reached. An aliquot of 500 μL was centrifuged at 16000×g for 5 minutes, resuspended in 50 μL of sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer (NZYTech), denatured in boiling water for 5 minutes and stored at -20°C – Non induced fraction. Cultures were induced

with isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1 mM and incubated overnight at 25°C with agitation (120 rpm) and an aliquot of 500 μL was saved and treated as before – Induced fraction. Cells were centrifuged at 5400×g for 7 minutes at 4°C, washed once with 20 mL of equilibration buffer (50 mM $Na_2PO_4$ pH 7,4) and resuspended in 30 mL of equilibration buffer. Cells were lysed by two cycles of French Press at 1000 psi and the lysate was centrifuged at 20100×g for 20 minutes at 4°C. Supernatant was stored at -20°C. Pellet was resuspended in 20 mL of resuspension buffer (20 mM $Na_2PO_4$ pH 7,4, 500 mM NaCl and 8 M urea), by pipetting, stirring, vortexing and was left on a rocker in cold room for 48 to 60 hours until homogenization and then diluted to 4 M urea by addition of 20 mL of dilution buffer (20 mM $Na_2PO_4$ pH 7,4, 500 mM NaCl) and left on the rocker in cold room for further 24 hours. Resuspended pellet was centrifuged at 17600×g for 10 minutes at 4°C several times, until no pellet remained. An aliquot of 500 μL of the supernatant was stored at -20°C.

A Talon® metal affinity resin (Clontech laboratories) (2,5 mL of resin with 2,5 mL of 20% ethanol) was equilibrated by washing 3 times with 5 mL of wash solution (50 mM $Na_2PO_4$ pH 7,4, 300 mM NaCl and 4 M urea) and recovered by centrifugation at 1000×g for 5 minutes. The supernatant obtained from homogenization was incubated with 5 mL of equilibrated resin for 30 minutes on a rocker in cold room – the histidine tag of the proteins binds to the nickel of the resin. The Talon resin was recovered by centrifugation at 1000×g for 5 minutes at 4°C, supernatant was stored (if it was still coloured, a new purification could be done from it) and pellet mixed with 30 mL of wash solution for 5 minutes. After new centrifugation, an aliquot of 500 μL of the supernatant was saved and pellet was mixed with 5 mL of wash solution and transferred to elution column. Resin was washed twice with 5 mL of wash solution, once with 10 mL of 5 mM imidazole wash solution, once with 10 mM imidazole wash solution and then protein was eluted with 3 mL of elution buffer (50 mM Na2PO4 pH 7,4, 300 mM NaCl, 150 mM imidazole) – imidazole leads to histidine tag elution, and consequently the purified protein. All flow throughs were stored at -20°C.

Eluted protein was dialysed overnight against 5 L of phosphate-buffered saline (PBS) 1x pH 6,0, and aliquots were frozen with liquid nitrogen and stored at -80°C. Quantification was done using Nanodrop and a protein purity assay was done by SDS-PAGE.

## 4.2. Analysis of PGRP binding to mycobacteria by fluorescence microscopy

Purified PGRP aliquots were defrosted and centrifuged at 30000×g for 15 minutes. Quantification was done by reading of 280nm absorbance in NanoDrop. Once this equipment is set to albumin aromatic amino acids readings, values were corrected using the molar absorptivity factor of each protein (Table 3), obtained by peptide sequence analysis (Protean - Lasergene). Four different PGRPs were tested (Table 3) and *M. smegmatis* was pre-treated with isoniazid (¼ MIC) and ethambutol (½ MIC) as previously described.

Treated and non-treated (control) mycobacteria were incubated at 37°C with agitation (180 rpm) until mid to late-log phase ($OD_{600nm}$ 0,8-1), and 500 µL of the treated culture was centrifuged at 16300×g for 3 minutes. Pellets were washed with 500 µL of sterile 1x PBS. After a new centrifugation, pellet was resuspended in PBS and PGRPs were added at 0,3 mg/mL to a final volume of 200 µL. A control of each culture was done, without the addition of PGRPs (200 µL of PBS). After 5 minutes incubation at room temperature with agitation, tubes were centrifuged at 10000×g for 3 minutes, washed with 20 µL PBS, resuspended in the remaining supernatant and 5 µL of each sample applied in a 1,2% agarose patch. Observation was done in a Zeiss Axio Observer Z1 microscope equipped with a Plan-Apochromat objective (100x/1.4 Oil Ph3; Zeiss) and a Photometrics CoolSNAP HQ2 camera (Roper Scientific).

Phase and fluorescence images were analysed using Metamorph (Meta Imaging series 7.5) and ImageJ softwares.

Table 3 - Recognition patterns of each PGRP used and respective correction factors, calculated from its molar absorptivity factor (Protean-Lasergene) and reference.

| Protein | Peptidoglycan-type recognition | Correction factor | Reference |
|---|---|---|---|
| **PGRP-SA** | Lys-type/DAP-type | 0,71 | (Vaz *et al.*, 2019) |
| **PGRP-SD** | DAP-type | 0,83 | (Leone *et al.*, 2008) |
| **PGRP-LC** | DAP-type | 0,64 | (Vaz *et al.*, 2019) |
| **PGRG-LE** | DAP-type | 0,75 | (Dziarski, 2004) |

## 4.3.Mycobacterial PG purification

The purification of mycobacterial PG, an adaptation of previously described methods (Draper, Kandler and Darbre, 1987; Mahapatra *et al.*, 2005), was done from a mid-log phase culture of *M. smegmatis* $mc^2155$.

Cells were centrifuged at 16000×g for 5 minutes and washed with PBS. After resuspension on 10 mL of PBS, cells were disrupted by three passages in a FrenchPress at 1000 psi and 2% of Triton X-100 and 20mM de $MgSO_4$ were added. The sonicate was digested with 10 μg/mL of DNase and 50 μg/mL RNase for 2 h at 37°C and a cell wall-enriched fraction was obtained by centrifugation at 27000×g for 30 minutes. Pellet was resuspended in 80 mL of a 2% sodium dodecyl sulfate (SDS) solution, previously heated to 50°C, incubated for 1 hour at the same temperature with constant stirring and centrifuged at 27000×g for 30 minutes. Supernatant was discarded and the process was repeated twice. Resulting pellet was resuspended in 80 mL of PBS containing 1% SDS and 0,1 mg/mL of proteinase K and incubated at 45°C for 1 hour with constant stirring, then heated at 90°C for 1 hour and centrifuged at 27000×g for 30 minutes. Two washes were done with 1% SDS to remove proteinase K, two more with PBS and six with deionized water do remove SDS. Resulting mAGP was aliquoted in weighted tubes, extracted with ethanol-diethyl ether (1:1), dried under vacuum and weighted again to determine the yield.

To hydrolyze MA, the mAGP fraction was resuspended in 0,5% KOH in methanol and stirred at 37°C for 4 days. The mixture was centrifuged, and the pellet was washed twice with methanol and twice with diethyl ether and dried under vacuum. After yield determination, 2 mL of concentrated hydrofluoric acid (48% HF) was added to 10 mg of mAGP and incubated at 4°C for 48 hours. This was done in a 15 mL plastic falcon tube, since HF is a very corrosive and volatile acid. All manipulations were done in a fume hood, with protective wear and at 4°C to avoid fume formation. After incubation, sample was diluted by filling the tube with 100 mM Tris-HCl pH 7,0; transferred to a centrifuge tube, diluted again with the same solution and centrifuged at 22700×g at 4ºC for 30 minutes. Supernatant was discarded to a plastic bottle and pellet was resuspended in 100 mM Tris-HCl pH 7,0, centrifuged again at 17600×g at 4ºC for 30 minutes until sample pH was about 7,0-7,5, washed twice with MilliQ water and resuspended in a small volume of water and transferred to pre-weighted tubes with punctured caps. Samples were

lyophilized overnight by vacuum, without heat. After yield determination, sample was resuspended to a final concentration of 10 mg/mL and stored at -20ºC.

## 4.4.Mycobacterial PG-PGRP co-precipitation

In order to determine the binding affinity of *Drosophila* PGRPs to mycobacterial PG, pull down assays were done with purified PG from *M. smegmatis*, *S. aureus*, and *E. coli*.

PGRPs were defrosted and centrifuged for 20 minutes at 20000×g. Supernatants were transferred to new tubes and quantification was done by 280nm absorbance reading at NanoDrop (ND-100 spectrophotometer) and correction by the molar absorptivity factor (Table 3). Proteins were maintained on ice until incubation.

Purified PG from *E. coli*, *S. aureus* and *M. smegmatis* were defrosted and incubated at 30°C and 1000 rpm for 30 minutes (Eppendorf TermoMixer C) and vortexed for better homogenization.

Reaction mixes were made with PG at 200 μg/mL, protein at 0,3 μg/mL and PBS 1x pH 6,0 to a final volume of 200 μL. As unbound control, each protein was incubated with 200 μL of purified water. Mixes were incubated for 25 minutes at 25°C at 1000 rpm and centrifuged for 10 minutes at 850×g. Supernatants were transferred to new tubes (unbound fraction) and 30 μL were mixed with 30 μL of sample loading buffer 2x (NZYTech). Pellets were washed twice with 300 μL of PBS, first for 5 minutes at 3400×g and last for 2 minutes at 16400×g, and finally resuspended in 30 μL of sample loading buffer 1x. Samples were denaturated for 10 minutes at 100°C and after spin down, 20 μL of each sample were loaded in a 0,75 mm 10% acrylamide SDS gel, with a protein ladder mass marker (NZYColour Protein Marker II NZYTech). Electrophoresis was done at 80V for 25-30 minutes, or until samples reached resolving gel, and at 120V for 45-60 minutes, or until samples reached the end of the gel. Gels were stained with Bluesafe 1x (NZYTech) and photographed on ChemiDoc. Binding was quantified using Image Lab software (BioRad).

# Results and Discussion

## 1. Determination of MIC and MBC of antibiotics that target the mycobacterial cell wall biosynthesis

As some recent studies have been exploring the effect of β-lactams on *Mtb* (Hugonnet *et al.*, 2009; Gonzalo and Drobniewski, 2013; Cohen *et al.*, 2016), the aim of this thesis is to study if mycobacteria susceptibility to β-lactams is enhanced after exposure to 1st-line anti-TB drugs that target the cell envelope biosynthesis, such as isoniazid and ethambutol. Four mycobacteria species were used as model: two slow-growing and avirulent species – *M. tuberculosis* H37Ra and *M. bovis* BCG – and two rapidly growing that are non-pathogenic or opportunistic pathogens – *M. smegmatis* $mc^2155$ and *M. fortuitum* ATCC 6841. Firstly, MIC and MBC of the different antibiotics were determined by the microdilution broth method and the micro cfu's assay. At least three assays for each mycobacteria species were done with the following antibiotics: amoxicillin, a penicillin derivative; cefotaxime, a third generation cephalosporin; imipenem and meropenem, carbapenems; ethambutol and isoniazid, 1st-line anti-TB drugs that are inhibitors of AG and MA biosynthesis, respectively. All four β-lactams were tested with and without the β-lactamase inhibitor clavulanate. The results were read visually and the lowest antibiotic concentration for which no detectable bacterial growth was observed, was annotated as the MIC or MBC; the median values, ranges and the reason between MBC and MIC were calculated. Median and ranges were chosen over mean and standard deviation because the concentrations tested were pre-defined discrete values and the median parameter is not as skewed by outliers values as the mean might be (Bill Brown, 2018). The MBC determination is used to classify the antibiotic as bactericidal or bacteriostatic, which is the case of the MBC/MIC ratio being higher than 4 (Wallace, Dalovisio and Pankey, 1979).

EUCAST does not have an established reference methodology to test mycobacterial breakpoints of susceptibility to β-lactams, therefore PK-PD breakpoints were used (Table 4) (EUCAST: European Committee on Antimicrobial Susceptibility Testing, 2019). These are non-species related values, used when no species-specific breakpoints are available. Breakpoints for the combination with clavulanate are only available for

amoxicillin and are the same as the antibiotic alone, therefore are not shown. These values were used to classify mycobacteria as susceptible or resistant to the β-lactams, although it is important to note that assays with mycobacteria have an increased variability due to lack of synchrony in the culture and slow division time. For isoniazid and ethambutol, critical concentrations for *Mtb* determined in 7H10 media were used as reference (Schön *et al.*, 2017). Once 1:2 dilutions of the antibiotics were made, concentrations are presented in a $\log_2$ scale, and comparisons between results were made by the difference of dilutions/logs.

Table 4 - EUCAST PK-PD breakpoints for β-lactams and critical concentrations for isoniazid and ethambutol. All concentrations are in μg/mL. S – susceptible; R – resistant.

| | Breakpoints/Critical Concentration (μg/mL) | |
|---|---|---|
| **Antibiotics** | **S** | **R** |
| Amoxicillin | 2 | 8 |
| Cefotaxime | 1 | 2 |
| Meropenem | 2 | 4 |
| Imipenem | 2 | 8 |
| Isoniazid | 0,2 | |
| Ethambutol | 5 | |

## 1.1. *M. smegmatis* mc²155

*M. smegmatis* is a non-pathogenic rapidly growing mycobacteria, used as a model for mycobacteria gene function studies, with the advantage of having a short doubling time when compared to *Mtb*. In addition, *M. smegmatis* does not have to be handled in a BSL-3 laboratory; does not form clumps or cell aggregates, as most of slow-growing mycobacteria, and in the case of the strain mc²155, has a higher transformability efficiency (Etienne *et al.*, 2005).

*M. smegmatis* colonies were inoculated in 7H9 media supplemented with OADC and tyloxapol, incubated at 37°C with agitation for 24 to 48 hours and then diluted from 10 to 200x, according to growth phase and $OD_{600nm}$. The sub-culture was incubated overnight, and when culture was in the mid to late-log phase (corresponding to an $OD_{600nm}$ between 0,7 and 1), bacterial suspensions were done to be used in the MIC assay, done as described in the Material and Methods section. 96-well microtiter plates were prepared with β-lactams antibiotics concentrations ranging between 0,5 and 256 μg/mL, with and without clavulanate at 2,5 μg/mL, and isoniazid and ethambutol concentrations ranging from 0,125 to 64 μg/mL; plates were incubated for 24 hours at 37°C.

After MIC determination, the micro cfu's assay was done for MBC determination. Agar plates were incubated for two to five days at 37°C. At least three independent MIC and MBC assays for each antibiotic were done and median and ranges were defined.

Table 5 shows the MIC and MBC medians and the reason between MBC and MIC determined for *M. smegmatis* $mc^2155$. To have a visual perspective of the antibiotic effect, the MIC values were coloured in green when MIC ≤ susceptible breakpoint/critical concentration and orange when MIC ≥ resistant breakpoint/critical concentration. Figure 20 shows the graphical representation of the MIC and MBC medians with ranges, showing the variability of the results obtained for the different antibiotics. In the graph, left bars of each group correspond to antibiotics without clavulanate and right bars to antibiotic conjugation with the β-lactamase inhibitor (not applicable to isoniazid and ethambutol). Bars with a "*" at the top mean that growth was observed in all concentrations tested, corresponding to values such as > 256 μg/mL. In these cases, it is not possible to define a MIC (or MBC) unless new assays with higher antibiotic concentrations are done.

MIC determination for *M. smegmatis* was straightforward, since opaque light-yellow colonies were deposited on the bottom of the microtiter plates, being abundant in the positive control, descending with higher concentrations of antibiotic and with clear distinction between growth and no growth wells. In the case of 7H10 agar medium plates used for MBC determination, reading was also very clear, with considerable size colonies formed after two days of incubation.

Table 5 - MIC and MBC median values of amoxicillin, cefotaxime, meropenem, imipenem, isoniazid and ethambutol for *M. smegmatis* $mc^2155$ in μg/mL. β-lactams were tested with and without clavulanate at 2,5 μg/mL. MIC values coloured green mean that the bacteria was susceptible to the antibiotic and orange means resistant. MBC values are presented in concentrations and as the reason to MIC.

| | **without clavulanate** | | | **with clavulanate** | | |
|---|---|---|---|---|---|---|
| **Antibiotic** | **MIC (μg/mL)** | **MBC (μg/mL)** | **MBC/MIC** | **MIC (μg/mL)** | **MBC (μg/mL)** | **MBC/MIC** |
| **Amoxicillin** | 32 | 32 | 1 | 2 | > 8 | > 4 |
| **Cefotaxime** | 128 | > 256 | > 2 | 32 | > 64 | 4 |
| **Meropenem** | 1 | 4 | 4 | 1 | > 4 | > 4 |
| **Imipenem** | 0,5 | 2 | 4 | 2 | 8 | 4 |
| **Isoniazid** | 8 | 32 | 4 | | | |
| **Ethambutol** | 2 | > 8 | > 4 | | | |

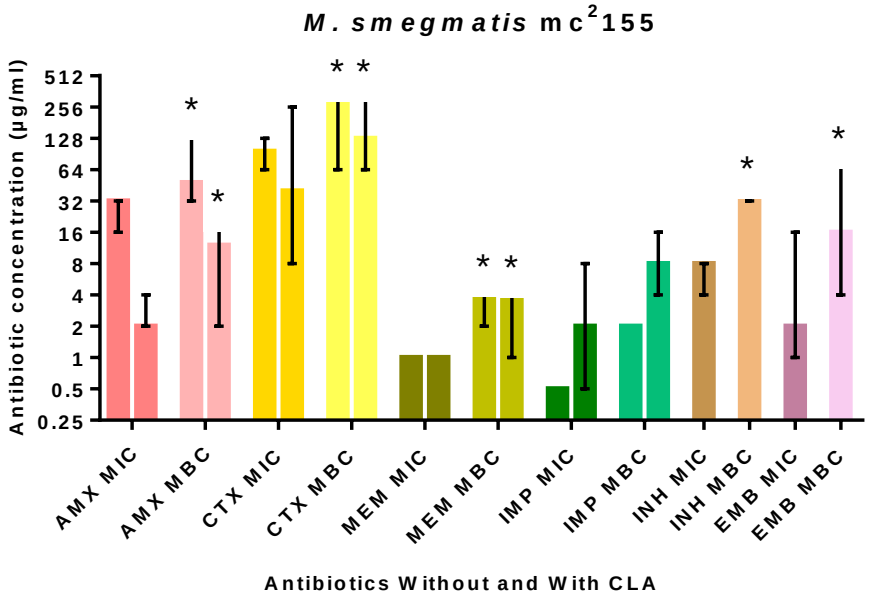


Figure 20 - Graphical representation of *M. smegmatis* mc²155 MIC and MBC medians and ranges for amoxicillin (AMX), cefotaxime (CTX), meropenem (MEM), imipenem (IMP), isoniazid (INH) and ethambutol (EMB). β-lactams were tested without (left bars) and with clavulanate (right bars) (CLA) at 2,5 µg/mL. "*****" at the top of the bars means that growth was observed in all concentrations tested.

Considering EUCAST PD-PK breakpoints for β-lactams and the critical concentrations for anti-TB drugs, *M. smegmatis* can be classified as susceptible to amoxicillin with clavulanate, both carbapenems with and without the β-lactamase inhibitor, and ethambutol; and resistant to amoxicillin without clavulanate, cefotaxime with and without the β-lactamase inhibitor, and to isoniazid.

The addition of clavulanate to amoxicillin lead to a 4-log decrease of the MIC (from 32 to 2 µg/mL), but a bacteriostatic effect was observed. The classification of *M. smegmatis* changed to susceptible, demonstrating that the inhibition of β-lactamases is essential for the action of amoxicillin. Cefotaxime was the antibiotic with higher MIC and MBC values (128 and > 256 µg/mL, respectively), being classified as bacteriostatic, once the highest concentration had grown on solid media. Clavulanate caused no significant decrease of the MIC of this antibiotic, and the classification of *M. smegmatis* as resistant remained.

Both carbapenems, meropenem and imipenem, showed low MIC values (1 µg/mL for meropenem and 0,5 µg/mL for imipenem), below the susceptible breakpoints from EUCAST. The high efficacy of this antibiotics alone was expected, once carbapenems are specially resistant to β-lactamase hydrolysis (Papp-Wallace *et al.*, 2011). However,

unexpectedly, clavulanate conjunction with meropenem did not increase the efficacy of the antibiotic, and in the case of imipenem, the presence of clavulanate have significantly decreased the efficacy of the antibiotic. This subclass of β-lactams had a low bactericidal effect on *M. smegmatis*.

Isoniazid is considered a highly bactericidal antibiotic to *Mtb* but had a low bactericidal effect on *M. smegmatis* (MIC of 8 μg/mL and an MBC 4x higher the MIC). This could be due to the fast-growing rate of *M. smegmatis*, which decreases the antibiotic action; also, the cell envelope composition of this species differs from *Mtb* (Etienne *et al.*, 2005). Ethambutol MIC determination had a higher variability between assays, with a median MIC value of 2 μg/mL and a bacteriostatic effect (MBC was more than 4 times higher than the MIC in all assays).

In conclusion, these results show that *M. smegmatis* is: i) highly resistant to cefotaxime, a third-generation cephalosporin; ii) susceptible to both meropenem and imipenem, with or without clavulanate, and clavulanate is essential for the efficacy of amoxicillin but not for the carbapenems. Having determined the MIC/MBC to different antibiotics the next step was to conjugate different classes of antibiotics, in order to determine if cell envelope biosynthesis inhibition increases the accessibility of PG to β-lactams.

### 1.1.1. Antibiotics synergies

To address how β-lactams efficacy is dependent on PG accessibility, three different conditions where the bacteria was exposed to antibiotics that inhibit cell envelope biosynthesis were tested (see Figure 19 and Table 2):

i) Mycobacteria were pre-exposed to sub-MIC of the 1$^{st}$-line anti-TB drug (isoniazid or ethambutol) and β-lactams MIC/MBC determination was done in the absence of isoniazid/ethambutol

ii) Exposure of mycobacteria to isoniazid or ethambutol was constant before and during the β-lactams MIC determination.

iii) Mycobacteria were not pre-exposed to isoniazid or ethambutol and the β-lactams MIC determination was done in the presence of sub-MIC of isoniazid/ethambutol.

Considering the previous MIC determination results, two sub-MIC of isoniazid and ethambutol were selected (¼ MIC and ½ MIC) and the pre-exposure was defined as addition of antibiotics when the culture was on the early-log phase ($OD_{600nm}$ between 0,3

and 0,4). For *M. smegmatis*, isoniazid median MIC was 8 μg/mL, and therefore the concentrations used for these assays were 2 μg/mL (¼ MIC) and 4 μg/mL (½ MIC). Ethambutol median MIC was 2 μg/mL, and the selected sub-MIC were 0,5 μg/mL (¼ MIC) and 1 μg/mL (½ MIC). The β-lactams tested were amoxicillin (a D, D-transpeptidases inhibitor) and meropenem (a L, D-transpeptidases inhibitor) ranging between 0,25 and 128 μg/mL, with and without clavulanate at 2,5 μg/mL; cefotaxime and imipenem were not used on these assays due to the lack of efficacy and instability, respectively.

Pre-exposure to isoniazid/ethambutol was done by inoculating mycobacteria in 7H9 media, with a shorter time of sub-culture incubation – about 4 to 6 hours for *M. smegmatis* – and when desired $OD_{600nm}$ was reached (between 0,3 and 0,4), antibiotics where added (½ MIC and ¼ MIC) and a control without antibiotic was included. Incubation was continued until mid to late-log phase (or overnight in the case of *M. smegmatis*). The MIC assay was done as described in the Material and Methods section, according to the conditions mentioned before. 96-well microplates were incubated for 24 hours and MBC assays were also done as described in the Material and Methods section, and 7H10 agar plates were incubated for 2 to 5 days at 37°C. At least three independent assays for each condition were done and median values and ranges were defined.

Amoxicillin and meropenem MIC determination was done in parallel (Table 6 and Figure 21) with the antibiotic synergies' experiments. Some variability was observed, when compared to the previous results, probably due to: i) non-syncronized cultures; ii) preparation of bacterial suspensions, that could sometimes carry clumps or more active bacteria; iii) genetic differences in mycobacteria inoculated – more than one colony was inoculated for each assay; iv) assays were done with independent cultures; v) the quality of antibiotics batches – quality tests by the MIC assay for *S. aureus* showed some variability between batches (Annex 1) which could influence the antibiotic activity.

Table 6 - MIC and MBC median values of amoxicillin and meropenem with and without clavulanate at 2,5 μg/mL for *M. smegmatis* $mc^2155$, in μg/mL. The reason MBC/MIC is used to define the effect of the antibiotics – bacteriostatic or bactericidal. MIC values are coloured according to breakpoints from EUCAST – green means susceptible, orange means resistant.

| | **without clavulanate** | | | **with clavulanate** | | |
|---|---|---|---|---|---|---|
| **Antibiotic** | **MIC (μg/mL)** | **MBC (μg/mL)** | **MBC/MIC** | **MIC (μg/mL)** | **MBC (μg/mL)** | **MBC/MIC** |
| **Amoxicillin** | 64 | 128 | 2 | 16 | 32 | 2 |
| **Meropenem** | 4 | 8 | 2 | 4 | 8 | 2 |

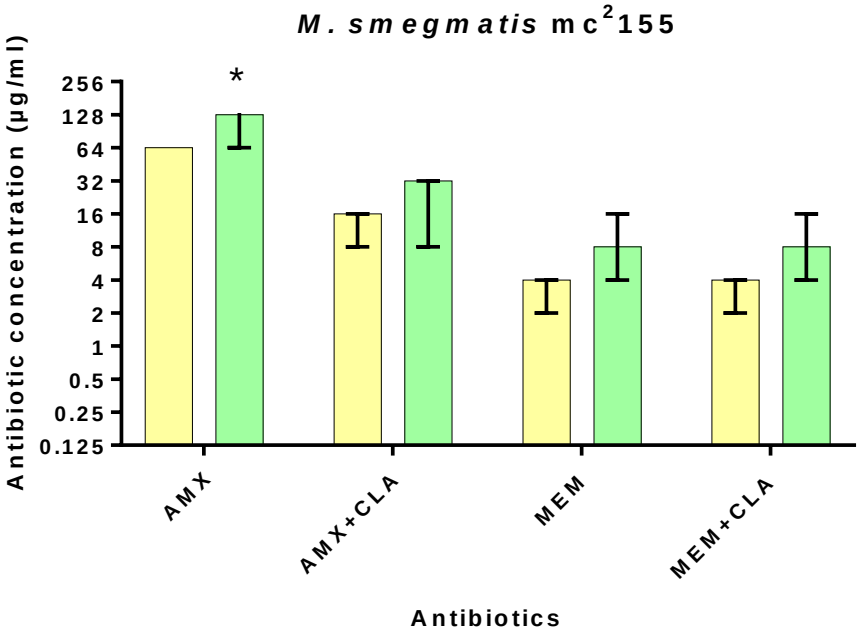


Figure 21 - Graphical representation of *M. smegmatis* mc²155 MIC and MBC medians and ranges (µg/mL) for amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL. Yellow bars are MIC results and green bars are MBC results. "*" means that growth was observed in all concentrations tested.

MIC values of these assays were higher than the MIC values previously determined, changing the classification for amoxicillin with clavulanate from susceptible to resistant according to EUCAST PK-PD breakpoints (16 µg/mL, 3-log higher than the previous assays median). However, the presence of clavulanate still had an enhancer effect on amoxicillin activity, with a MIC 2-log lower than the antibiotic alone. Amoxicillin MBCs was two times higher than MIC with and without clavulanate, and therefore the antibiotic is considered bactericidal.

MIC and MBC results for meropenem in these assays were higher than previous results (4 and 8 µg/mL, respectively), but classification as susceptible according to EUCAST PK-PD breakpoints remained, the addition of clavulanate was still not relevant for the efficacy of this carbapenem, and the bactericidal effect was constant.

#### 1.1.1.1. Efficacy of β-lactams after *M. smegmatis* mc²155 exposure to isoniazid in sub-MIC

Isoniazid is an anti-TB antibiotic, that targets the MA biosynthesis by inhibition of InhA, a reductase that participates in the MA elongation, after activation by mycobacterial KatG, causing oxidative stress and consequent death of the bacteria (Rawat, Whitty and

Tonge, 2003). This is a bactericidal antibiotic for *Mtb*, with major importance on TB treatment (Rather and Maqbool, 2017). Given the inhibitory effect of isoniazid over MA, an increased β-lactams activity is expected, due to a better accessibility to PG.

Table 7 summarizes the MIC and MBC results for the combination of isoniazid at 2 µg/mL (¼ MIC) and 4 µg/mL (½ MIC) with β-lactams, for the three conditions tested (described in the Material and Methods section). MBC values are presented in concentrations (µg/mL) and as the reason MBC/MIC. Figure 22 have the graphical representation of these values, with ranges.

MIC determination of β-lactams in combination with isoniazid was more difficult to establish as the morphology of the mycobacteria was altered and therefore has a deficient growth. Nevertheless, the distinction from growth/no growth on wells was still clear, and the morphology of colonies and growth on 7H10 agar plates was not significantly altered.

Table 7 - MIC and MBC median values of amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL for *M. smegmatis* $mc^2155$ in µg/mL. Three different conditions with two sub-MIC of isoniazid (INH - 2 and 4 µg/mL) were tested, and control results previously determined are represented for comparison. MBC values are presented in concentrations and as the MBC/MIC reason. MIC values are coloured in green when equal or lower than susceptible EUCAST PD-PK breakpoints, orange when equal or higher than resistant breakpoints, and uncoloured when intermediate.

| | | | | **Condition of INH exposure** | | |
|---|---|---|---|---|---|---|
| **INH (µg/mL)** | **Antibiotic** | **Value (µg/mL)** | **Control** | **Before β-lactam MIC determination** | **Before and during β-lactam MIC determination** | **During β-lactam MIC determination** |
| **2** | **AMX** | **MIC** | 64 | 32 | 32 | 32 |
| | | **MBC** | 128 | 64 | 32 | 64 |
| | | **MBC/ MIC** | 2 | 2 | 1 | 2 |
| | **AMX+ CLA** | **MIC** | 16 | 8 | 2 | 4 |
| | | **MBC** | 32 | 8 | 8 | 8 |
| | | **MBC/ MIC** | 2 | 1 | 4 | 2 |
| **4** | **AMX** | **MIC** | 64 | 32 | 16 | 32 |
| | | **MBC** | 128 | 32 | 16 | 32 |
| | | **MBC/ MIC** | 2 | 1 | 1 | 1 |
| | **AMX+ CLA** | **MIC** | 16 | 4 | 1 | 1 |
| | | **MBC** | 32 | 8 | 4 | 4 |
| | | **MBC/ MIC** | 2 | 2 | 4 | 4 |
| **2** | **MEM** | **MIC** | 4 | 4 | 2 | 4 |
| | | **MBC** | 8 | 4 | 4 | 4 |
| | | **MBC/ MIC** | 2 | 1 | 2 | 1 |
| | **MEM+** | **MIC** | 4 | 4 | 2 | 2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | **CLA** | **MBC** | 8 | 4 | 4 | 4 |
| | | **MBC/ MIC** | 2 | 1 | 2 | 2 |
| **4** | **MEM** | **MIC** | 4 | 4 | 1 | 2 |
| | | **MBC** | 8 | 4 | 4 | 4 |
| | | **MBC/ MIC** | 2 | 1 | 4 | 2 |
| | **MEM+ CLA** | **MIC** | 4 | 2 | 1 | 1 |
| | | **MBC** | 8 | 4 | 4 | 4 |
| | | **MBC/ MIC** | 2 | 2 | 4 | 4 |

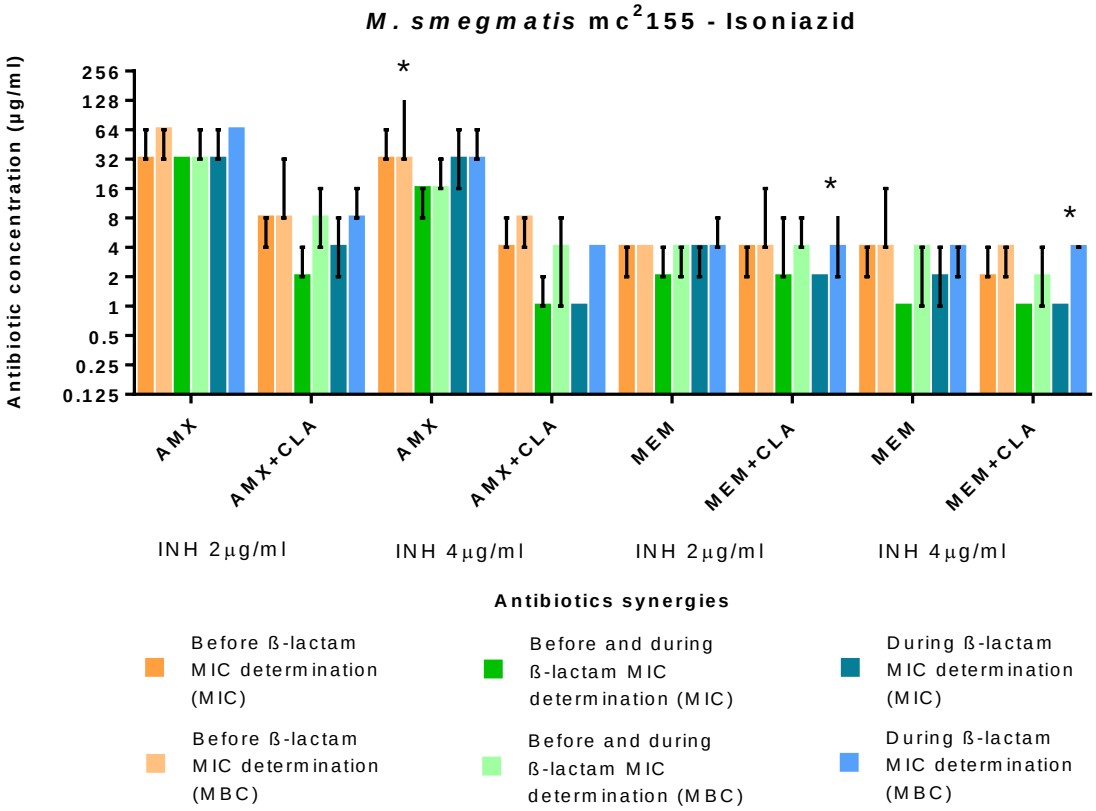


Figure 22 - Graphical representation of *M. smegmatis* mc²155 MIC and MBC values for amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL, when treated with isoniazid in sub-MIC (INH - 2 and 4 µg/mL) in different conditions. Dark colour bars are MIC results and light colour bars are MBC results. "*"

When *M. smegmatis* was exposed to isoniazid, no significant changes on the MIC of amoxicillin alone were observed, but the antibiotic had a bactericidal effect, especially in combination with a higher concentration of isoniazid (4 µg/mL). When amoxicillin was conjugated with clavulanate, and isoniazid at 4 µg/mL was present on the assay plates (both with and without pre-treatment), amoxicillin MIC decreased from 16 to 1 µg/mL, reverting the classification to susceptible, although with a less bactericidal effect. This

outcome corroborates the hypothesis that the exposure of mycobacteria to isoniazid enhances β-lactams accessibility to PG.

This observation is interesting and leads to the question why amoxicillin is less bactericidal to *M. smegmatis* when conjugated with a β-lactamase inhibitor. The decrease of the MIC when clavulanate is present is expected, since it enhances amoxicillin's activity, by inhibition of β-lactamases, and a lower concentration of the antibiotic is needed to inhibit mycobacteria growth. However, when mycobacteria are inoculated in solid media for MBC determination, it is most likely that some resistant mycobacteria are present and capable of growing without antibiotics. In the case of amoxicillin without clavulanate, a higher concentration is needed to inhibit growth, and therefore the possibility of resistant bacteria survival and recovering on solid media is minor.

Meropenem MIC values were not significantly altered when *M. smegmatis* was exposed to isoniazid at 2 μg/mL. In the case of isoniazid at 4 μg/mL, a decrease of 2-log (from 4 to 1 μg/mL) was observed when isoniazid was maintained during the β-lactams MIC determination. However, a less bactericidal effect was observed, and values were identical to the first assays done with β-lactams. For meropenem, the conjugation with isoniazid does not seem to have a great impact on antibiotic efficacy, and this carbapenem *per se* is likely to have good effectiveness against *M. smegmatis*, a rapidly growing, non-pathogenic mycobacteria.

#### 1.1.1.2. Efficacy of β-lactams after *M. smegmatis* mc$^2$155 exposure to ethambutol in sub-MIC

Ethambutol is a 1$^{st}$-line anti-TB drug that prevents AG polymerization by inhibition of EmbA and EmbB, two of four mycobacterial arabinosil transferases (Rather and Maqbool, 2017). This antibiotic is bacteriostatic for *Mtb*, but significantly compromises the cell envelope, which possibly leaves breaches for another antibiotic's activity (Schubert *et al.*, 2017). Like for isoniazid, it is expected that ethambutol will weaken the cell envelope outer layer, exposing PG for β-lactams activity.

The concentrations of ethambutol selected for *M. smegmatis* treatment were 0,5 μg/mL (¼ MIC) and 1 μg/mL (½ MIC). The MIC and MBC results for the combination of this antibiotic with amoxicillin and meropenem, with and without clavulanate, are summarized at Table 8 and Figure 23.

*M. smegmatis* cultures that were exposed to ethambutol had an altered morphology and slower growth on 96 well plates, but the distinction between growth/no growth wells was still clear, and colonies on 7H10 agar plates from MBC determination were not significantly altered.

Table 8 - MIC and MBC median values of amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL for *M. smegmatis* $mc^2155$ in µg/mL. Three different conditions with two sub-MIC of ethambutol (EMB - 0,5 and 1 µg/mL) were tested, and control results previously determined are represented for comparison. MBC values are presented in concentrations and as the MBC/MIC reason. MIC values are coloured in green when equal or lower than susceptible EUCAST PD-PK breakpoints, orange when equal or higher than resistant breakpoints and uncoloured when intermediate.

| | | | | **Condition of EMB exposure** | | |
|---|---|---|---|---|---|---|
| **EMB (µg/mL)** | **Antibiotic** | **Value (µg/mL)** | **Control** | **Before β-lactam MIC determination** | **Before and during β-lactam MIC determination** | **During β-lactam MIC determination** |
| **0,5** | **AMX** | **MIC** | 64 | 64 | 16 | 16 |
| | | **MBC** | 128 | 64 | 16 | 64 |
| | | **MBC/ MIC** | 2 | 1 | 1 | 4 |
| | **AMX + CLA** | **MIC** | 16 | 8 | 1 | 2 |
| | | **MBC** | 32 | 8 | 1 | 2 |
| | | **MBC/ MIC** | 2 | 1 | 1 | 1 |
| **1** | **AMX** | **MIC** | 64 | 32 | 2 | 8 |
| | | **MBC** | 128 | 32 | 2 | 8 |
| | | **MBC/ MIC** | 2 | 1 | 1 | 1 |
| | **AMX + CLA** | **MIC** | 16 | 4 | 0,25 | 2 |
| | | **MBC** | 32 | 8 | 0,25 | 2 |
| | | **MBC/ MIC** | 2 | 2 | 1 | 1 |
| **0,5** | **MEM** | **MIC** | 4 | 4 | 1 | 2 |
| | | **MBC** | 8 | 4 | 1 | 2 |
| | | **MBC/ MIC** | 2 | 1 | 1 | 1 |
| | **MEM+ CLA** | **MIC** | 4 | 4 | 1 | 1 |
| | | **MBC** | 8 | 4 | 1 | 1 |
| | | **MBC/ MIC** | 2 | 1 | 1 | 1 |
| **1** | **MEM** | **MIC** | 4 | 2 | 0,5 | 0,5 |
| | | **MBC** | 8 | 2 | 1 | 0,5 |
| | | **MBC/ MIC** | 2 | 1 | 1 | 1 |
| | **MEM+ CLA** | **MIC** | 4 | 2 | <0,25 | 0,5 |
| | | **MBC** | 8 | 4 | 0,25 | 0,5 |
| | | **MBC/ MIC** | 2 | 2 | 1 | 1 |

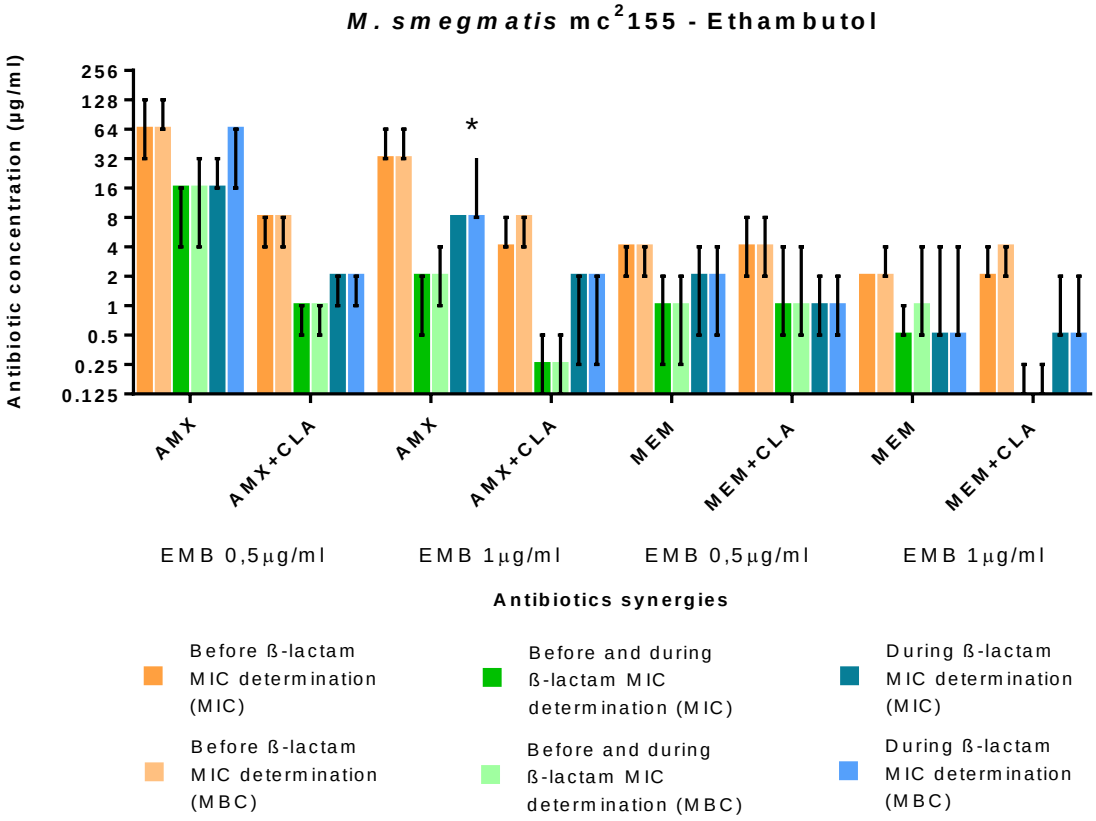


Figure 23 - Graphical representation of *M. smegmatis* mc²155 MIC and MBC values for amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL, when treated with ethambutol in sub-MIC (EMB – 0,5 and 1 µg/mL) in different conditions. Dark colour bars are MIC results and light colour bars are MBC results. "*" means that growth was observed in all concentrations tested.

For amoxicillin, the combination with ethambutol resulted in an accentuated bactericidal effect, and a decrease of up to 5-log of the MIC (from 64 to 2 µg/mL). Lower amoxicillin MICs were obtained when mycobacteria was treated with ethambutol 1 µg/mL, with or without pre-exposure. In the case of the combination of amoxicillin with clavulanate, a decrease was also observed, with a 5-log difference when compared to the control (32 to 1 µg/mL) and a strong bactericidal effect. For *M. smegmatis*, the combination of amoxicillin with ethambutol seems to have a synergistic effect, changing its classification from resistant to susceptible, suggesting a better accessibility of amoxicillin to its target, the PG.

For meropenem, the combination with ethambutol lead to a MIC decrease up to 4-log (4 to <0,25 µg/mL). In the condition of pre-treatment and assay with ethambutol at 1 µg/mL, no growth was observed at the lowest concentration tested for meropenem with clavulanate, meaning that the MIC was lower than 0,25 µg/mL. So, unlike the

combination with isoniazid (to which *M. smegmatis* was resistant), exposing this mycobacterium to sub-MICs of ethambutol leads to a significant decrease of meropenem MIC and MBC values. This effect is promising, since both antibiotics are bacteriostatic or slightly bactericidal when administered alone and the ethambutol/meropenem combination leads to a bactericidal effect even at low concentrations of the antibiotics.

## 1.2.*M. fortuitum* ATCC 6841

*M. fortuitum* is a rapidly growing mycobacteria that is considered as an opportunistic pathogen even though it can be handled at a BSL-2 laboratory. Inclusion of this mycobacteria species on this study had the aim to study the differences of antibiotic activity on mycobacteria with distinct cell envelope composition and importance to human health.

Incubation of *M. fortuitum* was done in the same conditions described for *M. smegmatis*, but the pre-inoculum was incubated for three to four days and the sub-culture was incubated for two days before reaching mid to log-phase. 96-well microtiter plates were prepared as described in the Material and Methods section, and plates were incubated for five days at 37°C before MIC determination and MBC assay. 7H10 agar plates used for MBC determination were incubated for 7 to 10 days at 37°C and at least three independent MIC and MBC assays were done for each antibiotic, and median and ranges were defined.

Table 9 summarizes the MIC and MBC results in µg/mL and the MBC/MIC value. MIC results are coloured according to EUCAST PD-PK breakpoints, with green for susceptible classifications and orange for resistant. Figure 27 is the graphical representation of MIC and MBC median and ranges of the antibiotics tested for *M. fortuitum*.

MIC determination for *M. fortuitum* was straightforward, with light-yellow colonies growing on the bottom of microtiter plates, and irregular shaped colonies on 7H10 agar plates. Comparing the MIC results with EUCAST PD-PK breakpoints and critical concentrations represented in Table 4, *M. fortuitum* is classified as susceptible to meropenem and resistant to the remaining antibiotics tested.

Table 9 - MIC and MBC median values of amoxicillin, cefotaxime, meropenem, imipenem, isoniazid and ethambutol for *M. fortuitum* ATCC 6841 in µg/mL. β-lactams were tested with and without clavulanate at 2,5 µg/mL. MIC values coloured green mean that the bacteria was susceptible to the antibiotic and orange means resistant. MBC values are presented in concentrations and as the reason to MIC.

| | without clavulanate | | | with clavulanate | | |
|---|---|---|---|---|---|---|
| | **MIC (µg/mL)** | **MBC (µg/mL)** | **MBC/MIC** | **MIC (µg/mL)** | **MBC (µg/mL)** | **MBC/MIC** |
| **Amoxicillin** | 8 | 32 | 4 | 8 | 8 | 1 |
| **Cefotaxime** | > 256 | > 256 | 1 | 32 | 32 | 1 |
| **Meropenem** | 4 | 4 | 1 | 4 | 8 | 2 |
| **Imipenem** | 128 | 128 | 1 | 64 | 64 | 1 |
| **Isoniazid** | 4 | > 16 | > 4 | | | |
| **Ethambutol** | 16 | > 32 | > 2 | | | |

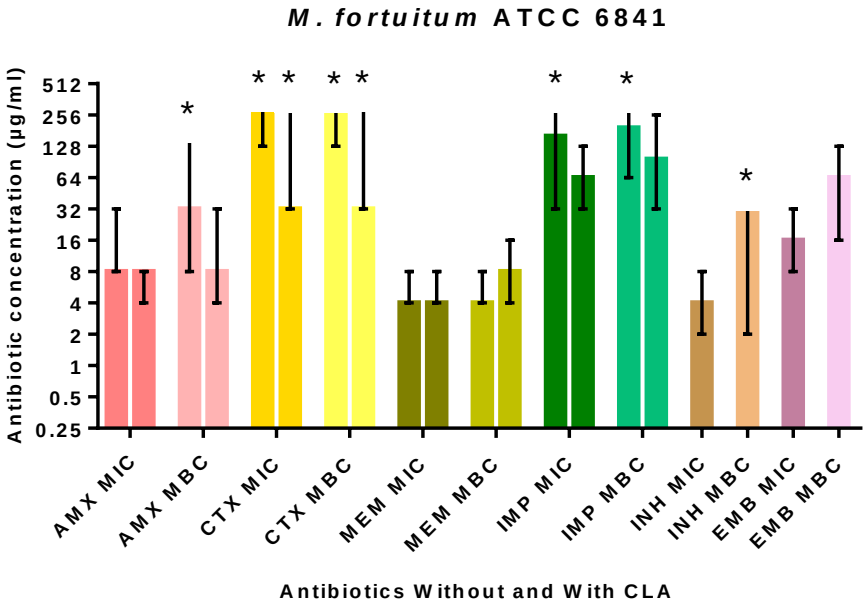


Figure 24 - Graphical representation of *M. fortuitum* ATCC 6841 MIC and MBC medians and ranges for amoxicillin (AMX), cefotaxime (CTX), meropenem (MEM), imipenem (IMP), isoniazid (INH) and ethambutol (EMB). β-lactams were tested without (left bars) and with clavulanate (right bars) (CLA) at 2,5 µg/mL. "*" at the top of the bars means that growth was observed in all concentrations tested.

Amoxicillin combined with clavulanate had a more bactericidal effect, although the MIC was equal to amoxicillin alone – 8 µg/mL. Cefotaxime had the highest MIC values, being out of reach to be defined, but conjunction with clavulanate lowered the MIC at least 3-log, (>256 µg/mL to 32 µg/mL), with a bactericidal effect.

Meropenem was the only antibiotic to which *M. fortuitum* could be considered as susceptible to. With a median MIC value of 4 µg/mL, a bactericidal effect and with some variance on MBC values when combined with clavulanate. This result might have to do with the observed variability between assays. Imipenem had a bactericidal effect, but high

MIC values (128 μg/mL) and in some assays, bacteria have grown in all concentrations tested. The addition of clavulanate to this carbapenem leads to a decrease of only 1-log of the MIC (64 μg/mL).

Isoniazid and ethambutol were both bacteriostatic for *M. fortuitum* with MICs of 4 and 16 μg/mL, respectively, with a major variability between assays for isoniazid. MA structure on the mycobacterial cell envelope is correlated with its virulence (Marrakchi, Lanéelle and Daffé, 2014), therefore rapidly-growing mycobacteria, having a distinct profile of MA, might have distinct susceptibility to isoniazid and ethambutol action (Laval *et al.*, 2001). In conclusion, *M. fortuitum* seems to be particularly resistant to cefotaxime, imipenem and ethambutol, and the addition of clavulanate is advantageous in combination with amoxicillin and cefotaxime, but not for meropenem.

### 1.2.1. Antibiotic synergies

The same conditions described for *M. smegmatis* were tested for *M. fortuitum*, considering the median MIC values previously obtained for isoniazid, 4 μg/mL, and ethambutol, 16 μg/mL. The β-lactams tested were amoxicillin and meropenem ranging between 0,25 and 128 μg/mL, with and without clavulanate at 2,5 μg/mL.

Table 10 and Figure 24 summarize the results of amoxicillin and meropenem assays that were done for *M. fortuitum*, simultaneously to combinations with isoniazid and ethambutol.

Table 10 - MIC and MBC median values of amoxicillin and meropenem with and without clavulanate at 2,5 μg/mL for *M fortuitum* ATCC 6841, in μg/mL. The reason MBC/MIC is used to define the effect of the antibiotics – bacteriostatic or bactericidal. MIC values are coloured according to breakpoints from EUCAST – green means susceptible and orange means resistant.

| | **without clavulanate** | | | **with clavulanate** | | |
|---|---|---|---|---|---|---|
| **Antibiotic** | **MIC (μg/mL)** | **MBC (μg/mL)** | **MBC/MIC** | **MIC (μg/mL)** | **MBC (μg/mL)** | **MBC/MIC** |
| **Amoxicillin** | 32 | 32 | 1 | 8 | 8 | 1 |
| **Meropenem** | 4 | 4 | 1 | 4 | 4 | 1 |

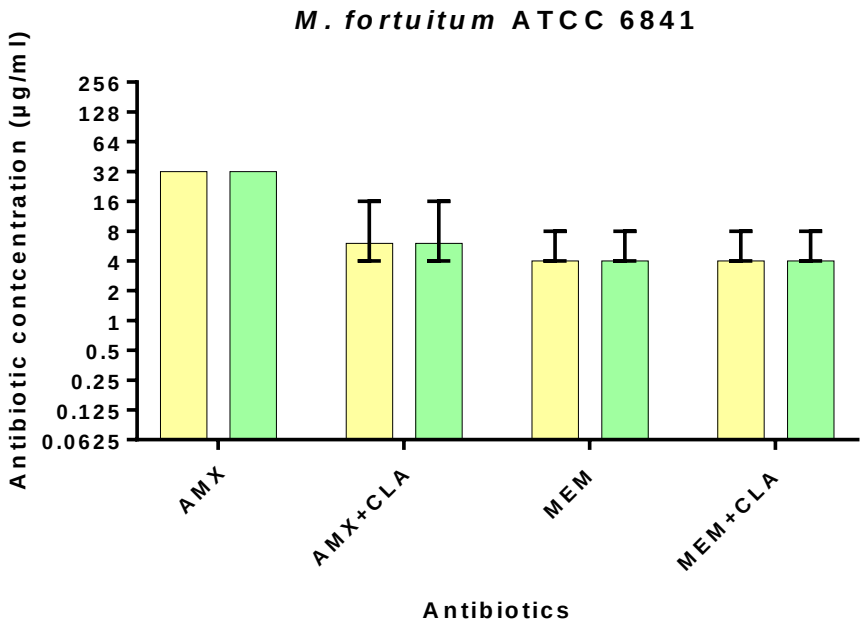


Figure 25 - Graphical representation of *M. fortuitum* ATCC 6841 MIC and MBC medians and ranges (µg/mL) for amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL. Yellow bars are MIC results and green bars are MBC results. "*" means that growth was observed in all concentrations tested.

As observed in previous assays, and according to EUCAST PD-PK breakpoints, *M. fortuitum* is classified as resistant to amoxicillin and susceptible to meropenem. However, the MIC of amoxicillin was 2-log higher than the previous determination – 32 µg/mL, with a more bactericidal effect than before. This discrepancy was only observed for amoxicillin alone and might be due to: i) non-syncronized cultures; ii) preparation of bacterial suspensions, that could sometimes carry clumps or more active bacteria; iii) the quality of antibiotics batches (Annex 1), which could influence the antibiotic activity.

Like for *M. smegmatis*, different conditions of pre-treatment with sub-MIC of isoniazid or ethambutol were tested and MIC and MBC assay for amoxicillin and meropenem (with and without clavulanate at 2,5 µg/mL) were performed.

#### 1.2.1.1. Efficacy of β-lactams after *M. fortuitum* ATCC 6841 exposure to isoniazid in sub-MIC

Table 11 and Figure 26 summarizes the β-lactams MIC results for *M. fortuitum* in combination with isoniazid, at 1 µg/mL (¼ MIC) and 2 µg/mL (½ MIC), with and without clavulanate at 2,5 µg/mL.

Table 11 - MIC and MBC median values of amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL for *M. fortuitum* ATCC 6841 in µg/mL. Three different conditions with two sub-MIC of isoniazid (INH - 1 and 2 µg/mL) were tested, and control results previously determined are represented for comparison. MBC values are presented in concentrations and as the MBC/MIC reason. MIC values are coloured in green when equal or lower than susceptible EUCAST PD-PK breakpoints, orange when equal or higher than resistant breakpoints and uncoloured when intermediate.

| | | | | Condition of INH exposure | | |
|---|---|---|---|---|---|---|
| **INH (µg/mL)** | **Antibiotic** | **Value (µg/mL)** | **Control** | **Before β-lactam MIC determination** | **Before and during β-lactam MIC determination** | **During β-lactam MIC determination** |
| **1** | **AMX** | **MIC** | 32 | 32 | 16 | 16 |
| | | **MBC** | 32 | 32 | 16 | 16 |
| | | **MBC/ MIC** | 1 | 1 | 1 | 1 |
| | **AMX + CLA** | **MIC** | 8 | 8 | 4 | 4 |
| | | **MBC** | 8 | 8 | 4 | 4 |
| | | **MBC/ MIC** | 1 | 1 | 1 | 1 |
| **2** | **AMX** | **MIC** | 32 | 32 | 1 | 2 |
| | | **MBC** | 32 | 32 | 2 | 2 |
| | | **MBC/ MIC** | 1 | 1 | 2 | 1 |
| | **AMX + CLA** | **MIC** | 8 | 4 | 1 | 2 |
| | | **MBC** | 8 | 8 | 1 | 2 |
| | | **MBC/ MIC** | 1 | 2 | 1 | 1 |
| **1** | **MEM** | **MIC** | 4 | 4 | 4 | 4 |
| | | **MBC** | 4 | 4 | 16 | 4 |
| | | **MBC/ MIC** | 1 | 1 | 4 | 1 |
| | **MEM + CLA** | **MIC** | 4 | 4 | 4 | 4 |
| | | **MBC** | 4 | 8 | 8 | 4 |
| | | **MBC/ MIC** | 1 | 2 | 2 | 1 |
| **2** | **MEM** | **MIC** | 4 | 4 | 1 | 1 |
| | | **MBC** | 4 | 4 | 4 | 1 |
| | | **MBC/ MIC** | 1 | 1 | 4 | 1 |
| | **MEM + CLA** | **MIC** | 4 | 2 | 1 | 2 |
| | | **MBC** | 4 | 4 | 2 | 2 |
| | | **MBC/ MIC** | 1 | 2 | 2 | 1 |

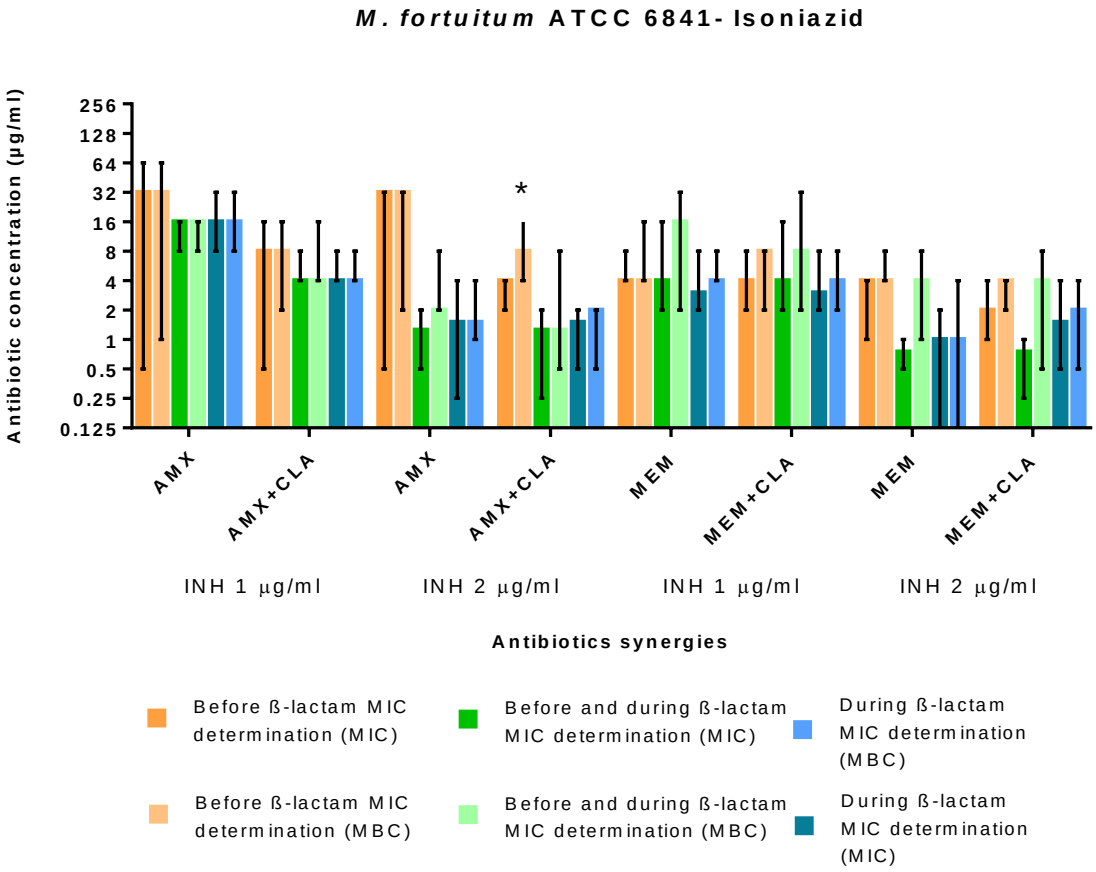


Figure 26 - Graphical representation of *M. fortuitum* ATCC 6841 and MBC values for amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL, when treated with isoniazid in sub-MIC (INH - 1 and 2 µg/mL) in different conditions. Dark colour bars are MIC results and light colour bars are MBC results. "*" means that growth was observed in all concentrations tested.

Growth rate and colonies morphology of *M. fortuitum* after pre-exposure to isoniazid were highly affected and a great variability between assays was observed. When pre-treatment was performed with isoniazid at 1 µg/mL (¼ MIC) no significant changes on MICs of β-lactams without clavulanate were observed. When isoniazid at 1 µg/mL was present on the microtiter plates (before and during or only during the MIC determination) amoxicillin without and with clavulanate had a bactericidal effect and a 1-log MIC decrease, comparing to control MIC (16 and 4 µg/mL, respectively). When isoniazid was present at 2 µg/mL (½ MIC) both before and during the β-lactams MIC determination, a decrease of 5 and 3-log was observed for amoxicillin without and with clavulanate (from 32 and 8 µg/mL to 1 µg/mL), respectively, with a bactericidal effect and a susceptible classification.

Combination of meropenem with isoniazid at 1 μg/mL showed no increased efficacy of its activity. A less bactericidal effect of meropenem was observed when isoniazid was present before and during the β-lactams MIC determination, indicating that resistance mechanisms might have been developed under this condition. When *M. fortuitum* was exposed to isoniazid at 2 μg/mL, meropenem MIC decreased up to 2-log (from 4 to 1 μg/mL), with a less bactericidal effect and a susceptible classification.

For *M. fortuitum*, isoniazid seems to have a synergistic effect with both amoxicillin and meropenem and β-lactams efficacy is enhanced by clavulanate conjunction. Again, mycobacterial exposure to a 1st line anti-TB drugs that target the MA biosynthesis seems to enhance β-lactams efficacy, possibly due to a higher PG accessibility.

#### 1.2.1.2. Efficacy of β-lactams after *M. fortuitum* ATCC 6841 exposure to ethambutol in sub-MIC

The same conditions described for isoniazid were used for ethambutol at 4 μg/mL (¼ MIC) and 8 μg/mL (½ MIC). Table 12 and Figure 27 summarize the MIC and MBC results for all the conditions tested and the classification of bactericidal effect of each combination. *M. fortuitum* growth was affected by exposure to ethambutol, but not as much as when isoniazid was present during culture growth.

Table 12 - MIC and MBC median values of amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 μg/mL for *M. fortuitum* ATCC 6841 in μg/mL. Three different conditions with two sub-MIC of ethambutol (EMB - 4 and 8 μg/mL) were tested, and control results previously determined are represented for comparison. MBC values are presented in concentrations and as the MBC/MIC reason. MIC values are coloured in green when equal or lower than susceptible EUCAST PD-PK breakpoints, orange when equal or higher than resistant breakpoints and uncoloured when intermediate.

| | | | | Condition of EMB exposure | | |
|---|---|---|---|---|---|---|
| **EMB (μg/mL)** | **Antibiotic** | **Value (μg/mL)** | **Control** | **Before β-lactam MIC determination** | **Before and during β-lactam MIC determination** | **During β-lactam MIC determination** |
| **4** | **AMX** | **MIC** | 32 | 32 | 4 | 4 |
| | | **MBC** | 32 | 32 | 4 | 4 |
| | | **MBC/ MIC** | 1 | 1 | 1 | 1 |
| | **AMX + CLA** | **MIC** | 8 | 32 | 1 | 2 |
| | | **MBC** | 8 | 32 | 2 | 4 |
| | | **MBC/ MIC** | 1 | 1 | 2 | 2 |
| **8** | **AMX** | **MIC** | 32 | 32 | 0,25 | 1 |
| | | **MBC** | 32 | 32 | 1 | 1 |
| | | **MBC/ MIC** | 1 | 1 | 4 | 1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | **AMX + CLA** | **MIC** | 8 | 16 | 0,25 | 1 |
| | | **MBC** | 8 | 16 | 0,25 | 1 |
| | | **MBC/ MIC** | 1 | 1 | 1 | 1 |
| **4** | **MEM** | **MIC** | 4 | 4 | 2 | 2 |
| | | **MBC** | 4 | 4 | 2 | 4 |
| | | **MBC/ MIC** | 1 | 1 | 1 | 2 |
| | **MEM + CLA** | **MIC** | 4 | 4 | 1 | 2 |
| | | **MBC** | 4 | 4 | 1 | 2 |
| | | **MBC/ MIC** | 1 | 1 | 1 | 1 |
| **8** | **MEM** | **MIC** | 4 | 4 | 1 | 1 |
| | | **MBC** | 4 | 4 | 1 | 1 |
| | | **MBC/ MIC** | 1 | 1 | 1 | 1 |
| | **MEM + CLA** | **MIC** | 4 | 4 | 0,25 | 1 |
| | | **MBC** | 4 | 8 | 1 | 1 |
| | | **MBC/ MIC** | 1 | 2 | 4 | 1 |

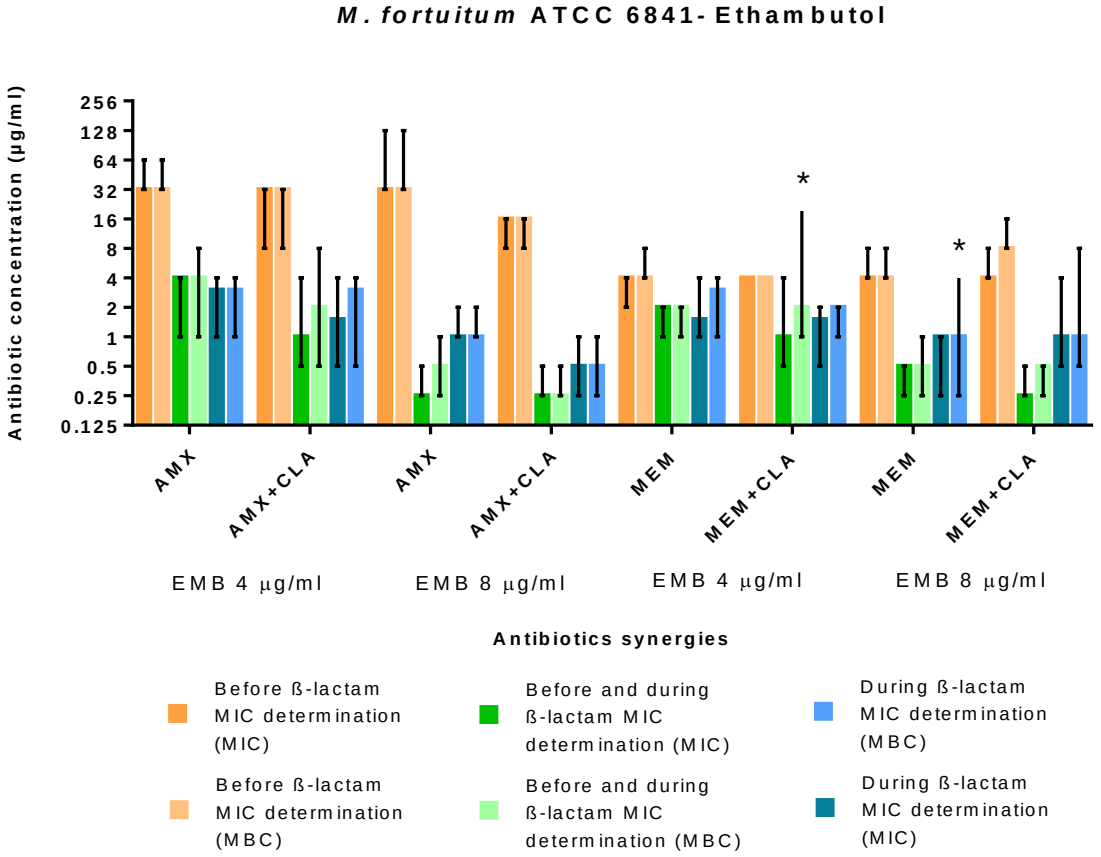


Figure 27 - Graphical representation of *M. fortuitum* ATCC 6841 MIC and MBC values for amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL, when treated with ethambutol in sub-MIC (EMB – 4 and 8 µg/mL) in different conditions. Dark colour bars are MIC results and light colour bars are MBC results. "*" means that growth was observed in all concentrations tested.

As observed for isoniazid, *M. fortuitum* exposure to ethambutol before but not during the MIC assay, did not decrease the β-lactams MIC values. In the case of amoxicillin with clavulanate, MIC values were higher than without the exposure to ethambutol. This outcome suggests that only pre-exposure of *M. fortuitum* to sub-MIC of ethambutol might lead to the emergence of resistance mechanisms when β-lactams MIC determination assays are done.

When ethambutol at 4 μg/mL (¼ MIC) was present during the β-lactams MIC determination, the MIC for amoxicillin decreased 3-log (from 32 μg/mL to 4 μg/mL, without clavulanate, and from 8 μg/mL to 1 μg/mL with clavulanate). In the case of combination with ethambutol at 8 μg/mL, a decrease of up to 7-log (from 32 μg/mL to 0,25 μg/mL), was observed, with a bactericidal effect. The presence of ethambutol seems to have an enhancing effect over amoxicillin's activity, changing *M. fortuitum* classification from resistant to susceptible, according to EUCAST PD-PK breakpoints. However, it is essential that antibiotics are given simultaneously, as consecutive treatment leads to no significant decrease of amoxicillin MIC.

When ethambutol was present at 4 μg/mL during meropenem MIC determination, a decrease of 1 and 2-log (from 4 μg/mL to 2 and 1 μg/mL) was observed, and up to 4-log (0,25 μg/mL) when ethambutol at 8 μg/mL was used. All combinations had a bactericidal effect, and *M. fortuitum* could be classified as susceptible to meropenem. Once again, the combination of ethambutol with meropenem seems to increase the efficacy of this carbapenem, which can be promising for *M. fortuitum* infections treatment.

### 1.3.*M. bovis* BCG

*M. bovis* BCG is the attenuated form of *M. bovis*, used globally as vaccine which provides protection against disseminated tuberculosis on new-borns. This is a slow growing mycobacteria that should be handled at a BSL-2 laboratory. To perform MIC assays, colonies from 7H10 agar plates were inoculated in 7H9 media supplemented with OADC and tyloxapol and incubated at 37°C with 5% $CO_2$, without agitation for two to three weeks. To prevent clumps formation cultures were regularly vortexed at low speeds. Sub-cultures were done by diluting pre-inoculum from 10 to 100 times and incubated at the same conditions until desired $OD_{600nm}$ (0,7-1) was reached. Bacterial suspensions were prepared as described in the Material and Methods section, and 96-well microtiter

plates were incubated for 7 to 10 days at 37°C. After MIC readings, the micro-cfu's assay was done for MBC determination, which had an incubation period from 2 to 3 weeks.

Table 13 and Figure 28 summarizes the MIC and MBC results obtained for β-lactams with and without clavulanate at 2,5 µg/mL and the two 1st-line anti-TB drugs, tested at concentrations between 0,125 and 64 µg/mL.

BCG grows on microtiter plates as a light layer of mycobacteria at the bottom of the wells, growing only at the well edges with higher concentrations of antibiotics. After being transferred to 7H10 agar plates for MBC determination, round, dry and light-yellow colonies start to appear after 7 to 10 days at 37°C.

Table 13 - MIC and MBC median values of amoxicillin, cefotaxime, meropenem, imipenem, isoniazid and ethambutol for *M. bovis* BCG in µg/mL. β-lactams were tested with and without clavulanate at 2,5 µg/mL. MIC values coloured green mean that the bacteria was susceptible to the antibiotic and orange means resistant. MBC values are presented in concentrations and as the reason to MIC.

| | **without clavulanate** | | | **with clavulanate** | | |
|---|---|---|---|---|---|---|
| **Antibiotic** | **MIC (µg/mL)** | **MBC (µg/mL)** | **MBC/MIC** | **MIC (µg/mL)** | **MBC (µg/mL)** | **MBC/MIC** |
| **Amoxicillin** | 32 | > 64 | > 2 | 4 | 16 | 4 |
| **Cefotaxime** | > 64 | > 64 | 1 | > 64 | > 64 | 1 |
| **Meropenem** | 2 | > 8 | > 4 | 0,25 | > 1 | > 4 |
| **Imipenem** | 1 | > 4 | > 4 | 0,25 | > 1 | > 4 |
| **Isoniazid** | 0,25 | 0,25 | 1 | | | |
| **Ethambutol** | 4 | 4 | 1 | | | |

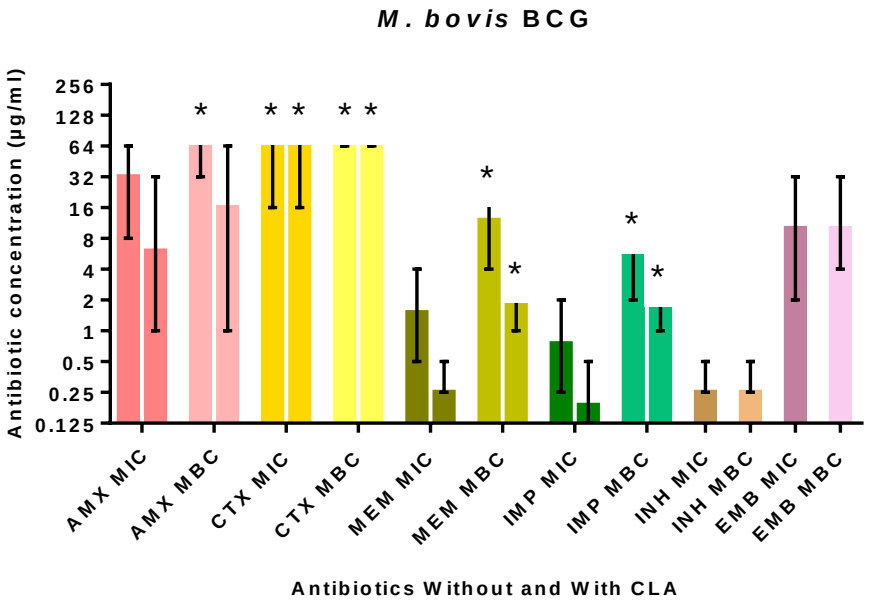


Figure 28 - Graphical representation of *M. bovis* BCG MIC and MBC medians and ranges for amoxicillin (AMX), cefotaxime (CTX), meropenem (MEM), imipenem (IMP), isoniazid (INH) and ethambutol (EMB). β-lactams were tested without (left bars) and with clavulanate (right bars) (CLA) at 2,5 µg/mL. "*" at the top of the bars means that growth was observed in all concentrations tested.

According to EUCAST breakpoints (Table 4), BCG can be classified as resistant to amoxicillin and cefotaxime, intermediate to amoxicillin with clavulanate and susceptible to the carbapenems. As for the anti-TB drugs, BCG is susceptible to ethambutol and the MIC of isoniazid is close to the critical concentration, and therefore BCG is classified as having a low resistance to this antibiotic (coloured as susceptible in Table 13). The conjunction with clavulanate improved the efficacy of amoxicillin, meropenem and imipenem, but had no significant effect on the activity of cefotaxime (the bacteria have grown on all concentrations tested for this antibiotic). All β-lactams, both without and with clavulanate, have shown a bacteriostatic effect, and isoniazid and ethambutol were bactericidal to BCG.

### 1.3.1. Antibiotic synergies

To do the antibiotics synergies assays, as described previously for the rapidly-growing mycobacteria, BCG cultures in the early-log phase (sub-culture incubated for 5 to 7 days, with an $OD_{600nm}$ 0,3-0,4) were subjected to three different conditions where the bacteria was exposed to the action of antibiotics that inhibit cell envelope biosynthesis (see Material an Methods, page 33) and incubated until $OD_{600m}$ 0,7-1, or for 7 days, at 37°C. As BCG is a slow growing mycobacteria, we observed that the presence of these antibiotics halted normal growth, and some cultures were not able to reach the late log-phase. When cultures have grown to or close to the desired $OD_{600nm}$, the β-lactams MIC assays were done as described in the Material and Methods section, by preparing the bacterial suspension with replacement of the media, and ultrasounds were used for clumps removing. The MIC assay had to be repeated several times, as growth was frequently not observed on the control wells of the microtiter plates. Considering that the cell envelope of mycobacteria was damaged by isoniazid/ethambutol, the ultrasounds treatment might have been too aggressive and caused further damage or killed most of the bacteria.

Table 14 and Figure 29 summarize the results for β-lactams MIC control assays that were done in parallel to the β-lactams MIC determination in the presence of isoniazid and ethambutol.

Table 14 - MIC and MBC median values of amoxicillin and meropenem with and without clavulanate at 2,5 µg/mL for *M bovis* BCG, in µg/mL. The reason MBC/MIC is used to define the effect of the antibiotics – bacteriostatic or bactericidal. MIC values are coloured according to breakpoints from EUCAST – green means susceptible and orange means resistant.

| | without clavulanate | | | with clavulanate | | |
|---|---|---|---|---|---|---|
| **Antibiotic** | **MIC (µg/mL)** | **MBC (µg/mL)** | **MBC/MIC** | **MIC (µg/mL)** | **MBC (µg/mL)** | **MBC/MIC** |
| **Amoxicillin** | > 64 | > 64 | 1 | 32 | > 64 | > 2 |
| **Meropenem** | 8 | > 32 | > 4 | 4 | 4 | 1 |

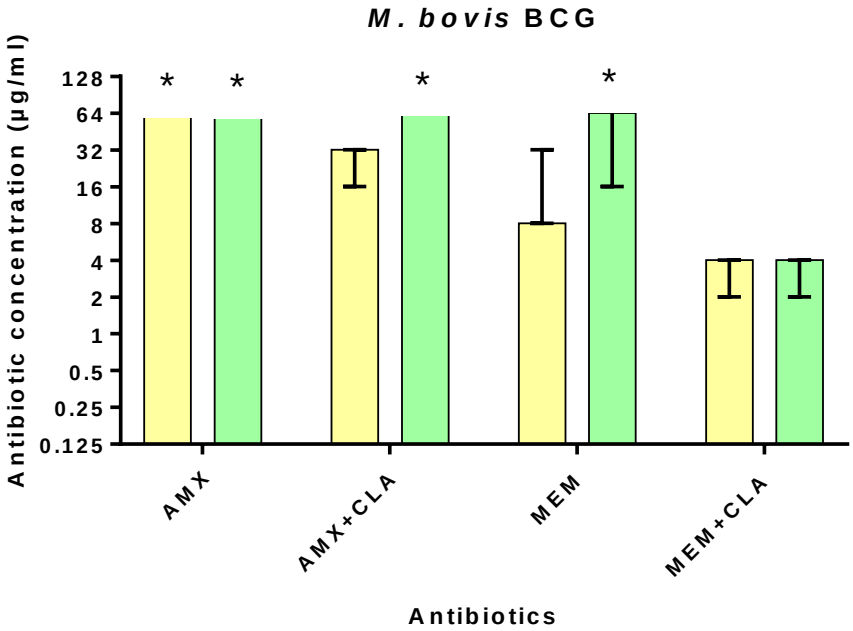


Figure 29 - Graphical representation of *M. bovis* BCG MIC and MBC medians and ranges (µg/mL) for amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL. Yellow bars are MIC results and green bars are MBC results. "*" means that growth was observed in all concentrations tested.

Amoxicillin MIC was higher than previous assays, at least 1-log for amoxicillin alone (from 32 to > 64 µg/mL) and 3-log when combined with clavulanate (from 4 to 32 µg/mL), shifting BCG to a resistant classification according to EUCAST PD-PK breakpoints. Meropenem also had higher MIC results, with a 2-log difference for meropenem alone (from 2 to 8 µg/mL), reverting the bacteria classification from susceptible to resistant, and a 4-log increase for meropenem with clavulanate (from 0,25 to 4 µg/mL). On the contrary from previous assays, meropenem with clavulanate had a bactericidal effect over BCG. This outcome might have to do with variability of mycobacteria cultures or the quality of antibiotic's batches.

#### 1.3.1.1. Efficacy of β-lactams after *M. bovis* BCG exposure to isoniazid in sub-MIC

When mycobacteria were exposed to isoniazid, growth was halted, and some cultures have not grown above an $OD_{600nm}$ of 0,6. In addition, the ultrasounds treatment when the bacterial suspensions were prepared might have caused bacterial lysis.

BCG did not grow on positive control wells for conditions where isoniazid was present on the 96-wells microtiter plates during the β-lactams MIC determination. This observation suggests that mycobacteria were killed, and assays should be repeated without the ultra-sound step or with lower concentrations of isoniazid. Table 15 and Figure 30 summarize the MIC and MBC results obtained for BCG when β-lactams were combined with isoniazid.

Table 15 - MIC and MBC median values of amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL for *M. bovis* BCG in µg/mL. Three different conditions with two sub-MIC of isoniazid (INH – 0,0625 and 0,125 µg/mL) were tested, and control results previously determined are represented for comparison. MBC values are presented in concentrations and as the MBC/MIC reason. MIC values are coloured in green when equal or lower than susceptible EUCAST PD-PK breakpoints, and orange when equal or higher than resistant breakpoints.

| | | | | **Condition of INH exposure** | | |
|---|---|---|---|---|---|---|
| **INH (µg/mL)** | **Antibiotic** | **Value (µg/mL)** | **Control** | **Before β-lactam MIC determination** | **Before and during β-lactam MIC determination** | **During β-lactam MIC determination** |
| **0,0625** | **AMX** | **MIC** | > 64 | > 64 | - | - |
| | | **MBC** | > 64 | > 64 | - | - |
| | | **MBC/ MIC** | 1 | 1 | - | - |
| | **AMX + CLA** | **MIC** | 32 | 8 | - | - |
| | | **MBC** | > 64 | 32 | - | - |
| | | **MBC/ MIC** | > 2 | 4 | - | - |
| **0,125** | **AMX** | **MIC** | > 64 | > 64 | - | - |
| | | **MBC** | > 64 | > 64 | - | - |
| | | **MBC/ MIC** | 1 | 1 | - | - |
| | **AMX + CLA** | **MIC** | 32 | 8 | - | - |
| | | **MBC** | > 64 | 32 | - | - |
| | | **MBC/ MIC** | > 2 | 4 | - | - |
| **0,0625** | **MEM** | **MIC** | 8 | 2 | - | - |
| | | **MBC** | > 32 | > 8 | - | - |
| | | **MBC/ MIC** | > 4 | > 4 | - | - |
| | **MEM + CLA** | **MIC** | 4 | 0,25 | - | - |
| | | **MBC** | 4 | > 1 | - | - |
| | | **MBC/ MIC** | 1 | > 4 | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| **0,125** | **MEM** | **MIC** | 8 | 4 | - | - |
| | | **MBC** | > 32 | > 8 | - | - |
| | | **MBC/ MIC** | > 4 | > 2 | - | - |
| | **MEM + CLA** | **MIC** | 4 | 0,25 | - | - |
| | | **MBC** | 4 | > 1 | - | - |
| | | **MBC/ MIC** | 1 | > 4 | - | - |

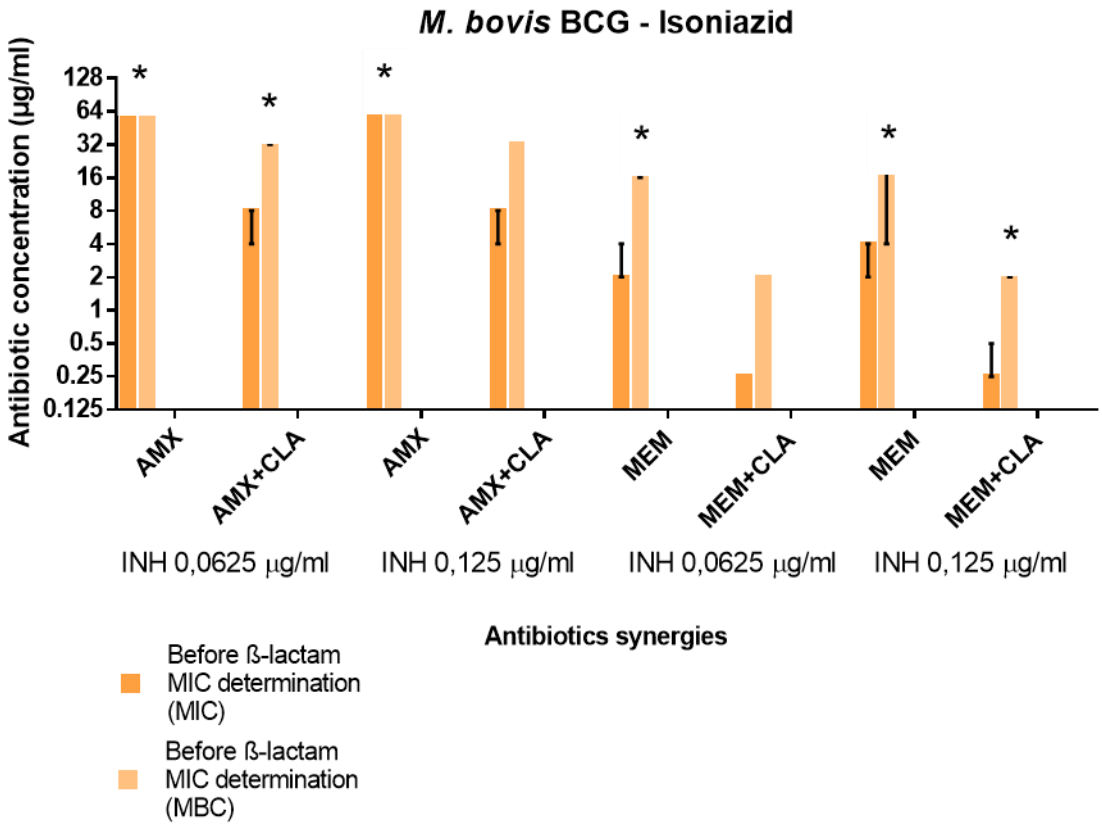


Figure 30 - Graphical representation of *M. bovis* BCG MIC and MBC values for amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL, when treated with isoniazid in sub-MIC (INH – 0,0625 and 0,0125 µg/mL) in different conditions. Dark colour bars are MIC results and light colour bars are MBC results. "*" means that growth was observed in all concentrations tested.

Results were only obtained for the condition where BCG was only exposed to isoniazid before the β-lactams MIC determination. For amoxicillin without clavulanate BCG have grown on all concentrations tested, as happened without exposure to isoniazid. When clavulanate was present, a decrease of 2-log was observed comparing to control (from 32 to 8 µg/mL) even though BCG remained resistant, according to EUCAST PD-PK breakpoints. These results suggest that the inhibition of MA biosynthesis by isoniazid during BCG early growth does not significantly increase amoxicillin's efficacy on BCG.

Meropenem without clavulanate MIC decreased (from 8 µg/mL to 4 and 2 µg/mL) when compared to the control condition (with no isoniazid exposure). When in

combination with the β-lactamase inhibitor, a decrease of 4-log was observed (from 4 to 0,25 μg/mL). After exposure to isoniazid, *M. bovis* BCG is classified of susceptible to meropenem both with and without clavulanate, according to EUCAST PD-PK breakpoints (Table 4).

For BCG, isoniazid highly alters the cell morphology and growth. Accordingly, MIC assays where isoniazid is present during β-lactams MIC determination should be repeated with lower concentrations of this antibiotic or without the ultrasounds step. It is expected to have a significant decrease on β-lactams MIC when in combination with sub-MIC of isoniazid.

#### 1.3.1.2. Efficacy of β-lactams after *M. bovis* BCG exposure to ethambutol in sub-MIC

BCG exposure to ethambutol did not affect bacterial growth as much as observed for isoniazid, but treated cultures had a slower growth rate when compared to the control (without ethambutol). Table 16 and Figure 31 summarize the results for β-lactams MIC performed after treatment with ethambutol at 1 μg/mL (¼ MIC) and 2 μg/mL (½ MIC).

Table 16 - MIC and MBC median values of amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 μg/mL for *M. bovis* BCG in μg/mL. Three different conditions with two sub-MIC of ethambutol (EMB - 1 and 2 μg/mL) were tested, and control results previously determined are represented for comparison. MBC values are presented in concentrations and as the MBC/MIC reason. MIC values are coloured in green when equal or lower than susceptible EUCAST PD-PK breakpoints, orange when equal or higher than resistant breakpoints and uncoloured when intermediate.

| | | | | Condition of EMB exposure | | |
|---|---|---|---|---|---|---|
| **EMB (μg/mL)** | **Antibiotic** | **Value (μg/mL)** | **Control** | **Before β-lactam MIC determination** | **Before and during β-lactam MIC determination** | **During β-lactam MIC determination** |
| **1** | **AMX** | **MIC** | > 64 | > 64 | > 64 | > 64 |
| | | **MBC** | > 64 | > 64 | > 64 | > 64 |
| | | **MBC/ MIC** | 1 | 1 | 1 | 1 |
| | **AMX + CLA** | **MIC** | 32 | 4 | 16 | 32 |
| | | **MBC** | > 64 | > 16 | 16 | 32 |
| | | **MBC/ MIC** | > 2 | > 4 | 1 | 1 |
| **2** | **AMX** | **MIC** | > 64 | > 64 | 1 | 2 |
| | | **MBC** | > 64 | > 64 | 0,5 | 2 |
| | | **MBC/ MIC** | 1 | 1 | 1 | 1 |
| | **AMX + CLA** | **MIC** | 32 | 4 | - | - |
| | | **MBC** | > 64 | > 16 | - | - |
| | | **MBC/ MIC** | > 2 | > 4 | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| **1** | **MEM** | **MIC** | 8 | 2 | 16 | 16 |
| | | **MBC** | > 32 | > 8 | 16 | 16 |
| | | **MBC/ MIC** | > 4 | > 4 | 1 | 1 |
| | **MEM + CLA** | **MIC** | 4 | 0,25 | 2 | 4 |
| | | **MBC** | 4 | > 1 | 2,0 | 4 |
| | | **MBC/ MIC** | 1 | > 4 | 1 | 1 |
| **2** | **MEM** | **MIC** | 8 | 2 | 0,25 | 1 |
| | | **MBC** | > 32 | > 8 | 0,25 | 1 |
| | | **MBC/ MIC** | > 4 | > 4 | 1 | 1 |
| | **MEM + CLA** | **MIC** | 4 | 0,25 | - | - |
| | | **MBC** | 4 | > 1 | - | - |
| | | **MBC/ MIC** | 1 | > 4 | - | - |

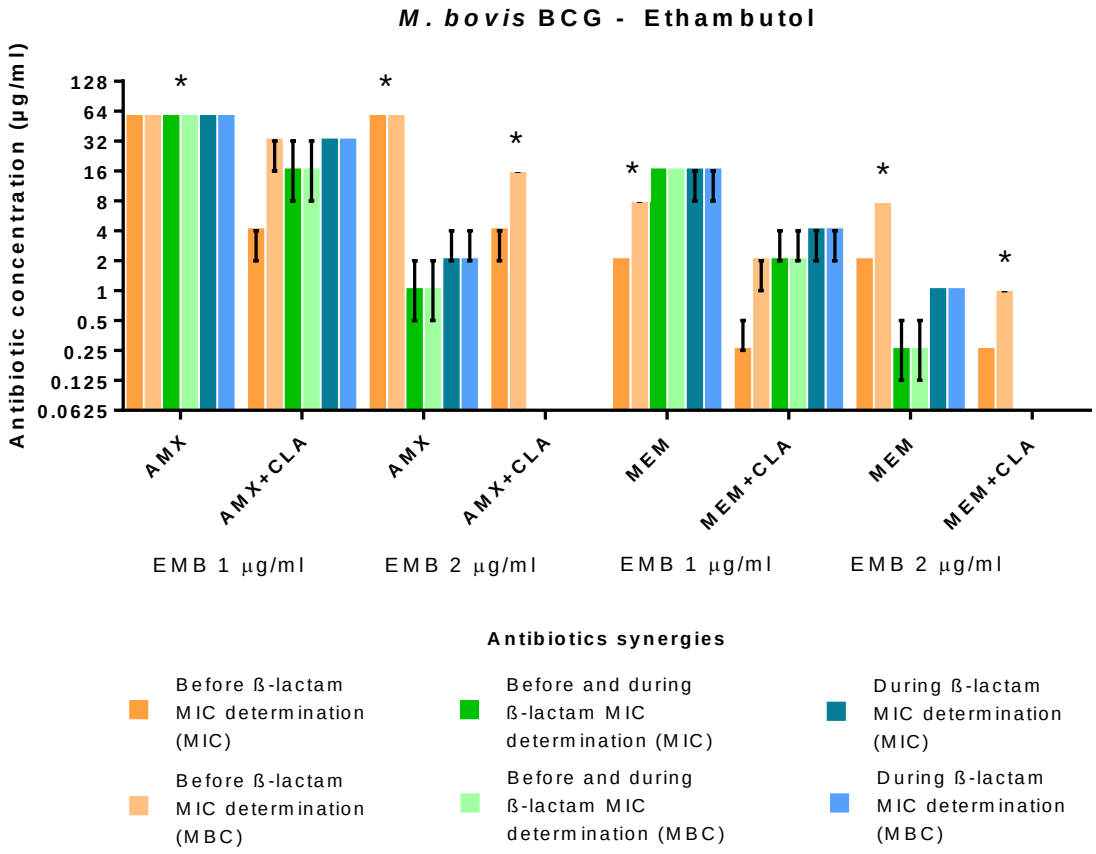


Figure 31 - Graphical representation of *M. bovis* BCG MIC and MBC values for amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL, when treated with ethambutol in sub-MIC (EMB – 1 and 2 µg/mL) in different conditions. Dark colour bars are MIC results and light colour bars are MBC results. "*" means that growth was observed in all concentrations tested.

Exposure of BCG to ethambutol at 1 µg/mL (¼ MIC), did not decrease the MIC of amoxicillin, and growth was observed in all concentrations, for the three conditions tested. In the case of amoxicillin conjugated with clavulanate, a MIC decrease of 3-log

(from 32 to 4 μg/mL) was observed, with a bacteriostatic effect and the resistant classification shifting to intermediate, according to EUCAST PD-PK breakpoints. For the other two conditions, where ethambutol was present during the β-lactams MIC determination, amoxicillin had a bactericidal effect, but no significant decrease in the MIC was observed.

When ethambutol at 2 μg/mL (½ MIC) was present during the β-lactams MIC assay, amoxicillin MIC decreased at least 5-log (from > 64 to 2 and 1 μg/mL) with a bactericidal effect, and under these circumstances BCG could be classified as susceptible to amoxicillin. Conjunction of amoxicillin with clavulanate lead to a decrease of 3-log, (from 32 to 4 μg/mL), as what happened when ethambutol was present at 1 μg/mL. In the presence of ethambutol during the MIC assay, BCG growth was halted, as observed with isoniazid. This may be due to the ultrasound's treatment of the bacterial suspension, that further weakens the cell envelope, or with some interaction between ethambutol and clavulanate.

When BCG was exposed to ethambutol at 1 μg/mL but it was not present on the β-lactams MIC assay, meropenem MIC decreased 2-log (from 8 to 2 μg/mL), with a bacteriostatic effect, reverting the classification of BCG to susceptible to the carbapenem. When the conjunction of meropenem with ethambutol (1 μg/mL) was done during the β-lactams MIC assay, no decrease of meropenem MIC was observed, even though a bactericidal effect of meropenem was observed. Conjunction of meropenem with clavulanate lead to a MIC decrease of 4-log (from 4 to 0,25 μg/mL) and a bacteriostatic effect. For the condition where ethambutol was present before and during the β-lactams MIC assay, no significant variations of the MIC were observed.

Exposure of BCG to ethambutol at 2 μg/mL during the β-lactams MIC assays lead to a decrease of meropenem MIC up to 5-log with a bactericidal effect – MICs between 0,25 and 2 μg/mL – suggesting a synergistic interaction between meropenem and ethambutol. Conjunction with clavulanate, resulted in a MIC decrease of 4-log when compared to the control – from 4 to 0,25 μg/mL, but meropenem had a bacteriostatic effect. When ethambutol was also present during the meropenem MIC assay, no growth was observed, as observed for amoxicillin with clavulanate. This observation reinforces the possibility of a possible interaction between the β-lactamase inhibitor with the anti-TB drug, when at higher concentrations (this effect is not observed when ethambutol is used at 1 μg/mL).

Exposure of BCG to sub-MIC of ethambutol in conjunction with β-lactams seems to increase the efficacy of the latter antibiotics, as the bacteria becomes susceptible to β-lactams, according to EUCAST breakpoints.

## 1.4.*M. tuberculosis* H37Ra

Among the four mycobacterial species tested, *M. tuberculosis* H37Ra is the closest model for *Mtb* antibiotic susceptibility, the mycobacteria with the highest relevance for human health. Heinrichs and colleagues have shown that MIC values for the avirulent strain H37Ra are equivalent and can be extrapolated for *Mtb* H37Rv, the virulent reference strain (2017). This is a slow growing mycobacteria, and biosafety level and incubation times used are the same as described previously for BCG.

Table 17 summarizes MIC and MBC results in μg/mL and MBC is presented as the reason MBC/MIC for the β-lactams and 1st-line anti-TB drugs tested for *Mtb* H37Ra. The cells are coloured according to the susceptibility of the bacteria to the antibiotics, with green meaning susceptible, orange meaning resistant and non-coloured meaning intermediate resistance. Figure 32 has the graphical representation of MIC and MBC median and ranges for the antibiotics tested.

Table 17 - MIC and MBC median values of amoxicillin, cefotaxime, meropenem, imipenem, isoniazid and ethambutol for *M. tuberculosis* H37Ra in μg/mL. β-lactams were tested with and without clavulanate at 2,5 μg/mL. MIC values coloured green mean that the bacteria was susceptible to the antibiotic and orange means resistant. MBC values are presented in concentrations and as the reason to MIC.

| | **without clavulanate** | | | **with clavulanate** | | |
|---|---|---|---|---|---|---|
| **Antibiotic** | **MIC (μg/mL)** | **MBC (μg/mL)** | **MBC/MIC** | **MIC (μg/mL)** | **MBC (μg/mL)** | **MBC/MIC** |
| **Amoxicillin** | 32 | > 64 | > 2 | 2 | > 4 | > 2 |
| **Cefotaxime** | 4 | > 8 | > 2 | 2 | > 4 | > 2 |
| **Meropenem** | 8 | > 32 | > 4 | 1 | > 4 | > 4 |
| **Imipenem** | 8 | > 32 | > 4 | 1 | > 4 | > 4 |
| **Isoniazid** | 0,25 | 0,25 | 1 | | | |
| **Ethambutol** | 2 | 4 | 2 | | | |

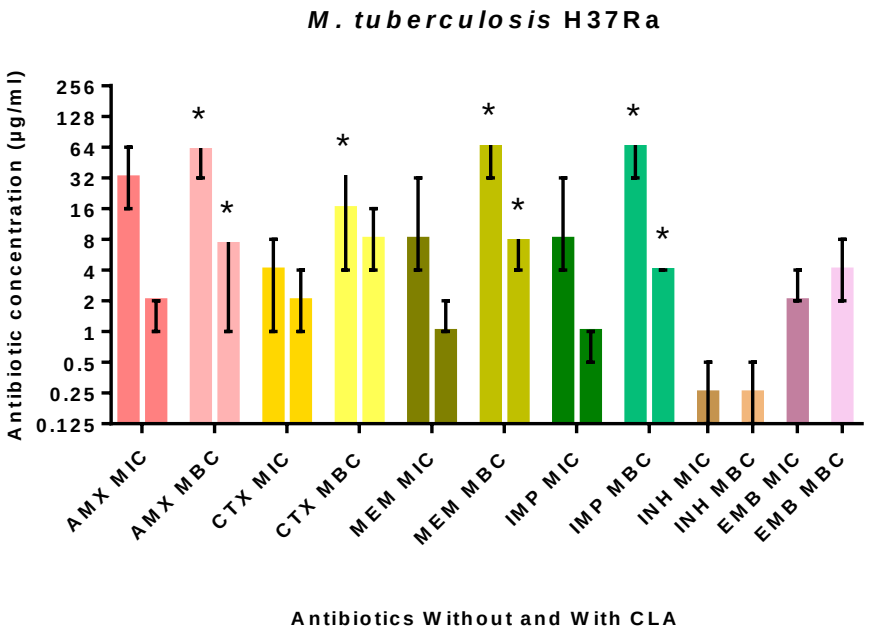


Figure 32 - Graphical representation of *M. tuberculosis* H37Ra MIC and MBC medians and ranges for amoxicillin (AMX), cefotaxime (CTX), meropenem (MEM), imipenem (IMP), isoniazid (INH) and ethambutol (EMB). β-lactams were tested without (left bars) and with clavulanate (right bars) (CLA) at 2,5 µg/mL. "*" at the top of the bars means that growth was observed in all concentrations tested.

According to EUCAST breakpoints (Table 4), *Mtb* H37Ra can be classified as resistant to all four β-lactams when alone, and susceptible when the antibiotics were conjugated with the β-lactamase inhibitor clavulanate, which have led to decreases of up to 4-log (from 32 to 2 µg/mL, in the case of amoxicillin) of the MICs. All β-lactams tested were bacteriostatic for *Mtb* H37Ra.

*Mtb* H37Ra has a low-level resistance to isoniazid, represented as susceptible, which has a bactericidal effect, and is susceptible to ethambutol, also with a bactericidal effect.

### 1.4.1. Antibiotic synergies

As described previously, MIC assays using combinations between the 1st-line anti-TB drugs and β-lactams were performed. Culture preparation and incubation times were the same as described for BCG. Table 18 and Figure 33 summarize the results for the control assays, that determined amoxicillin and meropenem MIC values with and without clavulanate at 2,5 µg/mL for *Mtb* H37Ra.

Table 18 - MIC and MBC median values of amoxicillin and meropenem with and without clavulanate at 2,5 µg/mL for *M tuberculosis* H37Ra, in µg/mL. The reason MBC/MIC is used to define the effect of the antibiotics – bacteriostatic or bactericidal. MIC values are coloured according to breakpoints from EUCAST – green means susceptible, orange means resistant and uncoloured values mean an intermediate resistance.

| | **without clavulanate** | | | **with clavulanate** | | |
|---|---|---|---|---|---|---|
| **Antibiotic** | **MIC (µg/mL)** | **MBC (µg/mL)** | **MBC/MIC** | **MIC (µg/mL)** | **MBC (µg/mL)** | **MBC/MIC** |
| **Amoxicillin** | > 64 | > 64 | 1 | 4 | > 16 | > 4 |
| **Meropenem** | 32 | > 64 | > 2 | 4 | 16 | 4 |

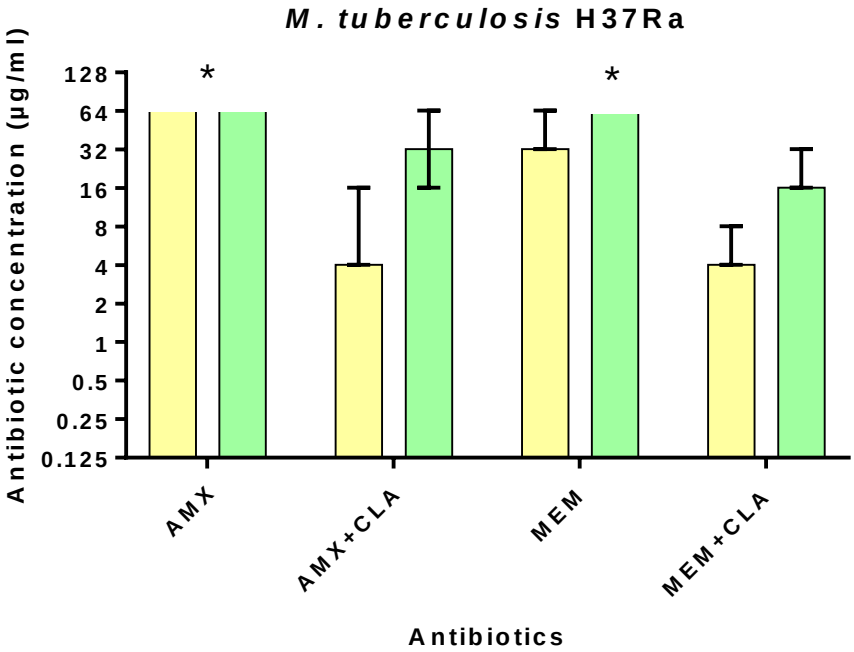


Figure 33 - Graphical representation of *M. tuberculosis* H37Ra MIC and MBC medians and ranges (µg/mL) for amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL. Yellow bars are MIC results and green bars are MBC results. "*" means that growth was observed in all concentrations tested.

Amoxicillin MIC was at least 1-log higher than previous assays and *Mtb* H37Ra have grown on all concentrations tested. When combined with clavulanate, the MIC was also 1-log higher (4 µg/mL), with a bacteriostatic effect and *Mtb* H37Ra is classified as having an intermediate resistance to amoxicillin. Meropenem MIC value increased 2-log when compared to the previous results, both without and with clavulanate (from 8 to 32 µg/mL and 1 to 4 µg/mL, respectively). According to EUCAST breakpoints, *Mtb* H37Ra is classified as resistant to meropenem alone, and susceptible when combined with the β-lactamase inhibitor.

#### 1.4.1.1. Efficacy of β-lactams after *M. tuberculosis* H37Ra exposure to isoniazid in sub-MIC

As observed for BCG, *Mtb* H37Ra cultures that were exposed to sub-MIC of isoniazid had a slower growth rate, and the addition of the antibiotic halted the culture growth. β-lactams MIC assays were done for cultures that reached an $OD_{600nm}$ of at least 0,6 and when isoniazid was present in the 96-well microtiter plates, no growth was observed on positive control wells and therefore results are not available (Table 19 and Figure 34). This suggests that the effect of *Mtb* H37Ra exposure to isoniazid is significant, and these assays should be repeated with lower isoniazid sub-MIC or without ultrasounds treatment of bacterial cultures.

Table 19 - MIC and MBC median values of amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL for *M. M tuberculosis* H37Ra in µg/mL. Three different conditions with two sub-MIC of isoniazid (INH – 0,0625 and 0,125 µg/mL) were tested, and control results previously determined are represented for comparison. MBC values are presented in concentrations and as the MBC/MIC reason. MIC values are coloured in green when equal or lower than susceptible EUCAST PD-PK breakpoints, and orange when equal or higher than resistant breakpoints.

| | | | | Condition of INH exposure | | |
|---|---|---|---|---|---|---|
| **INH (µg/mL)** | **Antibiotic** | **Value (µg/mL)** | **Control** | **Before β-lactam MIC determination** | **Before and during β-lactam MIC determination** | **During β-lactam MIC determination** |
| **0,0625** | **AMX** | **MIC** | > 64 | > 64 | - | - |
| | | **MBC** | > 64 | > 64 | - | - |
| | | **MBC/ MIC** | 1 | 1 | - | - |
| | **AMX + CLA** | **MIC** | 4 | 4 | - | - |
| | | **MBC** | > 16 | > 16 | - | - |
| | | **MBC/ MIC** | > 4 | > 4 | - | - |
| **0,125** | **AMX** | **MIC** | > 64 | > 64 | - | - |
| | | **MBC** | > 64 | > 64 | - | - |
| | | **MBC/ MIC** | 1 | 1 | - | - |
| | **AMX + CLA** | **MIC** | 4 | 4 | - | - |
| | | **MBC** | > 16 | 16 | - | - |
| | | **MBC/ MIC** | > 4 | 4 | - | - |
| **0,0625** | **MEM** | **MIC** | 32 | 32 | - | - |
| | | **MBC** | > 64 | > 64 | - | - |
| | | **MBC/ MIC** | > 2 | > 2 | - | - |
| | **MEM + CLA** | **MIC** | 4 | 4 | - | - |
| | | **MBC** | 16 | > 16 | - | - |
| | | **MBC/ MIC** | 4 | > 4 | - | - |
| **0,125** | **MEM** | **MIC** | 32 | 32 | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | **MBC** | > 64 | > 64 | - | - |
| | | **MBC/ MIC** | > 2 | > 2 | - | - |
| | **MEM + CLA** | **MIC** | 4 | 2 | - | - |
| | | **MBC** | 16 | > 8 | - | - |
| | | **MBC/ MIC** | 4 | > 4 | - | - |

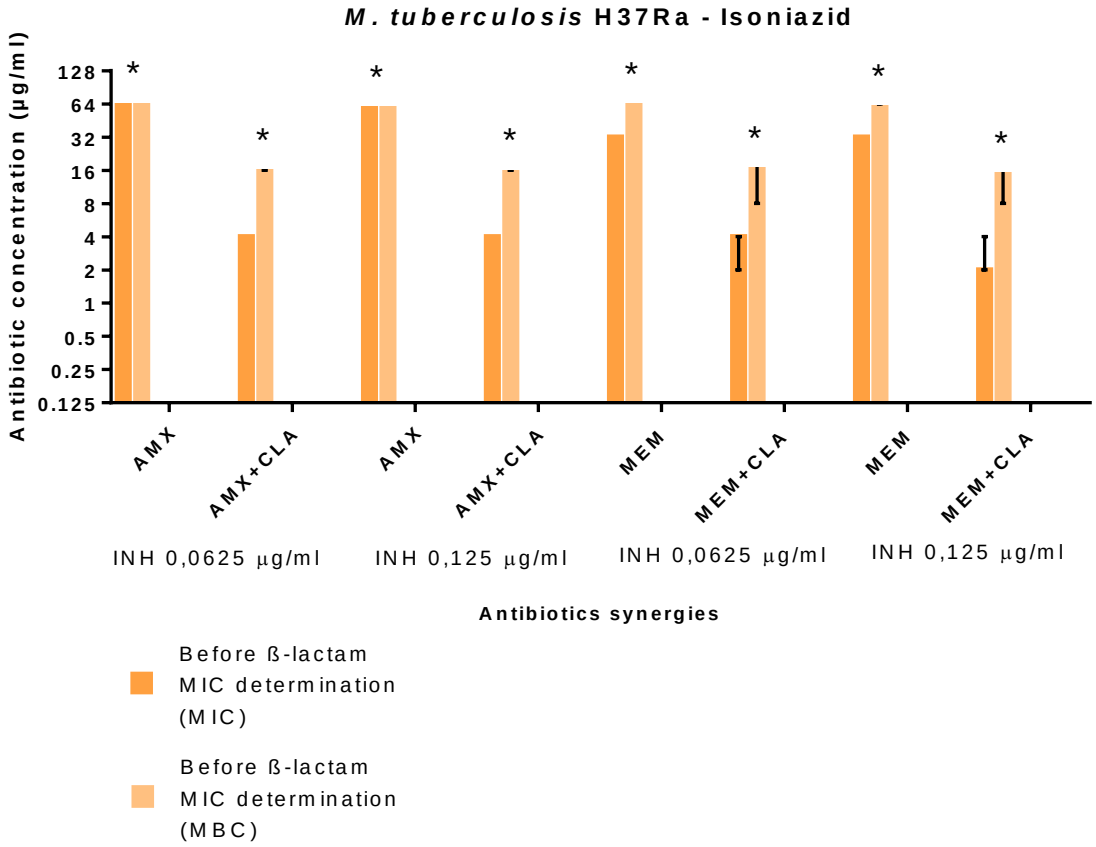


Figure 34 - Graphical representation of *M. tuberculosis* H37Ra and MBC values for amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL, when treated with isoniazid in sub-MIC (INH – 0,0625 and 0,125 µg/mL) in different conditions. Dark colour bars are MIC results and light colour bars are MBC results. "*" means that growth was observed in all concentrations tested.

Amoxicillin MIC and MBC values for *M. tuberculosis* H37Ra, with and without clavulanate, were not altered by pre-exposure to isoniazid. The same result was observed for meropenem, after *Mtb* H37Ra treatment with isoniazid at 0,0625 µg/mL (¼ MIC). The only combination that resulted in a MIC decrease was when *Mtb* H37Ra was pre-exposed to isoniazid at 0,125 µg/mL and the assay was done with meropenem combined with clavulanate – from 4 to 2 µg/mL – and the classification as susceptible to the carbapenem, according to EUCAST PD-PK breakpoints, remained. This outcome suggests that the cell wall inhibition caused by isoniazid during pre-exposure of the bacteria to the antibiotic is not enough to increase β-lactams efficacy. These assays should

be repeated with lower sub-MIC of isoniazid, without the ultrasounds step on bacterial suspension preparation and *Mtb* H37Ra exposure to this antibiotic should be maintained during the β-lactams MIC determination.

### 1.4.1.2. Efficacy of β-lactams after *M. tuberculosis* H37Ra exposure to ethambutol in sub-MIC

Ethambutol did not cause a growth slowdown as significant as isoniazid, and it was possible to collect data for all conditions tested. Table 20 and Figure 35 summarize amoxicillin and meropenem MIC and MBC results for *M. tuberculosis* H37Ra, in combination with sub-MIC of ethambutol in different conditions.

Table 20 - MIC and MBC median values of amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 μg/mL for *M. tuberculosis* H37Ra in μg/mL. Three different conditions with two sub-MIC of ethambutol (EMB – 0,5 and 1 μg/mL) were tested, and control results previously determined are represented for comparison. MBC values are presented in concentrations and as the MBC/MIC reason. MIC values are coloured in green when equal or lower than susceptible EUCAST PD-PK breakpoints, and orange when equal or higher than resistant breakpoints.

| | | | | Condition of EMB exposure | | |
|---|---|---|---|---|---|---|
| **EMB (μg/mL)** | **Antibiotic** | **Value (μg/mL)** | **Control** | **Before β-lactam MIC determination** | **Before and during β-lactam MIC determination** | **During β-lactam MIC determination** |
| **0,5** | **AMX** | **MIC** | > 64 | > 64 | 16 | 32 |
| | | **MBC** | > 64 | > 64 | 16 | 32 |
| | | **MBC/ MIC** | 1 | 1 | 1 | 1 |
| | **AMX + CLA** | **MIC** | 4 | 4 | 1 | 2 |
| | | **MBC** | > 16 | > 16 | 1 | 2 |
| | | **MBC/ MIC** | > 4 | > 4 | 1 | 1 |
| **1** | **AMX** | **MIC** | > 64 | > 64 | 16 | 16 |
| | | **MBC** | > 64 | > 64 | 16 | 16 |
| | | **MBC/ MIC** | 1 | 1 | 1 | 1 |
| | **AMX + CLA** | **MIC** | 4 | 4 | 0,5 | 2 |
| | | **MBC** | > 16 | > 16 | 0,5 | 2 |
| | | **MBC/ MIC** | > 4 | > 4 | 1 | 1 |
| **0,5** | **MEM** | **MIC** | 32 | 16 | 4 | 8 |
| | | **MBC** | > 64 | 64 | 4 | 8 |
| | | **MBC/ MIC** | > 2 | 4 | 1 | 1 |
| | **MEM + CLA** | **MIC** | 4 | 2 | 2 | 4 |
| | | **MBC** | 16 | > 8 | 2 | 4 |
| | | **MBC/ MIC** | 4 | > 4 | 1 | 1 |
| **1** | **MEM** | **MIC** | 32 | 16 | 2 | 1 |
| | | **MBC** | > 64 | 64 | 2 | 1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | **MBC/ MIC** | > 2 | 4 | 1 | 1 |
| | **MEM + CLA** | **MIC** | 4 | 2 | 1 | 2 |
| | | **MBC** | 16 | > 8 | 1 | 2 |
| | | **MBC/ MIC** | 4 | > 4 | 1 | 1 |

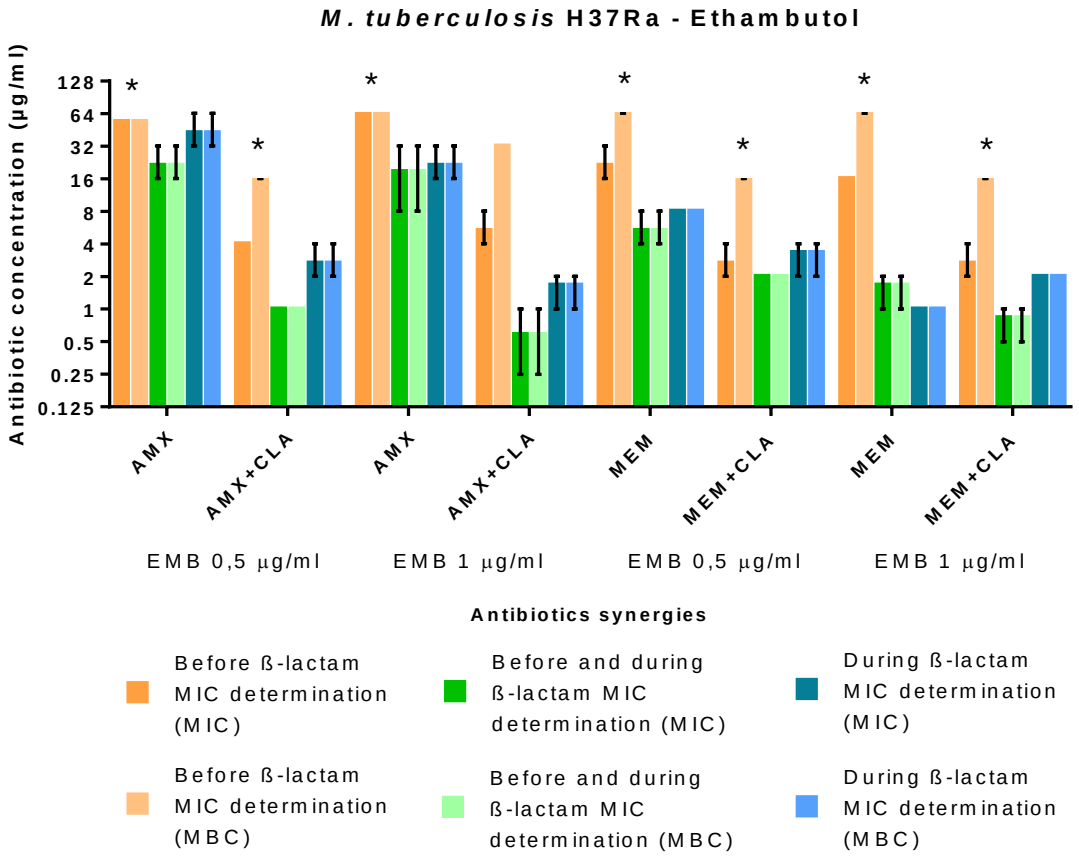


Figure 35 - Graphical representation of *M. tuberculosis* H37Ra MIC and MBC values for amoxicillin (AMX) and meropenem (MEM) with and without clavulanate (CLA) at 2,5 µg/mL, when treated with ethambutol in sub-MIC (EMB – 0,5 and 1 µg/mL) in different conditions. Dark colour bars are MIC results and light colour bars are MBC results. "*" means that growth was observed in all concentrations tested.

When *Mtb* H37Ra was exposed to ethambutol (¼ MIC and ½ MIC) before the β-lactams MIC assay, MICs for amoxicillin, without and with clavulanate, had no variations comparing to the control (without ethambutol treatment). When ethambutol was present during the MIC assay, amoxicillin MIC decreased at least 2-log (from > 64 to 16 µg/mL), and a bactericidal effect was observed, even though the resistant classification of the bacteria to this antibiotic remained. When amoxicillin was combined with clavulanate, a MIC decrease up to 4-log was observed, being higher with ethambutol 1 µg/mL (from 8 to 0,5 µg/mL), and a bactericidal effect was observed. In these cases, *M. tuberculosis* H37Ra can be classified as susceptible to amoxicillin.

For meropenem, exposure to ethambutol only before the MIC assay, lead to a MIC decrease of 1-log both without and with clavulanate (from 32 to 16 μg/mL and 4 to 2 μg/mL, respectively), and had always a bacteriostatic effect. For the conditions where *Mtb* H37Ra was simultaneously exposed to ethambutol and meropenem, more significant MIC decreases were observed, with bactericidal effect. *Mtb* H37Ra can be classified as susceptible to meropenem when combined with clavulanate, and when ethambutol is present at 1 μg/mL (MICs of 1 and 2 μg/mL for meropenem).

In conclusion, ethambutol at 1 μg/mL showed an interesting synergistic effect when combined with β-lactams and the β-lactamase inhibitor clavulanate for *Mtb* H37Ra. This corroborates the hypothesis that when ethambutol inhibits the AG biosynthesis of the cell envelope, PG becomes more accessible for β-lactams activity, as proposed by (Schubert *et al.*, 2017), and clavulanate plays an important role on enhancing its activity.

Accounting the results collected with the four distinct species of mycobacteria, the exposure to sub-MIC of 1$^{st}$-line anti-TB drugs that target the cell envelope biosynthesis seems to overall enhance β-lactams efficacy and therefore accessibility to mycobacterial PG. The combinations here presented, and the positive outcomes consist in a promising tool for TB therapeutics, that with the emergence of MDR and XDR strains requires fast and powerful new approaches, both antibiotics and new targets to be explored. The application of β-lactams on TB treatment has been revisited in the last years (Hugonnet *et al.*, 2009; Gonzalo and Drobniewski, 2013) and seems to be a way of exploring new *Mtb* vulnerabilities.

## 2. Mycobacterial peptidoglycan recognition assays

The results from MIC determination assays show an increased susceptibility of mycobacteria to β-lactams, antibiotics that target the biosynthesis of PG, when in combination with sub-MIC of isoniazid or ethambutol, 1$^{st}$-line anti-TB drugs that target MA and AG biosynthesis. These data supports the hypothesis, also observed for *Corynebacterium glutamicum* (Schubert et al.2017), that 1$^{st}$-line anti-TB drugs which inhibit proper biosynthesis of the components of the mAGP complex, remove the outer layers of the mAGP and consequently increase the accessibility of PG for β-lactams activity in ethambutol/isoniazid-treated cells.

The next aim of the work was to confirm that after treatment with isoniazid and ethambutol, mycobacterial PG was more exposed/accessible and recognition by specific receptors could be enhanced. To test this, specific PG receptors (PGRPs) from *Drosophila* were used in co-precipitation assays in order to address the affinity of different PGRPs to purified and quantified mycobacterial PG. In addition, fluorescent derivatives of these PGRPs were used on binding assays with live mycobacteria, before and after treatment with isoniazid or ethambutol.

## 2.1. PGRP-PG co-precipitation assays

In order to quantify the affinity of four *Drosophila* PGRPs (-SA, -SD, -LC and -LE) to mycobacterial PG, co-precipitation assays were done as described in the Material and Methods section (Vaz *et al.*, 2019).

Purified PG from *E. coli, S. aureus* and *M. smegmatis*, were incubated at 25°C for 30 minutes with the four PGRPs (at a final concentration of 0,3 mg/mL), centrifuged and washed. Once PG is an insoluble macromolecule, all the protein that is bound will be harvested with it after centrifugation (Vaz *et al.*, 2019). The pellets were boiled and applied in an SDS-PAGE gel. Quantification of the resulting bands, which appeared between 48 and 35 KDa molecular weight marker bands, was performed using Image Lab (6.0.1, BioRad).

Figure 36 shows the results of the quantification of PGRP co-precipitation with PG from *E. coli, S. aureus* and *M. smegmatis*. For *M. smegmatis*, two different samples were tested: PG purified with KOH and HF (as described in the Methods and Material section), and a mAGP sample that was not submitted to the step of MA hydrolysis by KOH. The purified PG sample will expectedly be better recognized by PGRPs.

*E. coli* is a Gram-negative bacteria, with a DAP-type PG, with mostly direct (3→4) cross-link (Yu *et al.*, 2010), corresponding to type A1, according to the classification system established by Schleifer and Kandler (1972). *S. aureus* is a Gram-positive bacteria with Lys-type PG and (3→4) cross-links with a monocarboxylic acid bridge, classified as type A3 (Schleifer and Kandler, 1972; Vaz *et al.*, 2019) and *M. smegmatis* is classified as Gram-positive, having a highly amidated DAP-type PG, included in the type A1 of cross-linking (Schleifer and Kandler, 1972; Jankute *et al.*, 2015).

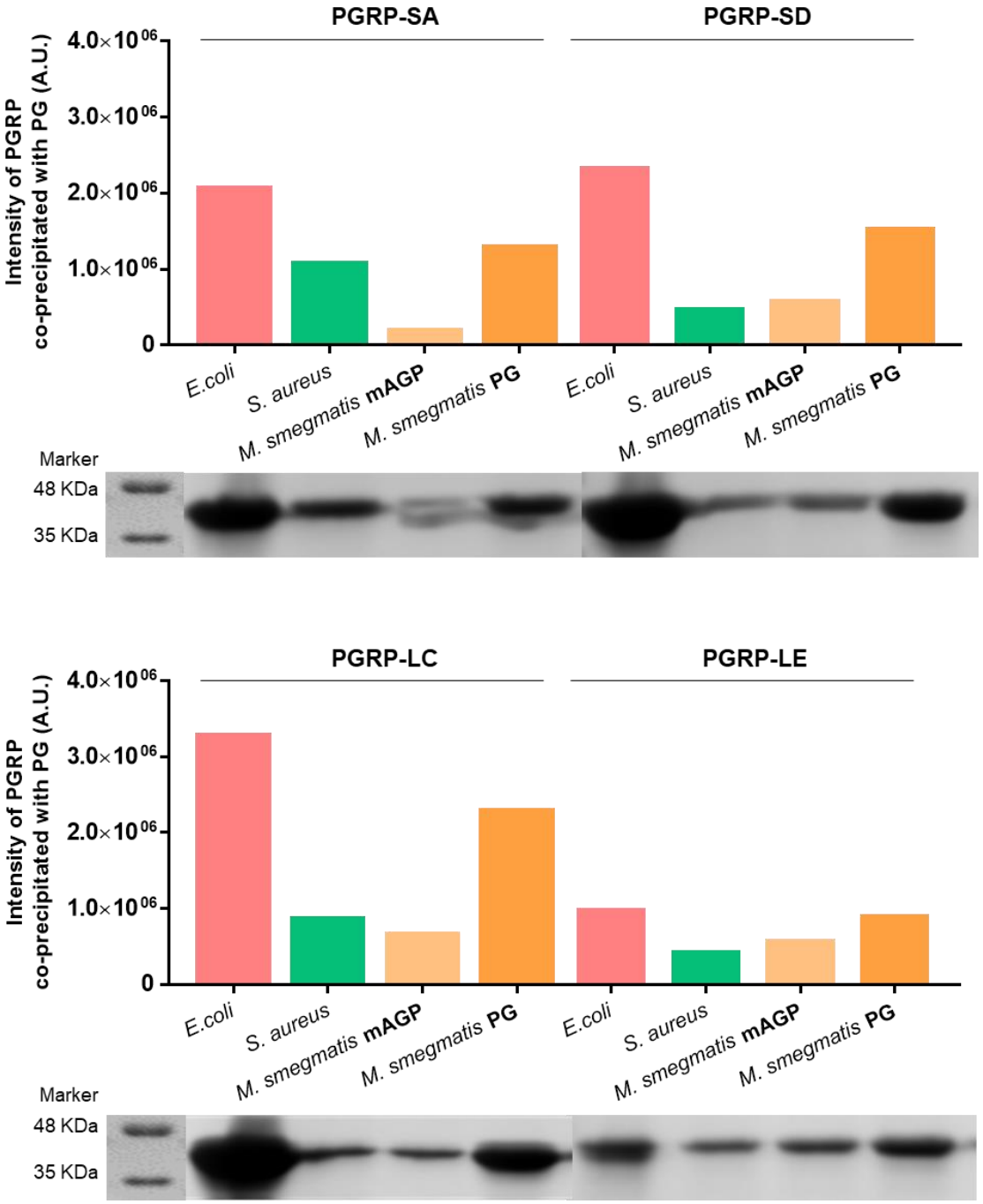


Figure 36 - Binding of *Drosophila* PGRPs (-SA, -SD, -LC and -LE) to purified and quantified PG from *E. coli, S. aureus* and *M. smegmatis*, and mAGP from *M. smegmatis.* The resulting bands from SDS-PAGE gels (between markers of 48 and 35 KDa) were quantified using Image Lab and results are graphically represented in arbitrary units (A.U.).

PGRP-SA showed preference to *E. coli* PG ($2\times10^6$), intermediate affinity to *S. aureus* and *M. smegmatis* PG ($1\times10^6$ and $1{,}2\times10^6$, respectively) and lower binding to *M. smegmatis* mAGP ($1{,}7\times10^5$). According to previous studies, PGRP-SA binds to both Lys-type and non-amidated DAP-type PG (Chang *et al.*, 2004; Vaz *et al.*, 2019), but no significant affinity to *S. aureus* PG (Lys-type) was observed. Although mycobacterial PG is amidated, a significant recognition of *M. smegmatis* PG by PGRP-SA was observed.

With PGRP-SD, the co-precipitation with *E. coli* and *M. smegmatis* PG was similar to the one from PGRP-SA ($2{,}3\times10^6$ and $1{,}5\times10^6$, respectively), a weaker recognition was obtained for *S. aureus* PG ($4{,}7\times10^5$) and an intermediate recognition was noted for *M. smegmatis* mAGP ($5{,}8\times10^5$). PGRP-SD was expected to show higher affinity to DAP-type PG, and additional affinity to amidated PG should be noted (Leone *et al.*, 2008).

PGRP-LC have showed a higher intensity of co-precipitation with *E. coli* and *M. smegmatis* PG ($3{,}3\times10^6$ and $2{,}3\times10^6$, respectively). Intermediate recognition of *S. aureus* and *M. smegmatis* mAGP was observed ($8{,}3\times10^5$ and $5{,}3\times10^5$, respectively). This protein is known to recognize DAP-type PG from both Gram-positive and Gram-negative bacteria, but activates a distinct immune response of *Drosophila*, the Imd pathway (Dziarski and Gupta, 2006).

With PGRP-LE, lower levels of co-precipitation were observed for *E. coli* and *M. smegmatis* PG ($9{,}7\times10^5$ and $9\times10^5$, respectively). For *S. aureus* and *M. smegmatis* mAGP weak affinity was observed, similar to the other PGRPs ($4{,}2\times10^5$ and $5{,}7\times10^5$, respectively). It was expected that PGRP-LE would have higher affinity for DAP-type PGs, but the mechanisms of PGRP-LE recognition have not yet been demonstrated, and it is assumed to function as a intra or extracellular receptor that cooperates with other PGRPs, which could mean a lower binding affinity of its own (Dziarski, 2004; Dziarski and Gupta, 2006)

In summary, the co-precipitation assays showed that PGRPs have distinct patterns of PG recognition, and mycobacterial PG is susceptible of being recognized by the four PRGPs tested (strong affinity for PGRP-LC, and intermediate affinity for PGRP-SA, -SD and -LE).

When images were treated on Image lab software, the background was equally subtracted to all lanes. Considering that *E. coli* PG have shown strong affinity to PGRPs, the high intensity of these bands might have caused a under estimation of the biding to the other PGs, and results for *M. smegmatis* might be clearer when the assays are optimized and repeated.

The PGRPs were also tested with whole cells of *M. smegmatis*, without any treatment, and with exposure to sub-MIC of isoniazid and ethambutol.

## 2.2.PGRP-live mycobacteria fluorescence microscopy assays

Having demonstrated that mycobacterial PG is recognized by *Drosophila* PGRPs, the fluorescent derivatives of these proteins were incubated with whole cells of *M. smegmatis* and observed by fluorescence microscopy. From the treatment conditions tested before, one sub-MIC of each 1st-line anti-TB drug was selected, and a sample from each treated culture and a control (culture without antibiotics) was incubated with each of the four PGRPs. After 5 minutes of incubation, samples were centrifuged and washed, and binding was observed in a Zeiss Axio Observer Z1 microscope and phase and fluorescence images were analysed using ImageJ software.

The concentration of isoniazid used on early-log phase cultures of *M. smegmatis* was 2 μg/mL (¼ MIC), because on the antibiotics synergistic assays it was enough for a significant decrease of the β-lactams MIC (see section 1.1.1.1, page 45) – therefore an increased accessibility of the PG – and a higher concentration (4 μg/mL) could lead to cell lysis. For ethambutol, the concentration selected was 1 μg/mL (½ MIC), because the lower concentration of this antibiotic (0,5 μg/mL) could not be enough for PG exposure – on the antibiotics synergies assays, both concentrations lead to decrease of the β-lactams MIC, but was significantly higher for ethambutol at 1 μg/mL (see section 1.1.1.2, page 48). It should be noted that for the mycobacterial cultures no autofluorescence was observed, and fluorescent signals come from the fluorescent PGRPs that were bound to mycobacterial PG. Figure 37 shows the phase contrast and fluorescence images obtained for treated and non-treated cultures of *M. smegmatis* after incubation with the four PGRPs mCherry derivatives, and only with PBS, for control. Fluorescence images where acquired using a TexasRed filter with 100 milliseconds of exposition.

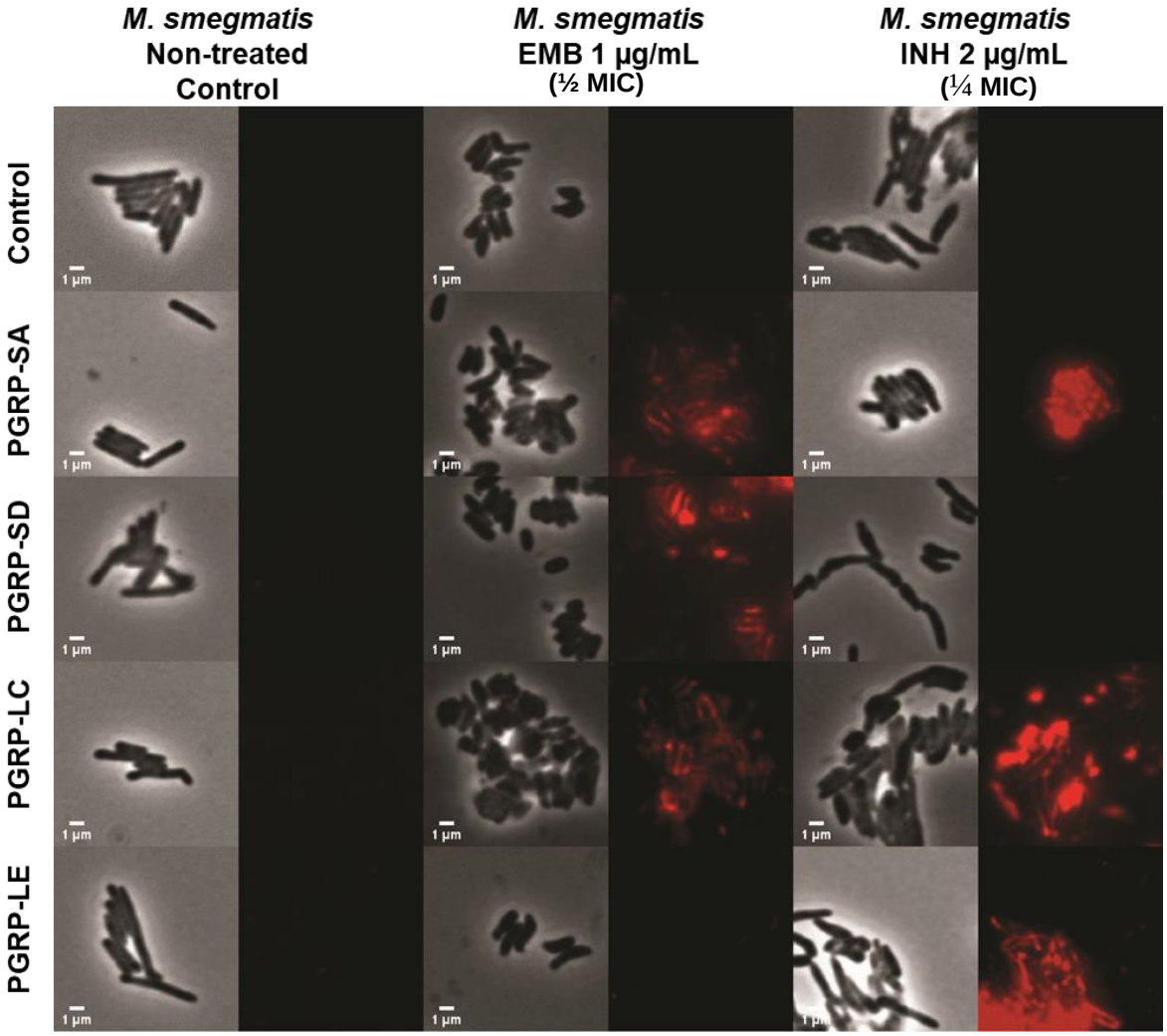


Figure 37 – Binding of fluorescent derivatives PGRPs (mCherry) to *M. smegmatis* whole cells *in vitro*. Three conditions were tested: control with no antibiotic exposure, ethambutol (EMB) added at 1 µg/mL during early-log phase, and isoniazid (INH) added at 2 µg/mL during early-log phase. Cultures were grown overnight, and after incubation with the PGRPs, observations were done in a Zeiss Axio Observer Z1 microscope. A no-binding control was included, where bacteria were incubated only with PBS. Fluorescence mCherry channel images were captured with 100 milliseconds of exposition.

As expected, no fluorescence signal was obtained for the non-treated culture, and for the cultures incubated with PBS, without PGRP. The culture of *M. smegmatis* that was not exposed to the antibiotics showed elongated bacilli, that had the tendency to form little clumps and isolated bacteria was not often observed.

When *M. smegmatis* was treated with ethambutol at 1 µg/mL (½ MIC), the morphology of the bacteria was clearly altered, with a less elongated form and tendency to form larger clumps. Significant binding was observed with PGRP-SA, -SD and LC, where the fluorescence clearly corresponds to bacterial cell envelope outlining. This

result goes in accordance with the co-precipitation assay with purified *M. smegmatis* PG, where affinity was stronger with PGRP-LC and lower to PGRP-SA and PGRP-SD. These data supports the hypothesis that ethambutol causes cell wall synthesis inhibition and consequent accessibility of PG. The effect of exposure to ethambutol was tested for *Corynebacterium glutamicum* (Schubert et al.2017), and the observation of deficient growth and division was also observed, with the suggestion that combinations between ethambutol and β-lactams are synergistic for bacteria with MA on its cell envelope.

In the case of the isoniazid treated culture, *M. smegmatis* morphology does not seem significantly altered, although there was some cell lysis due to the antibiotic addition. Binding was observed with PGRP-SA, -LC and -LE. A study realized by Campanerut-Sá and colleagues (2016) have also used the approach of exposing early-log phase mycobacterial cultures (*Mtb* H37Rv) to sub-MIC of isoniazid, and showed, by scanning electron microscopy and proteomic analysis, that bacterial division, shape and virulence factors expression was altered. These changes are interpreted as defence mechanisms from *Mtb* but can also mean increased susceptibility to other targets, which should be explored.

In conclusion, optimizations of antibiotic treatment and PGRPs concentration will be done in the near future. However, the data shown here support the hypothesis that combinations of 1$^{st}$ line anti-TB drugs and β-lactams could act synergistically in mycobacteria. Further work will be done with *Mtb*, with the possibility of using synergistic combinations of cell envelope targeting antibiotics and β-lactams which have a limited access to their target, PG, in normally growing mycobacteria.

# Final Considerations

The main objective of this thesis was to study how mycobacteria susceptibility to β-lactams is affected and could be improved after exposition to the 1st-line anti-TB drugs, isoniazid and ethambutol. β-lactams are not usually included on TB therapeutics because of the intricate cell envelope of *Mtb*, which is associated with its high level of intrinsic resistance, and the production of the highly active β-lactamase BlaC, capable of hydrolyse most β-lactams (Catalão, Filipe and Pimentel, 2019). In addition, according to the WHO, carbapenems are included in group D3, which indicates that its use against TB has restricted safety and efficacy supporting information (World Health Organization, 2011).

The emergence of MDR and XDR-TB urge the need for new approaches on fast TB diagnosis, and shorter periods of therapeutics. The data obtained in this thesis are preliminary but serve as model for *Mtb*, and the evidence presented here shows how promising the strategy of combining β-lactams with isoniazid and ethambutol can be for TB treatment.

MIC and MBC results of different β-lactams for four different species of mycobacteria showed that, all mycobacteria can be classified as susceptible to at least one of the β-lactams tested. Clavulanate was overall essential for enhanced activity of β-lactams, meaning that it effectively inhibits mycobacterial β-lactamases. In addition, antibiotics synergistic assays were done with combinations of the 1st-line anti-TB drugs (isoniazid or ethambutol) with β-lactams (amoxicillin and meropenem). The condition where mycobacteria were treated with sub-MIC of isoniazid or ethambutol before and during the β-lactams MIC assays lead to decreases of up to 7-log comparing to the control (without exposure to isoniazid or ethambutol) and mycobacteria classification frequently changed from resistant to susceptible to amoxicillin and meropenem. These observations suggests that anti-TB drugs that target the biosynthesis of the outer layer of the mycobacterial cell envelope are increasing the PG accessibility for β-lactams activity. Therefore, a synergistic effect between the two different classes of antibiotics is observed in mycobacteria, as already described for *Corynebacterium* (Schubert et al 2017).

The higher accessibility of mycobacterial PG after treatment with isoniazid or ethambutol was confirmed by fluorescence microscopy assays, where PGRP from *Drosophila* only showed affinity to mycobacteria that were treated with isoniazid/ethambutol, comparing to control culture (without treatment). This approach

may be a promising tool for studies of mycobacterial cell envelope integrity. In the future, these assays will be performed with fluorescent derivatives of human receptors that recognize PG, namely TLR-2, TLR-4, NOD-1 and NOD-2 in order to study how human immune response activaton might be altered when mycobacterial cell envelope is weakened by the 1$^{st}$-line anti-TB drugs. Also, assays of macrophage infection and inflammatory response will be performed, in order to assess if treatment with antibiotics that inhibit the biosynthesis of mycobacterial cell envelope alters the host immune response, increasing mycobacterial recognition and killing.

This work will be performed with clinical isolates of *Mtb*, including MDR and XDR strains, and the tools have already been developed for an optimized study of how an old class of antibiotics can be applied for improvement of TB treatment.

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# Annex

## 1. Quality control of β-lactams – MIC determination for *S. aureus*

As mentioned in the Material and Methods section, stocks of all antibiotics were done in purified water (and phosphate buffer for clavulanate) and kept at -80°C for 6 months maximum. In order to assure the quality of amoxicillin, cefotaxime and meropenem, MIC assays were performed with two strains of *S. aureus*, one that was susceptible to all β-lactams – FF219, and a resistant one (MRSA) – ATCC 43300. Table 21 has the result for each assay, that was realized three times for amoxicillin and meropenem, and twice for cefotaxime. EUCAST (EUCAST: European Committee on Antimicrobial Susceptibility Testing, 2019) has published MIC values of some antibiotics specific for *Staphylococcus spp*, but EUCAST PD-PK breakpoints were used for better agreement. MIC values are coloured according to the classification, with green when the bacteria is susceptible and orange when resistant. Imipenem was not tested due to its instability.

Table 21 – MIC results in µg/mL for amoxicillin, meropenem and cefotaxime, with two strains of *S. aureus* – FF219 and ATCC 43300. One assay was done each time a new stock of antibiotics was prepared. MIC values are coloured according to EUCAST PD-PK breakpoints, green for susceptible and orange for resistant.

| **MIC (µg/mL)** | **Amoxicillin** | | | | | | **Meropenem** | | | | | | **Cefotaxime** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | without CLA | | | with CLA | | | without CLA | | | with CLA | | | without CLA | | with CLA | |
| FF219 | 0,5 | 0,5 | 0,25 | 0,125 | 0,5 | 0,5 | 1 | 0,5 | 0,5 | 0,25 | 0,5 | 0,5 | 4 | 4 | 2 | 4 |
| ATCC 43300 | 64 | 8 | 8 | 32 | 1 | 4 | 8 | 0,5 | 1 | 8 | 0,25 | 1 | 8 | 1 | 8 | 1 |

*S. aureus* FF219 was isolated at the Faculty of Pharmacy of the University of Lisbon. Results of different antibiotics stocks were concordant for this strain that is classified as susceptible to amoxicillin and meropenem, both with and without clavulanate, and resistant to cefotaxime with and without the β-lactamase inhibitor.

In the case of the MRSA strain, more variability was observed between the different stocks that were tested. For amoxicillin without clavulanate, despite some variability, the resistant classification was constant. However, for all the other antibiotic combinations, the first assay had values significantly higher than the replicates. These results might indicate i) a higher activity of the antibiotics from second and third stocks, compared to the first; ii) loss of resistance from the bacteria, which is conferred by the *mecA* gene; iii) inoculation of a susceptible colony from TSA plates.