Modelling *Mycobacterium tuberculosis* cultures for understanding antibiotic mechanisms

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Abstract

Tuberculosis (TB) is an infectious disease caused by the bacterium Mycobacterium tuberculosis (Mtb). It is currently one of the three main causes of death from infectious disease worldwide. Development of new drugs and combinations of antibiotics to deal with resistant, multiresistant and extremely resistant strains has become a major challenge in the treatment of TB. In experimental research, in vitro cultures are a simple way to evaluate the interaction between antibiotics and the bacterium.

The aim of this project is to improve the understanding of the in vitro dynamics of Mtb with and without the presence of antibiotics by means of modelling and simulation techniques. This work arose from a collaboration established between the Computational Biology and Complex Systems (BIOCOM-SC) research group from the UPC and the tuberculosis research group at the Hospital Clinic de Barcelona, led by Dr. Julià González. The goal of the study is to develop tools that enable us to better understand the interactions antibiotic-bacterium, helping researchers to improve the design of in vitro experiments, reducing their cost and duration.

In this work, an individual-based model (IBM) has been developed and implemented in NetLogo. The model was then parameterized using experimental data provided by the University Medical Centre Rotterdam together with the Dutch National Tuberculosis Reference Laboratory. The simulations performed were presented in time-kill curves. In these curves, the evolution of the bacterial concentration (on a logarithmic axis) over time is plotted depending on the antibiotic concentration.

First of all, the developed model was fitted to Mtb growth without antibiotic. Secondly, functions and parameters were included to reproduce the effect of different rifampicin concentrations in the functionality of the bacterium and to study the emergent dynamics. Once the model was implemented, multiple virtual experiments were generated, the results of which have been analysed.

The most important factors of the Mtb cell cycle were first identified, allowing an accurate simulation of in vitro growth without antibiotic. Subsequently, the introduction of the effects of rifampicin in the model led us to several conclusions. It has been proven that the effect of rifampicin is successfully simulated by introducing multiple combined effects on the functionality of the bacterium. We found that the ability of some non-genetic resistant strains to survive a prolonged exposure to rifampicin is associated with lower cellular maintenance energies. This observation seems to match hypotheses from previous studies, which associated this resistance to the diverse metabolic activities found in Mtb population. Furthermore, after fitting the model to experimental data, we were able to assess the order of magnitude of several biological parameters which are difficult to measure experimentally.

In future studies, we expect to introduce the effect of other antibiotics (and the combination of them) into the developed model. Finally, we will study the possibility of implementing this model into other more powerful platforms in order to reduce the duration of the simulations.
Resum

La tuberculosis (TB) és una malaltia infecciosa causada pel bacteri *Mycobacterium tuberculosis* (*Mtb*). Actualment és una de les 3 principals causes de mort per malaltia infecciosa a nivell mundial. Un dels reptes més importants actualment és el desenvolupament de nous fàrmacs i combinacions d’antibiòtics per fer front a les soques resistentes, multiresistentes i extremadament resistentes. En recerca experimental, els cultius in vitro són una forma senzilla d’avaluar la interacció directa entre *Mtb* i l’antibiòtic.

L’objectiu d’aquest projecte ha estat millorar la comprensió de la dinàmica de *Mtb* en cultius in vitro amb i sense la presència d’antibiòtics mitjançant tècniques de modelització i simulació. Aquest treball s’emmarca dins d’una col·laboració entre el grup de recerca en Biologia Computacional i Sistemes Complexes (BIOCOM-SC) de la UPC i el grup de recerca en tuberculosi de l’Hospital Clínic de Barcelona, liderat pel Dr. Julià González. L’objectiu del treball ha estat desenvolupar eines que ens permetin entendre millor les interaccions bacteri-antibiòtic i que ens ajudin a dissenyar experiments in vitro de menor cost i durada.

En aquest treball s’ha desenvolupat un model basat en l’individu (IBM) que ha estat implementat en la plataforma NetLogo. Posteriorment, el model ha estat parametritzat utilitzant dades experimentals proporcionades per la University Medical Centre Rotterdam juntament amb el Dutch National Tuberculosis Reference Laboratory. Dels cultius simulats s’han obtingut les corbes de letalitat. En aquelles corbes es representa l’evolució de la concentració bacteriana (en un eix logarítmic) al llarg del temps depenent de la concentració d’antibiòtic.

En primer lloc, s’ha ajustat el model desenvolupat al creixement de *Mtb* sense antibiotic. Posteriorment, s’han inclòs funcions i paràmetres per poder reproduir l’efecte de diverses concentracions de rifampicina en la funcionalitat del bacteri i estudiar-ne la dinàmica emergent. Un cop el model ha estat implementat, s’han generat múltiples experiments virtuals, els resultats dels quals han estat contrastats i analitzats.

D’entrada s’han identificat aquells factors més rellevants en el cicle cel·lular de *Mtb*, permetent així una simulació acurada del creixement in vitro sense antibiotic. Posteriorment, la introducció dels efectes de la rifampicina en el model han permès arribar a diferents conclusions. S’ha comprovat que l’efecte de la rifampicina es simula satisfactòriament mitjançant la introducció de múltiples efectes sobreposats en la funcionalitat del bacteri. S’ha detectat que la capacitat no genètica d’alguns bacteris de sobreviure a l’exposició prolongada de rifampicina està associada a menors necessitats de manteniment cel·lular. Aquesta observació, sembla coincidir amb hipòtesis d’estudis previs, els quals associaven la resistència no-genètica amb la diversitat metabòlica del cultiu microbià. A més a més, després d’ajustar el model a les dades experimentals, s’ha pogut avaluar l’ordre de magnitud de diversos paràmetres biològics dificilment mesurables experimentalment. En estudis posteriors, es pretén introduir en el model desenvolupat l’efecte d’altres antibiòtics (i combinacions d’aquests). Per últim, s’estudiarà la possibilitat d’implementar aquest model en altres plataforma més potents que redueixin la durada de les simulacions.
Resumen

La tuberculosis (TB) es una enfermedad infecciosa causada por la bacteria *Mycobacterium tuberculosis* (*Mtbt*). Actualmente es una de las 3 principales causas de muerte por enfermedad infecciosa a nivel mundial. Uno de los retos más importantes en la actualidad es el desarrollo de nuevos fármacos y combinaciones de antibióticos para hacer frente a cepas resistentes, multiresistentes y extremadamente resistentes. En investigación experimental, los cultivos *in vitro* son una forma simple de evaluar la interacción directa entre *Mtbt* y el antibiótico.

El objetivo de este proyecto ha sido mejorar la comprensión de la dinámica de *Mtbt* en cultivos *in vitro* con y sin la presencia de antibióticos mediante técnicas de modelización y simulación. Este trabajo se enmarca dentro del trabajo de colaboración entre el grupo de investigación en Biología Computacional y Sistemas Complejos (BIOCOM-SC) de la UPC y el grupo de investigación en tuberculosis del Hospital Clinic de Barcelona, liderado por el Dr. Julià González. El objetivo del trabajo ha sido desarrollar herramientas que nos permitan entender mejor las interacciones bacteria-antibiótico y que nos ayuden a diseñar experimentos *in vitro* de menor coste y duración.

En este trabajo se ha desarrollado un modelo basado en el individuo (IBM) que ha sido implementado en la plataforma NetLogo. Posteriormente, el modelo ha sido parametrizado utilizando datos experimentales proporcionados por la University Medical Centre Rotterdam junto con el Dutch National Tuberculosis Reference Laboratory. De los cultivos simulados se han obtenido las curvas de letalidad. En estas curvas se representa la evolución de la concentración bacteriana (en un eje logarítmico) a lo largo del tiempo dependiendo de la concentración de antibiótico.

En primer lugar, se ha ajustado el modelo desarrollado al crecimiento de *Mtbt* sin antibiótico. A continuación, se han incluido funciones y parámetros para poder reproducir el efecto de varias concentraciones de rifampicina en la funcionalidad de la bacteria y estudiar la dinámica emergente. Una vez el modelo ha sido implementado, se han generado experimentos virtuales posteriormente contrastados y analizados.

Inicialmente se han identificado aquellos factores más relevantes en el ciclo celular de *Mtbt*, permitiendo así una simulación precisa del crecimiento *in vitro* sin antibiótico. Posteriormente, la introducción de los efectos de la rifampicina en el modelo ha permitido llegar a diferentes conclusiones. Se ha comprobado que el efecto de la rifampicina se simula satisfactoriamente mediante la introducción de múltiples efectos sobrepuestos en la funcionalidad de la bacteria. Se ha detectado que la capacidad no genética de algunas bacterias de sobrevivir a la exposición prolongada de rifampicina está asociada a menores necesidades de mantenimiento celular. Esta observación, parece coincidir con hipótesis de estudios previos, en los cuales se asociaba la resistencia no-genética con la diversidad metabólica del cultivo microbiano. Además, después de ajustar el modelo a los datos experimentales, se ha podido evaluar el orden de magnitud de varios parámetros biológicos difíciles de evaluar experimentalmente. En estudios posteriores, se pretende introducir el efecto de otros antibióticos (y combinaciones de éstos) en el modelo desarrollado. Por último, se estudiará la posibilidad de implementar el modelo en otras plataformas más potentes para así reducir la duración de las simulaciones.
Acknowledgments

I would like to first thank Dr. Daniel López and Dr. Clara Prats for giving me the opportunity to work under their supervision. This experience has been extremely enriching for me, and would not have been possible without their constant personal and educational guidance. This project has given me the chance to discover the field of mathematical modelling, towards which I have decided to continue with my future studies.

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Moreover, I would like to thank the Department of Medical Microbiology and Infectious Disease from the University Medical Centre Rotterdam for kindly providing essential experimental data for the development of this project.

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Lastly, I would like to thank my family, for their support and encouragement during all the stages of my life.
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1. INTRODUCTION

1.1 Tuberculosis

Tuberculosis (TB) is one of the most ancient infectious diseases of mankind, with molecular evidence going back to over 17,000 years (1). The causative organism of this disease, *Mycobacterium tuberculosis* (*Mtbc*), is a gram-positive bacterium, that was first reported by Robert Koch in 1882.

During the last century, scientific and clinical progress in our societies has resulted in a number of achievements related to its diagnosis, the development of efficient drug therapies and an unprecedented comprehension of the interactions between *Mtbc* and the host body, which has sharply fallen TB mortality. In spite of these unquestionable developments, nowadays TB is still one of the top 10 causes of death worldwide, and during the last years, we have witnessed a worsening of the efficacy of control manifested by a rising prevalence of drug resistance strains in many countries (2).

According to the World Health Organization (WHO), TB is considered a worldwide pandemic. In 2015, 10.4 million new (incident) cases were estimated. In Figure 1.1 the annual number of incident TB cases is shown relative to population size in different countries worldwide.

![Estimated TB incidence rates, 2015](image)

*Figure 1.1:* Estimated TB incidence rates in 2015 (5).
1.1.1 Epidemiology and resistance situation

**Infection dynamics**

The World Health Organization estimates that about one-third of the world’s population is infected with *Mtb* (2-3 billion people). Once an infection occurs, it can mainly remain as a latent infection or it can eventually evolve towards an active TB disease. About 90 percent of *Mtb* infections remain as Latent Tuberculosis Infection (LTBI). In latent infection, *Mtb* does not grow significantly due to the capacity of the immune system to form granulomas and control the spread out of the bacteria. When the host immune system is able to keep the bacterium under control, a balanced situation is established, where the infected individual is not contagious and does not show any apparent symptoms (3). This latent situation may persist for months, years, or even decades without turning into the active form and making its host ill. Nevertheless, 10 percent of those with latent infection do develop the active form during their lifetime (90 percent of whom live in low- or middle-income countries).

The probability of developing the active form of the disease, significantly increases among people with weakened immune systems, such as young children and individuals who have HIV or are undergoing chemotherapy. When the bacterium outpace the immune system, active TB starts, followed by rapid multiplication and spread out of bacilli to attack the organs of the host (4). The active *Mtb* can affect several organs of the host body, but as it prefers environments rich in oxygen most patients exhibit a pulmonary variety of the disease. Most infections are acquired via an aerosol route from active TB patients. The infection can occur by breathing just a few *Mtb* bacteria released into the air when an active individual coughs spits or talks. Furthermore, in some cases, an individual can also become infected by ingestion of milk or meat from an infected animal (3) (4). The classic symptoms of the disease are; a chronic cough with blood-containing sputum, night sweats, fever and weight loss (5).

**History of treatment**

For many centuries, there was no way to combat tuberculosis. In its early days, TB was commonly known as consumption, due to the weight loss of its victims. Between 1600 and 1800 TB increased dramatically in Europe and North America and resulted in a large number of deaths. This devastating effect was linked to the appalling socioeconomic conditions that people suffered during that period (overcrowding, poor nutrition, lack of hygiene and sanitation, dearth of medical care), which affected their immunologic system, obliged them to live in small and overcrowded places, and sharply raised death rates (2).

In the beginning of the nineteenth century, socioeconomic conditions improved, giving rise to better nutrition and living standards. Moreover, the first public health measures started, and there was a dawning realisation that TB could be an infectious disease, which led to isolating the affected individuals in sanatoriums. As a result of these factors, death rates began to fall in 1800. Long after TB mortality had begun its remarkable downward trend, in 1882 Robert Koch discovered *Mtb*, and in 1895 Wilhelm Konrad Röntgen discovered X-rays, which had a tremendous subsequent impact on the diagnosis and management of the disease (2).
During the twentieth century, with the development of antibiotics and other clinical triumphs, scientists began to gain on the disease. In 1921, Albert Calmette and Camille Guérin developed a vaccine (BCG vaccine) that entered into public use. However, this vaccine only conferred protection against severe childhood forms (4). In January 1944, Schatz, Bugle and Waksman reported the discovery of streptomycin, which was an effective drug against 22 different species of bacteria, including Mtb (6). In spite of inhibiting Mtb activity, streptomycin caused some side effects. After the discovery of streptomycin, a rapid succession of better antibiotics were developed in the following years, enabling those countries that had the necessary money and infrastructure to significantly reduce TB incidence.

**Resistant strains**

The current TB standard treatment was developed in the 1960s, and consists of four antibiotics, which seem to be extremely effective against active drug-susceptible (non-resistant strains) TB, as long as patients are compliant to the entire six-to-nine-month treatment. However, if patients do not complete the full protocol or use antibiotics inconsistently, it gives time to the bacterium to evolve into a drug-resistant strain. The fact that some patients stopped the treatment, whether because they started to feel better or because their drug supply was interrupted for some reason, led to one of the major challenges in the history of TB, the emergence of resistant strains (4).

The emergence of drug resistance is explained by the “fall and rise” effect. If a patient with active TB is treated with only one anti-TB drug, the subpopulation of susceptible bacilli (those bacilli affected by the drug action) will suddenly decline. However, if there were enough bacteria in the host, and no other antibiotic was given, spontaneously resistant bacilli would be selected and resistant subpopulation would increase (7). This is the reason why TB treatment consists in a combination of different drugs and must follow a strictly periodic administration of anti-TB drugs. Otherwise, it would give bacteria the chance to become resistant.

The inadequate management of patients with susceptible TB, poor TB infection control measures and persistent high prevalence of HIV has resulted in a number of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB cases (XDR-TB), becoming a tremendous challenge for clinical and programmatic TB management and control (7).

According to the WHO’s Tuberculosis Report 2016, the global number of cases with MDR-TB or rifampicin-resistant TB (MDR/ RR-TB) detected and notified in 2015 was 132,120, from which 124,990 enrolled on treatment. Nevertheless, the estimated number of MDR/RR-TB cases in 2015 was 580,000, accounting for about 250,000 deaths. This big difference between reported and estimated cases may be caused by the lack of laboratory-confirmed diagnostics or incomplete reporting of laboratory data. In addition, in many low-income countries, the ratio of enrolled to diagnosed cases is very low due to the economic unavailability to provide treatment. The cost of treatment per drug-susceptible TB patient ranges between US$100-10,000, while in the case of MDR-TB the cost is in the range of US$2,000-20,000 (5).
1.1.2 Drug treatment of tuberculosis

*Mycobacterium tuberculosis* is a rod-shaped pathogenic bacillus with a unique cell wall. Its smart stealth behaviour to get away from host defence, together with metabolic fine tuning in hostile environment, makes it the world’s most successful pathogen in action (3). Therefore, since the discovery of the first anti-TB drug (Streptomycin), many other antibiotics and treatments have been required and developed in order to combat the bacteria.

Anti-TB drugs are classified into five groups depending on their efficacy, potency, drug class and experience of use. Such categorization is shown in Table 1.1. Moreover, those drugs recommended for the treatment of drug susceptible TB are called first-line anti-TB drugs (corresponding to those from Group 1). Second-line anti-TB drugs are used in drug-resistant strains of *Mtb*and include Groups 2, 3 and 4. Finally, third-line anti TB drugs (Group 5) have unclear efficacy or undefined roles (8).

**Table 1.1:** Classification of anti-TB drugs. Adapted from Alimuddin Zumla, 2013 (8).

<table>
<thead>
<tr>
<th>First-line drugs</th>
<th>Second-line drugs</th>
<th>Group 2</th>
<th>Injectable aminoglycosides: streptomycin (S/STM), kanamycin (KM), amikacin (AMK). Injectable polypeptides: capreomycin (CM), viomycin (VIM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Oral: isoniazid (H/INH), rifampicin/rifampin (R/RIF), pyrazinamide (Z/PZA), ethambutol (E/EMB), rifapentine (P/RPT) or rifabutin (RFB).</td>
<td>Group 3</td>
<td>Oral and injectable fluoroquinolones: ciprofloxacin (CFX), levofloxacin (LFX), moxifloxacin (MXF) ofloxacin (OFX), gatifloxacin (GFX).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Group 4</td>
<td>Oral: para-aminosalicylic acid (PAS), cycloserine (DCS), terizidone (TRD), ethionamide (ETO), prothionamide (PRO), thioacetazone (THZ), linezolid (LZD).</td>
</tr>
<tr>
<td>Third-line drugs</td>
<td>Clofazimine (CFZ), linezolid (LZD), amoxicillin plus clavulanate (AMX/CLV), imipenem plus cilastatin (IPM/CLN), clarithromycin (CLR).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Multidrug-resistant tuberculosis is defined as tuberculosis that is resistant to at least rifampicin and isoniazid, and extensively drug-resistant TB is defined as a particular type of MDR-TB that is also resistant to fluoroquinolone and any second-line injectable anti-TB drug (7).

Currently, the most common treatment (and recommended by the WHO, 2016) for drug susceptible TB is a 6-month regimen of four first-line drugs: isoniazid, rifampicin, ethambutol and pyrazinamide. This treatment results successful in at least 85% of new cases of drug-susceptible TB. However, the main treatment for those patients with MDR-TB and XDR-TB is much longer (20 months) and requires more expensive and toxic drugs (second-line anti-TB drugs), with the consequent side effects. Nevertheless, a shorter (but more expensive) MDR-TB treatment (9-12 months) is recently being recommended for all patients with pulmonary MDR-TB that do not have XDR-TB (5).
As mentioned above, the BCG vaccine prevents severe forms of TB in children, and it is being used worldwide. Although there is not yet any available vaccine capable of preventing the disease in adults, 13 new TB vaccines are in Phase I, II or III trials. In addition, new anti-TB drugs are emerging from the pipeline, and many combinations are being tested in clinical trials (5). In Table 1.2 targets and effects of the main anti-TB drugs in clinical use are presented.

**Anti-TB mechanisms of first line drugs**

In the present study, data from the main first-line anti-TB drugs has been analysed, and therefore an introduction to their targets and effects is required.

**Table 1.2:** Mechanisms of action of several anti-TB drugs (8)

<table>
<thead>
<tr>
<th>Drug (year of discovery)</th>
<th>Target</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First-line drugs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoniazid (1952)</td>
<td>Enoyl-[acyl-carrier-protein] reductase</td>
<td>Inhibits mycolic acid synthesis</td>
</tr>
<tr>
<td>Rifampicin (1963)</td>
<td>RNA polymerase, beta subunit</td>
<td>Inhibits transcription</td>
</tr>
<tr>
<td>Pyrazinamide (1954)</td>
<td>S1 component of 30S ribosomal subunit</td>
<td>Inhibits translation and trans-translation, acidifies cytoplasm</td>
</tr>
<tr>
<td>Ethambutol (1961)</td>
<td>Arabinosyl transferases</td>
<td>Inhibits arabinogalactan biosynthesis</td>
</tr>
<tr>
<td><strong>Second-line drugs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Para-aminosalicylic acid (1948)</td>
<td>Dihydropteroate synthase</td>
<td>Inhibits folate biosynthesis</td>
</tr>
<tr>
<td>Streptomycin (1944)</td>
<td>S12 and 16S rRNA components of 30S ribosomal subunit</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td>Ofloxacin (1980)</td>
<td>DNA gyrase and DNA topoisomerase</td>
<td>Inhibits DNA supercoiling</td>
</tr>
<tr>
<td>Capreomycin (1963)</td>
<td>Interbridge B2a between 30S and 50S ribosomal subunits</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td>Kanamycin (1957)</td>
<td>30S ribosomal subunit</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td>Amikacin (1972)</td>
<td>30S ribosomal subunit</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td>Cycloserine (1955)</td>
<td>D-alanine racemase and ligase</td>
<td>Inhibits peptidoglycan synthesis</td>
</tr>
</tbody>
</table>

**Rifampicin**

Rifampicin (RIF) was discovered in 1963, and it is an essential, first-line anti-TB drug for the treatment of TB, but it is also effective against a wide range of microorganisms. It is considered a bactericidal agent against *Mtb*, and is active against both growing and stationary phase bacilli with low metabolic activity. Its minimum inhibitory concentration (lowest concentration of a chemical that prevents visible growth of a bacterium, MIC (9)) ranges from 0.05 to 1 µg/mL on solid or liquid media. Strains with MICs > 1 µg/mL in liquid or agar medium are considered resistant strains (10). According to a study from J.V.Piddock (11), rifampicin penetrates the cell wall and balances the plasmatic concentration with the medium in 1-2 minutes.
RIF binds to and inhibits the $\beta$ subunit of the DNA-dependent RNA polymerase (which carries out the transcription process) and physically blocks elongation of the RNAm chain (which is the responsible of the later translation to a protein). The $\beta$-subunit of the RNA polymerase, which is shown in Figure 1.2, contains the major part of the active centre responsible of the RNAm synthesis, and therefore, $\beta$-subunit inhibition results in no-protein synthesis. The $\beta$-subunit is encoded by the gen $rpoB$ (12).

Moreover, once RIF has bound to the $\beta$-subunit, it induces hydroxyl radical formation (10). An excessive concentration of hydroxyl radicals, make these molecules interact with and damage proteins, lipids, carbohydrates and nucleic acids. Consequently, it produces membrane lipid peroxidation, protein cross-linking and degradation, and DNA fragmentation (13).

Other rifamycins include rifambutin (RFB) and rifapentine (RPT). RFB is used to substitute RIF in TB-HIV patients, and RPT may be used in the treatment of RIF-resistant TB (7).

In $Mtb$, resistance to RIF occurs at a frequency of $10^{-7}$ to $10^{-8}$. As in other bacteria, the main cause of RIF’s resistance (96% in $Mtb$) are mutations in a defined region of the $rpoB$ gene (10).

**Isoniazid**

Isoniazid (INH) was discovered in 1952 and it is a drug only active against mycobacteria, and mainly against $Mtb$. Nevertheless, it is the most widely used first-line anti-TB drug and has the strongest early bactericidal activity. However, it is only active against growing tubercle bacilli, and not effective against non-replicating bacilli or under anaerobic conditions. INH low MIC (0.02–0.2 $\mu$g/mL) proves the high susceptibility of $Mtb$ against INH (10).

INH is a prodrug that needs to be activated by the catalase-peroxidase enzyme (KatG) encoded by the $katG$ gene. Once it is activated, it generates a wide range of highly reactive species that attack multiple targets in $Mtb$. The main target of INH consists in the inhibition of the synthesis of the mycolic acids from the cell wall. Mycolic acids are the major component of $Mtb$ cell wall, and their biosynthesis is crucial for $Mtb$ survival and parthenogenesis. Therefore, inhibition of mycolic acids synthesis can result in the lysis and death of the bacterium. Resistance to INH occurs more frequently than for most anti-TB drugs, at a frequency $10^5$ to $10^6$. INH-resistant $Mtb$ mechanisms often consist in losing the catalase and peroxidase enzyme, without which INH cannot be activated (10).
**Ethambutol**

Ethambutol (EMB) is a first-line drug discovered in 1961 and it is only active against mycobacteria (10).

EMB is considered a bacteriostatic agent that is active for growing bacilli but has no effect on non-replicating *Mtb* (MIC = 0.5-2 µg/mL). However, despite its lack of bactericidal effect, EMB is an essential drug in the treatment of drug-susceptible TB because, when used in combination with INH, RIF and PZA, it minimizes the risk of emergence of drug resistant *Mtb* strains (10).

EMB interferes in mycolic acid synthesis by targeting the polymerization of arabinogalactan (an essential component of the mycobacterial cell wall), and it also triggers a cascade of changes in lipid metabolism of bacteria, leading to the disaggregation of bacteria clumps into smaller clusters. Mutation to EMB resistance occurs at a frequency of $10^{-5}$ (14).

**Pyrazinamide**

Pyrazinamide (PZA) is an important first-line drug discovered in 1954. It is active only against mycobacteria, and used along INH and RIF. PZA’s importance lays in its unique capacity to kill a persistent population of bacilli in acidic pH environment, which is commonly found in lesions. PZA’s activity reduces TB treatment from 9-12 months to 6 months. PZA is only active at acid pH (~ 5.5), but even in these environments it has a poor bactericidal activity, with MICs ranging from 6.25 to 50 µg/mL (10). Nevertheless, PZA has a powerful sterilizing activity on non-growing intracellular bacilli in acidic environments, which enables the shortening of the treatment.

As well as INH, PZA is a prodrug that needs to be converted into the active form (pyrazinoic acid (POA)) by the pyrazinamidase/nicotinamidase enzyme encoded by the *pncA* gene. When the *Mtb* extracellular environment is acid, accumulation of POA disrupts the membrane potential. Although the specific target of PZA remains to be identified, it is known that it is related to membrane energy metabolism. Resistance to PZA in *Mtb* is linked to the loss of pyrazinamidase/nicotinamidase activity (15).

### 1.1.3 *In vitro* cultures for drug development

The field of biomedicine considers three main experimental models when referring to antibiotic research:

- **In vitro models:**

  *In vitro* models are those experiments performed outside the common biological context of an organism. In antibiotic research a common type of *in vitro* models are *in vitro* pharmacokinetic/pharmacodynamic (PK/PD)* models.

  *In vitro* PK/PD model refers to a static or dynamic system that can mimic *in vivo* conditions of changing drug concentrations. They are designed to evaluate the efficacy of antibiotics *in vitro* (16).
* **Pharmacokinetics (PK):** Describes the time course of concentration of a drug resulting from administration of a dosage regimen and accounts for its absorption, distribution, metabolism and excretion in the body.

* **Pharmacodynamics (PD):** Describes the intensity of drug effect in relation to its concentration. In the context of anti-infective therapy, it defines the effect of the drug on the pathogen residing in the host organism.

- **Ex vivo models:**

  *Ex vivo* literally means outside of the living body. In biomedicine, *ex vivo* models refer to a procedure where the cells are first taken from the living body, then treated with the minimum modification of their natural context, and finally returned to the living body (17).

- **In vivo models:**

  *In vivo* models are those that are tested within a living organism, whether it is an animal, human or plant in order to study the effect of a particular agent. In the field of antibiotic research, animal testing and clinical trials are crucial elements that involve *in vivo* models (18).

Due to the increasing incidence of MDR and XDR-TB worldwide, it is essential to develop new anti-TB drugs in order to study new possible combinations and therapies that block the increasing drug resistance and shorten TB therapy. The importance of *in vitro* models lies in its capacity to study and evaluate the activity of drugs and therapies under several scenarios and controlled conditions.

As *in vitro* experiments do not take into account the interaction between the antibiotic and the human body, it allow researchers to evaluate the specific effect of the drug on the bacterium. Nevertheless, these models have several simplifications and come with certain drawbacks that will be discussed later.

Some of the most useful models in antibiotic research are *in vitro* PK/PD models. These have many favourable characteristics compared with *in vivo* models, such as more flexibility in study design, adaptability, relatively low cost, and no ethical concerns (19). Especially in *Mtb*, the interactions between the pathogen and the anti-TB agents can be measured more precisely than *in vivo*, since the immune system is ignored in *in vitro* PK/PD models. Based on drug exposure and bacterial concentration, *in vitro* PK/PD models can be classified into static and dynamic models.

- **Classification of *in vitro* PK/PD models:**

- **Static models**

  Static *in vitro* PK/PD models evaluate how a bacterial culture responds to a constant environment with a fixed antibiotic exposure. In these systems, bacterial growth is limited by nutrition, space, aeration, toxic metabolites, and the effect of the antibiotic (20). Static *in vitro* PK/PD experiments are the most common way to determine MIC, a routinely used PD parameter to track antibacterial resistance in clinical isolates (16).
One of the main limitations that these models present is their inability to provide information about the bacterial growth when varying antibiotic concentrations. Noteworthy is that such situation is found in vivo when a patient follows a periodic antibiotic treatment and the drug is removed by clearance mechanisms.

A very common way to study the effects of a drug in static PK/PD models is by carrying out different experiments with a range of constant antibiotic concentrations over a period of time. Tested concentrations include values above and below MIC, and often a 2-fold scaling between concentrations is used. The data taken from such experiments is usually represented in time-kill curves, which are profiles that follow the microbial killing and growth as a function of both time and antibiotic concentration (16). In Figure 1.3 an example of these curves can be observed for Mtb cultures with RIF.

- **Dynamic models**

As previously mentioned, anti-TB treatments involve periodic administration of antibiotics with varying drug concentration at the effect site, which provokes several physical phenomena within the host body such as absorption of the drug, spatial distribution, and particularly clearance mechanisms. These phenomena directly affect the bacterial reaction to the changing environment (16).

In order to mimic this situation, in vitro PK/PD dynamic models expose the bacterial culture to a constantly changing antibiotic concentration in a controlled environment (usually in a bioreactor). However, these models need more advanced technologies, and different methodologies are used. In Vaddady et al. (16), such dynamic models are differentiated as follows:

- **Type 1**: One of the main targets of in vitro dynamic models is to simulate the drug clearance. In type 1 models, this is achieved by diluting the medium. Moreover, a specific volume of the medium is constantly removed and substituted by fresh medium. However, one of the main drawbacks of these systems is the loss of bacteria by dilution. In rapid-growing bacteria, this loss can be corrected using mathematical equations. However, the slow-growth of Mtb leads to a bias that cannot be corrected mathematically. Therefore, type 1 dynamic models have not been reported for the study of anti-TB agents.
✓ Type 2: These models use filters or membranes in order to avoid bacterial loss. One of the most common type 2 models uses a filter and a stirring to prevent blockage of the filter membrane and homogeneity. This model has become popular in the study of anti-TB agents. In Figure 1.4 the results of a dynamic model with periodic administration of INH in Mycobacterium bovis can be observed.

![Figure 1.4](image)

**Figure 1.4**: Panel A shows INH concentration-time profiles. Panel B shows kill-curves of *Mycobacterium bovis* under the INH concentrations of panel A. Source: Budha et al. (26).

✓ Type 3: One of the main problems of type 2 dynamic models is the eventual membrane pore blockage. In order to overcome this problem, a more advanced and costly model has been employed by several groups. These *in vitro* models control the desired concentration profiles in the medium by using diffusion or dialysis systems. These are two-compartment models with semi-permeable hollow fibres, with pore sizes that selectively allow transfer of nutrients, drugs and bacterial metabolites but restrict bacteria from leaving their compartment. Gumbo et al. (21) published some studies of RIF, INH, PZA and MXF using a hollow-fibre bioreactor system. Although these models represent a cutting-edge technique when controlling antibiotic concentrations in a medium, these bioreactors are complex and difficult to sterilize, making the studies with such technology very costly.

- **Drawbacks of *in vitro* models**

As it has been discussed, *in vitro* models are extremely useful tools when studying interactions between bacteria and antibiotics. However, they come with some simplifications of *in vivo* scenarios that cannot be ignored.

One of the main differences, refers to the lack of immune system. Most of the times, *in vivo* infections are controlled by the capacity of the host immune system to kill the bacterium with macrophages or to form granulomas, which are tissue nodules that contain and prevent the dissemination of *Mtbc*. *In vitro* PK/PD models lack the ability to mimic this complex host-defence mechanism. One limitation of these models is noticed when analysing PZA’s activity. As mentioned above, PZA is only active in acidic pHs that are thought to be found inside the activated macrophages. This situation is technically difficult to simulate *in vitro* and therefore, PZA’s activity differs significantly compared with *in vivo* models. As a result, *in vitro* models are more likely to approximate a situation of an immunodeficient patient (16).
Furthermore, differences in growth rate between *in vitro* and *in vivo* scenarios have been reported. These differences could be a significant limitation for *in vitro* models due to the fact that antibiotic activity in an *in vitro* model is highly related to bacterial growth (21). In addition, growth rates differ within each lesion, which varies the susceptibility of *Mtb* to a specific anti-TB agent. This variety of environments and growth rates is also hard to be simulated *in vitro* (16).

Finally, another situation that seems challenging to simulate *in vitro* is the drug distribution. Although *in vitro* models can approximate elimination processes of a drug, it can differ from the *in vivo* distribution due to co-existing disease condition or the presence of other drugs.

### 1.2 Modelling and simulation

Modelling and simulation represent great tools in the study of TB. Until we truly comprehend the interactions between *Mtb* and antibiotics, new drugs will remain elusive. In order to face this challenge, modelling and simulation techniques are used to maximize the information gained from experimental data and to predict new possible situations.

A mathematical model is a description of a system by means of mathematical concepts and language. These models can be categorized into different classes depending on some of their characteristics. The following, are some of their main classifications:

- Depending on the treatment of variables we have **continuous and discrete** models. If the variables of the system can be represented at every point in the time and space interval, it is considered a continuous model. These are usually represented by differential equations. On the other hand, if variables are tracked discretely (e.g. by integer values), then it is considered a discrete model (22).

- Depending on their representation and target there are **empirical and mechanistic** models. Empirical models attempt to represent the system in a descriptive way, without explaining the reasons of its behaviour, whereas mechanistic models aim to reflect and understand the structure and relations between the variables of the system.

- If there is temporal evolution in the system, then the model is called **time-dependent** model, while if the model does not consider changes over the time it is considered a **stationary** model (22).

- Depending on randomness inclusion we have **deterministic** and **stochastic models**. Deterministic models are those which output is only dependent on the initial conditions, and therefore have a unique output for each input. Stochastic models’ dynamics are not determined due to the presence of randomness and variability in their simulations, as a result, its outputs cannot be predicted with precision (23).
There are two main approaches when modelling a system:

The most used and known is the “**Top-down approach**”. When using this strategy, the system and its parameters are considered globally. They aim to understand the intrinsic characteristics of the system by looking at the dynamics of the macroscopic variables. In this case, the most used equations are continuous.

The other modelling strategy is the “**Bottom-up approach**”, which establishes procedural rules for the individuals and their interactions, and then studies the emergent properties of the system as a whole (24).

All the mentioned models and strategies have their advantages and drawbacks. The decision of which one to use will depend on the goal of each particular case. In Figure 1.5 we can observe a schematic summary of the most common models used at the different biological scales of *Mtb* infection, from molecular and cellular to anatomical and population levels.

**Figure 1.5:** Main modelling strategies of *Mtb* infection at different biological scales. Source: Young *et al.* (23).
1.2.1 Modelling and simulation in microbiology

The field of mathematical modelling and simulation has been successfully applied in many areas of microbiology, such as waste control and treatment, microbial ecology, pharmaceutical industry or predictive microbiology in foods, among many others.

These models are becoming increasingly popular in the fields of computational biology and systems biology. The majority of the models that have been used in microbiology describe the kinetics within the cell through continuous models by means of differential equations, and through stochastic discrete models when modelling at the molecular level (24).

Those models that use differential equations to study microbial communities describe the population as a whole and usually deal with macroscopic variables. They can be classified into 3 main groups:

- **Primary models**: Which represent the microbial growth over the time maintaining environmental conditions constant. Some of these are represented in Figure 1.6 A.

- **Secondary models**: Which study microbial growth as a function of some variables of its environment. Represented in Figure 1.6 B.

- **Tertiary models**: Those models result from the union between primary and secondary models in a user-friendly software.

![Figure 1.6](image.png)

**Figure 1.6**: A) Some primary models that measure the response of a microorganism. Source: *et al.* McKellar (36). B) An example of secondary model, where growth rate is represented as a function of glucose concentration.

**Modelling and simulation of in vitro PK/PD cultures**

Modelling and simulation techniques are also used in order to analyse the data from *in vitro* PK/PD experiments. Mathematical modelling of the data from these experiments provides further insight into the mechanisms of drug effects and allows for simulations in order to design studies or even derive clinical treatment strategies (16). Being able to build predictive PK/PD *in vitro* models by means of simulation techniques would allow researchers to estimate optimal dosing regimens without testing all potential combinations in costly and time-consuming laboratory experiments.
The main types of models that have been used in this field can be grouped into mechanistic and empirical models:

- **Mechanistic models in PK/PD:** These models are commonly used to estimate different PK/PD parameters and they have to include at least 3 submodels: one simulating replication of the microorganism (1), another simulating the antimicrobial drug effects (2), and finally a submodel for changing drug concentrations (3).

- The most common submodel when representing microbial replication is the following one:

\[
\frac{dN}{dt} = N \cdot k_{\text{replication}} - N \cdot k_{\text{death}} \quad \text{Equation 1.1}
\]

Where N is the number of cells, and \( k_{\text{replication}} \) and \( k_{\text{death}} \) represent the natural replication and death rates of microorganisms without antimicrobial effects. One of the main limitations of this model is the assumption that the microbial population is homogeneous. However, Nielsens et al. (25) differentiated between growing (S) and resting (R) populations of MTB:

\[
\frac{dS}{dt} = S \cdot k_{\text{growth}} - S \cdot k_{\text{death}} - S \cdot k_{SR} \quad \text{Equation 1.2}
\]

\[
\frac{dR}{dt} = S \cdot k_{SR} - R \cdot k_{\text{death}} \quad \text{Equation 1.3}
\]

Where \( k_{SR} \) represent the rate at which growing population is transferred to the resting population.

- A common representation of the submodel for antimicrobial effect of the drug simulation is often described as:

\[
\frac{dN}{dt} = E_{\text{replication}} \cdot N \cdot k_{\text{replication}} - N \cdot k_{\text{death}} \cdot E_{\text{death}} \quad \text{Equation 1.4}
\]

Where \( E_{\text{replication}} \) and \( E_{\text{death}} \) are the variables that represent the effect of the antibiotic in the microbial growth (bacteriostatic effect) and in bactericidal effect respectively. Noteworthy is that these variables are a function of drug concentration. For more detailed information about \( E_{\text{replication}} \) and \( E_{\text{death}} \) dynamics see et al. Vaddady 2010 (16).

- Finally, a general form for the drug concentration submodel in one compartmental in vitro models can be described by the integrated Equation 1.5:

\[
C(t) = A \cdot e^{-\alpha \cdot t} \quad \text{Equation 1.5}
\]

Where A is a correlation coefficient and \( \alpha \) the first-order rate constant.

- **Empirical models in PK/PD:** Empirical models are useful tools to predict future outcomes as they normally fit the experimental data better than mechanistic models. However, they do not take the biological mechanisms into account and usually have limited predictability beyond the experimental conditions. In spite of these drawbacks, these mathematical models have been used for determining different PK/PD parameters for the first-line anti-TB agents INH, RIF and PZA (26).
1.2.2 Individual-based modelling

Despite the popularity of the previously mentioned models, which study systems structure and dynamics from a “Top-down approach”, the usage of “Bottom-up” modelling techniques is on the rise. Individual-Based Models (IBMs), allow researchers to study how the structure and function of biological systems at a big scale emerge from the underlying characteristics of individual organisms. Grimm et al. (27) defined IBMs as “simulation models that treat individuals as unique and discrete entities which have at least one property in addition to age that changes during their life cycle”.

One of the major fields where IBMs have been applied is ecology. Nevertheless, during the last decade, some studies have applied IBMs to model microbial populations. Microbial IBMs consider microorganisms as discrete entities that interact between them and with their environment according to a set of behavioural rules. This unique approach opens the possibility to analyse the interrelationships between micro-level microorganism interactions and the emergent population characteristics (28).

Compared to models with a “Top-down approach”, using IBMs in microbiology provide some substantial advantages. First of all, more realistic assumptions at single-cell scale can be carried out. Procedural rules are established for each individual of the system instead of working with population parameters. In addition, the presence of randomness and individual variability allow to simulate the diversity found on real microbial cultures (24).

In spite of these clear advantages, IBMs also face some drawbacks. Due to the presence of randomness and the unpredicted outcomes, their representation is usually less clear and intuitive than in continuous models. Furthermore, it is hard to determine the appropriate degree of complexity to introduce in an IBM, as they offer the possibility to start from the smallest scales, but excessive complexity can come with greater uncertainty and errors. Thus, it is essential that those handling IBMs apply the parsimony principle, which states that the model used to reproduce a system should be as simple as possible. Therefore, the modeller should identify those processes that have big/small impact on the outputs, and evaluate the consequences of removing/simplifying them. Moreover, parametrization of IBMs usually becomes more difficult than continuous models. Many of IBMs parameters are found at individual scale, and therefore are more difficult to measure experimentally than population parameters. This fact is especially important in the case of microbiology, where some specific microbial characteristics might be difficult to find out (such as the rate that a bacteria metabolize a specific compound).
1.3 Objectives

In order to reap the full benefits of the cutting-edge techniques that are being used in the field of biomedicine, we need tools capable of dealing with datasets generated in laboratory experiments. The good news is that we are making progress on that front. Apart from giving insights into the dynamics of Mtb by processing experimental data, we hope that simulation techniques will enable us to understand those interactions between the bacilli and different anti-TB drugs.

We start with the following long-term goals:

- Identifying the key factors that define the bacterium-antibiotic interactions observable in the experimental results from antibiotic research.

- Once these factors have been identified, we aim to improve the understanding of the interaction between Mtb and anti-TB drugs by means of simulation and modelling techniques.

- In the near future, we expect to develop tools capable of helping researchers when designing in vitro experiments, reducing their cost and duration.

In this particular project we have set the following goals:

- To develop an individual-based model capable of dealing with the experimental data generated during in vitro experiments.

- To reproduce the evolution of Mtb cells without antibiotic to better understand the dynamics of the bacilli, which would allow researchers to approximate the order of magnitude and significance of some intrinsic parameters within the bacterium.

- To simulate the activity of rifampicin in order to better understand the emergent dynamics of Mtb population depending on RIF mechanisms within the cell.

The tasks carried out in this work have been the following:

1. Bibliographic research of anti-TB drugs effects in Mtb at a molecular and cellular level.
3. Development of an individual-based model that simulates Mtb activity.
4. Implementation and parametrization of the model without antibiotic in NetLogo software.
5. Introduction and evaluation of rifampicin’s effect in the model developed.
6. Discussion of the introduction of other anti-TB drugs in the model and the possible implementation into more powerful platforms.
1.4 Context

This project has been developed under the supervision of the Computational Biology and Complex Systems (BIOCOM-SC, http://biocomsc.upc.edu/en) research group at the Universitat Politècnica de Catalunya. The project is carried out in collaboration with the research group led by Dr. Julià González Martin from the Center of Biomedical Diagnosis in the Hospital Clinic de Barcelona. In addition, this last year another important collaboration has been established with the Department of Medical Microbiology and Infectious Diseases at the University Medical Centre of Rotterdam, which provided us essential experimental data in order to fit several parameters of our model. These collaborations, enable research groups to work in an interdisciplinary environment. Interchanging experimental and simulated data allow to better understand the disease and to approach those emergent setbacks from microbiology and biomedicine with numerical tools and techniques.

This work starts with a preliminary version of an individual-based model that simulates the activity of $Mtb$. This model analysed in a qualitative way (without parametrization) how these processes affect the growth of $Mtb$. In this project, the preliminary model was used as a first approach towards the study of antibiotics effect after a careful revision and improvements of its basic version.
2. MATERIALS AND METHODS

2.1 Experimental data

Experimental data from in vitro cultures have been essential in order to evaluate and compare the behaviour of the in silico model developed in this study with real data. As previously mentioned, there are many classes of in vitro PK/PD experiments. Therefore, depending on the environment that the experiments have been developed, it will be necessary to consider the processes that are relevant for this model and leave out the others.

This study has been based on experimental data reported in the paper by Steenwinkel et al. (29) from the University Medical Centre Rotterdam in collaboration with the Dutch National Tuberculosis Reference Laboratory. All the original data shown in that study was requested to that research group and kindly provided to BIOCOM-SC. In these experiments the concentration dependence and time-dependent killing capacity of the currently used Rif, INH, EMB and AMK against Mtb were assessed. The study was conducted under static in vitro PK/PD conditions. A wide range of static drug concentrations (2-fold scaling between concentrations) were evaluated, and the number of colony-forming units (CFUs) was reported at different time points. The data collected from these experiments was represented in time-killing curves as shown in Figure 2.1.

![Figure 2.1: Time-kill curves of rifampicin on susceptible Mtb under different static concentrations (2-fold increasing). Quantitative counting was performed on days 1, 2, 3 and 6. Source: Steenwinkel et al. (29).](image)
In order to study the concentration and time-dependent activity of anti-TB drugs, a commonly strain of susceptible *Mtb* (H37Rv) in early logarithmic phase (a period characterized by constant cell doubling) was used. The time-killing experiments were carried out in Erlenmeyer flasks with Middlebrook 7H9 broth under shaking conditions at 37°C. In each experiment, samples were taken for CFU counting at days 1, 2, 3 and 6. For more methodological details see Steenwinkel et al. (29).

In order to generate a realistic model, important characteristics of the bacterium were also taken from other experiments. The range of *Mtb* volumes was reported according to Cardona et al. (3). Bacterial volumes and mass distribution were considered to be bell shaped skewed to the right (shown in Figure 2.2) as reported by Kubitschek et al. (30) and many others later.

### 2.2 NetLogo platform

The model developed in this work was implemented in NetLogo, which is a free open-source multi-agent programmable modelling environment. NetLogo is well suited for modelling a wide range of Individual/Agent-based systems. Moreover, it has a user-friendly interface that allows non-experts to run simulations and perform virtual experiments. This user-friendly interface clearly facilitates the control of input values as well as visualization of the outputs from the model. This feature makes NetLogo especially helpful when working in multidisciplinary projects like this. Its users can come from a variety of fields, without the need of in-depth experience on programming or modelling.

Nevertheless, NetLogo’s main limitations come when working with large populations (around 500,000 individuals), where its simulations become very time-consuming. As an example, our experimental data deal with populations up to $10^6$ CFUs/mL contained in Erlenmeyer flasks of 125mL. Therefore, it is necessary to proportionally reduce the space and number of CFUs in order to maintain the concentrations observed in the experiments.
3. MODEL

The model developed is described in this section following the IBM standardized protocol ODD (Overview, Design concepts and Details) (31). This protocol consists in formalizing the IBM with three main blocks, so that the basic features and the details of the IBMs can be clearly communicated.

3.1 Overview

Purpose

The purpose of this IBM is to analyse and understand the dynamics of Mtb during *in vitro* experiments, first without antibiotic, and later with different concentrations of RIF in the medium. Specifically, it has been developed as a first-step towards the generation of an *in silico* model capable of simulating the response of Mtb in combination therapy.

Entities, state variables and scales

The fundamental entity of this IBM is the bacillus (*Mtb*). Although most models built in NetLogo use *turtles* (individuals) and *patches* (spatial cells), in this case only the *turtles* are taken into account. As the bacterium is considered to be under constant shaking conditions, the medium is homogeneous and all the spatial cells have the same characteristics. The space is represented with periodic boundary conditions.

The time scale was established as 1h per each step, which is small enough to allow the simulation of the bacilli growth cycle (around 24 hours). Discretization of time in 1 min per time step was analysed in order to evaluate any possible different behaviours. However, the dynamics of the bacterium were approximately the same using both time scales. Nevertheless, we noticed that the smaller the time scale, the less computationally feasible the simulations are. The simulated period in this study was 6 days, as the data from the experimental group was taken until day 6.

NetLogo code structure allows programmers to clearly differentiate between variables of turtles, patches and global variables. In this IBM, the variables related to the turtles (*Mtb* bacteria) are those linked with bacterial mass and volume, inner nutrient concentration, reproduction cycle, maintenance energy and cell damage. Global variables include characteristics of the bacterial population as a whole, such as means or growth rates. Other global variables are those concerning the characteristics of the medium, antibiotic concentrations and all the deviations related to normal distributions.
**Process overview and scheduling**

As mentioned above, NetLogo has a user-friendly interface that allows an easy-usage of the model. In this interface, users can change the input values as shown in Figure 3.1. The simulation starts with definition of the initial configuration, in which a specific number of *Mtb* are randomly distributed in the space. This initial number of bacilli can be chosen by the user. At each time-step (1h) all the turtles execute a series of actions, and their variables, as well as the global variables, are updated.

![Screenshot of the model's interface.](image)

**Figure 3.1:** Screenshot of the model’s interface.

The actions for the individuals are, when possible: move (although it has no effect on the calculation, it represents the stirring medium), nutrient and antibiotic uptake, growth (mass and volume increase), damage (due to the lack of nutrient or antibiotic effects), reproduction and death (when the cell has too much damage).

Due to the nutrient consumption by the bacterial culture, the medium nutrient concentration decreases at each step. Another important action available in the model is the possibility of taking a specific number of cells and transferring them to a new medium, which we have named “Re-inoculation”. This process allows the users of this model to start the simulation with bacteria in exponential phase (where bacteria have already adapted to the growth conditions and are mature). Figure 3.2 shows the flow diagram for the computational model.
3.2 Design concepts

Basic principles

The model developed is based on the principles and theories of the INDISIM model, *An Individual-based Discrete Simulation Model to Study Bacterial Cultures* from Ginovart *et al* (32). However, some modifications were conducted in order to adapt the principles from INDISIM to our particular experimental environment. Moreover, PK/PD principles of RIF were taken from a variety of bibliographical sources, as well as the recommendations of researchers from the Hospital Clinic de Barcelona.

Emergence

Emergent patterns of the population as a whole are strongly dependent on the individual characteristics of the bacteria. Therefore, variations in bacterial yield, maximum inner concentration, or maintenance energy among others, result in significant changes of the population dynamics.

However, changes in medium nutrient concentration does not seem to have significant effects on growth rate as long as there is enough nutrient to satisfy the bacterial minimum requirements. Nonetheless, if the medium nutrient concentration is below a threshold, it has a devastating effect on the bacterial culture. Thus, the total nutrient concentration in the medium seems to act as a switch.

Another essential factor of the model is the simulation of antibiotic effects, which significantly alter the dynamics of the population. These effects have been simulated as a combination of two factors. On one hand, hydroxyl radicals induced by RIF have been simulated as a global damage for the bacteria. When global damage increases, the bactericidal effect of RIF emerges. On the other hand, RIF’s effect on RNA polymerase has been simulated as an effect on the inner membrane and cell wall functionality, limiting the inner nutrient concentration of the bacteria.

Interaction

Local interactions between bacteria or between patches and bacteria are not considered in this model. As bacteria are in constant stirring conditions, no cellular aggregation was observed during the experiments, and interactions between cells do not seem feasible. In fact, and as a consequence of homogeneity arising from stirring, interactions between the bacterium and the local medium are captured by the interaction between bacteria and the global nutrient (and antibiotic) concentration. The most important interaction is the one between bacteria and antibiotic concentration, which has a strong effect on bacterial dynamics.

Stochasticity

Randomness is introduced at all levels of the simulation. All parameters from the model are affected by Gaussian noise. Thus, each action is associated with a certain probability around a mean stated value. In addition, the initial distribution of the individual properties is randomly executed according to input distributions.
Figure 3.2: Flow diagram of the computational code. * Antibiotic uptake is considered to reach steady state concentration within the bacterium in few minutes. Thus, antibiotic uptake is not introduced in the computational code, and the antibiotic effect is directly dependent on the antibiotic concentration in the medium.
3.3 Details

Initialization

The user can change some initial conditions at the beginning of the simulation. The input parameters from the interface are shown in Table 3.1. At the beginning of the simulation, those variables related to bacterial characteristics are updated as well as the global variables (nutrient and antibiotic concentration, growth and mass rates etc.). Once reaching the Re-inoculation time, a number of initial bacteria remain in the medium while the others are eliminated, simulating the transferring of some bacteria to a new medium, starting to grow exponentially from the beginning.

Sub-Models

Several processes are performed by each bacterium at each time step:

- To move

All bacilli rotate randomly from 0 to 360° and move 10 spatial cells forward. Although this process has no-effect on the calculus, it represents the constantly stirring medium. Therefore the experimental stirring speed has no influence in this system as long as the bacteria are distributed homogeneously.

- Nutrient and antibiotic uptake

In this process, bacilli inner nutrient is evaluated. We consider the bacterium to reach nutrient concentrations at the equilibrium in less than 1 hour. Inner nutrient concentration can be either the maximum inner concentration settled in the interface, or the medium concentration (if nutrient is limiting bacterial growth) depending on the available nutrient. After defining bacterial inner concentration, it is multiplied by the bacterium volume in order to calculate the amount of inner nutrient (in mass simulation units). In one time step (1 hour), this process can be repeated more than once by the uptake_repetitions parameter. This parameter allow us to simulate how many times the process of equalising concentrations and increasing bacterial mass occurs in 1 hour.

As reported in recent studies (11) rifampicin reaches a steady-state concentration within Mtb in less than 2 minutes. Therefore, the uptake process by which antibiotic enters to the cell has been omitted in this model. The effect of RIF within the bacterium is then only dependent on the concentration of Rif in the medium. As a result, the uptake is considered immediately and has not been included in this model.

- Cell damage

This process simulates cellular damage occurred by hydroxyl radicals formation or the lack of nutrient. This fact can occur either because a certain concentration of antibiotic is present or the nutrient has been consumed. Cell damage is introduced as a counter. If cell damage reaches a certain threshold (critical cell damage), then the bacterium dies. If damage is occurred by the effect of hydroxyl radical formation, the counter increases a certain quantity at each step (unless the bacterium is able to recover the damage). This specific quantity is set at the interface with the parameter Cell Damage Effect when the modeller wants to simulate one of the effects of the antibiotic.
This kind of bacterial damage is considered to be the main cause of the bactericidal antibiotic effect (so only bactericidal antibiotics should induce this effect). If the bacterium does not have enough nutrient to satisfy its maintenance energy, the counter cell damage increases 1 unit each time that maintenance energy is not satisfied. If bacterial damage occurs, the bacterium can still recover its damage if it has enough nutrient in the following time steps.

- **Growth**

During this process, the maintenance energy needed for the bacterium to carry out its activities and enzymes synthesis is calculated. In order to define the maintenance energy we have used the following formula:

\[
\text{MaintenanceEnergy} = C_{\text{mant}} \cdot \text{mass} \cdot (1 + \text{factor } C_{\text{mant}} \cdot \text{CellDamage}) \quad \text{Equation 3.1}
\]

Thus, maintenance energy depends on a constant \(C_{\text{mant}}\), bacterial mass, cell damage, and a factor that determines the effect of cell damage on maintenance energy \((\text{factor } C_{\text{mant}})\).

Then, one part of the inner nutrient is used to maintain bacterial activity, whereas another part of the remaining nutrient is transformed into bacterial biomass \((\text{inner nutrient} - \text{maintenance energy})\), according to a specific yield named “\(Y\)”. This process is also limited by the possible effect of the antibiotic on the cell wall and membrane (bacteriostatic antibiotic effect). The maintenance energy can exceed the bacterial inner nutrient due to 2 factors: medium nutrient depletion, or antibiotic effect on the membrane and cell wall. The effect of antibiotic limiting inner nutrient is simulated by multiplying the inner nutrient by a factor from 0 to 1 ("Membrane Functionality" (MF)). If the bacterium does not have enough nutrient to maintain itself, cell damage occurs, increasing 1 unit the cell damage counter. The effect of antibiotic on MF has a delayed effect in order to simulate a progressive degradation of the cell wall and membrane. This delay is controlled in the model by the parameter \(alpha\), which effect is further studied in section 4.2.1.

- **Death**

If bacterial damage exceeds a critical point tolerable by the bacilli (critical cell damage), the bacterium dies.

- **Reproduce**

The bacterial volume is updated at each time step. When the bacterial volume reaches a critical value (start volume), the bacilli starts its duplication cycle. When bacteria have been a certain period within the duplication cycle (\(trdi\) seconds), they are divided into 2 daughter cells by bipartition. As \(Mtb\) division is asymmetrical (33), daughter cells are considered to have different volumes. Once a bacterium is divided, the volume of one of the daughter cells is assigned randomly according to a normal distribution with a mean of: original cell volume / 2. Then, the other daughter cell acquires the difference between the volume of the original cell and the first daughter cell.
Once the bacterium is divided, most of the parameters of the daughter cells are recalculated such as: reproduction volume, trd, Cmant, maximum inner concentration, and critical cell damage. All these variables are assigned randomly with a normal distribution, which means have been introduced at the interface. However, cell damage is considered to be inheritable from the original bacterium by the daughter cells, which is crucial for the dynamics of the population.

Table 3.1: Summarized information about the parameters found in the interface of the IBM.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>initial_bacteria</td>
<td>n° bacteria</td>
<td>Number of bacteria at the beginning of the simulation</td>
</tr>
<tr>
<td>initial_total_nutrient</td>
<td>msu**</td>
<td>Amount of initial nutrient present in the medium</td>
</tr>
<tr>
<td>Y</td>
<td>-</td>
<td>Proportion of the absorbed nutrient that is fixed as bacterial biomass. It can be understood as a metabolic yield</td>
</tr>
<tr>
<td>mean_C_max</td>
<td>msu/m³</td>
<td>Maximum intracellular nutrient concentration</td>
</tr>
<tr>
<td>C_mant_density</td>
<td>msu</td>
<td>Constant related to the bacterial maintenance energy</td>
</tr>
<tr>
<td>factor_C_mant</td>
<td>-</td>
<td>Factor related to the effect of cellular damage to the maintenance energy</td>
</tr>
<tr>
<td>uptake_repetitions</td>
<td>-</td>
<td>Number of times that uptake process is repeated in one time step</td>
</tr>
<tr>
<td>Medium_volume</td>
<td>m³</td>
<td>Volume of the medium</td>
</tr>
<tr>
<td>reproduction_volume</td>
<td>m³</td>
<td>Average volume at which one bacteria starts the duplication cycle</td>
</tr>
<tr>
<td>mean_initial_age</td>
<td>seconds</td>
<td>Stage at which initial culture cells are found at the beginning of the simulation</td>
</tr>
<tr>
<td>time_step</td>
<td>seconds</td>
<td>Time increased at each repetition</td>
</tr>
<tr>
<td>trd</td>
<td>seconds</td>
<td>Time that one bacteria remains within the duplication cycle once the reproduction volume is achieved</td>
</tr>
<tr>
<td>Re_inoculation</td>
<td>hours</td>
<td>Time at which a specific number of bacteria are taken and transferred to a new medium</td>
</tr>
<tr>
<td>SimulationTime</td>
<td>seconds</td>
<td>Time at which the simulation ends</td>
</tr>
<tr>
<td>Membrane functionality</td>
<td>%</td>
<td>Antibiotic effect on the inner membrane and cell wall function</td>
</tr>
<tr>
<td>Cell Damage Effect</td>
<td>-</td>
<td>Antibiotic global effect on the bacterium. Similar as the one produced by hydroxyl radicals' formation</td>
</tr>
<tr>
<td>Alpha</td>
<td>hours⁻¹</td>
<td>Delay of the antibiotic effect on the membrane and cell wall function</td>
</tr>
<tr>
<td>Critical_Cell_Damage</td>
<td>-</td>
<td>Average damage at which the bacterium dies</td>
</tr>
</tbody>
</table>
4. RESULTS AND DISCUSSION

4.1 Model dynamics without antibiotic

4.1.1 Calibration with control group data

Once the model was implemented, the next step was to calibrate its parameters with real data. First of all, the model was fitted to in vitro data without antibiotic. Experimental data of growth without antibiotic are represented in Figure 4.1 (grey lines). These data, which were published in Steenwinkel et al. 2010(29), were provided by the University Medical Centre Rotterdam and the Dutch National Tuberculosis Reference Laboratory. In the model developed, one Colony Forming Unit (CFU) has been considered to be equal to 1 simulated bacteria.

![Graph showing growth curves](image)

**Figure 4.1**: Evolution of *Mtb* cultures without antibiotic. CFU counting is performed at days 0, 1, 2 and 3. Grey lines represent the data measured during different in vitro experiments under the same conditions. The red line is the average of simulated data after 30 simulations.

In order to prepare experimental data for being compared with simulation outcomes, we have calculated the average of the 5 experimental curves and fitted a continuous exponential bacterial growth model according to Equations 4.1 and 4.2 (differential and integral forms):

\[
\frac{dN}{dt} = \mu_m \cdot N \quad \text{Equation 4.1}
\]

\[
N(t) = N_0 \cdot e^{\mu_m \cdot t} \quad \text{Equation 4.2}
\]
Where $N$ is the number of bacteria at a certain time, $\mu_m$ is the bacterial maximum growth rate and $N_0$ the initial number of bacteria.

The exponential fitted curve to experimental results is shown in Figure 4.2:

![Graph showing exponential growth of bacteria](graph.png)

**Figure 4.2:** Each grey dot represent the average of the number of CFU between the 5 experimental curves of Figure 4.1. The exponential model fitted to the grey dots is shown in black.

From the fitted equation, we can first notice the high value of the coefficient of determination ($R^2$). $R^2$ ranges from 0 to 1, and the higher the $R^2$ the better the fitting of the model to the experimental data. Therefore, we can assume these bacteria to grow exponentially. However, high concentrations of CFU’s tend to produce aggregations of *Mtb* in the culture. Therefore, we can detect a slight slowdown in the slope of the experimental curves from Figure 4.1 from day 2 to 3. From the fitted equation from Figure 4.2 we can extract the following parameters:

$$N_0 = 674,375 \text{ CFU/mL}$$

$$\mu_m = 1.85 \text{ days}^{-1}$$

In order to obtain the simulated curve shown in Figure 4.1 (red line), many simulations were systematically performed by varying the values of the different parameters. The obtained values for the parameters, which would be used to simulate *Mtb* growth without antibiotic, are shown in Table 4.1. As a starting point, we set the values for those parameters for which we had experimental data, such as medium volume, initial number of bacteria, density and reproduction volume. Simulation time, or the time step equivalence, do not have biological meaning, and are set up to the modeller needs. The other parameters have been either estimated, recommended or fitted with the model.
It is important to note that the combination of parameters that we have obtained is one solution, and other combinations of parameters that fit experimental data could be found. However, most parameters and variables have biological meaning, and we have based their fitting on biologically reasonable ranges. In order to reduce the number of possible solutions we would need to obtain more experimental data of the value of these variables.

The parameters related to bacterial damage are: membrane functionality, alpha, cell damage effect, maintenance factor and critical cell damage. Damage can occur either because of the lack of nutrient or due to the antibiotics effect. However, this is considered to be a non-limiting nutrient and free-antibiotic medium. Therefore, these parameters have been set in a way in which they have no effect on bacterial growth. Re-inoculation time was included with the aim that the re-inoculated bacteria started to grow exponentially since the beginning of the experiment. Nevertheless, we had no information related to other parameters such as metabolic yield, maintenance constant, and trd among others. Therefore, we have run and processed many simulations with different values for these unknown parameters. After processing the data from these simulations, we have obtained a combination of parameters that reproduced satisfactory the experimental data. Moreover, in the next section we have analysed which parameters have greater effect on the model’s responses.

| Table 4.1: Input values used in the fitted curve without antibiotic and their source. * E=estimated or fitted, R=recommended by field experts, D=decided/eligible by the user. **Mass simulation units. |
|----------------|--------|------|-----|
| **Parameter** | **Value** | **Units** | **Source** |
| Initial number of bacteria | 674 | n⁰ bacteria | (29) |
| Initial nutrient | 9·10⁷ | msu** | E |
| Metabolic yield (Y) | 0.59 | - | E |
| Maximum inner nutrient concentration | 743 | msu/m³ | E |
| Maintenance Constant Density | 0.599 | msu | E |
| Maintenance factor | 1000 | msu/m³ | R |
| Uptake repetitions | 0.2 | - | E |
| Medium volume | 150 | - | E |
| Reproduction volume | 10⁹ | m³ | (29) |
| Mean initial age | 3.96·10⁹ | m³ | (3) |
| Time step | 3600 | seconds | R |
| Time in reproduction cycle (trd) | 3600 | seconds | D |
| Re-inoculation time | 50 | hours | D |
| Simulation time | - | seconds | D |
| Membrane functionality | 100 | % | E |
| Cell damage effect | 0 | - | E |
| Alpha | 1 | hours | E |
| Critical cell damage | 10 | - | E |

In order to start our simulations with the same concentration of bacteria than experimental CFU’s, we have set:

\[ N₀ = 674 \text{ bacteria} \]

\[ \text{Medium volume} = 10⁹\text{m}³ = 10³\text{mL} \]
Obtaining a bacterial concentration of:

674,000 bacteria/mL

We have also tried to perform simulations with more bacteria and smaller medium volumes. However, when we work with large populations, NetLogo’s execution time becomes a big limitation.

![Graph showing the relationship between experimental fitting (CFU/10^9/mL) and model outcome (nBacteria/10^6)/mL]

y = 0.9964x
R² = 1

**Figure 4.3:** In the horizontal axis, the number of bacteria is plotted at each hour from 0 to 72h according to the values obtained in NetLogo simulations as an average of 30 runs. In the vertical axis, the equation found in Figure 4.2 has been fitted to the experimental data with N₀=674375 and t=0-72h.

The perfectly representative model would be the one that matches all the experimental values at each time that data is collected. As a consequence, if we plot the experimental vs simulated data and fit a linear model, we would obtain a straight line with the expression: y=x. Nevertheless, each of the experimental time-points have several measurements, according to the number of repetitions (5 experimental curves). In addition, although simulations may provide the bacterial concentration at each time step, experimental data are only available at the measurement time-points. Therefore, in order to better compare experimental data with simulation outcomes, we have used the exponential fitted curve to represent experimental data (Figure 4.2) and the mean of the concentration of the 30 runs at each hour to represent the simulation outcome. These data are shown in Figure 4.3, together with the regression analysis between them. As shown, we have obtained an R²=1 and slope of 0.9964, which is also closed to 1, indicating an almost perfect correlation between the exponential growth equation and the calibrated IBM.
As shown in Figure 2.2 the volumes distributions for most bacterial species have been reported to be bell shaped skewed to the right. Therefore, in Figure 4.4 a histogram to represent volumes distribution has been plotted by means of MATLAB.

![Histogram of bacterial volumes](image)

**Figure 4.4:** Distribution of cell volumes obtained from the IBM developed.

From the histogram presented, we can observe a distribution of volumes with a similar shape to those from Figure 2.2. The mean volume observed in the simulations is approximately $2.7 \cdot 10^{-19}$ m$^3$, which seems reasonable considering a reproduction volume of $3.96 \cdot 10^{-19}$ m$^3$.

As a result of the analysis performed in this section, we have considered that the values shown in Table 4.1 simulate the experimental data convincingly good. However, in order to better use and understand the model developed, we consider that a sensitivity analysis is necessary.

### 4.1.2 Sensitivity analysis

In order to find out which parameters of the model have more effect on the output, a sensitivity analysis has been performed. In this analysis, we have explored the values of 5 parameters:

- Maximum inner nutrient concentration
- Maintenance constant
- Time within the duplication cycle once reproduction mass is achieved (trd)
- Metabolic yield (Y)
- Re-inoculation time
Other parameters from the interface have not been included in this analysis, either because we have obtained reliable information about their value (such as bacterial volume) or because they are defined by the experimental data (e.g. number of bacteria or medium volume). Initial nutrient has not been taken into account in this analysis because we have considered the bacterium to be in a non-limiting nutrient medium. Their growth is then dependent to the maximum nutrient inner concentration. Moreover, membrane functionality, cell damage effect and alpha parameters will be discussed in the following chapter.

The sensitivity analysis has been performed by varying the analysed parameters up and down by a 10% and comparing the variation in the number of bacilli found after 2 days. We have performed 30 executions with each parameter value, and calculated the mean and standard deviation in this variable. We have presented the results in a box-plot diagram, which is a useful resource to identify variations in the means and deviations. This representation is shown in Figure 4.5, and has been plotted using Minitab software. A box plot diagram is based on quartiles (values that divide the observations in 4 equal parts). The limits of the boxes are the 1st and 3rd quartiles, and contain 50% of the observations. The line inside each box represents the median (or second quartile). The extension of the verticals lines are the maximum/minimum value, or 1.5 times the difference between the 1st and 3rd quartile (interquartile range, IQR). Finally, outliers are represented by asterisks. Numerical data used to plot these diagrams can be found in appendix A.

In Figure 4.5a we can see the high sensitivity of the model to variations in the maintenance constant. The lower the maintenance constant, the larger the number of bacteria found. Noteworthy is that the number of bacteria are measured after 2 days of exponential growth. Therefore, small variations in growth rate can result in great differences on the number of bacteria.

The most sensitive parameter analysed is the maximum inner concentration that bacteria can have in their cytoplasm (Figure 4.5b). Variations in this parameter seems to have a significant effect on the number of bacteria measured.

One interesting result is found on Figure 4.5c, where the number of bacteria seems to increase as time in reproduction cycle (trd) increases. This parameter represents the time spent by the bacterium once the reproduction mass (or volume) is achieved until division occurs. However, once a bacterium reaches this reproduction mass, its mass still increases until trd is achieved. Therefore, the larger the trd, the greater is the mass at which bacteria divide. As a result, the greater the mass of the daughter cells, the sooner the bacterium starts the duplication cycle again. However, trd seems to be less sensitive than the previous parameters.

Re-inoculation time represents the time at which a number of bacteria (usually during exponential growth) are taken from their environment and transferred into a new medium. This process allows researchers to start simulations with bacteria already adapted to their medium and without the presence of a lag phase. Re-inoculation time has previously been fitted at 50 hours. After this time, most global variables (mean bacterial volume, mean maintenance energy, growth rate etc.) tend to remain around a constant value. As a consequence, small variations in Re-inoculation time produce non-effect on bacterial growth.
However, the smaller the Re-inoculation time, the higher the possible number of global variables in a transitory state. Therefore, in Figure 4.5d the smallest Re-inoculation time seems to have the highest variability.

Finally, another sensitive parameter is the proportion of inner nutrient that the bacterium fixes as biomass (Y), or metabolic yield. As shown in Figure 4.5e, the number of bacteria measured after 48 hours seems to be significantly higher as Y increases.

**Figure 4.5**: Results obtained from the sensitivity analysis for: (a) Maintenance energy constant, (b) Maximum inner concentration, (c) Duration of the duplication cycle once the critical mass is achieved (trd), (d) Re-inoculation time, (e) Metabolic yield (Y).
However, if bacteria increase their mass excessively at each step, the mass at which they divide can also increase at each step, since they will no longer have to reach a reproduction mass. Therefore, in our analysis, we have detected that a high Y results in an uncontrolled growth with a constant increase of bacterial mean volume.

Overall, we can notice that in all graphs, larger populations result in higher deviations. This emergence, is due to the fact that bacterial growth is exponential. Thus, the same deviation in bacterial growth rate, will result in larger deviations in the number of bacteria when the growth rate is higher.

The values for the parameters found in the previous section seem to match empirical available data satisfactorily. However, after having performed the sensitivity analysis, a more accurate value for the most sensitive parameters could be studied in future projects.

4.2 Model dynamics with antibiotic

Another target of this project was to reproduce the effect of rifampicin by means of the IBM developed. In Figure 4.6 experimental time-kill curves of RIF have been plotted from the data reported in Steenwinkel et.al (29). All these data have also been kindly provided by the University Medical Centre Rotterdam and the Dutch National Tuberculosis Reference Laboratory. The study from Steenwinkel et.al investigated the killing capacity of rifampicin, isoniazid, ethambutol and amikacin depending on the metabolic activity (growth phase) of Mtb cells. Therefore, they plotted Mtb time-kill curves with a range of 2-fold increasing concentrations of anti-TB drugs against metabolically active (replicative state) bacilli.

![Figure 4.6: Concentration- and time-dependent effect of RIF on Mtb. Each curve represents the evolution of the number of CFU under a particular RIF's concentration. The concentration corresponding to each curve is shown in the legend in mgRIF/L. Due to aggregation of Mtb in the culture, after 6 days of exposure to low concentrations, accurate CFU counts could not be performed. The graph is adapted from Steenwinkel et.al 2010 (29).](image)
These data have been analysed and processed in order to generate an IBM capable to simulate the dynamics of *in vitro* *Mtb* under the presence of RIF.

Each time-kill curve from Figure 4.6 has been drawn from one single experiment. Therefore, we have considered that the calibration analysis should be performed in a qualitative way by means of graphical analysis. This analysis would allow us to detect general behavioural patterns depending on RIF’s concentration. However, to draw quantitative conclusions we would need some repetitions of each experimental curve in order to overcome the unavoidable deviations.

The effect of RIF on *Mtb* cells has been simulated in our IBM by means of two main parameters, as detailed in Chapter 3: membrane functionality and cell damage effect. As previously mentioned, the effect of RIF on membrane functionality aims to reproduce the effect that RIF has in the synthesis of proteins. Due to the fact that protein synthesis is limited by RIF, we have assumed that cell wall and inner membrane weaken. This effect, has been simulated in a way that, the lower the membrane functionality the lower the maximum inner concentration of nutrient that one cell can contain. Furthermore, RIF is also known to induce hydroxyl radical formation (10). As previously mentioned, these molecules have a global effect on bacterial activity, damaging all substances within the cell. Therefore, apart from the membrane functionality effect, we have introduced the cell damage effect. This last effect acts as a counter, increasing at each time step until a threshold is reached and the bacterium dies or recovers. In order to fit the IBM to the experimental results, both effects have been analysed first separately, and later combined.

### 4.2.1 Membrane functionality

Membrane functionality (MF) ranges from 100 to 0%. In mediums without antibiotic, MF is considered to be 100%. The lower the MF, the greater the effect of the antibiotic in limiting the inner nutrient. However, the effect that some concentrations of anti-TB drugs can have on MF is considered to be cumulative. In this way, if the MF parameter is set at 80%, at the beginning of the simulation the real MF of the bacterium will be 100%, and will decrease progressively until it reaches a value of 80%. In order to simulate this effect, we have generated the Equation 4.3. In this equation, $MF_r(t)$ (transitory membrane functionality) tends to MF over time. Moreover, the parameter $\alpha$, allows us to decide how much time is spent until $MF_r(t)$ reaches MF.

$$MF_r(t) = 100 - ((100 - MF) \cdot (1 - e^{-\alpha \cdot t}))$$  \hspace{1cm} \text{Equation 4.3}

In order to evaluate and understand the effect of $\alpha$ on membrane functionality, we have plotted the evolution of $MF_r(t)$ under different values of $\alpha$, maintaining MF constant to 80%. The results from this study can be observed in Figure 4.7. In this figure, we can see that the higher the $\alpha$'s value, the sooner the $MF_r(t)$ reaches its minimum (MF).

Based on expert’s recommendations and patterns observed from experimental results we have considered that values for $\alpha$ should be around 0.1-0.05 h\(^{-1}\) in order to properly reproduce the experimental behaviour. However, a specific value for $\alpha$ will be chosen during the calibration process, in the last section of this chapter.
Figure 4.7: Evolution of rifampicin’s effect on membrane functionality depending on $\alpha$. The parameter membrane functionality has been set as 80. However it takes some time to achieve this effect within the bacterium depending on $\alpha$.

Figure 4.8: Effect of membrane functionality on \textit{Mtb}. Each curve represents the evolution of the number of bacteria under a particular membrane functionality with $\alpha = 0.05$. The membrane functionality corresponding to each curve is shown in the legend in \%.
In Figure 4.8 we have analysed a range of values for membrane functionality with a constant $\alpha = 0.05$ h$^{-1}$. We can detect several behaviours from the curves drawn.

First of all, MF from 85-95% seems to allow bacteria to grow exponentially, although reducing their growth rates. This behaviour could be explained by the lower inner nutrient, which limits bacterial growth. In this range of MFs, bacteria seems to still have enough nutrient to satisfy their maintenance energy and increase their mass. However, this increase in mass is reduced by the decrease in MF.

We can also detect a similar behaviour in population dynamics for MFs from 70 to 80%. In these cases, $Mtb$ populations seem to grow during the first 24h. However, a similar decrease is observed in these curves from day 1 to 6. This up and down effect is due to the delay of MF$_{i}(t)$ to reach its minimum value (MF). From day 0 to 1 MF$_{i}(t)$ seems to still allow bacteria to grow and reproduce. However, from day 1 to 6 we can notice a decrease in $Mtb$ population caused by lower MF$_{i}(t)$. This decrease can be explained by the fact that most bacteria are not capable of satisfying their maintenance energy. As a result, bacterial damage occurs and, unless the bacterium recovers, it dies.

For MFs from 0 to 70% we can observe the same population behaviour than in the previous range (70-80%) but with faster killing capacities. MFs from 60 to 45% seem to kill all bacteria in 3 days, while lower values for MF kill the whole population in 2 days.

Although the majority of bacteria die with MFs between 70 to 80% other bacteria seem to remain active after 6 days. The fact that some bacteria remain while other die is also observed in the experimental curves. This behaviour can be explained by the diversity in metabolic activities within the population. Bacteria with lower metabolic activities seem to be more capable of surviving in mediums with antibiotic. In our IBM, those bacteria that remain while the majority of the population dies seem to have lower maintenance energies. Lower maintenance energies allow bacteria to better satisfy their requirements and make them more capable of recovering after damage occurs. Therefore, the heterogeneity in maintenance energies seem to be a crucial factor when reproducing the dynamics of $Mtb$ in our IBM.

Another important detectable fact from Figure 4.8 is that none of the MF explored values has been able to kill the population before 2 days. Moreover, all decreasing curves seem to have a significant change in their slope after the first day. The delay on MF effect is due to the alpha value. However, eliminating the alpha factor does not seem to better reproduce experimental data; this parameter is essential to produce changes in slopes, and many experimental curves present a change in their slope after some days of exposure ($\text{[RIF]}=0.001/0.0019$mg/mL Figure 4.6 ). Therefore, we introduced another factor to reproduce the early killing capacity of RIF, which is the cell damage effect.
4.2.2 Cell damage effect

As previously mentioned, the global damage on \textit{Mtb} cells caused by hydroxyl radical formation is simulated by means of the cell damage effect (CDE). Instead of causing cell damage by limiting the nutrient, CDE directly increases the amount of damage in each bacterium. Moreover, CDE is not considered to have a delayed effect. In Figure 4.9, a range of values for CDE from 0 to 3, has been analysed keeping a MF of 100\% (non-effect in membrane).

From the curves in Figure 4.9, several patterns can be pointed out on the dynamics of CDE. First of all, it is worth noting that as CDE increases the damage on each cell, it also increases the bacterial maintenance energy required. This increase in maintenance energy results in lower mass increases at each time step. Therefore, we can observe that CDE from 0.15 to 0.9 have a similar effect than MF from 85 to 95\%.

Moreover, CDEs around 1.2 produce a bacteriostatic effect on \textit{Mtb} population, as only few bacterial deaths were detected in these curves. Therefore, these bacteriostatic behaviours could be explained by the fact that CDEs around 1.2 increase maintenance energy to approximately the same inner nutrient, producing an almost null mass increase.

Time kill-curves with CDEs higher than 1.2 have a similar effect on \textit{Mtb} population. During the first 24h, we can detect an early bactericidal activity, significantly reducing the number of initial bacteria. However, after this period the number of bacteria seems to decrease very slowly.

Heterogeneity in maximum inner concentrations and maintenance energies seem to confer the capacity to recover to some bacteria while others die. The lack of a progressive increase in CDE forces it to work as a switch. Since CDE is the same at each time step, once a bacterium has the enough inner nutrient to recover it will rarely die. Nonetheless, those bacteria that are unable to recover the first time, will increase their maintenance energy needed, sharply reducing their possibilities to repair cell damage in the following step.

Those bacteria that can overcome CDE do not increase their cell damage. Therefore, they will only die if they reproduce (dividing their masses and changing their maintenance energies) or if randomness associated to CDE produces a much higher damage. However, as mass increase is very limited, they will only reproduce after a large period of time. As a result, those bacteria that can repair CDE seem to be extremely resilient.

Another important fact that should be noticed from Figure 4.9 is the big effect of noise in small bacterial populations. As previously mentioned, in order to adapt our simulations to NetLogo's execution time limitations, our simulations start with 674 bacteria in a medium of $10^3$ mL. Therefore, when $\log (n^o \text{ bacteria/ mL}) = 3$, it is equivalent to 1 bacteria in our simulation. This fact raises the importance of noise associated to individual variables when working with few bacteria. As a consequence, in this particular set of simulations we can observe that the curve for CDE of 2.55 (which kills all bacteria in 2 days) seems to result in a higher killing capacity than the one with a CDE of 2.85 (which still seems to have some bacteria after 6 days).
In this particular example (CDE 2.55 vs 2.85), the dynamics observed by the 2.85 CDE curve is due to one specific bacterium that seemed to have a lower maintenance energy, and increases its mass extremely slowly. Therefore, it is clear that, when working with very small populations, the noise associated to bacterial variables seem to be much more important.

Although more simulations could be performed in order to reproduce the average behaviour for each CDE, the noise observed in silico seem to be similar to the one from experimental curves.

In contrast with the simulations varying MF, from Figure 4.9 we can point out the clear early bactericidal capacity of CDE. Moreover, the remaining bacteria encountered in some CDE curves might die when combining CDE with the effect on MF. Therefore, in the following section we will analyse if the combination of CDE with a reduction on MF reproduces more precisely the behaviour of Mtb population under the presence of rifampicin in the medium.

**Figure 4.9:** Cell damage effect on Mtb. Each curve represents the evolution of the number of bacteria under a particular cell damage effect. The cell damage effect corresponding to each curve is shown in the legend.
4.2.3 Combined effect

After performing the analysis for CDE and MF effect separately, we expected that the combined effect would allow us to better simulate patterns observed experimentally. Moreover, we consider that the combination of diverse effects is a more mechanistic approach, as RIF is known to affect a variety of functions within *Mtb*.

In order to carry out this study, a first set of simulations was performed combining values of CDE from 0 to 3 with an increase of 0.15, with values of MF from 100 to 0% varying 1% at each simulation. Noteworthy is that these ranges contain 101 possible combinations for MF and 21 for CDE, resulting in a number of $101 \cdot 21 = 2121$ possible simulations. As a consequence, 2121 time-kill curves could be drawn from the study. However, analysing all these curves in one single graph simultaneously seemed an extremely hard and probably useless work.

As a first approach, and taking into account the previous analyses, we decided to draw two graphs with constant MFs of 90 and 85% with the whole range of CDEs values. Then, by comparing the Figures 4.10 with the previous graphs, we were able to have a first idea of the output of the combined effects in our IBM. In Figure 4.10a (MF = 90%) we can observe a similar pattern to that from Figure 4.9 (without MF effect). However, those bacteria that remained when MF was 100%, now seem to be less capable of surviving after a period of time. Moreover, both graphs from Figure 4.10 seem to overcome the fact of an abrupt change in the slope observed in Figure 4.8 (without CDE effect). Therefore, we can first notice that the combination of CDE and MF effect produce curves more similar to those observed during *in vitro* experiments.

If we now compare Figure 4.10a with 4.10b we can observe that a decrease of 5% in MF produces a significant reduction on *Mtb* growth rate. Moreover, curves from Figure 4.10b seem to have a significant change in their slope after day 1. This change is explained by the prevalence of the delay produced by the lower MF effect. Therefore, lower MFs produce greater change on the slope of time-kill curves. In this study, MF effect was performed with an $\alpha = 0.05h^{-1}$.

After having performed the 2121 simulations, we decided to generate a method capable of detecting those simulated curves that had a behaviour close to the one observed experimentally. With this analysis, we could relate some RIF’s concentrations to a particular simulated CDE and MF effect. In the next section, we present the methods and techniques used to calibrate membrane functionality and cell damage effects.
Figure 4.10: (a) Evolution of *Mtb* cells under a constant membrane functionality of 90% and a range of cell damage effects from 0 to 3. (b) Evolution of *Mtb* culture under a constant membrane functionality of 85% and a range of cell damage effects from 0 to 3. Both graphs were performed with α=0.05 h⁻¹.
4.2.4 Calibration of MF and CDE

In the previous section, we presented the results obtained from the analysis of membrane functionality and cell damage effect acting simultaneously. Afterwards, we wanted to numerically detect which combinations of CDE and MF better fit experimental time-kill curves.

In order to numerically analyse which of the 2121 simulations were similar to the experimental results we have developed a practical method. The method used started by grouping those experimental curves that behaved similar over time, and analysed them separately. We have formed 4 main groups. As we can observe in Figure 4.11, we have separated time-kill curves in 4 ranges of RIF’s concentration:

- 0 – 0.0019 mgRIF/mL (blue): This range include those curves that have shown increase in *Mtbd* population over time.

- 0.0038 – 2 mgRIF/mL (green): This range include those curves with RIF’s concentrations that triggered a decrease of the bacterial population but remained after 6 days.

- 4 – 64 mgRIF/mL (orange): This range of RIF’s concentrations kill the bacterial population between 3 and 6 days of exposure.

- 128 – 256 mgRIF/mL (red): These RIF’s concentrations kill the whole population in 1 day.

![Figure 4.11: Experimental time-kill curves with different concentrations of RIF grouped in 4 main classes in order to calibrate the model. The specific concentration of rifampicin corresponding to each curve is shown in the legend in mgRIF/mL.](image)
We have started by fitting the time-kill curves that decreased over time (0.0038-256 mgRIF/mL), as these were the most challenging to calibrate and limit the subsequent parametrization.

*Time-kill curves with [RIF] between 0.0038 and 2 mg/mL*

First of all, we have calculated the range of log (cfu)/mL each day for the group of RIF’s concentrations between 0.0038 and 2 mg/mL. Subsequently, we have detected those simulated curves that ranged similarly to the ones with RIF’s concentrations between 0.0038 and 2 mg/mL. As we can see in Figure 4.11, this group of green curves range between values of log (cfu)/mL of 4.8 and 5.5 the first day, 4.2-5.2 the second day, 3.9-4.5 the third day, and 2.7-3.9 the sixth day. Then, the next step was to implement specific algorithms in an Excel sheet to detect simulated curves that resulted in the same range of log (n° bacteria)/mL each day to the experimental results. Once this analysis was performed, we knew how many and which of the 2121 simulated curves precisely reproduced the experimental behaviour.

Furthermore, we also used this method to assess the goodness of $\alpha$ value. We considered that the larger the number of curves resulting in the previous range, the better the $\alpha$ value would be. Then, we repeated the analysis of 2121 simulations with different values for $\alpha$, and calculated how many curves produced results between the ranges previously measured. As a result, we obtained that the $\alpha$ value that reproduced a larger number of experimental curves was equal to $0.05 h^{-1}$. However, this methodology requires a very large number of simulations and cannot be considered to be very precise. Therefore, more accurate values for $\alpha$ and better methodologies to assess its value can be performed in future studies.

After the first set of 2121 simulations with $\alpha = 0.05 h^{-1}$, we detected that simulated curves with MFs around 88% and CDEs of 1, where similar to those with RIF’s concentrations between 0.0038 and 2 mg/mL. A deeper study with lower increases in MFs (0.1) around 81% showed that simulated curves with MFs of 87.9, 88 and 88.1% reproduced the experimental behaviour fairly good.

As shown in Figure 4.12b, these curves have a significant noise effect due to the small number of simulated bacteria that remained after 6 days. In other studies, we realised that repeated simulations with the same MF effect (88%) and CDE (1) can differ from 3 to 4 log (cfu)/mL at day 6. Therefore, we have also performed the same experiment increasing the number of initial bacteria from 674 to 67438 and the medium volume from $10^3$ to $10^4$ mL. This way, we had a larger number of bacteria but keeping the same log (cfu)/mL. As a result, much lower variations were detected.

However, if we look at the experimental data from Figure 4.12a, we can observe that, for concentrations of RIF from 0.0038 to 2 mg/mL, there is also a significant noise. For instance, time-kill curve of [RIF] =0.0038 mg/mL ends with a number of 2.75 log (cfu)/mL, while the curve for [RIF] =2 mg/mL has approximately 3.6 log (cfu)/mL at the same time point. Therefore, we have considered that the noise observed in IBM simulations with an initial number of 674 bacteria, was similar to the noise observed experimentally in this range of concentrations.
Nevertheless, another interesting option to consider in future studies with more experimental data would be to work with larger populations and consider a linear increase on *Mtb* population effect with RIF’s concentrations between 0.0038 and 2 mg/mL.

**Figure 4.12:** (a) Experimental time-kill curves of for different concentrations of RIF. The specific concentration of rifampicin corresponding to each curve is shown in the legend in mgRIF/mL. Data adapted from Steenwinkel et al. (30)(b) 10 curves corresponding to different simulations with CDE of 1 and MFs from 87.9 to 88.1%.
Another important point to notice when comparing experimental vs simulated graphs from Figure 4.12 is that simulated curves are limited to a number of 3 log (n° bacteria)/mL. Since we considered 3 log (n° bacteria)/mL to be equal to 1 bacteria in the simulation, it is not possible to find values between 0 and 3 log (n° bacteria)/mL in these simulations. Although this is a limitation, something similar happens in the *in vitro* experiments.

In laboratory experiments, when counting the number of CFU/mL, it is necessary to have a range of 30-300 CFU in agar plates. Therefore, to ensure that a sample will yield CFU in this range, a number of dilutions of the sample is needed. Typically, ten-fold dilutions are used, but it depends on each experiment. This fact is also limiting when counting a range of low concentrations of CFU/mL. As a consequence, we can see from Figure 4.11 that no data are reported between 0 and 2.5 log (cfu)/mL.

Overall, we have considered that *in silico* time-kill curves with MFs between 87.9 and 88.1 combined with CDE of 1 reproduced the experimental curves with RIF’s concentrations between 0.0038 and 2 mg/mL satisfactorily.

*Time-kill curves with [RIF] between 4 and 64 mg/mL*

Soon after detecting which CDEs and MFs reproduced rifampicin concentrations from 0.0038 to 2 mg/mL we applied the same methodology to detect curves that simulate concentrations between 4 and 64 mg/mL.

We first detected that those simulations with CDEs of 1.3 and MFs around 86% produced curves similar to those observed in Figure 4.13a. After repeating the analysis with small variations in MF, we concluded that the MF value that better reproduced the experimental behaviour was 86.5%. Nevertheless, as we are working with a very small number of bacteria, noise associated to variables from *in silico* individuals become a big setback when fitting the model. Several repetitions with a constant MF of 86.5% and CDE of 1.3 were performed. The vast majority of these curves killed all bacteria between 3 and 6 days. However, few of them still had one bacterium after 6 days. In Figure 4.13b, we have plotted 5 curves (the same than experimentally) with a constant MF of 86.5% and CDE of 1.3. We have decided to plot 5 of the most common curves observed when setting these MF and CDE values, but it is important to note that in some simulations, one might obtain curves with one bacterium after 6 days.

We would like to find specific values for MF and CDE for each rifampicin concentration. In order to do that, we could perform our simulations with a larger number of initial bacteria. However, as each experimental curve was generated from one single experiment, experimental noise also seems to be significant. Therefore, we consider that this specific fitting will be better performed when obtaining more experimental data. As a result, in some cases, we have associated ranges of rifampicin concentrations to one specific value of MF and CDE.
Figure 4.13: (a) Experimental time-kill curves for 5 different concentrations of RIF. The specific concentration of rifampicin corresponding to each curve is shown in the legend in mgRIF/mL. (b) 5 curves corresponding to different simulations with CDE of 1.3 and MF of 86.5%.
The remaining ranges have been plotted together with all experimental and simulated curves in one single graph in Figure 4.14, as there were just a few number of curves in each range.

**Time-kill curves with [RIF] between 128 and 256 mg/mL**

In Figure 4.14a we can observe that experiments exposing *Mtb* to rifampicin concentrations of 128 mg/mL and 256mg/mL result in killing the whole *Mtb* population in 1 day. This behavior, is easily simulated with high values of CDE. Specifically, we have found that for a value of 85% in MF and 2.5 in CDE, all simulated curves killed bacteria in 1 day. One of these simulated curves can be observed in Figure 4.14b.

**Time-kill curves with [RIF] between 0.0005 and 0.0019 mg/mL**

Finally, we have found a combination of MF and CDE values that reproduced RIF concentrations from 0.0005 to 0.0019 mg/mL. These curves worked with high concentrations of bacteria, which avoids the problem of high variations in the simulated curves, and made possible to find specific values for MF and CDE. First, we can notice that the simulated curves that better reproduced the experimental behavior for these concentrations were mainly affected by the effect on MF. From the experimental curve with a RIF concentration of 0.001 mg/mL (Figure 4.14a, grey line) we observe how the number of bacteria increases during the first day, but then decreases its slope during the following days. The behavior is very well reproduced by a CD of 0.3 and MF of 89%. In this case, growth is limited after the first day due to the delayed effect in MF produced by alpha. Perhaps the least reproducible behavior in our IBM is the one produced by a RIF concentration of 0.0019mg/mL (yellow line). The early growth and later decrease in *Mtb* population might be explained by a delayed damage effect, such as the one that MF has. However, non-MF and CDE combination was capable of simulating this abrupt change. It is worth noting that experimental CFU counting also has an associated noise. Furthermore, the aim of this study was not to simulate each experimental curve accurately, but to reproduce and understand behaviours observed during *in vitro* experiments by means of individual based modelling.
Figure 4.14: (a) Experimental time-kill curves under different RIF’s concentrations from Steenwinkel et al (30). The specific concentration of RIF corresponding to each curve is shown in the legend in mgRIF/mL. (b) Simulated time kill curves depending on CDE and MF. The value for both parameters in each curve is shown in the legend. In the left side of the legend values for MF are expressed in %, and CDE in the right.
5. CONCLUSIONS

From the previous results we have obtained the following conclusions:

- Our initial goal was to generate an IBM that enable researchers to cope with the datasets generated from in vitro experiments. After analysing the results presented in the previous chapter, we conclude that we have developed a model that can be used to understand those experimental results from in vitro research.

- The model simulates the activity of Mtb cells without antibiotic by a number of individual variables that reproduce intrinsic biological characteristics of the bacilli. Therefore, it allows us to estimate the order of magnitude of some individual parameters that are hard to evaluate experimentally.

- Moreover, the model has reproduced the effects of rifampicin in Mtb population by means of two main parameters, Cell Damage Effect (CDE) and Membrane Functionality (MF). The combination of these parameters has been found to be crucial to reproduce the effect of RIF. Therefore, these results allow us to remark the importance of multiple effects when understanding the mechanisms of rifampicin within the cell.

From the results obtained in the simulations we have obtained several additional conclusions:

- The delay introduced to the effect on membrane functionality has accurately reproduced the dynamics of the bacterium with low concentrations of rifampicin. These results, reinforce the idea of a possible progressive degradation of Mtb membrane and cell wall.

- The heterogeneous metabolic activities experimentally found on non-resistant Mtb strains seem to confer the capacity to some bacteria to survive while others die. In our model, the survivors appear to be bacteria with lower maintenance energies. Thus, the model developed could allow us to assess the role of population heterogeneity in the survival of Mtb culture.

- By using the IBM developed, we could differentiate those experimental patterns that are caused by the emergence of genetic-resistant strains to the ones produced by population heterogeneity.

- Another important result is the one associated with the high noise found in mediums with low concentration of bacteria. As we observed, in this range we were unable to find a specific value of MF and CDE for each rifampicin concentration. However, this noise is also observed experimentally when working with high RIF concentrations. Therefore, the model has also given insights into possible explanations of experimental noise.
In general terms:

- The model developed has given insights into the intrinsic key factors that are responsible of the dynamics of the bacterial population as a whole. After the sensitivity analysis, we have detected a number of highly sensitive parameters. Moreover, CDE and MF have also proven to be crucial facts in the response of the bacterial culture to RIF’s exposure.

- The analysis of these important factors has allowed us to improve our understanding of the interactions between *Mtb* and rifampicin. In further studies, we believe that CDE and MF will allow us to introduce the effect of other anti-TB drugs in this model, improving our comprehension of their mechanisms and effects.

- For a number of initial bacteria and non-excessive rifampicin concentration, the simulated results seem to match empirical patterns very accurately. Therefore, this tool could also be used to reduce the time and cost of *in vitro* experiments by predicting the evolution of the population from a certain time point.

6. FUTURE PERSPECTIVES

In future studies, we hope that more *in vitro* data will enable us to generate a more specific correlation between the concentration of rifampicin, CDE, and MF. We could also study the possibility of using other calibration methods to better fit those sensitive parameters.

Moreover, by means of CDE and MF, we hope to reproduce the effect of other antibiotics used in the treatment of tuberculosis. However, as the mechanisms by which antibiotics affect *Mtb* are diverse, it might be necessary to introduce other effects apparat from CDE and MF.

In addition, by fitting its parameters, the model could be adapted to simulate other *Mtb* strains. In future projects, we also expect to introduce the dynamics of resistant *Mtb* strains.

Another important field of study is the simulation of the effect of combining different antibiotics, which is very common in *in vitro* experiments.

Finally, we have also found that working with larger populations would significantly reduce the noise of the simulations. However, the larger the population, the more limiting NetLogo’s execution time becomes. Therefore, we consider that the implementation of the current model to a more powerful software would reduce the time spent in the simulations performed. Other similar models have already shown a significant improvement when introducing NetLogo’s models to Python.
BIBLIOGRAPHY


APPENDIX A

Table A1: Numerical results of the sensitivity analysis:

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APPENDIX B

SOURCE CODE

;; DEFINICIO DE VARIABLES GLOBALS I DELS TURTLES

globals [
  total_nutrient
  total_biomass
  bacteria_t-1
  bacteria_t
  biomass_t-1
  biomass_t
  rate_bacteria
  rate_biomass
  devr
  devv
  devtrd
  devage
  dev_C_max
  dev_C_mant
]
dev_Ant_nut
dev_CellDamageEffect

max_mass
devs_time
nº_duplic
dupl_time
mean_duplic_time
resembra
concentracio_med
mu
mean_interior_nutrient
mean_maintenance_energy
morts
Antnut

mean_volume
C_max_average

x0
x1
x2
x3
x6
]

turtles-own [ volume
start_volume
maintenance_energy
trdi
Cell_Damage
s_int
age
mass
reproduction_time
C_max
C_int
C_mant_real
damage_death
Antnutindividual
CellDamageEffectIndividual
]

;; INICIALITZACIÓ
to setup
clear-all

;; desviacions típiques:
set dev_c_max 0 * mean_c_max
set dev_c_mant 0.08 * c_mant
set devr 0.2 * reproduction_volume
set devv 0.8 * critical_cell_damage
set devtrd 0.1 * trd
set devage 0.20 * mean_initial_age

;; Bucle que creen els turtles i defineixen el medi:

setup-bacteria
setup-medium

set morts 0
set time 0
set resembra 0;

do-plotting

file-close
let spacer ","
if file-exists?
(word "TB_senseAntib" behaviorspace-run-number ".csv") [ file-delete (word "TB_senseAntib" behaviorspace-run-number ".csv")]
file-open (word "TB_senseAntib" behaviorspace-run-number "_.csv")
file-print (list spacer "Temps" spacer "Nº bacteris" spacer "Volum total" spacer "Energia de manteniment"

spacerr trdi spacer "Velocitat mitjana de consum del nutrient" spacer "Edat" spacer "Temps de reproduccio"

spacerr Nutrient total spacer "Biomassa total" spacer "Temps de duplicacio"

file-close

reset-ticks
to setup-bacteria

set-default-shape turtles "bacil"
create-turtles initial_bacteria

[ setxy random-pxcor random-pycor

let volume_ini abs ( random-normal reproduction_volume devr ) / 2

set volume volume_ini
set start_volume abs ( random-normal trd devtrd )
set trdi abs ( random-normal trd devtrd )
set age abs ( random-normal mean_initial_age devage )
;set Cell_Damage 0
set maintenance_energy 0
set reproduction_time 0
set color pink
set total_biomass total_biomass + mass
set s_int 0
set mass volume * density
set C_max abs ( random-normal mean_C_max dev_C_max)
set C_int 0

set C_mant_real random-normal C_mant dev_C_mant
while [C_mant_real < 0]
[set C_mant_real random-normal C_mant dev_C_mant ]
;set C_mant_real abs ( random-normal C_mant dev_C_mant )

set damage_death random-normal Critical_Cell_Damage devv
while [damage_death <= 0 or damage_death >= Critical_Cell_Damage * 4]
[set damage_death random-normal Critical_Cell_Damage devv]
]

end

to setup-medium

set total_nutrient initial_total_nutrient
if total_nutrient < 0 [ set total_nutrient 0 ]

set concentracio_medi total_nutrient / Medium_volume

end

;; Bucle principal:

    to go

       set time time_step + time
       if bacteria_t != 0
       [ set mu ln (bacteria_t / initial_bacteria) * (1 / (time / (3600 * 24))) ]

;;;;RESEMBRA;;;;

   if resembla = 0
   [ if (time / 3600) = Re_inoculation
       [ set time 0
       let x (bacteria_t - initial_bacteria)
       ask n-of x turtles [die]
       set resembla 1
       ] ]

  export-files
      set bacteria_t-1 count turtles
      set biomass_t-1 sum [mass] of turtles

      set n°_dupl 0
      set dupl_time 0

      set concentracio_medi total_nutrient / Medium_volume

      ask turtles [ set age age + time_step]
      ifelse resembla = 0
[  
  set Antnut 1  
]

[  
  set Antnut (Membranefunction / 100)  
  set dev_Ant_nut Antnut * 0.001  
  set dev_CellDamageEffect CellDamageEffect * 0.001  
]

ask turtles [move uptake]

;;;;; MOVE UPTAKE

ifelse resembla = 0

[

  set total_nutrient initial_total_nutrient  
  if total_nutrient < 0 [set total_nutrient 0]
]

[
  set total_nutrient initial_total_nutrient - (sum [s_int] of turtles)  
  if total_nutrient < 0 [set total_nutrient 0]
]

  set mean_interior_nutrient sum [s_int] of turtles / count turtles  

  set C_max_average sum [C_max] of turtles / count turtles  

ask turtles [Damage growth Death reproduce]

;;;;; DAMAGE GROWTH DEATH REPRODUCE

  set mean_maintenance_energy sum [maintenance_energy] of turtles / count turtles  

  set mean_volume (sum [volume] of turtles) / (count turtles)

  ifelse n°_dupl > 0 [ set mean_dupl_time dupl_time / n°_dupl ][ set mean_dupl_time 0 ]
set total_biomass sum [mass] of turtles

set bacteria_t count turtles
set biomass_t sum [mass] of turtles

; ; ; ; EXPORT TIME-KILLING CURVE

IF resembla = 1
[
if (time / 3600 = 0) and (count turtles != 0)
[ set x0 (log (10 * count turtles) 10)]

if (time / 3600 = 24) and (count turtles != 0)
[ set x1 (log (10 * count turtles) 10) ]

if (time / 3600 = 48) and (count turtles != 0)
[ set x2 (log (10 * count turtles) 10)]

if (time / 3600 = 72) and (count turtles != 0)
[ set x3 (log (10 * count turtles) 10)]

if (time / 3600 = 144) and (count turtles != 0)
[ set x6 (log (10 * count turtles) 10)]
]

set rate_bacteria (bacteria_t - bacteria_t-1) / bacteria_t-1
set rate_biomass (biomass_t - biomass_t-1) / biomass_t-1

if bacteria_t = 0 [ stop ]

if resembla = 1 [if time > SimulationTime [stop]]

tick

if resembla = 1 [do-plotting] ;or resembla = 0 [do-plotting]
end ;; END GO

to move ;;MOVE
   right random 360
   forward 10
end

to uptake ;; UPTAKE

ifelse resembra = 1
[set Antnutindividual (abs (random-normal Antnut dev_Ant_nut))
 set Antnutindividual (1 - ( (1 - Antnutindividual) * (1 - exp( - alpha * (time / 360)))))
]
[set Antnutindividual 1]
ifelse concentracio_medi >= C_max
 [set C_int C_max * Antnutindividual]
 [set C_int concentracio_medi ]

set s_int (C_int * volume) * uptake_repetitions

end
to Damage

if resembra = 1
[
    set CellDamageEffectIndividual abs( random-normal CellDamageEffect dev_CellDamageEffect)
    ;set CellDamageEffectIndividual CellDamageEffectIndividual * ( 1 - exp( - alpha * (time / 360)))); si consideressim que CDE també és acumulatiu
set Cell_Damage Cell_Damage + CellDamageEffectIndividual
]
end

to growth ;; GROWTH

set mass volume * density

set maintenance_energy C_mant_real * mass * uptake_repetitions

set maintenance_energy maintenance_energy * (1 + factor_C_mant * Cell_Damage)

ifelse ((s_int ) > (maintenance_energy)) ;; ens
[ set Cell_Damage 0

let DV (((s_int - maintenance_energy) / uptake_repetitions ) / density) * Y ;;
set volume volume + DV
set mass volume * density
set s_int 0]

[ set Cell_Damage Cell_Damage + 1

set s_int 0
]
end

to Death ;; DEATH

if Cell_Damage > damage_death

[ set morts morts + 1
die
]
end
to reproduce ;; REPRODUCTION

if volume > start_volume

[set reproduction_time reproduction_time + time_step]

if reproduction_time > trdi

[let volume1 (random-normal (volume / 2) ((volume / 2) * 0.15))
let volume2 volume - volume1
set volume volume1
set start_volume abs (random-normal reproduction_volume devr )
set trdi abs (random-normal trd devtrd )
set reproduction_time 0
set mass volume * density
set s_int 0
set maintenance_energy 0
;set Cell_Damage 0
set n°_dupl n°_dupl + 1
set dupl_time dupl_time + ( age / 3600)
set age 0

set C_mant_real random-normal C_mant dev_C_mant
while [C_mant_real < 0]
[set C_mant_real random-normal C_mant dev_C_mant ]
;set C_mant_real abs (random-normal C_mant dev_C_mant)

while [damage_death <= 0 or damage_death >= Critical_Cell_Damage * 4]
[set damage_death random-normal Critical_Cell_Damage devv]
set C_max (random-normal mean_C_max dev_C_max)

hatch 1 [set volume volume2
set start_volume abs (random-normal reproduction_volume devr )
set trdi abs (random-normal trd devtrd )
set age 0
set mass volume * density
set reproduction_time 0
set maintenance_energy 0
set s_int 0]
set C_mant_real random-normal C_mant dev_C_mant

while [C_mant_real < 0]
    [set C_mant_real random-normal C_mant dev_C_mant ]
;set C_mant_real abs (random-normal C_mant dev_C_mant)

while [damage_death <= 0 or damage_death >= Critical_Cell_Damage * 4]
    [set damage_death random-normal Critical_Cell_Damage devv]
;set Cell_Damage 0
set C_max (random-normal mean_C_max dev_C_max)
]

end

to do-plotting ;; PLOTTING

set-current-plot "Nutrient"
set-current-plot-pen "total_nutrient"
pplot total_nutrient

set-current-plot "Viable bacteria"
set-current-plot-pen "turtles"
pplot count turtles

set-current-plot "Total viable biomass"
set-current-plot-pen "total_biomass"
pplot total_biomass

end

to export-files

;;; write the information to the file
    let spacer ","
    file-open (word "TB_senseAntib" behaviorspace-run-number ".csv")

file-close

end