UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA VEGETAL

SimulATe: A Simulator of Antibiotic Therapy Effects on the Dynamics of Bacterial Populations

Pedro Henrique Chorão David

MESTRADO EM BIOINFORMÁTICA E BIOLOGIA COMPUTACIONAL
ESPECIALIZAÇÃO EM BIOINFORMÁTICA

Dissertação orientada por:
Doutor Teresa Nogueira
Prof. Doutor Francisco Dionísio

2018

Agradecimentos

Quero começar por agradecer aos meus orientadores por terem aceitado acompanhar-me durante o decorrer deste trabalho. À Teresa Nogueira, pela constante motivação e entusiasmo, pela enorme disponibilidade e pelo esforço investido em organizar reuniões com grupos e investigadores potencialmente interessados no simulador fruto deste trabalho. Ao Francisco Dionísio, por tornar amontoados de números e letras gregas em algo inteligível e por estar sempre disponível para me explicar todos os conceitos de que necessitei. À Xana Sá Pinto, minha orientadora não-oficial, pelos altamente motivantes "Isto é muita fixe!", pelo ponto de vista educacional e pela ajuda em certos aspectos do trabalho.

Gostaria de agradecer ao Luís Borda de Água, por me ter facultado um MAC onde pude testar o simulador. Gostaria de agradecer aos Professores que, de bom grado, estiveram disponíveis na minha primeira apresentação do simulador, tanto pela presença como pelo "feedback" prestado, sendo eles Sara Aboim, André Rodrigues, Lucinda Motta e Xana Sá Pinto. Gostaria também de agradecer à Professora Rita Ponce, por ter conspirado benevolamente em conjunto com a Teresa Nogueira para organizar uma apresentação minha do simulador aos seus alunos de Licenciatura na Escola Superior de Tecnologia do Barreiro, que acabou por ser uma experiência óptima e bastante enriquecedora (estendo assim também os meus agradecimentos aos alunos presentes pelo escrutínio e questões colocadas).

Os maiores agradecimentos são dirigidos à minha família, aos meus pais e irmã, que sem eles não teria como prosseguir com os meus estudos.

Agradeço ao "gang" do almoço, Zé, Stufu, Sofia, Vanessa, Chico e Inês, pela companhia, dicas, desabafos e corte e costura. Destes, agradeço ao Zé por me fazer rir com as suas "noobices", ao Stufu pelas suas hilariantes demandas emocionais, à Sofia pelas risadas de altos decibéis que alegram qualquer um, à Vanessa pelas suas intrincadas histórias de vida e ao Chico pela sua constante análise psicoterapêutica do grupo. Agradeço também ao Matias por ter desenhado um ícone para o simulador de completa boa vontade.

Um agradecimento muito especial à Inês Pacheco, que esteve sempre presente durante todo o decorrer da tese, tanto nos momentos altos como nos baixos, pelas refeições, bolos, passeios e afectos e, porque se não fosse ela, teria de certeza entregue a tese mais cedo.

Um grande agradecimento ao meu amigo de longa data Fernando Alves, pela constante pressão para conclusão da tese e pelos comentários deixados depois de ter sido a primeira pessoa a ler uma das primeiras versões da tese.

Por último, a todos os que me ajudaram a testar o simulador e a todos aqueles que contribuíram com a sua opinião, agradeço a disponibilidade.

Resumo

Um antibiótico é definido como uma substância química, tanto produzida por microrganismos como sintetizada artificialmente, que inibe o crescimento ou mata outros microrganismos. Estas substâncias são produzidas naturalmente por muitas bactérias, fungos e plantas. Já desde os tempos pré-históricos da humanidade que, inconscientemente, se exploram as vantagens dos antibióticos com o fim de tratar infeções. Mais recentemente, durante o século XX, certos investigadores começaram a identificar, refinar e sintetizar artificialmente antibióticos específicos. Foi o caso da penicilina, o primeiro antibiótico da história, específico de bactérias, a ser produzido em massa. Estes eventos contribuíram para dar origem à era dos antibióticos e, no decorrer do resto do século, uma multitude de novas classes de antibióticos foi descoberta e aprovada para uso humano. No entanto, a taxa de descoberta de novos antibióticos tem vindo a diminuir constantemente ao longo dos anos, sendo que nenhuma nova classe de antibióticos foi descoberta desde 1997. Além disso, bactérias possuidoras de resistências são geralmente detetadas logo após a descoberta de um novo antibiótico, o que aconteceu para cada antibiótico atualmente conhecido. A existência de resistências a antibióticos constitui assim um grande problema para os cuidados de saúde humanos, pois limita a eficácia de um medicamento que, de outra forma, é altamente eficaz no combate à infeção bacteriana.

As resistências a antibióticos podem surgir através de mutações genéticas, ou trocadas entre bactérias (transferência horizontal de genes). Estas resistências são depois tornadas mais prevalentes como produto da pressão seletiva exercida pelos antibióticos: matando as bactérias sensíveis e, consequentemente, aumentando a frequência relativa das bactérias resistentes. Múltiplas vias podem conferir uma resistência idêntica ao mesmo antibiótico, nomeadamente através de: modificações da molécula de antibiótico, diminuição da sua penetração através da membrana celular, aumento do efluxo da molécula para fora da célula ou alteração dos locais alvo do antibiótico. Os antibióticos tendem a ser mal utilizados de amplas maneiras, como por ingestão em excesso, prescrição inadequada e uso não controlado na agricultura, bem como devido à falta de conhecimento sobre o uso adequado de antibióticos pelo público em geral, fazendo com que as resistências aumentem a sua prevalência e se espalhem a uma taxa muito mais rápida do que a esperada e até mesmo causando a génese de variedades de bactérias resistentes a múltiplos fármacos simultaneamente.

Embora existam tratamentos alternativos aos antibióticos, estes têm um efeito muito mais limitado quando comparado com os antibióticos, ou ainda têm de superar o uso de antibióticos como a abordagem dominante no tratamento de infeções bacterianas. No entanto, um antibiótico nem sempre é necessário no tratamento de uma infeção. Uma pessoa saudável possui um sistema imunitário capaz de reconhecer e eliminar a maioria dos agentes estranhos ao corpo, como bactérias patogénicas, sem necessidade de ajuda externa, como a de um antibiótico. No entanto, uma infeção pode ocasionalmente ser tão grave ou invasiva que os antibióticos, ou outros tratamentos, deverão ser fornecidos para prevenir condições de risco de vida para o paciente. Um antibiótico também pode não ser suficiente para eliminar todas as bactérias patogénicas. No entanto, ao eliminar uma parcela substancial das bactérias suscetíveis aos seus efeitos, o sistema imunitário pode então mais facilmente reduzir a carga microbiana, eliminando as bactérias patogénicas remanescentes, mesmo aquelas que sejam resistentes ao antibiótico aplicado. A combinação dos efeitos de um antibiótico com o funcionamento normal do sistema imunitário deve, portanto, produzir uma eliminação facilitada e mais rápida de uma infeção do que se qualquer um dos sistemas atuasse sozinho, além de reduzir a probabilidade de qualquer bactéria resistente ao antibiótico sobreviver ao tratamento. Por isso, um sistema imunitário funcional é crucial para a sobrevivência de

qualquer pessoa. A deterioração do sistema imunitário pode afectar perigosamente o bem-estar de um indivíduo, uma vez que qualquer infeção, por menor que seja, pode crescer rapidamente para uma situação perigosa, mesmo quando um antibiótico é administrado, como acontece no caso de indivíduos infetados pelo VIH.

Embora já existam muitas maneiras de agir contra a questão do uso inadequado de antibióticos, o que escolhemos abordar neste trabalho assenta no ensino do público em geral sobre a forma como os antibióticos funcionam e as causas para o aparecimento e/ou aumento de resistências bacterianas, nomeadamente como estas podem aumentar a sua prevalência em resultado do referido uso inadequado de antibióticos. Alguns exemplos do que já foi feito em todo o mundo por esta causa incluem a consciencialização de profissionais de saúde, incorporação de informação relacionada em livros escolares e ensino desta problemática na escola. Ainda assim o problema persiste e as resistências a antibióticos continuam a ser um problema importante, especialmente em hospitais ou outras instalações de saúde. A investigação sobre as resistências a antibióticos e os meios para as superar tornou-se cada vez mais popular ao longo dos anos. Este trabalho pretende contribuir para a simplificação do processo de investigação através do desenvolvimento de novas ferramentas e tecnologias, e poderá ajudar os investigadores a testar mais rapidamente os seus modelos do desenvolvimento da resistência a antibióticos, a encontrar novas resistências a antibióticos e a criar novas metodologias de combate a essas resistências. Acreditamos que qualquer contribuição feita, tanto para a disseminação de boas práticas no uso de antibióticos como para o conhecimento sobre resistências a antibióticos, bem como para o avanço da investigação relativa à resistência a antibióticos, são significativos.

Com este trabalho, procurámos desenvolver uma ferramenta digital de simulação que pudesse ser utilizada em dois cenários diferentes: a) por professores, como recurso didático para o ensino das ciências e exploração da problemática da resistência a antibióticos, nomeadamente dos seus impactos, formas de prevenir a sua génese e propagação e interação com o sistema imunitário humano; e b) por investigadores, para ajudar no teste de hipóteses sobre o desenvolvimento de resistências a antibióticos. Em ambos os casos o uso de programas de simulação pode ser vantajoso, pois permitem a visualização e manipulação de variáveis com base em situações reais em ambiente controlado. Esta ferramenta digital, que faz uso das tecnologias atuais, poderá permitir aos alunos exercitar competências científicas fundamentais para o desenvolvimento da sua literacia científica e simultaneamente compreender a problemática da resistência a antibióticos e o impacto das escolhas individuais neste fenómeno e na saúde individual. Estes objetivos de aprendizagem vão ao encontro das diretivas curriculares e programáticas e de metas curriculares em vigor para diversas disciplinas lecionadas ao longo do percurso escolar dos alunos em Portugal. Simultaneamente a inclusão de diversos parâmetros reais permite também a simulação de contextos reais com potencial para serem usados em investigação científica, proporcionando assim aos investigadores um grau elevado de liberdade e controlo sobre as suas simulações.

Em linha com estes objetivos, desenvolvemos o *SimulATe*, um simulador dos efeitos da antibioterapia na dinâmica de populações bacterianas. Este possui uma interface de usuário gráfica e permite a simulação de dois cenários distintos: o primeiro simula os efeitos de um antibiótico numa única população bacteriana em conjunto com o sistema imunitário humano; o segundo simula o equilíbrio natural do microbioma intestinal humano e os efeitos que uma antibioterapia pode ter na sua estabilidade. É um simulador altamente configurável que funciona em tempo real e permite a simulação de uma ampla gama de cenários de administração de antibióticos. Estes tipos de simulações não são possíveis de obter com outras aplicações existentes atualmente, já que estas são ou muito específicas ou não abrangem todos os casos que nos propusemos abordar.

Palavras-Chave: Simulação, Resistência a antibiótico, População bacteriana, Microbioma

Abstract

Antibiotics are substances either produced by microorganisms or artificial synthesized, which, above certain concentrations, can inhibit the growth or kill other microorganisms. Humanity has been exploiting antibiotics since pre-history times, but only in the 20th century did mass-production begin, allowing for more widespread usage. Nowadays, antibiotics are used extensively worldwide to treat all sorts of infections, especially those caused by bacteria. However, the effectiveness of antibiotics is severely hindered by the existence of antibiotic resistances. These resistances can emerge in bacteria in a variety of different ways, mainly as a result of genetic mutations or horizontal gene transfer and persist due to the selective pressure caused by antibiotics. Multi-resistant strains of bacteria can arise and are a major cause of concern in many health care facilities, the primary source of these strains. Coupled with the fact that the rate of discovery of new antibiotic classes has been steadily declining over the past decades, the existence of antibiotic resistances constitutes one of the most serious health care crises of the 21st century.

Arguably, the main cause of antibiotic resistances persistence in nature is antibiotic misuse, such as via overusing, inappropriate prescribing and uncontrolled use in agriculture as well as due to the lack of knowledge on appropriate antibiotic usage by the public. Several approaches can be adopted to combat antibiotic misuse, including raising awareness among medical professionals, incorporating related information in schoolbooks and teaching these issues at school, the latter approach being the one we decided to tackle with this work.

We developed *SimulATe*, a simulator of antibiotic therapy effects on the dynamics of bacteria populations, with the purpose of being used as an educational tool in the teaching of science, exploring antibiotic resistance and the impacts of antibiotic misuse. *SimulATe* allows the simulation of two distinct scenarios: the first simulates the effects of an antibiotic on a single bacteria population alongside the human immune system; the second simulates the natural equilibrium of the human gut microbiome and the effects an antibiotic therapy can have on its stability. Being a highly configurable real time simulator, which allows the simulation of a broad range of antibiotic therapy administration scenarios, *SimulATe* can also be used by both researchers and medical institutions to test antibiotic usage scenarios or the development of an infection under antibiotic therapy.

Keywords: Simulation, Antibiotic resistance, Bacteria population, Microbiome

Contents

List of Figures

List of Tables

Glossary

ECDC	European Centre for Disease Prevention and Control.
MIC	Minimum Inhibitory Concentration.
Infective Dose	Number of pathogenic bacteria necessary for an infection to take hold.
Microbiome	Community of microorganisms that share the same habitat.
Enterotype	Specific stable composition of the human gut microbiome.
CD4+ Cells	Helper T cells; Regulate the overall innate and adaptive immune responses.
Naïve T Cells	Precursor lymphocytes that have not yet identified foreign pathogens.
CD8+ cells	Cytotoxic T cells; Killer cells; Effector cells. Eliminate identified threats to the human body.
Memory T Cells	Lymphocytes which preserve antigens of previously identified threats.
MHC	Major Histocompatibility Complex.
OS	Operating System.
IDE	Integrated Development Environment.
Kivy Framework	Python graphical user interface library.
Widget	Control element in a graphical user interface.
API	Application Programming Interface.
VCS	Version Control System.
XML	Extensible Markup Language, defines rules for encoding documents.

1 Introduction

An antibiotic is defined as a chemical substance, produced by some living organisms or artificially, which, above a certain concentration, either inhibits the growth or kills bacteria. These substances are naturally produced by many bacteria, fungi and plants, providing an advantage against competing microorganisms and infections [1–5]. Humanity has been unknowingly exploiting the advantages of antibiotics since pre-historical times, and records of ancient civilizations, such as Egypt, China, Serbia, Greece and Rome, describe the application of antibiotic producing moulds and plants to treat wounds and infections [6 7]. During the twentieth century, specific antibiotic agents began being identified and refined as well as artificially synthesized by researchers, as was the case with penicillin, the first mass produced bacteria-specific antibiotic in history [8 9]. During the Second World War the large-scale production of penicillin became a necessity, mainly to help the war effort, a decade after its initial discovery in 1928. Meanwhile, in 1932, the first *sulphonamide* based antibiotic drug, *prontosil*, was discovered and proved to be an effective antibiotic against *streptococcal* and *staphylococcal* bacterial infections [10]. These events contributed to effectively give rise to the antibiotic golden age and, throughout the rest of the century, a multitude of new antibiotic classes were discovered and approved for human use. All currently known antibiotics can be grouped together in classes (Table 1.1) based on the physiological effect (Figure 1.1) they have on their target bacteria as well as whether they exert bactericidal (killing) or bacteriostatic (halting growth) effects [11].

Table 1.1 Antibiotic Classes.
Mechanism of action of each class of antibiotic, year of discovery, year introduced to the public and year of the first observation of a microorganism resistant to it. Adapted from K. Lewis et al [11].

Class of Antibiotic	Mechanism of Action	Year Discovered	Year Introduced	Year Resistance Observed
β-lactams (Penicillin)	Inhibition of cell wall biosynthesis	1928	1938	1945
Sulphonamides (Prontosil)	Inhibition of dihydropteroate synthetase	1932	1936	1942
Aminoglycosides	Binding of 30S ribosomal subunit	1943	1946	1946
Tetracyclines	Binding of 30S ribosomal subunit	1944	1952	1950
Chloramphenicols	Binding of 50S ribosomal subunit	1946	1948	1950
Macrolides	Binding of 50S ribosomal subunit	1948	1951	1955
Fidaxomicin	Inhibition of RNA polymerase	1948	2011	1977
Glycopeptides	Inhibition of cell wall biosynthesis	1953	1958	1960
Oxazolidinones	Binding of 50S ribosomal subunit	1955	2000	2001

Rifamycins	Binding of RNA polymerase β-subunit	1957	1958	1962
Quinolones	Inhibition of DNA synthesis	1961	1968	1968
Streptogramins	Binding of 50S ribosomal subunit	1963	1998	1964
Lipopetides	Depolarization of cell membrane	1986	2003	1987
Diarylquinolines	Inhibition of F1Fo ATPase	1997	2012	2006

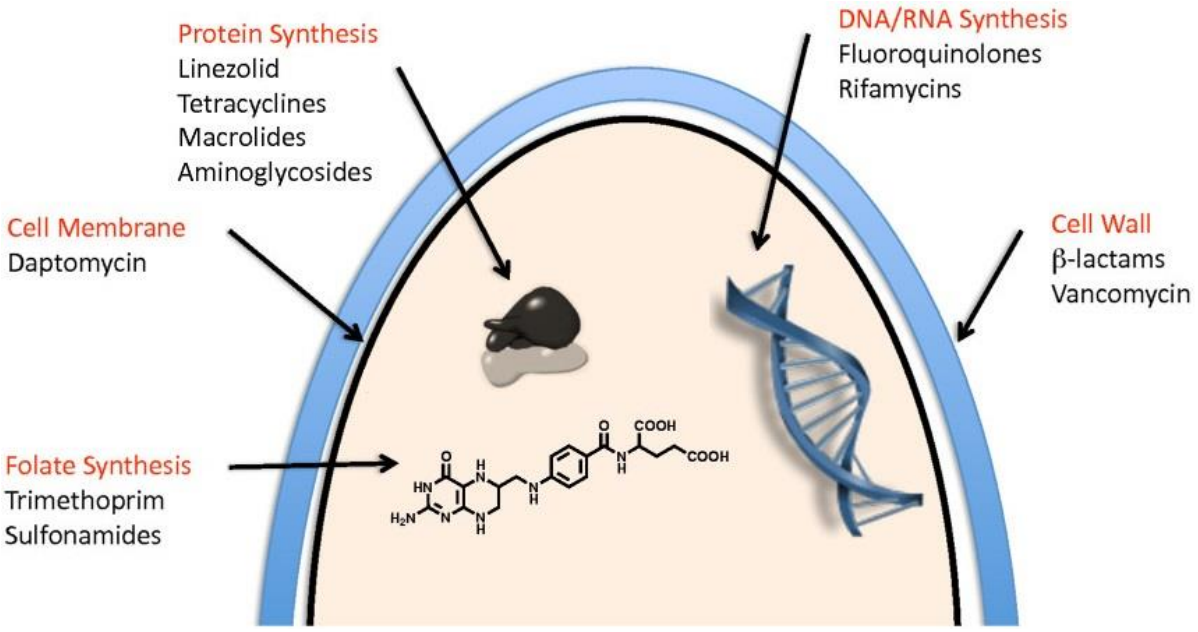

Figure 1.1 Antibiotic targets.
Antibiotic classes and their main targets inside the cell, specifically: cell wall synthesis, cell membrane disruption, DNA and RNA synthesis, folic acid metabolism and ribosome functioning. Adapted from G. D. Wright et al [12], distributed under the CC license.

However, the rate of discovery of new antibiotics has been steadily declining over the years, with almost no new antibiotic classes discovered since 1997 [11], with the exception of Neofiscalin A [13] and Teixobactin [14]. In addition, resistant bacteria are usually detected shortly after the discovery of a new antibiotic, which happened to each currently known antibiotic. The existence of antibiotic resistances, therefore, constitutes a major setback to human healthcare [11] [15], as it limits the effectiveness of an otherwise highly effective drug. These antibiotic resistances can emerge naturally, mainly via random gene mutations, or traded between individuals via horizontal gene transfers [16–18], and can be made more prevalent as a product of the selective pressure exerted by an antibiotic.

Antibiotic resistances can be achieved through many different biochemical pathways and, usually, multiple pathways can confer a similar resistance to the same antibiotic, notably by modifying the antibiotic molecule, decreasing the antibiotic penetration and efflux power or changing the antibiotic target sites [16]. Antibiotics misuse, namely by overusing, inappropriate prescribing and uncontrolled use in

agriculture [19] as well as due to the lack of knowledge on appropriate antibiotic usage [20], has been selecting resistant and even multidrug-resistant strains of bacteria [21], which has been spreading at a much faster rate than expected. This problem, previously predicted by the discoverer of penicillin, Alexander Fleming [22], knows no frontiers and is becoming one of the most pressing human healthcare problems. Although there are possible alternative treatments to antibiotics, such as passive immunization [23] or phage therapy [24], these have a much narrower range of effect than antibiotics or have yet to overcome antibiotic usage as the mainstream approach in dealing with bacterial infections. A healthy person possesses an immune system capable of recognizing and eliminating most foreign agents to the body, such as pathogenic bacteria, without the need for any external help [25], like that of an antibiotic. Nonetheless, occasionally, an infection can be so severe or evasive to the immune system that antibiotics, or other treatments, must be supplied to prevent life threatening conditions. Antibiotics alone may not suffice at clearing every single infecting bacterium. However, by actively eliminating a very large portion of the antibiotic susceptible bacteria, the immune system may more easily, and non-specifically, kill all the remaining bacteria, even those strains that are resistant to the antibiotic [26] [27]. Synergistically combining the effects of an antibiotic with that of the immune system should, therefore, yield a better and quicker elimination of an infection than if either acted alone, while also reducing the likelihood of any resistant bacteria surviving the treatment [26–29]. Hence, a functioning immune system is crucial to the survivability of any person. The deterioration of the immune system may dangerously impact the wellbeing of the individual, as any minor infection can quickly grow to an untreatable situation, even when an antibiotic is administered, as happens in the case of HIV infected individuals [30] [31].

1.1 Motivation

The importance of antibiotics in today's society is much too high for it to be jeopardized by the ever-increasing presence of antibiotic resistances. During the 40's and 50's the damages caused by antibiotic resistances were mitigated by the high discovery rate of new classes of antibiotics [19] [32], but as the rate declined, so did the assurance of new antibiotics having lower impact from antibiotic resistances. Due to this decline, the occurrence and spreading of antibiotic resistances developed into a serious issue [19] which, if not controlled, can have huge healthcare related repercussions.

To fight this problem some steps are necessary [33], from which the most essential are: a) to improve public scientific literacy on antibiotic usage and b) to support research on antibiotic development and clinical use.

As stated before, public misinformation and carelessness related to antibiotic use greatly contributed to the rise and spread of antibiotic resistances. Promoting public scientific literacy on this topic, namely public understanding of antibiotic effects, the impacts of antibiotic misuse and resistance evolution are thus essential to overcome this societal problem. Some examples of actions taken around the world to foster public scientific literacy on antibiotics usage include raising awareness among medical professionals [33], distributing leaflets, the inclusion of this problem, causes and effects in official school curricula [34] [35] and programs, and the development of educational materials to be used in schools [33] [36]. Still the problem remains, and antibiotic resistances continue to crop up, especially in hospitals or other healthcare facilities [19].

Research into antibiotic resistances and means by which to overcome them are, without a doubt, important now more than ever. Streamlining the research process by developing novel technologies and

tools can help scientists to more quickly test their models on antibiotic resistance development, finding new antibiotic resistances and creating new methodologies to better fight those resistances.

Therefore, we believe that any contribution made towards both the dissemination of good antibiotic usage practices and knowledge about antibiotic resistances as well as the further advancement of research regarding antibiotic resistance are significant and worth pursuing.

1.2 Objectives

With this work we aimed at developing a suitable simulation tool that could be used in two different scenarios: a) by professors, to help students develop scientific literacy and competences while exploring the problem that is antibiotic resistance and antibiotic misuse; and b) by researchers, to test hypothesis regarding antibiotic resistance and treatment efficacy. In both cases the use of simulator software can be advantageous, as it allows the manipulation and control of several important parameters and the observation of the expected outcomes of several biological scenarios.

To be useful in the teaching of science and the development of scientific literacy, an educational tool should promote opportunities for students: a) to learn scientific contents and how these can be applied in daily life, b) engage in scientific inquiry namely in posing questions and formulating hypothesis, planning and developing experiments, collecting, treating and interpreting data; c) understand how science is produced and the nature of science; d) engage in scientific debates using evidence to choose among distinct possibilities [37]. In this context, a digital simulator that uses available technology to model the expected outcomes of the evolution of antibiotic resistant bacteria, can be a wonderful educational tool as it allows: a) students to learn about antibiotic resistance, its causes, consequences and the impacts of daily life choices in both individual and community health, b) engage in scientific inquiry regarding the expected outcomes of procedures and biological scenarios in terms of the health of an individual and frequency of antibiotic resistant strains of bacteria, planning experiments and interpreting data; c) understand the nature of science by, for example, exploring how models are used in science, its limitations and potential [38], d) engage in scientific debate about effective practices of controlling infections and limiting the frequency increase and spread of antibiotic resistant strains of bacteria. These outcomes are aligned with the goals of education and, particularly, science education in Portugal [34 35 39 40 41].

When researching antibiotic resistance and treatment efficacy, researchers benefit from exploring the outcomes obtained in a simulated environment as these can provide guidance and feedback when designing real world solutions. By having access to a simulator designed specifically to model the effects of antibiotic therapies on bacterial populations, research in this area can be streamlined.

1.3 Contributions

The main contribution to arise from this dissertation is the development of a computer simulator, named *SimulATe*. This simulator is suitable for fostering students' scientific literacy on antibiotic resistance and promote the development of their scientific skills as well as to be used as a research tool by researchers and medical staff. Previously existing software were not suited for these purposes as they were either too specific in scope or did not include the required parameters to simulate realistic biologic scenarios.

This work was presented as a seminar to Bioinformatics students at the *Escola Superior de Tecnologia do Barreiro*. A poster was presented at the conference *Frontiers in E3: cE3c 4th Annual Meeting*. A book chapter regarding the human microbiome was written during the course of this thesis, borrowing some of the bibliographical research done for this work as well as the use of *SimulATe* itself (Appendix A). A mini review for the special issue of the *Drug Development and Research* Journal on *Overcoming Antibiotic Resistance* was written with contributions from this work and will be publicly available on September 2018. An educational activity, aimed at 9th grade students, was developed with the aim of exploring *SimulATe* (Appendix B). An article regarding the complete work done in this thesis is soon to be submitted to the Oxford's *Biology Methods & Protocols* journal.

1.4 Document Structure

The remaining document is structured as follows:

Chapter 2, Related Work: Establishes the state of the art and reviews relevant information on bacteria and microbiomes, the immune system, antibiotics, antibiotic resistances, the chemostat and existing simulation software solutions.

Chapter 3, Materials and Methods: Describes the chosen programming language, design decisions and the mathematical equations used.

Chapter 4, Implementation: Describes the overall implementation specifications of the program, code structure, layout design and components as well as testing procedures. Issues arising during development are also described.

Chapter 5, Results: Outlines usage cases of the program and describes feedback given by testers.

Chapter 6, Discussion: Discusses the purpose and usability of the program.

Chapter 7, Conclusions: Summarizes the work performed on this thesis and discusses future work.

Chapter 8, Source Code and License: Points to software hosting service and license.

2 Related Work

2.1 Antibiotics

Different antibiotics have different spectra of activity depending on the range of bacterial species affected and are usually designated as broad- or narrow-spectrum antibiotics accordingly. Certain molecular mechanisms, on which antibiotics rely to exert its effects, might not exist on every bacteria, therefore the spectrum of activity varies between antibiotics [42] [43]. Some antibiotics might encompass a wider range of bacterial species due to the existence of more common molecular mechanisms, as is the case with certain ribosome targeting antibiotics, while other antibiotics target uncommon targets, having a narrower spectrum of activity. For an antibiotic to have its effect maximised while also minimising the probability of resistances arising, the spectrum of activity of the antibiotic used should be taken into account while also having both the antibiotic user and the prescribing doctor adhere to some good-practises, as proposed by the *European Centre for Disease Prevention and Control* (ECDC) [19] [44]: a) avoid unnecessary prescriptions; b) strictly follow the antibiotic administration guidelines defined by the prescribing doctor or as written on the package; c) avoid large-spectrum antibiotics if narrow-spectrum are available for the same ailment; d) avoid narrow-spectrum antibiotics on non-susceptible bacteria; e) avoid over or under-dosages; f) avoid interrupting a treatment when symptoms begin to disappear, in other words, always follow through with a treatment until the end.

Even though the role of antibiotic misuse in antibiotic resistance development has been widely discussed, as stated in the Introduction of this document, the authors of a recent study have argued that it might not have the detrimental effects most medical doctors and researchers think they do [45].

All currently known antibiotic classes are already summarized in Table 1.1. The most frequently used antibiotics in Portugal during 2015, as calculated from ECDC's ESAC-Net data submitted to TESSy [46] in 2017, are the following:

Table 2.1 Antibiotic consumption per 1000 inhabitants per day in Portugal.
Consumption of antibiotics for systemic use expressed in DDD (the average maintenance dose per day for a drug used for its main indication in adults) per 1000 inhabitants per day during 2015, as reported by ECDC [44].

Antibiotics	Primary care sector	Hospital sector
Beta-lactams (penicillins)	12.2	0.54
Other beta-lactams	1.56	0.44
Tetracyclines	0.83	0.02
Macrolides, lincosamides and streptogramins	3.06	0.16
Quinolone	2.05	0.15
Sulphonamides and trimethoprim	0.43	0.07
Other substances	1.14	0.19

In Portugal, penicillins are still the most used antibiotics, both in hospitals and in the primary care sector. The most common antibiotic classes/types used in Portugal, as shown in Table 2.1, are: penicillins, tetracyclines, macrolides, lincosamides, streptogramins, quinolones, sulphonamides and trimethoprim.

There has been a growing effort in researching new antibiotics [11] which has resulted in new antibiotics, namely Neofiscalin A [13] and Teixobactin [14], that are still under study and testing and thus still not available for human use.

2.2 Antibiotic Resistance

Antibiotic resistances are organized in classes based on their mechanism of action. These classes include: Modification or destruction of the antibiotic molecule (ex: Beta-Lactamase), reduced antibiotic penetration and efflux (ex: Multidrug Transporters), changes in antibiotic target sites (ex: Vancomycin Resistance), and resistance due to global cell adaptations [16 47].

Antibiotic resistances are acquired either by random mutations or horizontal gene transfers, which can occur via several different mechanisms: transformation, transduction and conjugation [17]. Transformation is the process by which a competent individual bacterium obtains genetic material from the environment, usually originating from a bacterium of the same species, and recombines it with its own DNA; Transduction is the insertion of foreign DNA into a bacterium by means of a virus; Bacterial conjugation involves physical contact between a donor and a receiving bacterium, by means of a membrane extension called “sex pilus”, which allow the exchange of genetic material. A successful antibiotic resistance, exchanged between individuals via horizontal gene transfer, can become epidemic as more individuals acquire it and resist extermination [17]. Some environments and community settings can even act as reservoirs for certain antibiotic resistance genes, by allowing these resistances to be preserved in the population. Example reservoirs include hospitals, nursing homes, childcare facilities, paediatric populations, schools and farm animals [17].

An antibiotic resistance can have effects ranging from a small tolerance to complete resistance to certain antibiotics. A new resistance does not usually confer complete resistance to an antibiotic at first, it does, however, increase the minimum inhibitory concentration (MIC) for a certain antibiotic class or type [48]. Because of selective pressures caused by the antibiotic, more similar mutations tend to survive, and more genetic material can also be exchanged between individuals, increasing the number of resistant individuals and raising the MIC even more [49]. Ultimately, a bacteria strain can evolve complete resistance to an antibiotic this way.

Genes conferring antibiotic resistances tend to be costly for the bacteria [50], which means that, in the absence of antibiotics, these genes can become disadvantageous to bacteria by, for example, lowering their fitness. Therefore, the expectation is that these genes will tend not to remain in the gene pool if no selective pressure from an antibiotic is present. This means these genes should become scarce if the antibiotic source disappears. This is not always the case, however, as these genes can be maintained even in the absence of antibiotics due to the existence of additional compensatory mutations [51]. A major problem with agriculture today is precisely the constant large-spectrum antibiotics that are dumped into crop plantations and farm animal feed [19]. With antibiotics always present, these antibiotic resistance genes are, consequentially, also always present, and in the absence of antibiotics, they subsist in reservoirs, as previously mentioned.

2.3 Bacteria

Bacteria are one of the oldest forms of living organisms on earth, having existed for at least 3.8 billion years [52]. Bacteria exist in many shapes and sizes and span more habitats than any other life form, ranging from the deepest ocean trenches to the highest mountain peaks, as they can survive in extreme conditions. They have considerable influence over the habitats they inhabit by being both primary producers and decomposers [53] as well as having symbiotic relationships with most other organisms [53]. On the human body, symbiotic bacteria can help regulate the metabolism [54], teach and train the immune system [55] and digest nutrients that humans wouldn't otherwise be able to digest [54]. On the other hand, pathogenic bacteria can cause infections and diseases. The bacterial infective dose is the number of pathogenic bacteria necessary for an infection to take hold and varies between bacteria species, some species requiring millions of individuals, as is the case with *Vibrio cholerae,* while other require only a few individuals, as is the case with *Mycobacterium tuberculosis* [56]. One of the most effective ways of fighting these infectious bacteria is through the use of antibiotics. Still, not all bacteria are equally susceptible to the same antibiotics, on the contrary, the susceptibility of different species to the same antibiotics varies wildly, sometimes even between individuals of the same species, when mutations occur, or antibiotic resistance genes are picked up from the environment [16 32].

Bacteria species very rarely occupy a habitat alone, and are usually part of a bigger microbial community, defined as a microbiome.

2.4 Microbiomes

A microbiota is defined as a community of microorganisms that share the same habitat. The term microbiome can also be used to define the same concept [57], although, with the advent of high-throughput genome sequencing, scientists have adopted the word to define the total amount of genes present in a given ecosystem [58]. Nonetheless, in this work, we adopted the former microbiome definition.

The human body is home to a large number of different ecosystems and, inhabiting them, are different microbiomes, such as those of the oral cavity, nose, different parts of the skin, gut and so forth [59]. These microbiomes harbour symbiotic, commensal and pathogenic bacteria, some of the most common genera of which are *Streptococcus* in the oral cavity, *Propionibacterium* on the skin and nose, and *Bacteroides*, *Prevotella*, and *Ruminococcus* in the gut [60]. The bacterial species that compose these microbiomes exist in a dynamic equilibrium and are subject to change at the smallest adjustment in temperature, ambient pH and other factors including the addition or removal of certain bacterial species and the introduction or shortage of certain nutrients [59].

2.4.1 Human Gut Microbiome

The human gut microbiome is of particular interest to the scientific community as it can have major effects on the development and maintenance of the human body [60–64], with some studies reporting a direct relation between the health of a gut microbiome and the health of its human host [64]. It's believed that human foetuses have a sterile gut up until birth, when it is first colonized by microorganisms

originating from the mother [65 66], which can vary based on birth mode (caesarean or vaginal birth) [67]. The gut microbiome then matures along with the infant human, being affected by the initial feeding regime, milk-based or formula-based diet, and achieving a stable adult configuration at around 3 years of age [63 67]. The microbiome composition also varies based on geography [67]. The most obvious effects of the gut microbiome over its host are those related to diet and weight. The diet of an individual directly affects its gut microbiome's composition, and in doing so, the ability to digest certain nutrients may be hindered or gained [68–70]. This disruption may cause some instability in the normal functioning of the intestine, which may lead to long term effects, such as obesity, and more instantaneous effects, such as nausea and vomiting, the latter being a common occurrence in intercultural tourists [68 71]. In addition to these effects, the gut microbiome also greatly affects the immune system by stimulating its development and modulating certain immune pathways [55 72]. The perturbation of the normal functioning of the gut microbiome is associated with dysregulation of the immune system, higher susceptibility to disease and may lead to autoimmune diseases [55]. Prebiotics and probiotics both have similar effects on the gut microbiome, by allowing certain bacteria species to more easily grow or by introducing beneficial bacteria directly in the system [73 74]. On the other hand, even though they are used to fight off prejudicial bacteria, antibiotics cause deeper disruptions by killing symbiotic bacteria, altering the bacterial makeup of the gut and allowing the proliferation of other opportunistic bacteria, especially when administering wide spectrum antibiotics [75 76].

The bacterial composition of the human gut microbiome can be in one of three stable states, which are neither nation nor continent specific. These states, referred to as enterotypes, designate the group of bacterial genera which co-exist in equilibrium in the human gut, and are usually driven by one bacterial genus [77]. Different enterotypes foresee different reactions of the individual to diet and antibiotics, and can be used to predict the existence of numerous disorders such as diabetes or colon cancer [77]. An enterotype can also be disrupted by dietary changes, antibiotic administration, probiotics, prebiotics and other factors, but will always tend to recover if these abnormal situations are not too prolonged or drastic. Enterotypes are not a product of body weight, age or gender, but are instead driven by species composition, more specifically by the relative abundance of *Bacteroides*, *Prevotella* and *Ruminococcus* bacteria genera.

2.5 Immune System

The human immune system consists of several tissue groups, organ systems and specialized defence cells, which work together to protect the organism against foreign invaders and malfunctioning cells. The immune system can be divided in two separate but interconnected immune systems: The innate and the adaptive immune systems [78].

The innate immune system works as a non-specific and non-adaptive first line of defence against outside pathogens by physically preventing access to the inside of the body or quickly eliminating those threats before they can cause perceptible damage if they manage to surpass the initial physical barriers [78]. Epithelial surfaces make up the very first line of innate immune defences [78], the skin being the main physical barrier present in the human body, while any cavity connecting to the outside world - mouth, nose, anus, etc. - is lined with mucous membranes that incorporate antimicrobial proteins and other properties which help fight off potential infections. Other bodily secretions include acid from the skin, saliva, lacrimal fluid, stomach and vaginal secretions and mucus of the respiratory and digestive passageways. Inside the body, the internal innate immune system is composed of antimicrobial proteins

and, more predominantly, phagocytes, which indiscriminately and non-specifically ingest pathogens and foreign molecules [78]. The phagocytes are further divided in to different types of cells, each one with different characteristics, but all aiming at identifying foreign intruders: Macrophages are long-lived and free to migrate from the bloodstream to the tissue to better fight off infections and can trigger inflammation of infected sites [78]; Neutrophils ingest pathogens and die shortly after, creating pus in the process [78]; Natural killer cells roam the body identifying and killing abnormal cells, including the body's own infected or cancerous cells [78]. Macrophages can trigger inflammation if the physical barriers are breached, by dispersing cytokines and chemokines [78]. This reaction is caused locally due to this breach and causes vasodilation, increased temperature and metabolic rate of the local cells. When an infecting agent overruns the fighting phagocytes, a fever might be triggered, causing the whole body to react to a specific infection [78].

The adaptive immune system, on the other hand, targets specific intruders and the host's own infected or damaged cells while also keeping a record of previous infections [78]. The cells of the adaptive immune system must be specifically introduced to pathogens before it attacks. This is the foundation of vaccination, which relies on the adaptive immune system to recognize attenuated or dead versions of a specific pathogen to build up a resistance [79]. The adaptive immune system functions alongside the innate immune system while also regulating some aspects of it [78]. The adaptive immune system is mainly comprised of B lymphocytes and T lymphocytes which are, respectively, involved in the humoral immune responses and the cell-mediated immune responses [78].

The humoral immune response is based on antibodies and is performed by B lymphocytes, which roam the body in search of antigens. These lymphocytes possess two important characteristics: Immunocompetence, the ability to recognize and bind to specific antigens, and self-tolerance, the ability to recognize and not attack the body's own healthy cells [78]. These two characteristics are achieved by the existence of thousands of different antibodies bound to the lymphocyte's membrane outer-surface. These antibodies differ between individual lymphocytes, which allows for the identification of a vast number of foreign molecules and pathogens. When an antigen binds to an antibody on the lymphocyte's surface, it waits for a helper T cell to analyse the antigen [78]. After the antigen is analysed and identified as a threat by the helper T cell, the lymphocyte starts multiplying and, in the process, transforms into either effector cells or memory cells. The memory cells keep a record of the identified antigen while the effector cells produce large quantities of the same antibody, which effectively marks the infecting agent for destruction. These antibodies, besides marking the pathogen, can prevent it from binding to other cells and, in higher numbers, can cause the agglutination of several pathogen cells, which facilitates its ingestion by macrophages [78].

The cell-mediated immune response targets cells specifically, be it infected or cancerous cells. It is performed by T cells, which can be divided in to four main cell types: naïve T cells, cytotoxic T cells (CD8+ cells), helper T cells (CD4+ cells) and memory T cells [78]. Naïve T cells, also referred to as precursor cells, are lymphocytes that have not yet identified foreign particles or pathogens, and therefore, have not yet transformed into one of the other types of cells. CD8+ cells, cytotoxic T cells, killer cells or, more commonly, effector cells, effectively eliminate the identified threat, be it foreign entities or the body's own cells. CD4+ cells, helper T cells, regulate the overall innate and adaptive immune responses by analysing the antigens identified by B lymphocytes and other T cells and determining whether an immune response is necessary. Memory T cells are lymphocytes which preserve antigens of previously identified threats, so that responses to future infections can be more easily and quicker to trigger. For a cell-mediated immune response to occur a naïve T cell must first identify a Major Histocompatibility

Complex (MHC) receptor on a cell to be defective. For a MHC to be defective something must be affecting the cell, be it cancer, a viral infection or a bacterial infection. When this happens the naïve T cell begins multiplying and transforms into both cytotoxic T cells and memory cells [78]. Helper T cells recognize this activity and start producing cytokines, which signal other T cells to multiply. The cytotoxic T cells induce apoptosis of the affected cells while memory T cells, once again, retain antigens of the identified pathogen so that, in a future infection, the immune response can be faster and more aggressive at clearing the infection [78].

2.6 Self-limited Bacterial Infection

Most human bacterial infections are self-limited due to the effects and performance of the human innate and adaptive immune systems [29] [80]. The application of antibiotics is usually used as a way to reduce the magnitude and duration of an infection in individuals with healthy immune systems, which means its purpose is not to completely eliminate an infection but instead to help the immune system do it [80]. Immunosuppressed individuals are more susceptible to acute infections because they have to rely solely on manmade antibiotics to completely clear the infections.

2.7 Chemostat

A chemostat is an apparatus which allows a bacterial population to keep growing while staying within a stable concentration range [81–83]. This is achieved by having a constant flow of nutrients into the bacterial suspension container and an equal constant flow of suspension out of the container, keeping the bacterial suspension homogenous and at a constant volume. The nutrient mixture is composed of all the necessary growth factors for the specific bacteria population. By modelling the chemostat it is possible to simulate a stable bacteria population in an environment like that of the human gut. The generic chemostat model is defined by the following two equations [82]:

Bacteria density (N) (Equation 2.1)

$$\frac{dN}{dt} = \psi \cdot N \cdot \frac{C}{Q + C} - \omega \cdot N$$

Nutrient density (C) (Equation 2.2)

$$\frac{dC}{dT} = -\varepsilon \cdot \psi \cdot N \cdot \frac{C}{Q + C} + \omega \cdot C_0 - \omega \cdot C$$

Bacterial density and nutrient density in the chemostat at any given time are represented by equation 2.1 and equation 2.2, respectively. Bacteria growth rate is represented by ψ, multiplied by $\frac{C}{Q+C}$, which is the Monod equation, composed by the nutrient density C and the half saturation constant Q. This models the growth of microorganisms in aqueous environments with a limiting nutrient, where ω is the flow rate of nutrients and ε is the nutrient quantity necessary for bacteria duplication.

2.8 Existing Software Solutions

Software similar to *SimulATe* already exists, although not completely customizable in terms of parameters and, usually, not having the same goals in mind [84–88]. All the software we analysed had one feature in common: all could be used to simulate the effects of antibiotic resistance, in some way. Some were even aimed at being used to teach the concept of antibiotic resistance, but none of those allowed for a great customization of the simulation.

The most complex antibiotic resistance simulation we discovered was *ARES*, Antibiotic Resistance Evolution Simulator [89], which allows for the simulation of individual cell compartments and all the interactions between them, be it nutrients absorption, antibiotic effects, plasmid exchange and more. All elements of the simulation interact and evolve according to a set of predefined rules set by the user. While this allows for a great control over the simulation, it also imposes some hurdles to the more casual user, by requiring the setup of a cell's internal structure. It is also not a real time simulation, so the user can't follow the simulation along, only having access to the results when the simulation finishes running. This software is, therefore, a great tool for scientific work and could be used in conjunction with *SimulATe*, but not for the teaching of antibiotic resistance to students.

Nowadays there are many video games with an antibiotic resistance related theme that can be effective teaching tools [84–86]. They are more engaging to students and can be very useful if used in certain teaching scenarios, although very limited in scope and parameter customization. This prevents students from simulating distinct scenarios and testing their hypotheses regarding the expected outcomes of these simulations.

Other unpublished software exists, written in scripting languages, which have limited scope and functionality but still aim at simulating antibiotic resistance in some form: *10-day Stochastic Simulation of E. coli Antibiotic Resistance* [87] and *Antibiotic Resistance Simulation* [88].

3 Materials and Methods

SimulATe was completely written using the *Python* 2.7.13 [90] programming language and was developed primarily on a laptop running Windows 10 version 1607 through 1703 with a dual core processor and 8GB of RAM, while testing and debugging were mainly carried out on a machine running both Windows 7 SP1 and Ubuntu 16.04 with a quad core processor and 8GB of RAM. A MacBook, running MacOS Sierra with a dual core processor and 8GB of RAM, was used once to test and debug the program on MacOS systems. To help with code development, testing and debugging, we used *PyCharm Community Edition Integrated Development Environment* (IDE) version 2016.3 through 2017.1 [91].

3.1 Python Programming Language

The *Python* programming language is a free and open source general-purpose programming language with a considerably large standard library. *Python* functionality can be further extended by using third party modules. *SimulATe* is dependent on one such third party module used in the design of the user interface, *Kivy* version 1.10.0 [92], which is the backbone of the program. Another module was used during development to package *SimulATe* into a runnable executable on Windows systems, named *PyInstaller* version 3.2.1 [93].

3.2 Graphical User Interface Libraries

Three graphical user interface libraries were deemed as suitable to be used in the development of *SimulATe*:

- *Kivy Framework* [92]
- *Pygame* [94]
- *Tkinter* [95]

The *Kivy Framework* is a cross platform python library used for the development of application graphical user interfaces. It allows the creation of a user interface composed of different containers called *layouts*. These layouts can in turn contain other layouts and various general user interface elements, called widgets, such as buttons, sliders and text fields. Every element is a python class and can, therefore, be extended to have any functionality the developer desires. Furthermore, *Kivy* has its own layout design language which simplifies the application layout design development by streamlining the process of implementing the classes mentioned above.

The *Pygame* library is aimed at game development, and although it is not specifically designed for the implementation of user interfaces, it could be used to do so, albeit requiring a longer development time and much more code due to the lower level application programming interface (API). K*ivy* even depends on *Pygame* for some specific functionality but possesses the added benefits of being specifically designed for user interface development by having a whole collection of pre-defined widgets and behaviours readily available to the developer.

Tkinter is the standard python graphical user interface design package. It is similar to *Kivy* in many ways, although much older, and was set aside mainly because of the old look and feel of the widgets and graphics it provides.

Ultimately, we ended up selecting *Kivy* as the library with which to develop *SimulATe*'s user interface as it includes many built-in widgets and is relatively easy to learn and use. *Kivy*, akin to *Python*, also supports third party extensions. *SimulATe* makes use of one such package, the *graph* package [96], which defines custom widgets designed to display various kinds of plots and graphs generated in real time. *Matplotlib* [97] is a very comprehensive python-plotting library, and was considered as the API for the development of the graph generating capabilities of *SimulATe*, but the *graph* package was selected instead for its simplicity and seamless integration with the *Kivy Framework*.

3.3 Equations

SimulATe is a mathematical based simulation program and, therefore, makes use of several mathematical equations which include bacterial density, immune system dynamics, antibiotic dynamics and nutrient consumption equations. All equations used to define the bacteria-antibiotic-immune system interactions were based on a study by *Erida Gjini* and *Patricia Brito* [26]. Their differential equations were converted in difference equations, which are the discrete-time analogues of differential equations, using the Euler method. This is done as follows: Consider quantity X is governed by $\frac{dX}{dt} = f(X, \dots)$. This is then changed towards $\frac{\Delta X}{\Delta t} = f(X, \dots) => \Delta X = f(X, \dots) \cdot \Delta t$. But $\Delta X = X(t + \Delta t) - X(t)$. Therefore, the difference equation becomes: $X(t + \Delta t) - X(t) = f(X, \dots) \cdot \Delta t$ or $X(t + \Delta t) = X(t) + f(X, \dots) \cdot \Delta t$. The equations are the following:

Bacteria density (B) (Equation 3.1)

$$B(t + \Delta t) = B(t) + (rB(t) - dB(t)I - \delta B(t)\eta(t)A_m(t)) \cdot \Delta t$$

This equation yields a new bacteria density for a given time step, where t is time, Δt is change in time, r is the growth rate of the bacteria, d is the rate at which lymphocytes inhibit the bacteria, $I(t)$ is the number of total immune cells, δ is the rate at which antibiotic inhibits the bacteria, $\eta(t)$ is the rate at which antibiotic is consumed and $A_m(t)$ is the mean antibiotic concentration on the environment.

Naïve precursor cells density (N) (Equation 3.2)

$$N(t + \Delta t) = N(t) + \frac{-\sigma N(t)B(t)}{k + B(t)} \cdot \Delta t$$

This equation yields a new naïve precursor cell density for a given time step, where σ is the maximum proliferation rate of the immune cells and k is the bacteria density at which the immune response grows at half its maximum rate, all other parameters are already defined in Equation 3.1.

Effector cells density (E) (Equation 3.3)

$$E(t + \Delta t) = E(t) + \left((2\sigma N(t) + \sigma E(t)) \frac{B(t)}{k + B(t)} - hE(t)\left(1 - \frac{B(t)}{k + B(t)}\right)\right) \cdot \Delta t$$

This equation yields a new effector cells density (CD8+ cells) for a given time step, where h is the maximum decay rate of effector cells.

Memory cells density (M) (Equation 3.4)

$$M(t+\Delta t) = M(t) + \left(fE(t)h\left(1 - \frac{B(t)}{k + B(t)}\right)\right) \cdot \Delta t$$

This equation yields new memory cells density for a given time step, where f is the fraction of effector cells which convert to memory cells.

Antibiotic uptake (η) (Equation 3.5)

$$\eta(t) = \begin{cases} 1 \; if \; t_1 <= t <= t_1 + t_2 \\ 0 \; if \; t < t_1 \; or \; t > t_1 + t_2 \end{cases}$$

For the classic treatment case, where t_1 is the start of antibiotic treatment and t_2 is the treatment duration, or

Antibiotic uptake (η) (Equation 3.6)

$$\eta(t) = \begin{cases} 1 \; if \; B(t) \geq \Omega \\ 0 \; if \; B(t) < \Omega \end{cases}$$

for the adaptive treatment case, where Ω is the defined bacteria density threshold. Both these equations yield the state of the antibiotic administration at each time step as a Boolean value, either 0 (antibiotic is being administered) or 1 (no antibiotic is being administered).

Equations pertaining to each microbiome's individual genus growth dynamics were based on the chemostat equations (equation 2.1 and equation 2.2), and were used as a way to simulate all the different factors that are present when a bacteria population grows in the human gut, be it the interaction with other bacteria, the flow of nutrients through the gut, the effect of the immune system, the natural growth rate of the bacteria and more. These were defined as follows:

Nutrient concentration (C) (Equation 3.7)

$$C(t+\Delta t) = C(t) + \left(\left(\omega C_0 - \varepsilon r B(t) \cdot \frac{C(t)}{Q + C(t)} \right) - \omega C(t) \right) \cdot \Delta t$$

This equation yields a new nutrient concentration for a given time step, where ω is the rate of nutrient flow through the system, C_0 is the initial nutrient concentration and Q is the half saturation constant, which allows $\frac{C}{Q+C}$ (Monod equation) to be equal to $\frac{1}{2}$ when $Q = C$.

Microbiome bacteria density (B_m) (Equation 3.8)

$$B_m(t+\Delta t) = B_m(t) + \left(rB_m(t) \cdot \frac{C(t)}{Q + C(t)} - \omega B_m(t) - \; dB_m(t)I(t) \; - \; \delta B_m(t)\eta(t)A_m(t) \right) \cdot \Delta t$$

This equation is a modified version of the bacteria density equation (Equation 3.1) which yields the density of a bacteria belonging to a microbiome, differing on the implementation of the nutrient consumption and nutrient availability.

Nutrient quantity necessary for bacteria duplication (ε) (Equation 3.9)

$$\varepsilon = \frac{C_0(r-\omega) - Q\omega}{B_s(\psi - \omega)}$$

This equation yields the nutrient quantity necessary for a bacterium to duplicate, where B_s is the density at which a bacteria population is stable in the microbiome, where there is no immune system.

All parameters and default values used and obtained by the preceding equations are described in the table below in brief:

Table 3.1 Parameters and default values.
Short description, default value, range and unit of every parameter used in the preceding equations.

Symbol	Description	Default Value	Range	Unit
B(0)	Initial antibiotic sensitive bacterial density (B_s)	10	1 - 100	cell/µl
	Initial antibiotic resistant bacterial density (B_r)	2		
N(0)	Initial naïve precursor cells density	200	0 - 1500	cell/µl
E(0)	Initial effector cells density	0	fixed	cell/µl
M(0)	Initial memory cells density	0	fixed	cell/µl
η(0)	Initial antibiotic uptake	0	0 or 1	-
C(0)	Initial nutrient concentration	100	fixed	mg/l
ε	Nutrient quantity necessary for bacteria duplication	varied	0 - ∞	µg
$B_m(0)$	Initial microbiome bacteria density	varied	varied	cell/µl
r	Antibiotic sensitive bacteria growth rate (r_s)	3.3	0.1 – 8.0	day^{-1}
	Antibiotic resistant bacteria growth rate (r_r)	1.1	0.1 - r_s	
d	Bacteria lymphocyte inhibition	10^{-5}	10^{-5} - 10^{-4}	µl/cell/day
I	Number of total immune cells	varied	0 - ∞	cell/µl
δ	Antibiotic sensitive bacteria antibiotic inhibition (δ_s)	1	0 - 1	l/mg/day
	Antibiotic resistant bacteria antibiotic inhibition (δ_r)	0.1	0 - d_s	l/mg/day
A_m	Antibiotic mean concentration	6	1 - 120	mg/l
σ	Immune cells' maximum proliferation rate	2	1.2 – 3.0	day^{-1}
k	Bacteria density at which the immune response grows at half its maximum rate	10^5	10^4 - 10^5	cell/µl
h	Effector cells' maximum decay rate	0.35	0.1 – 0.8	day^{-1}
f	Fraction of effector cells which convert to memory cells	0.1	0.05 – 0.10	-
t_1	Start of antibiotic treatment	3.5	1 - 15	day
t_2	Treatment duration	7	3 - 15	day
Ω	Bacteria density threshold	10^6	10^3 - 10^7	cell/µl
ω	Rate of nutrient flow	0.1	fixed	day^{-1}
Q	Half saturation constant	5	fixed	-
B_s	Stable bacteria density in the microbiome	varied	0 - ∞	cell/µl

Most parameters preserve their default values and ranges from the original source study [26], but some were changed to allow for more realistic or broad ranged simulation scenarios. The initial naïve precursor cells density range was the changed from 15-1500 to 0-1500 to allow the exclusion of the immune system from the simulation, this way scenarios without the effects of the immune system can be simulated, including non-human *in vitro* cultures or conditions. The range of the antibiotic mean concentration was also changed, from 0.03-128 to 1-120, to allow for better selection of a value in the user interface by removing the decimal values.

3.4 Bacteria Dataset

We asked the authors of the *Enterotypes of the human gut microbiome* paper [77] for the dataset generated in the study with the objective of obtaining the relative frequencies of the bacteria available on the average human gut microbiome, and they kindly obliged. We filtered the available Sanger sequence data for the top ten most abundant bacteria genera available for each enterotype, excluding unidentified genera. We then calculated the relative frequency of each of the top ten genera. We obtained the bacteria genera depicted in the following table:

Table 3.2 Most abundant bacteria genera per enterotype.
Top ten most abundant bacteria genera per enterotype. Relative frequency calculated from the raw Sanger sequence dataset generated for the study 'Enterotypes of the human gut microbiome' [77], as kindly provided by its main author Arumugam M.

Enterotype 1		**Enterotype 2**		**Enterotype 3**	
Genus	**Relative Frequency**	**Genus**	**Relative Frequency**	**Genus**	**Relative Frequency**
Bacteroides	0.579311132	*Prevotella*	0.514100163	*Bacteroides*	0.25850528
Faecalibacterium	0.116691419	*Bacteroides*	0.159122669	*Bifidobacterium*	0.157105162
Roseburia	0.083290084	*Faecalibacterium*	0.077149738	*Faecalibacterium*	0.144911601
Bifidobacterium	0.062349593	*Lachnospiraceae*	0.07068057	*Lachnospiraceae*	0.091303254
Lachnospiraceae	0.046407791	*Roseburia*	0.046132131	*Alistipes*	0.090955237
Parabacteroides	0.032297551	*Collinsella*	0.038162789	*Akkermansia*	0.058831694
Alistipes	0.022191181	*Bifidobacterium*	0.025532778	*Ruminococcus*	0.055381611
Anaerostipes	0.020859591	*Alistipes*	0.024677992	*Collinsella*	0.050654524
Acidaminococcus	0.020827165	*Streptococcus*	0.023139472	*Blautia*	0.045579416
Collinsella	0.015774493	*Coprococcus*	0.021301699	*Roseburia*	0.046772221

4 Implementation

SimulATe contains in its graphical user interface a *parameters and options configuration* section, a *graph* section and a *flow control* section. The *parameters and options configuration* section allows the user to set a variety of parameters and options, mostly directly associated with the previously described equations, the *graph* section is where the simulations run in real time and the *flow control* section is a set of widgets that allows the user to start/pause/restart and control de speed of the simulation. This layout was implemented by making use of the *Kivy Framework* while the backend was written as a combination of python classes and standalone functions.

4.1 Program Layout

When running *SimulATe*, an initial screen is loaded which allows the user to change between the two available simulation scenarios - *Single Population* or *Microbiome* - by clicking the *Scenario* button; change between display languages - English or Portuguese - by clicking the *Language* button; or save useful data regarding the current simulation such as plot points, the options used and the current graph as an image, by clicking the *Save* button. Simulation flow control buttons are also displayed at the bottom of the window but are disabled until a simulation scenario is selected (Figure 4.1).

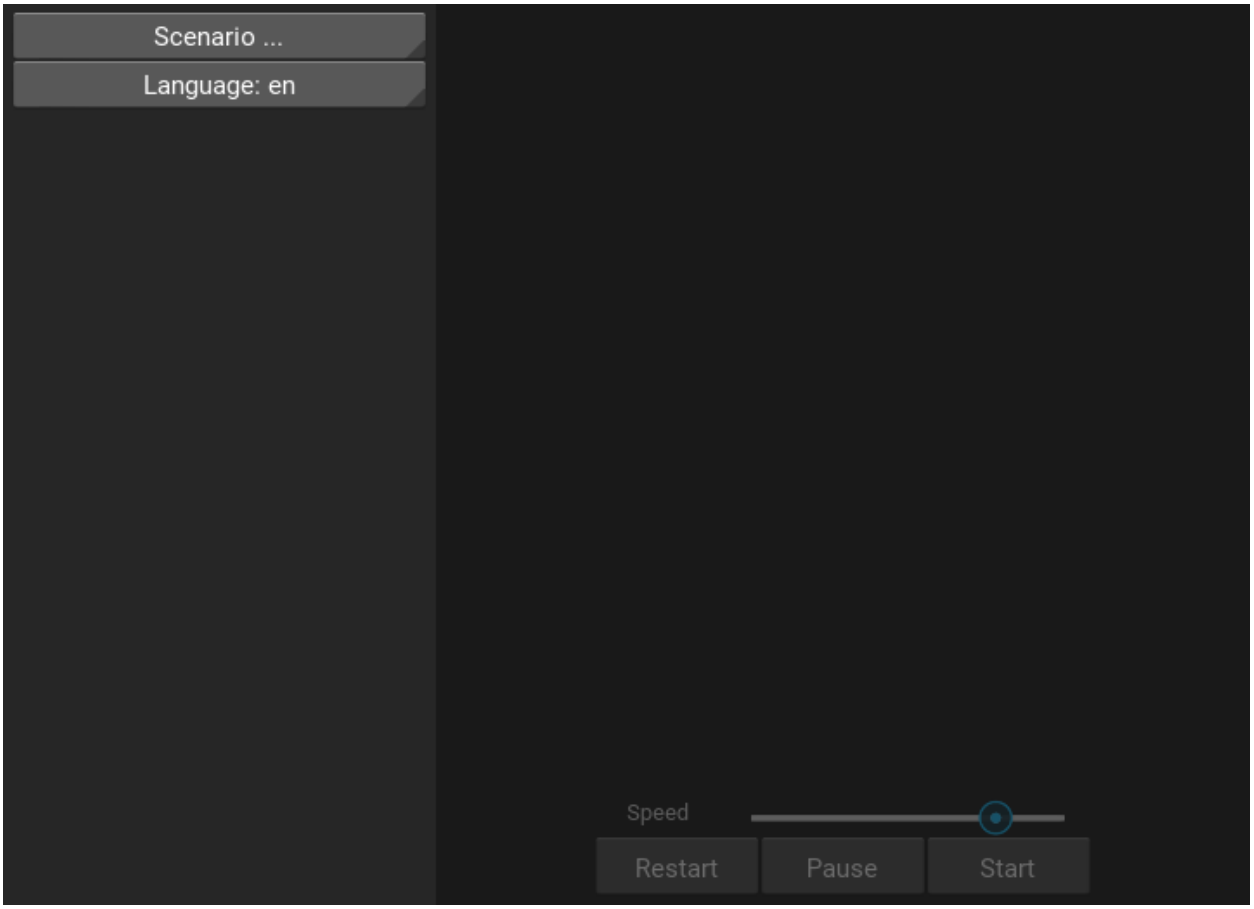

Figure 4.1 Initial program screen.
First screen that loads when SimulATe is executed, containing the scenario selection and the language selection buttons alongside the disabled flow control buttons.

From this point forward, the user will be presented with one of two relatively similar user interfaces, depending on the selected simulation scenario, which are both divided into 3 main sections: *Parameters and Options*, *Graphs* and *Simulation Flow Control*.

The Parameters and Options panel is located on the left-hand side of the user interface. It is a scrollable panel as there are a lot of parameters and options available in both scenarios, thus we determined that being able to scroll through the panel was the best approach to take which would allow us to preserve readability and graphical user interface space while still being able to show every parameter and option. Most of the differences between the layout of both scenarios occur on the parameters and options panel, which are further described on chapters 4.1.1 and 4.1.2.

The Graph sections of both scenarios are mostly identical. Located on the right-hand side of the user interface, this section is where the simulation output is represented in graph form (Figure 4.2).

a) b)

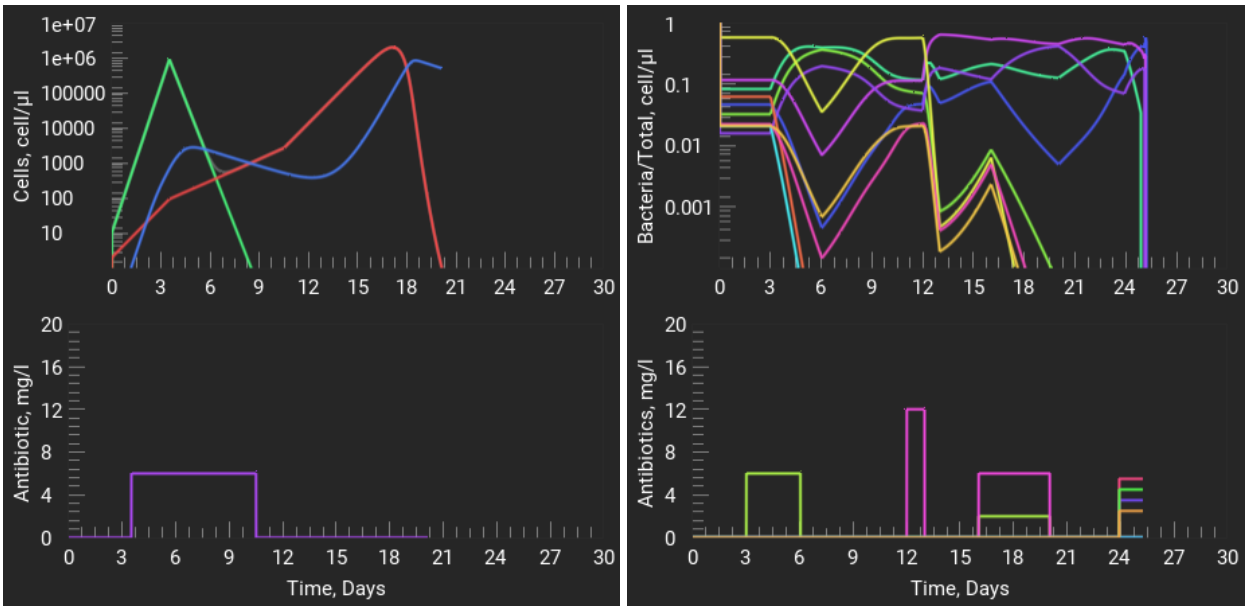

Figure 4.2 Graph section of both scenarios.
The graph section, in scenarios a) and b), consists of two graphs: A top graph representing densities of resistant and sensitive bacterial cells as well as immune system cells, in the single population scenario (a), and relative frequency of each bacteria in the microbiome scenario (b), both plotted in a linear x-axis and a logarithmic y-axis. The bottom graph, in scenarios a) and b), represents antibiotic concentration and is plotted in a linear x- and y-axis. The x-axis represents time for both graphs. The example plot a) is the result of running the simulation with default parameters and the classic treatment type, while plot b) is the result of running the simulation for the Gut Enterotype 1 with default antibiotic resistance values and the arbitrary administration of antibiotics through the course of a 25-day period until complete intestinal dysbiosis.

Two graphs are always present in each scenario: A top graph which represents cell densities on the single population scenario and the relative frequency of each bacteria on the microbiome scenario, while the bottom graph represents the antibiotic concentration for both scenarios. On the top graph, up to 4 plots can occur during a single population scenario simulation: both antibiotic resistant and sensitive bacteria plots, a plot representing the total bacteria density and the immune system plot. On a microbiome scenario simulation, up to ten plots can occur, one for each bacteria genus of the selected enterotype. The bottom graph always shows just the antibiotic concentration plot, although on the microbiome scenario up to 8 antibiotic concentration plots can be active at the same time. All these plots have predefined colours, each corresponding to a certain group of parameters in the parameters and options panel (refer to Figure 4.3 and Figure 4.4). All axes expand automatically when a plot reaches the limits of the graph, allowing the plots to be completely visible at any time. The x-axis of both the top and bottom graphs are synchronized and will expand equally as the simulation progresses. The y-axis of the top graph is represented in a logarithmic scale while the bottom y-axis is represented on a linear scale. The top graph, in the single population scenario, also shows a white line representing the *host death density* parameter whenever the y-axis expands enough to be able to show the defined value.

The flow control section of the program, as stated before, is located at the bottom of the user interface. This section is comprised of a Start, Pause and Restart button, which execute the expected operations of starting the simulation with the currently defined parameters and options, pausing/continue the simulation, and restarting the simulation, i.e., preparing the simulation for a new run. A slider is also

available which allows the speed of the simulation to be changed. The flow control buttons are exactly the same in both scenarios and have the same exact functionality.

4.1.1 Parameters and Options: Single Population Scenario

The parameters and options panel for the single population scenario is depicted in Figure 4.3. This panel allows the user to configure all parameters as well as several other available options, most of which are directly tied to parameters from the equations described in chapter 3.3, through the use of sliders. At the top of the panel the options available on the initial user interface screen are still visible, allowing the user to change scenario, language or save simulation data. Below these options are five groups of parameters, each group with an assigned colour which directly corresponds to a plot on the graph, doubling as a plot legend. All parameters are predefined to a default value as featured on Table 3.1. The first group corresponds to parameters related to antibiotic sensitive bacteria, represented by the colour green. Its parameters, *Initial Density*, *Growth Rate* and *Antibiotic Inhibition*, which correspond to the initial bacteria density (B_s), its growth rate (r_s) and the inhibition caused by antibiotics (d_s) respectively, are identical to the next group of parameters, which are related to antibiotic resistant bacteria (B_r, r_r and d_r), represented by the colour red, albeit different initial default values and upper limits of some parameters. The third group of parameters is a special group as it does not define a real entity in the program. It is a group which contains two parameters shared by both antibiotic sensitive and antibiotic resistant bacteria. Those parameters are *Lymphocyte Inhibition* and *Host Death Density*, the first being the non-specific immune system inhibition of the bacteria population (d) and the second represents an arbitrary bacteria density threshold that causes the death of the host, not directly related to any equation parameter. This group is represented by the colour grey and corresponds to a plot on the graph that allows the user to check the total bacteria density - antibiotic sensitive plus antibiotic resistant bacteria - at a glance. The fourth group of parameters corresponds to the immune system and is represented by the colour blue. It is comprised of 5 parameters which govern the dynamics of the immune system. The first parameter, *Initial Precursor Cell Density*, allows the user to set the initial density of the immune system (N) and disable the immune system by setting a value of 0, which is a method of simulating the absence of an immune system in non-animal environments. The following parameter, *Proliferation Rate* is the rate at which the precursor cells transform into effector cells (σ). The *Half Maximum Growth* parameter represents the bacteria density at which the immune response grows at half its maximum rate (k), i.e. the pathogen density at which the proliferation of precursor immune cells into effector cells is half of its maximum. *The Effector Cells Decay Rate* is the rate at which effector cells die (h) and, lastly, *Memory Cells Conversion* represent the fraction of effector cells that convert into memory cells per day (f). The fifth and last group of parameters corresponds to the antibiotic and is represented by the colour blue. It consists of the parameter *Mean Concentration* which represents the average antibiotic concentration during treatment in milligrams per litre (A_m), and the *Treatment Type* toggle buttons, which allow the user to select the type of treatment to be applied (η). There are three treatment types available, the *Classic* and *Adaptive*, as defined in the study by *Erida Gjini* and *Patricia Brito* [26], and a the *User* treatment type, defined by us. When selecting a treatment type, new related parameters become available as can be seen on the right-hand side of Figure 4.3. *Delay* and *Duration* are the two parameters available in the *Classic* treatment type, which represent both the number of days between the start of the infection and the beginning of treatment (t_1), and the number of days the patient is under antibiotic treatment (t_2), after the

initial delay. In the *Adaptive* treatment type there is only one parameter, *Symptoms at Density*, which represents the threshold of bacteria density at which symptoms occur and antibiotic is applied to the system (Ω). The *User* treatment type allows the user to administer antibiotic at will, by pressing a single ON/OFF button. At last, at the bottom of the panel is a button named *Default Values* which allows the user to reset every parameter to its initial default value.

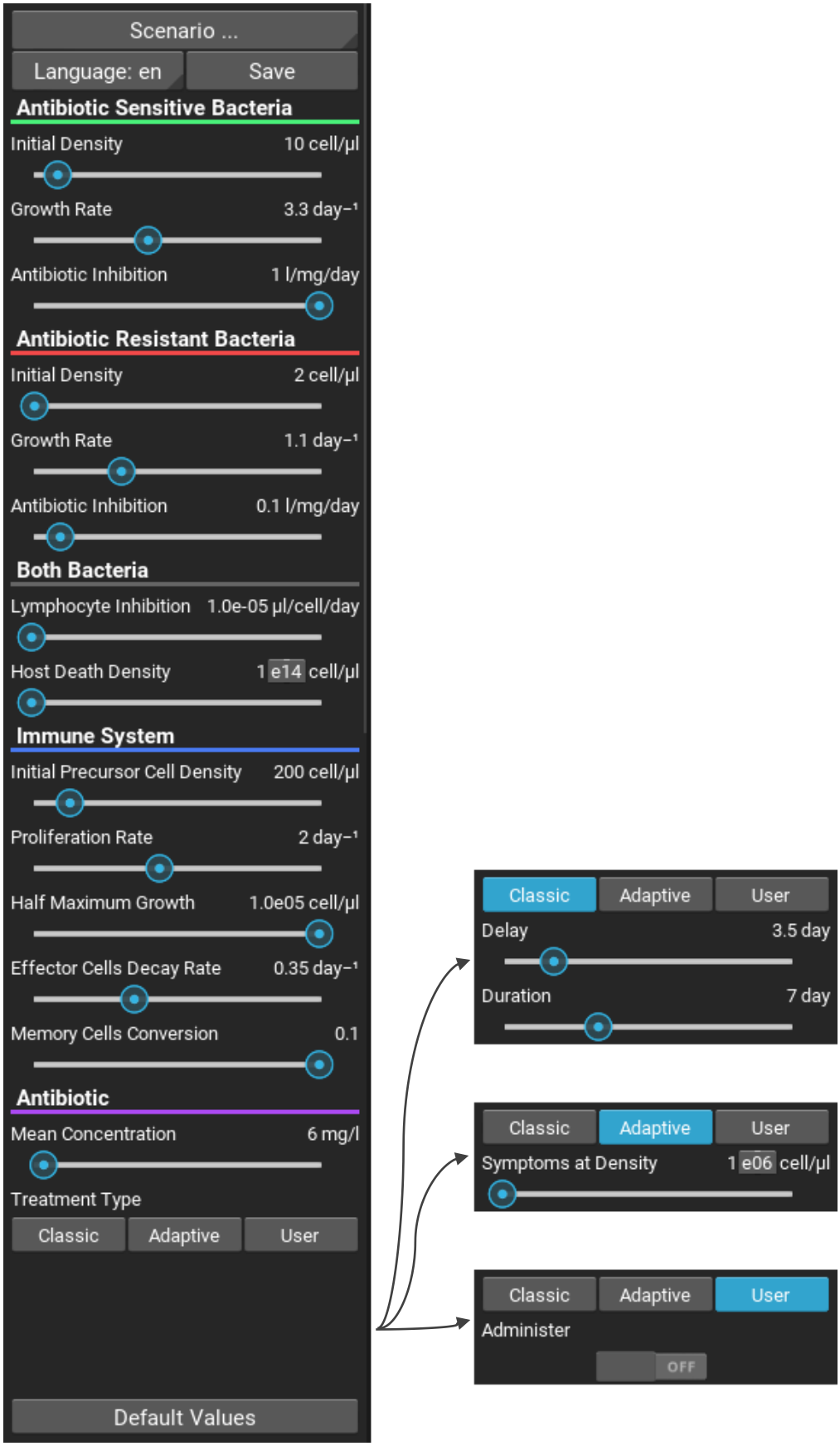
Scenario ...
Language: en
Save
Antibiotic Sensitive Bacteria
Initial Density 10 cell/µl
Growth Rate 3.3 day⁻¹
Antibiotic Inhibition 1 l/mg/day
Antibiotic Resistant Bacteria
Initial Density 2 cell/µl
Growth Rate 1.1 day⁻¹
Antibiotic Inhibition 0.1 l/mg/day
Both Bacteria
Lymphocyte Inhibition 1.0e-05 µl/cell/day
Host Death Density 1 e14 cell/µl
Immune System
Initial Precursor Cell Density 200 cell/µl
Proliferation Rate 2 day⁻¹
Half Maximum Growth 1.0e05 cell/µl
Effector Cells Decay Rate 0.35 day⁻¹
Memory Cells Conversion 0.1
Antibiotic
Mean Concentration 6 mg/l
Treatment Type
Classic Adaptive User
Default Values
Classic Adaptive User
Delay 3.5 day
Duration 7 day
Classic Adaptive User
Symptoms at Density 1 e06 cell/µl
Classic Adaptive User
Administer
OFF

Figure 4.3 Parameters and Options panel of the Single Population scenario.
The parameters panel for the single population scenario is organized in sections. The first section at the top is composed of buttons and drop-down menus akin to the File menu in most computer programs. The following sections contain all the available parameters with which to configure the simulation, each section represented by a colour which corresponds to a single plot line in the graph. The Antibiotic section presents different options depending on the type of treatment selected, shown on the right. At the bottom of the panel is a button named "Default Values" which allows the user to reset every parameter to its default initial value.

4.1.2 Parameters and Options: Microbiome Scenario

The parameters and options section for the microbiome scenario is similar to the single population scenario as it is divided in groups, albeit different parameters and options (Figure 4.4). Below the menu buttons at the top of the panel is the gut enterotype selection button which allows the user to select one of the three available human gut enterotypes. This is the only available button at first and all the remaining parameters and options, because they are related to a specific enterotype, only make themselves available after an enterotype is selected. After selecting an enterotype, more parameters and options become available below the enterotype selection button, starting with the *Antibiotic Inhibition* group of parameters which includes ten buttons, one for each bacteria genus of the current selected enterotype. Each of these buttons has an associated colour which relates to the plot on the graph and, when pressed, spawns a new set of sliders which allow the user to set the individual antibiotic resistance for each antibiotic. Each slider has a predefined value by default which was randomly generated. No value representative of reality was used here because these values could change drastically between individual humans. However, these can be updated with values better resembling reality in case an antibiogram is generated for one of the available bacteria, for example, in a personalized medicine treatment approach. At the bottom of the set of antibiotic resistance sliders is a button which resets the values back to their original default values. The next group of parameters, named *Antibiotic Concentrations*, allow the user to set each antibiotic concentration independently of the other antibiotics by using its respective slider and administer each antibiotic individually by using its respective ON/OFF button. Each of these antibiotics has an associated colour representative of it respective plot in the graph and will affect each bacteria genus differently depending on the set antibiotic resistance value. At the end of the panel are two buttons, which allow the user to reset both the antibiotic concentration and the administering status of all antibiotics simultaneously.


Scenario ...
Language: en
Save
Microbiome
Gut Enterotype 1
Antibiotic Inhibition
Bacteroides
Faecalibacterium
Roseburia
Bifidobacterium
Lachnospiraceae
Parabacteroides
Alistipes
Anaerostipes
Acidaminococcus
Collinsella
Antibiotic Concentrations
Lincosamides OFF 6 mg/l
Macrolides OFF 6 mg/l
Penicillins OFF 6 mg/l
Quinolones OFF 6 mg/l
Streptogramins OFF 6 mg/l
Sulfonamides OFF 6 mg/l
Tetacyclines OFF 6 mg/l
Trimethoprims OFF 6 mg/l
All Antibiotics:
Stop Administration
Default Concentrations
Gut Enterotype 1
Gut Enterotype 2
Gut Enterotype 3
Lincosamides 0.45 l/mg/day
Macrolides 0.77 l/mg/day
Penicillins 0.71 l/mg/day
Quinolones 0.73 l/mg/day
Streptogramins 0.43 l/mg/day
Sulfonamides 0.80 l/mg/day
Tetacyclines 0.53 l/mg/day
Trimethoprims 0.08 l/mg/day
Default Values

Figure 4.4 Parameters and Options panel of the Microbiome scenario.
The parameters panel for the microbiome scenario is also organized in sections. The first section at the top corresponds to the menu buttons exactly as it appears in the single population scenario. The following sections allows the user to select the active enterotype which, when selected, will load its corresponding options below it. The next section encompasses all ten available bacteria genera for the selected enterotype and allows the user to specify each antibiotic resistance for each genus individually. The last section contains all antibiotics available for use along with a slider to set its concentration and a button to activate each antibiotic. At the bottom of the panel is a set of buttons, "Stop Administration" and "Default Concentrations", which allow the user to reset both the antibiotic concentration and the administering status of all antibiotics at once.

4.2 Code Structure

Code and resource files of *SimulATe* were organized in a folder structure, as pictured on Figure 4.5.

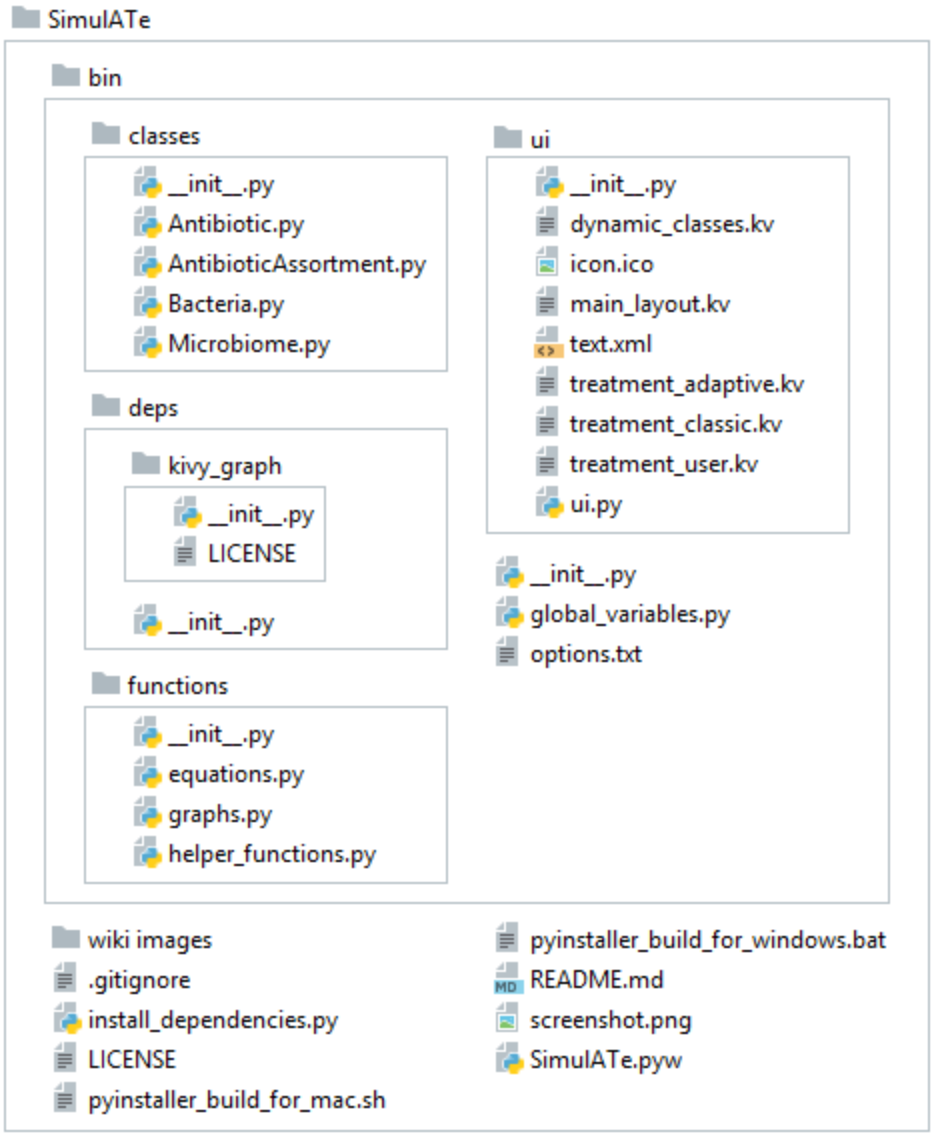

Figure 4.5 Folder structure of SimulATe's code and resource files.
All code, image files and text strings purposely developed for SimulATe are represented here.

The root directory – *SimulATe* folder - has two folders and an assortment of different files: The *.gitignore* file is related to the version control system (VCS) used, git [98]; the *LICENSE* and *README* files are both informative to the user as they describe both the license under which *SimulATe* is released as well as general information on how to install and run the program, the latter is intended to be used as the partial front page of the *GitHub* online repository of the program alongside the *screenshot.png*; both *pyinstaller_build_for_mac.sh* and *pyinstaller_build_for_windows.bat* files are used to build executable versions of *SimulATe* for MacOS and Windows system; *install_dependencies.py* is a python script used to install all the necessary dependencies needed to run *SimulATe*; the *SimulATe.pyw* script is the entry point of the program, i.e., it is the file the user should run to start *SimulATe*; the *wiki images* folder contains a variety of images and screenshots of *SimulATe* which are used exclusively on the wiki of the program, hosted online at the same *GitHub* repository. The remaining *bin* folder is where almost all the code is located.

The *bin* folder contains four folders, two .py files and one .txt file: The *options.txt* file is where *SimulATe* checks for saved configurations, in this case, the last language set by the user; the *__init__.py* is a python file that marks the current folder as a python package and enables python scripts within it to be used inside other python scripts via importing; the *global_variables.py* is the script that initializes most of the custom classes implemented for this program as well as other functions, specifically, it loads the text strings related to the selected language, initializes the necessary bacteria, antibiotics and immune system, initializes some extra plots and appends those to the main graph area. The four folders contained in *bin* are described in the following subchapters.

4.2.1 *classes* folder

This folder contains four classes, two related to bacteria, *Bacteria.py* and *Microbiome.py*, and the other two related to antibiotics, *Antibiotic.py* and *AntibioticAssortment.py*. The *Bacteria.py* and *Antibiotic.py* classes are intended to represent both individual bacteria and antibiotics respectively and are very similar, in that their instances are initialized with a name, a colour and a plot object and both have *get* methods that allow access to most of the initialized properties. *Microbiome.py* and *AntibioticAssortment.py* on the other hand are intended to represent groups of bacteria – microbiomes - and groups of antibiotics, respectively, and are also very similar, as instances of these classes initialize and maintain a group of bacteria or antibiotics respectively, create the graph section where every respective plot will be shown in the main program and initialize functions that detect when to expand the graph axes. Both classes also implement *get* methods for most of the initialized properties.

4.2.2 *deps* folder

This folder contains some third-party code dependencies needed for the graph generation capabilities of *SimulATe*. The *kivy_graph* folder contains the *graph Kivy* module, which implements a widget that can generate a variety of different plots and graphs and. Although most dependencies should already be installed before attempting to run the program, this dependency is explicitly included because the *garden.bat* file used to run the *Kivy* modules installer crashes when trying to launch from directories with

names that include white spaces. This has already been fixed on the project's *GitHub* page, but, as of the time of this writing, it was still not included in the main repository used when downloading *Kivy*, this means that the average user would still download the affected version and thus *SimulATe* might not be able to run. To avoid this problem, we decided to directly include the *graph* module with the *SimulATe*.

4.2.3 *functions* folder

This folder contains three scripts and a *__init__.py* file. The *equations.py* script contains all the equations discussed in chapter 3.3 implemented as python functions which simply return a value calculated from the inputted parameters. It also contains a couple of functions which calculate all the necessary equations simultaneously for a certain time step of a simulation. The *graphs.py* script includes a single function which implements the logic behind the expansion of the axes of the graph when a plot reaches its limits. Lastly, the *helper_functions.py* script implements two classes used in multiple places around the whole code. The first class, *NewColor*, generates non-overlapping colours for all bacteria and antibiotics. This class was implemented initially as way to get new randomly picked colours every time the program was started, but that was deemed as too confusing for users. Now a seed is set in the random generator so that, every time the program initializes, the same colours are generated. The second class, *XMLTextParser*, is a XML parser which analyses and extracts text strings from the language defining XML file. This parser is used at the program start to set the initial language and every time the user changes language.

4.2.4 *ui* folder

This folder contains five .kv files, a python file, a XML file, an icon and a *__init__.py*. The icon is just an image used as the program icon. The *text.xml* file contains all the text strings used by the program in two different languages. The .kv files are kv language files, a file structure parsed by Kivy, which define the program user interface. The *dynamic_classes.kv* defines general classes while the remaining .kv files make use of those classes to define the user interface layout and some function calls and user interface elements interaction logic. Lastly, the *ui.py* script is the main backbone of the program as it implements most of the main user interface classes used, initializes and keeps track of an enormous amounts of variables and defines most of the functions which characterize the interactivity and overall use of the program.

4.3 Hindrances During Development

During the development of *SimulATe* we came across a few issues. The first issue, which happened earlier in development, is related to the model of bacteria growth and interaction used. At first, we pursued the use of an agent based model [99] as the basis of our simulation instead of the mathematical based model that is now part of *SimulATe*. This earlier model consisted of a matrix of squares – agents - which would either be empty or inhabited by different bacteria populations. These bacteria could then grow to the adjacent squares or shrink by abandoning squares. We eventually deemed this approach not suitable for

the kinds of simulations we wanted to perform because we would need to implement a lot more characteristics of the simulation pertaining to the inherent 2D space of the simulation, than when compared to a mathematical based simulation.

Another issue we found was the fact that there was a lack of data regarding the duplication rate of the bacteria we wanted to simulate (refer to Table 3.1). We associated this lack of information to the type of bacteria in question: symbiotic human gut bacteria. Researchers tend to analyse the human microbiome as a whole and not each species or genus individually, which does not provide the individual growth rates for each species or genus. Also, most bacteria in the human gut are symbiotic and non-threatening to the human health, therefore there is not much interest in studying them when compared to other pathogenic bacteria. We tried to overcome this problem by making use of a tool called *Growthpred* [100], which tries to predict growth rates by analysing codon usage bias in the bacterial genomes, but we quickly found out that this tool required a data set of highly expressed genes in order to obtain a growth rate prediction, information which was virtually non-existent for the bacteria we wanted analysed.

4.4 Testing

SimulATe was tested primarily on a Windows machine with subsequent testing done on a Linux and a MacOS machine, as stated in the Materials and Methods chapter. Code testing was performed manually and through the use of the testing and debugging functionality of the chosen IDE, *PyCharm*. The usability of the program was tested by having volunteers use the program for the first time without prior knowledge of its functionalities. Questions, reactions and time lost in trying to understand the layout of the program were taken into account and helped to further develop the program.

5 Results

To overcome the limitations imposed by existing software-based learning and simulation tools related to antibiotic resistance we developed *SimulATe*, a computer program that simulates the effect of antibiotic therapy on bacterial populations and the role of antibiotic resistance on the sustainability of bacterial communities in the human gut. *SimulATe* allows the simulation of: a) bacterial growth under the effect of an antibiotic and the immune system; b) different antibiotic treatment protocols; c) the disruption of the human gut microbiome caused by the administration of antibiotics among those that are more commonly prescribed for human health. *SimulATe* runs these simulations in real time and allows a wide range of parameter configuration. *SimulATe* can be applied in developing the scientific literacy and skills of students and to explore the learning goals defined in the official Portuguese school programs for: 6th grade, Natural Sciences curriculum, when the appropriate use of antibiotics is taught; 9th grade, Natural Sciences, when the concept of antibiotic resistance is introduced and related to the misuse of antibiotics; 11th grade, Biology and Geology, when natural selection and artificial selection is introduced; 12th grade, Biology, when immunity is disease control is taught [34] [35] [101] [102]. We also developed an educational activity that makes use of *SimulATe* aimed at 9th grade students (Appendix B). This activity aims at developing scientific skill, critical thinking and engage students in scientific debates related with the misuse of antibiotics and the increase of antibiotic resistance. It also aims at fostering students' engagement and their active role in finding and implementing solutions to reduce the problem of bacteria resistance.

To better represent the range of situations this program can be used to simulate we performed some exemplifying simulations, described in the following subchapters.

5.1 Usage Cases

All usage cases were simulated using *SimulATe*. The final graphs were modified to include a small plot legend, better alignment of the values on the axes and the removal of the background colour.

5.1.1 Single Population Scenario Usage Cases

The first example, related to the first simulation scenario, represents the dynamics of a human gut infected by a regular pathogenic bacterial population and its interaction with the host's immune system - a self-limited bacterial infection - which corresponds to the default program parameters without any kind of treatment.

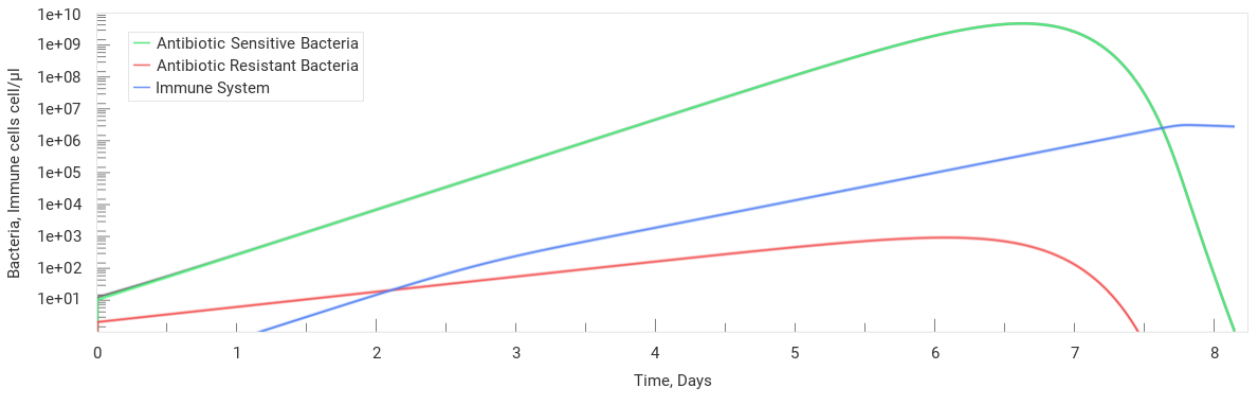

Figure 5.1 Dynamics of the infection by a bacterial population (self-limited bacterial infection).
Simulated dynamics of the infection by a regular bacterial population, consisting of both antibiotic sensitive (green) and antibiotic resistant (red) individuals, and its interaction with the immune system (blue) in a virtual human body environment. Values on the x-axis represent time measured in days since the beginning of the infection and values on the y-axis represent the density of both bacterial and immune system cells, measured in cells/µl. To obtain this graph, parameters in the SimulATe application were set to their default values and no treatment was selected (User Treatment set to OFF).

The infection starts at time 0 and the bacterial population, which includes antibiotic sensitive and antibiotic resistant sub-populations, begins to grow exponentially. Shortly after the first day of infection, the immune response is triggered. Six days after the start of the infection the bacteria reaches its peak density and starts to quickly succumb to the immune system, and at day 8 the infection is eliminated.

The second example compares the effect of a normal antibiotic therapy against the effect of the early termination of that same therapy.

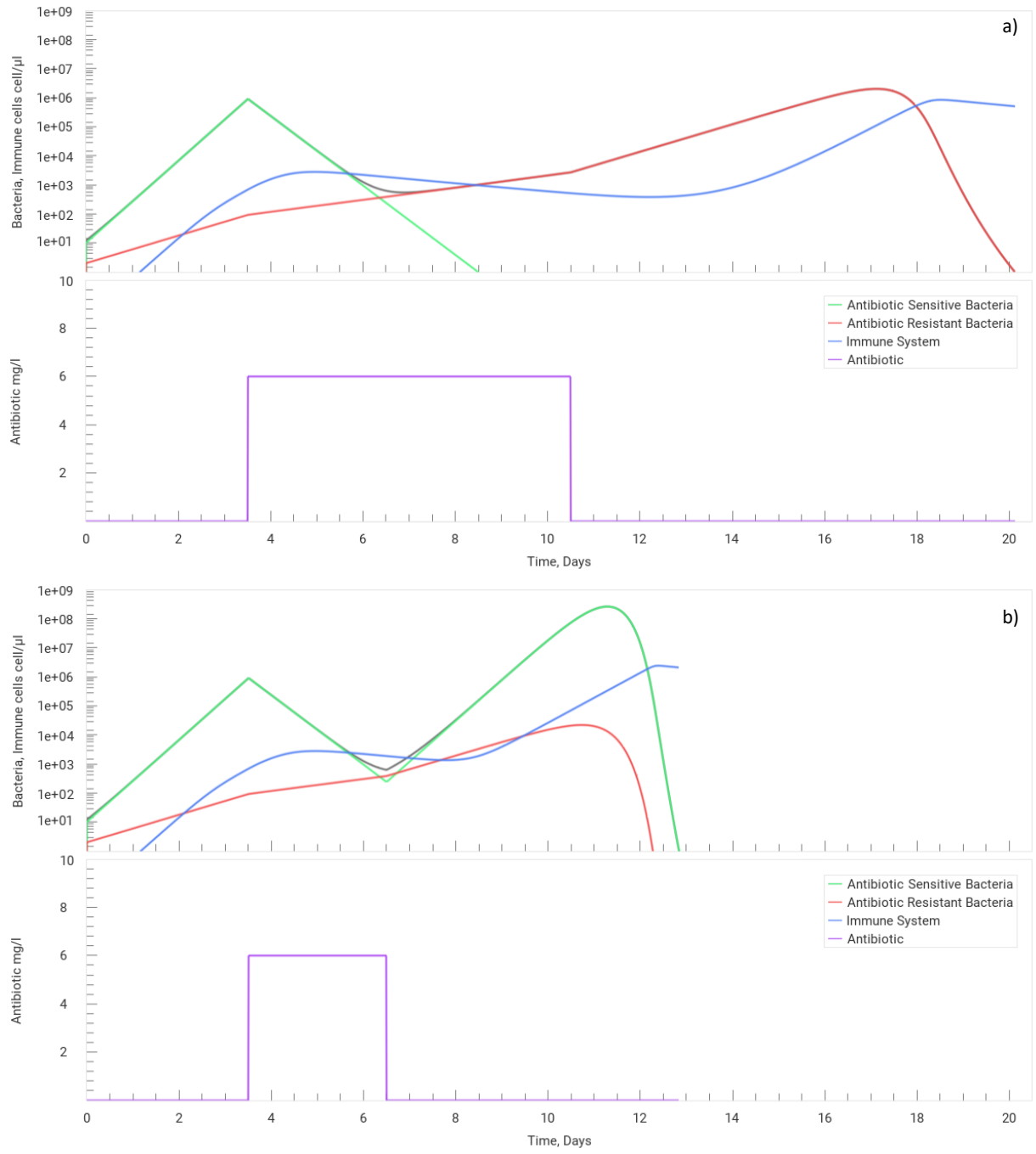

Figure 5.2 The effect of the early termination of antibiotic therapy.
Effects of the administration of an arbitrary antibiotic (purple). The top graph, for both a) and b), represents the same bacterial populations, immune system and axis as in Figure 5.1. The bottom graph's x-axis, for both a) and b), represent time measured in days since the beginning of the infection and values on the y-axis represent concentration of antibiotic in mg/L. In both a) and b) the antibiotic is administered at a concentration of 6 mg/L starting 3.5 days after infection. To obtain these graphs, parameters in the application were set to their default values, the classic treatment was selected, and the duration parameter was set differently for each scenario (a=7, b=3). In all cases, the simulation stops when both bacteria reach a density of 0.

Both infections are treated with the same antibiotic. In the case of Figure 5.2a) the antibiotic treatment is taken until the end (7 days), resulting in a light infection, which causes the elimination of the antibiotic

sensitive bacteria while the antibiotic resistant bacteria are eliminated solely by the immune system around ten days after. The total bacteria density never reaches a very high value. In the case of Figure 5.2b) the therapy is interrupted earlier, after just 3 days. This causes the infecting bacteria to reach higher densities and, thus, can possibly cause death of the host. In case the host survives, the infection is cleared much faster than Figure 5.2a) because the immune system is much more stimulated by the high levels of bacterial cells. This is an example of the most frequent case of antibiotic misuse, according to the ECDC [44], which is the cessation of the antibiotic therapy as soon as the symptoms disappear.

The third example addresses the effect of the delayed start of antibiotic treatment.

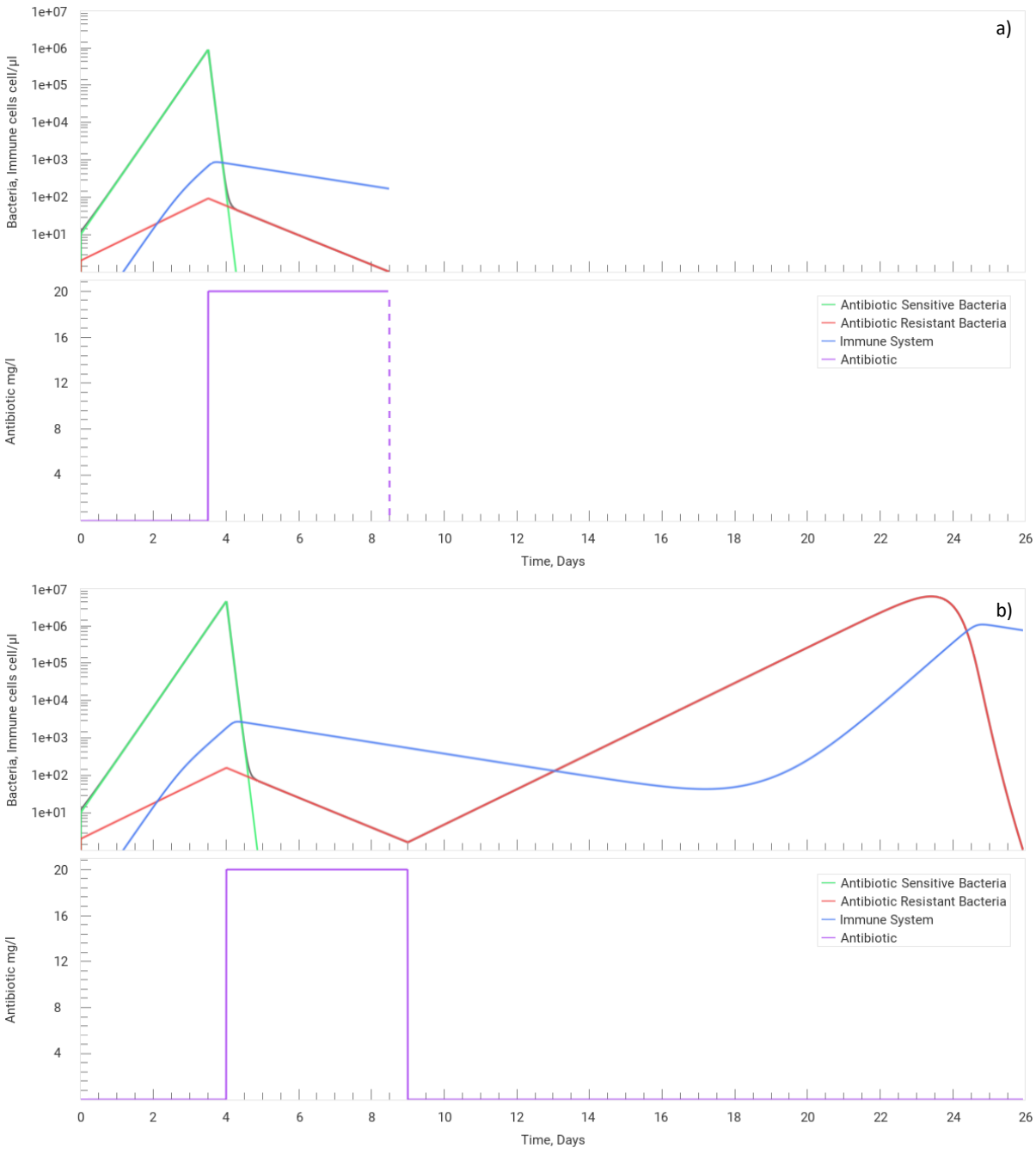

Figure 5.3 Effect of the delayed start of the antibiotic therapy.
Comparing the delayed versus "normal" start of the antibiotic therapy. The same bacterial populations, immune system and axis present in Figure 5.2 are also represented here. In a) treatment starts 3.5 days after infection and the treatment has a duration of 5 days (the full extent of the treatment is represented in a dashed line). In b) the treatment is delayed by just half a day and, with the same 5-day duration as in a). To obtain these graphs, parameters in the application were set to their default values except for the antibiotic mean concentration, which was set at 20 mg/L. The classic treatment was selected with a duration of 5 days and the delay parameter was set differently for each scenario (a=3.5, b=4). In all cases, the simulation stops when both bacteria reach a density of 0.

Both antibiotic therapies have the same duration but start at slightly different times. In Figure 5.3a) the treatment starts at three and a half days after infection and is enough to fight off the infection. In Figure

5.3b) with just a half day of delay when compared to Figure 5.3a), the infection is able to survive the treatment, causing a resurgence of antibiotic resistant bacteria a few weeks later, which rise to the same levels of density as at the start of the treatment, later eliminated by the immune system. Yet another example of one of the most common cases of antibiotic misuse, according to the ECDC, which is the delayed administration of antibiotics in critically ill patients.

The fourth example represents the use of antibiotics in immunosuppressed individuals.

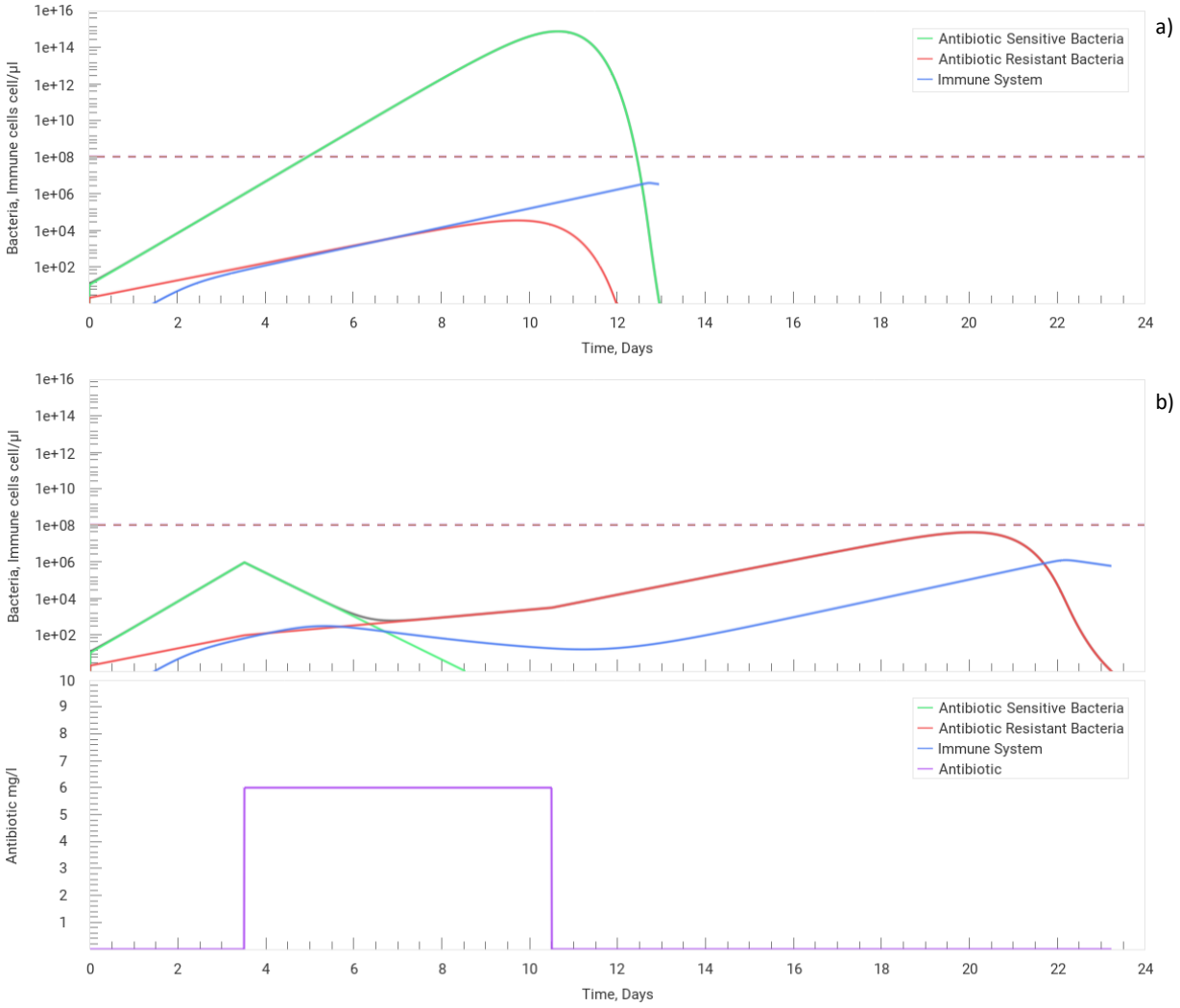

Figure 5.4 Administration of antibiotics in immunosuppressed individuals.
Potential risk of the administration of antibiotics in immunocompromised individuals. Same axis and plots as in Figure 5.2. In a) a bacterial infection runs its course without the interference of an antibiotic to hinder its growth, causing the host to die (death threshold represented by a brown dashed line at a density of 10^8 cell/µl). In b) and antibiotic therapy is applied, beginning 3.5 days after infection, where the host survives. b) can be further compared with Figure 5.2a), in which a normal immune system is in effect. To obtain these graphs, parameters in the application were set to their default values except for the immune system related parameters, which were set as follows: initial precursor cell density = 15 cell/µl, proliferation rate = 1.2 day^{-1} (min), half maximum growth = 10^4 cell/µl (min), effector cell decay rate = 0.8 day^{-1} (max), memory cells conversion = 0.05 (min). Host death density was also set to 9.99e^{14} cell/µl (max) to prevent host death. While no treatment was selected for a) (User treatment set to OFF), in b) the classic treatment was selected with default parameters. In all cases, the simulation stops when both bacteria reach a density of 0.

In the case of Figure 5.4a), because no antibiotic therapy is applied and as this specific individual has a compromised immune system, it is not able to subvert the bacterial development and dies 5 days after the initial infection because the infecting bacteria is able to reach extremely high densities, which easily causes the death of the host. In Figure 5.4b), although the immune system is still compromised, with the help of an antibiotic treatment it is able to fight off the infection, although really close to the death threshold.

5.1.2 Microbiome Scenario Usage Cases

The first case, related to the second simulation scenario, represents the effects of a combination antibiotic therapy on the human gut microbiome.

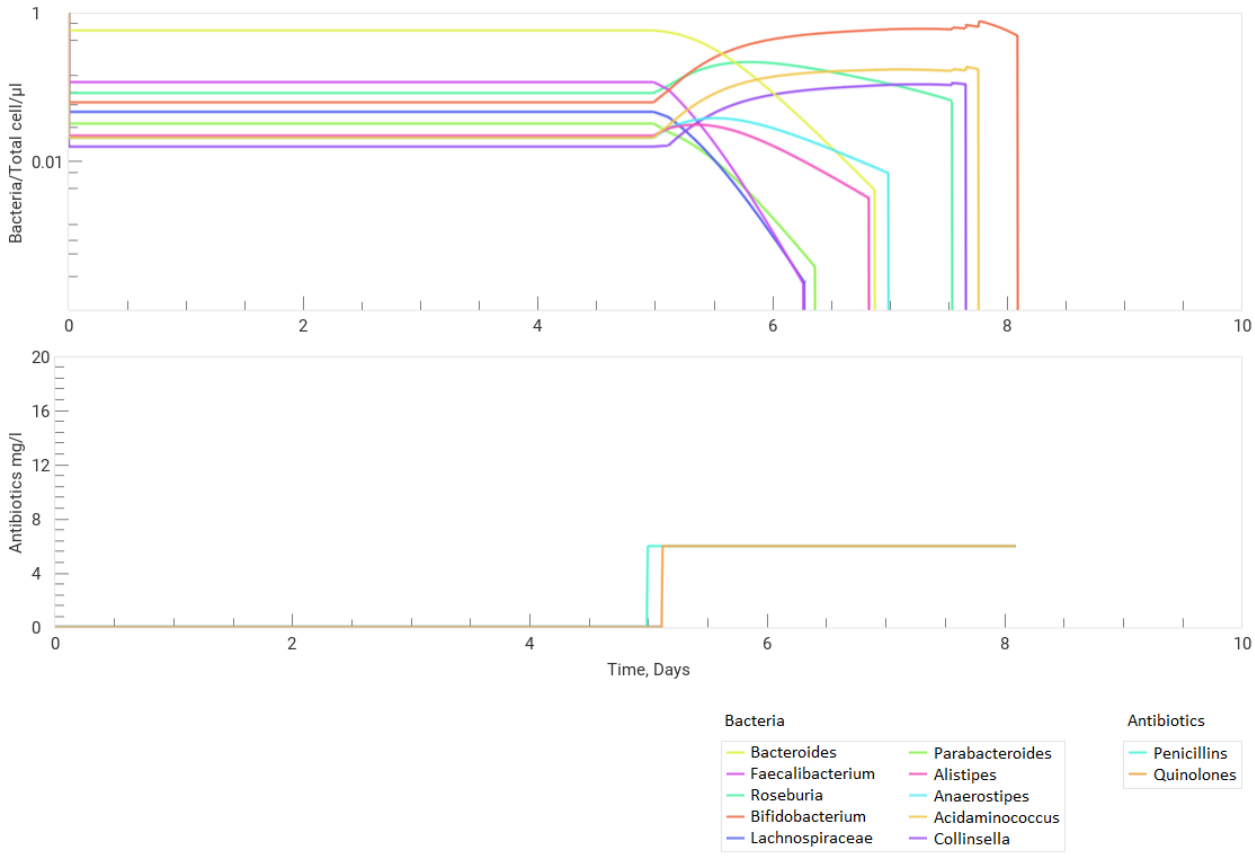

Figure 5.5 Effects of antibiotic combination therapy on the human gut microbiome.
Complete dysbiosis of the human gut caused by the administration of an antibiotic combination. The top graph contains 10 plots, each pertaining to a bacteria genus present in the human gut. The values in the y-axis represent the relative frequency of that genus in the human gut microbiome while values in the x-axis represent time. The bottom graph contains 2 plots, one for each of the antibiotics used. The x- and y-axis, in this case, represent time and antibiotic concentration in mg/L, respectively. A penicillin and quinolone antibiotics were used. All bacteria are stable at a predefined value, representing normal gut activity. During the first 5 days a bacterial infection (not shown) runs its course on the body of the individual. At day 5 an antibiotic combination therapy is administered, which immediately disrupts the microbiome. During the next 3 days each bacteria population present in

the gut dies under the combination antibiotic pressure. The time it takes for a bacteria population to die is determined by the strength of the antibiotic resistance it carries. Faecalibacterium and Lachnospiraceae are the first populations to die, while Bifidobacterium is the last. To obtain these graphs, the "Gut Enterotype 1" of the "Microbiome" scenario was chosen, all parameters were set to their default values, and at day 5 both penicillins and quinolones antibiotics were administered (with a slight delay to allow the plots to be better perceived). The simulation ended when all bacteria populations were eliminated.

This type of antibiotic combination is usually applied in cases of infection by multidrug-resistant bacteria [103]. In this example, we assume the patient is infected with such a multidrug-resistant bacterium. Five days after the initial infection the patient checks-in at the hospital or goes to a medical appointment and begins treatment with a combination of penicillin and quinolone antibiotics. During the next 3 days, while the treatment is active, all bacteria populations in the patient's gut, sensitive to any of the prescribed antibiotics, die one by one due to the effects of the combination antibiotics. Despite the outcome of the treatment on the target bacteria, the microbiome is severely affected and will require some time to recover. This does not happen frequently for most antibiotic combination therapies, as these antibiotics not always affect every bacteria genus present in the gut. Nonetheless, it is representative of what might happen for more aggressive combination antibiotic therapies that include several antibiotics.

The second case represents the disruption of the microbiome after the administration of an antibiotic and the eventual return to equilibrium after the end of the treatment.

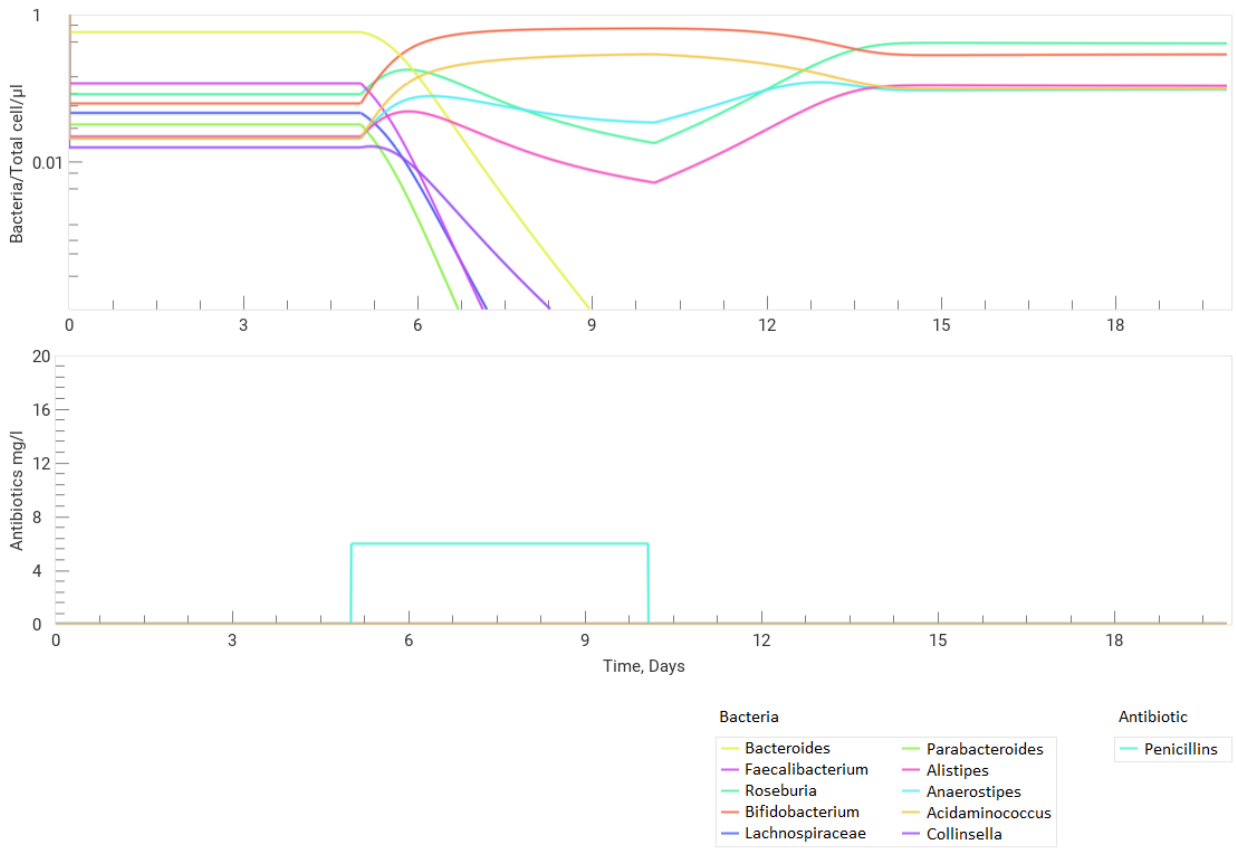

Figure 5.6 Disruption and return to equilibrium of the microbiome's dynamics after antibiotic therapy.
Ability of the human gut microbiome to return to its stable configuration after a major disruption. All plots and axis are the same as in Figure 5.5. A penicillin antibiotic was used. Again, the microbiome is stable and is disrupted by the administration of an

antibiotic, used to treat some infection. In this case, the antibiotic treatment has a duration of 5 days, from day 5 to day 10. During that period each bacteria genus in the microbiome is affected, 5 dying off completely. After the treatment is over, the remaining bacterial genus recover after a period of 4 days. Without any outside addition the dead bacteria won't appear again in the population. To obtain these graphs, the "Gut Enterotype 1" of the "Microbiome" scenario was chosen, all parameters were set to their default values, and at day 5 penicillins antibiotics were administered. The treatment ended at day 10.

The patient suffers from an infection which the medical personnel tries to treat with a penicillin antibiotic. As a side effect of this treatment, symbiotic sensitive bacteria in the gut die alongside the pathogenic bacteria. Some bacteria genera are completely eliminated from the microbiome. When the treatment ends, the surviving bacteria regrow and repopulate the gut, returning to a similar stable configuration as before, but with a now impaired microbial diversity, which can impact the patient's nutrient absorption mechanisms, lead to gut infections by undesirable or opportunistic bacteria and more.

5.2 Feedback

We obtained feedback from several sources throughout the development of the program. Apart from the mandatory friends and family criticism, the most important feedback was that given by a group of high school and university professors which kindly agreed to sit and watch a presentation along with a demonstration of *SimulATe*. Those professors were: Sara Aboim *PhD*, Professor at the *Escola Superior de Educação do Politécnico do Porto*, where she teaches Biology, Geology and Natural Sciences; André Rodrigues *MSc*, high school Professor; Lucinda Motta, Biology, Geology and Natural Sciences high school Professor and author of many high school science books; Xana Sá Pinto *PhD*, Professor at *Escola Superior de Educação do Politécnico do Porto*. Most of the criticism focused around the accessibility and interpretation of the program by high school students such as the usability and position of some buttons or the text description of some parameters. It was suggested several times, even by other people, that the sliders controlling the values of the parameters should be able to collapse so that only the text would be visible as compared to always showing everything, reducing the overall clutter of the user interface and making it much easier to interpret. Making the parameters section completely collapsible would also help by allowing the user to only show sections he deems as relevant at any given time. Having tooltips that would, at a glance, explain what each parameter, button and slider does would also be a much welcome feature that would diminish the reliance on an outside documentation. The existence of a y-axis on the right side of the graph area would also help with the interpretation of the simulated plots as it is being generated. The ability to load the saved data for a given simulation was also a main point of criticism, followed by the idea of having predetermined saved configurations for certain scenarios or bacteria profiles, which could then be loaded and used in a simulation. Some of these criticisms were built into the program but, because of the lack of time, we were not able implement some of the feedback that required more time to be implemented. The group of high school professors did show interest in the program and could recognize the teaching potential that *SimulATe* can have. They have also shown interest and have suggested that we provide continued education for teachers so that they may be able to use *SimulATe* as a science teaching tool.

A seminar was also given at the *Escola Superior de Tecnologia do Barreiro* to a group of Bioinformatics students, under the supervision of Rita Ponce *PhD*, lecturer at the same institution, which allowed for further discussion about several aspects of the program, as well as being the first time the program was ever presented to students of the same area of expertise.

People of different backgrounds, including Bioinformatics, Designers, Ecologists, Microbiologists and Software Engineers have also given their opinions and feedback. We tried to follow most of the advices and feedback given although some were too divergent from the original program idea to implement in such a late time in the development cycle.

6 Discussion

SimulATe consists of two separate simulation scenarios:

The first scenario - Single Population scenario - simulates the combined effects of antibiotic administration and the immune system on the densities of both resistant and sensitive bacteria in a single pathogenic bacterial population. This simulation does not take into account the medium on which the cells are developing, possible nutrients needed for growth or any other third-party interactions as these behaviours are not predicted by the equations used in this situation.

The second scenario - Microbiome scenario - was designed to simulate the effects of the most commonly administered antibiotics in humans, in Portugal during 2015, on different human gut microbiomes. As defined in a study by Arumugam et al [77], the human gut can harbour three different stable microbial compositions, named enterotypes, which were simulated in this scenario by having the top ten most abundant bacteria genera of each enterotype at their respective mean abundance levels in an artificial stable configuration. The ten available bacteria genera for each enterotype are in a stable configuration, which can be perturbed by the use of antibiotics. This simulation does not include the explicit effect of the immune system, as these are all gut dwelling, non-pathogenic and symbiotic bacteria, which do not warrant any specific immune response, and any existing residual effect associated with fluctuations in the bacterial composition are already taken into account when calculating the artificial stable configuration. This simulation does not consider repopulation of dead or extinct bacterial genera and there are no opportunistic infections by outside microorganisms.

In the first scenario (Figure 6.1) a bacteria population must first be defined along with the immune system by using the available parameters described in the Materials and Methods chapter, although all parameters are set to default values on start-up.

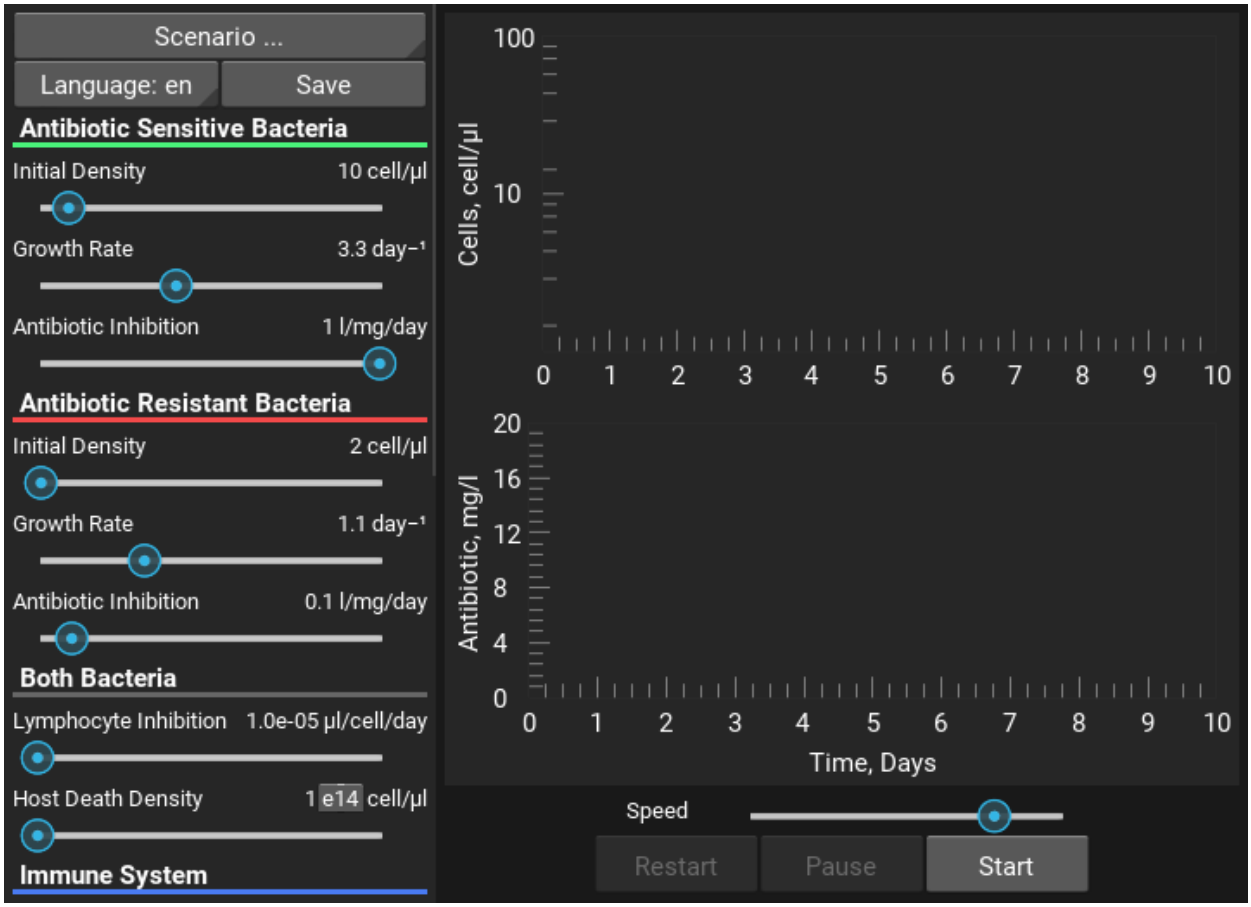

Figure 6.1 User interface of the Single Population scenario.
User interface for the single population scenario, composed of a scrollable "parameters and options" panel (described in Figure 4.3) on the left-hand side which contains all the parameters and options necessary to control the simulation; an initially empty graph canvas containing 2 graphs, one for bacterial and immune system cells (top) and another for antibiotic concentration (bottom) (described in Figure 4.2); and a flow control section which allows the user to start/pause/restart as well as control the speed of the simulation.

Then, one of the three available antibiotic treatments must be selected for the simulation to be able to proceed. The *Classic* treatment represents the situation where a person gets infected by a certain bacteria genus at time zero. After a few days (*delay*) that person starts to feel the symptoms of the infection, which prompts a visit to the hospital where a specific antibiotic treatment is prescribed for a predetermined amount of time (*duration*). Both the *delay* and the *duration* are variable and can be set by the user. The *Adaptive* treatment represents the treatment of a closely monitored patient, as is the case with patients in the intensive care unit of a hospital, where an antibiotic is administered every time the density of the infecting bacteria surpasses a certain predefined threshold, assuming the bacterial density can be determined accurately. The *User* treatment represents the case where a person has full control of the antibiotic dosage and timing of the administration, meaning that the antibiotic can be taken at will, at any time and for any amount of time. This case can represent the self-medication practice without any professional surveillance or guidelines. After all parameters and options are set, the user can finally start the simulation by clicking the *start* button which initializes the real-time generation of the graphs. These graphs represent the outcome of the equations that govern the simulation, explained in the Materials and

Methods chapter. This outcome can vary widely depending on both the initial conditions and on the conditions the user is able to alter during a simulation run (refer to the Usage Cases subchapter).

The second scenario simulates a human gut microbiome under the effect of one or more antibiotic treatments over time, assuming the individual is healthy, the microbiome is in a stable equilibrium with the immune system and does not harbour infectious bacteria.

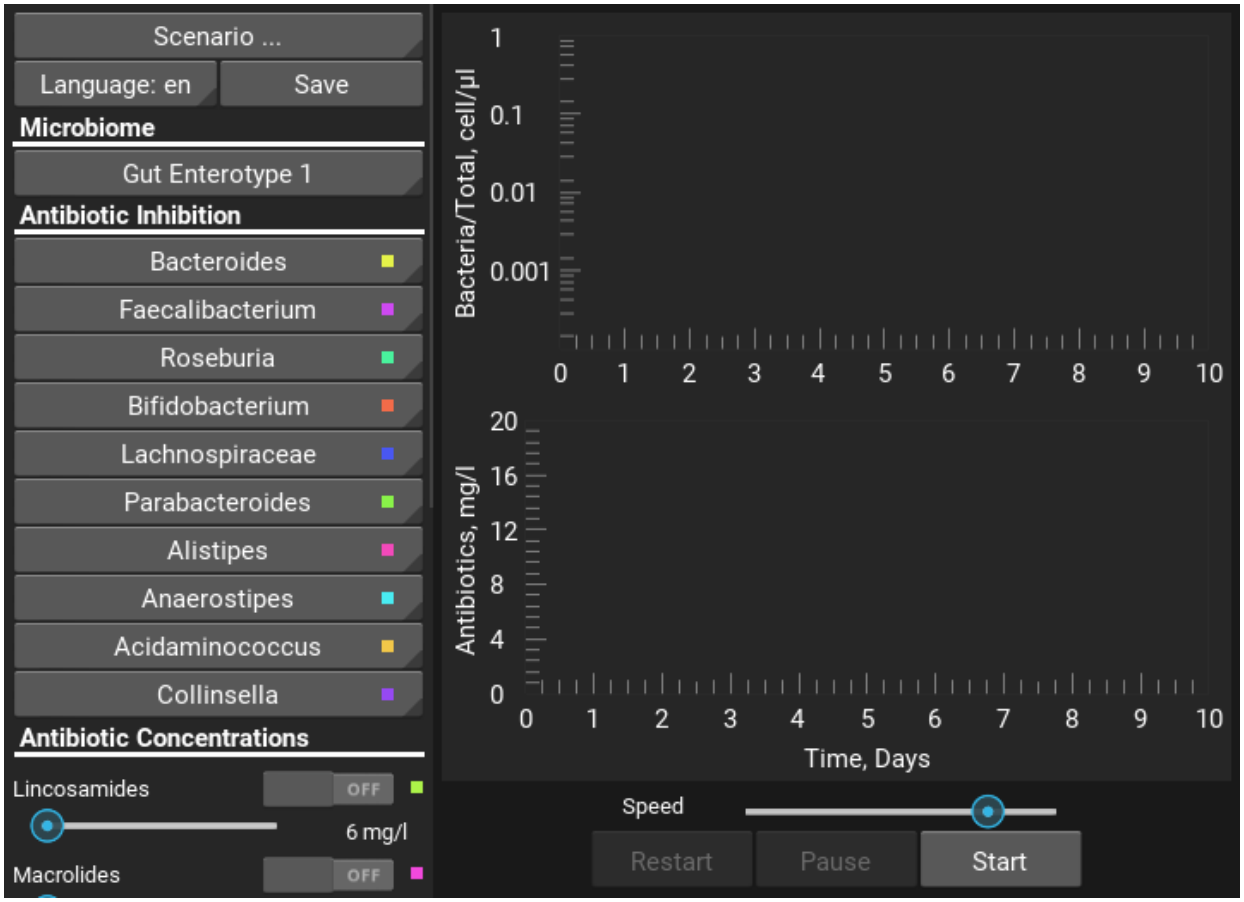

Figure 6.2 User interface of the Microbiome scenario.
User interface for the microbiome scenario, composed of a scrollable "parameters and options" panel (described in Figure 4.4) on the left-hand side which contains all the parameters and options necessary to control the simulation for each individual enterotype; an initially empty graph canvas containing 2 graphs (described in Figure 4.2), one for the relative frequency of each bacteria genus (top) and another for the antibiotic concentration of each available antibiotic (bottom); and a flow control section which allows the user to start/pause/restart as well as control the speed of the simulation.

This microbiome is defined by first selecting an enterotype, which defines the assortment of bacteria genera that are most prevalent, and then by selecting the resistance to each available antibiotic individually for each genus, although, once again, each bacteria genus has these values set to a randomly generated default at start-up. Each one of the three enterotype is its own separate simulation and its output graph represents the relative frequency of each one of the ten bacteria genera alongside the antibiotic concentration of each antibiotic.

The user can then start the simulation which, once again, initiates the real-time generation of the graph. At this time, the antibiotics can be administered at will. In this scenario, because the microbiome is in a state of equilibrium, the outcomes only vary if an antibiotic is administered at all. After stopping administering antibiotics, the microbiome will return to a steady state configuration similar to the initial state. In the cases where one or more bacteria genera dies, this steady state will differ from the initial state, as caused by the reduction in microbial diversity.

With the wide range of possible outcomes available for each scenario, the teaching possibilities are quite extensive. Be it the analysis of the effect of different antibiotic therapies on different gut microbiome compositions, the dynamics of different bacteria populations with different immune systems status, among others, *SimulATe* can be used to simulate it. This program allows for a more interactive teaching of antibiotic resistance and the effect of antibiotic therapies on bacterial communities. Users can follow a simulation in real time, stop it, resume it, change some values on the fly and watch the instant repercussions of those changes. With a real antibiogram and bacterial profile data, this program could even be used to somewhat foresee the evolution of certain infections and certain antibiotic treatments.

We could not find similar existing computer programs used in high school as educational tools. There are, however, a few antibiotic resistance simulators of bacteria, but they either add to much complexity to the simulation, as is the case with ARES [89], or are overly simplified.

Although the mathematical model used to perform the simulations is already a very suitable model and encompasses a handful of details not seen on other simpler models, it could still be improved with even more realistic parameters such as nutrient consumption rate, effect of the type of medium on which the bacteria proliferate, the impact of plasmids and quorum sensing on the growth speed of the bacteria, and more. A different and superior model could also be used altogether, if such model were to be developed.

As a final note we would like to emphasize that *SimulATe* makes use of a relatively simple model. There are many physiological characteristics that the model does not encompass. For example, it does not take into account horizontal gene transfer between sensitive and resistant bacteria nor the effect of the antibiotic concentration in the triggering of horizontal gene transfer. The first scenario only simulates one bacterial population, so interaction between different species are not considered. The second scenario assumes a constant microbiome, when in real life a human gut microbiome is much more malleable and is prone to colonization by outside bacteria or even recolonization by previously existing bacteria.

7 Conclusions

Antibiotics are still widely misused worldwide [20]. This is due, in part, to the misinformation that is still passed around the population despite the effort employed by many health organizations to combat that misinformation with actual reliable and fact-based information. In Portugal, the science curriculum includes the mandatory teaching of antibiotic resistance, but not many informatics-based teaching tools exist that help teach this subject. Therefore, this project focused on the development of a computer program, named *SimulATe*, with the aim of being used as a tool in the teaching of antibiotic resistance.

This program simulates the effect of an antibiotic therapy on bacteria populations. It allows two distinct simulation scenarios: one of more generic characteristics, enabling the configuration of several parameters for either the antibiotic therapy and a single bacteria population, allowing it to be used to simulate a large amplitude of antibiotic therapy scenarios; and another specific to the human gut microbiome, simulating the natural equilibrium of the microbiome and the effects a possible antibiotic therapy can have on its stability and phylogenetic diversity and composition. Besides being used to help teaching antibiotic resistance evolution to students, it could eventually be used by healthcare institutions, such as hospitals, to get a rough simulation predicting the effect a certain antibiotic could have on a certain infection.

We started developing *SimulATe* as an agent-based program [99]. We quickly ran into unexpected problems, which prompted us to change the mathematical model [26], as the foundation of *SimulATe*. The final product is a program that can simulate antibiotic-bacteria interactions in real time based on the provided parameters. We also tested some example scenarios in *SimulATe* which were all consistent with reality.

In summary, this work concluded with the creation of a simulation tool which will probably be useful in the teaching of antibiotic resistance.

7.1 Future Work

Further work can still be done to improve the program, namely the implementation of the options file in the more standardized *.cfg* config file format, instead of the current text file, along with the implementation of a read/write function by using the *ConfigParser* module available in the Python standard library. All configurations of the program would also be migrated to this config file and loaded at start-up. The behaviour of the *Save* button would be modified to save a config file with all the parameters of the current active scenario, instead of the current behaviour, which creates a text file containing all the parameters. A *Load* button would also be implemented which would allow a user to load the config files generated when saving a simulation.

A new treatment type option would be implemented in the *Single Population* scenario. This new treatment type would function like the *Classic* treatment type but instead of having a static mean antibiotic concentration when administered, the concentration would start at 0, grow to the defined value and then decrease based on pharmacokinetics data, in a sigmoid-like fashion. This new treatment would have the added benefit of allowing for the compatibility with certain bacterial behaviour such as the SOS response or heightened horizontal gene transfer rate when in sub-minimum inhibitory concentration of antibiotics, and would also be compatible with the enrichment of pre-existing mutations in susceptible bacteria as well

as the selection of *de novo* mutations and increased mutagenesis rate [49]. These latter additions would also be implemented.

The *Microbiome* scenario would be made more robust, with the addition of several biological interactions between species within the same microbiome and the possibility of gene exchange via horizontal gene transfer.

We would like to be able to have real values pertaining to the antibiotic susceptibility of each bacteria genus in the *Microbiome* scenario. As it stands now, each bacteria population is given a random susceptibility value to each antibiotic, but this could be changed if we were able to get the necessary data to define those values. Unfortunately, this is very unlikely, as most of the research done in antibiotic resistance is focused on pathogenic bacteria, which do not normally inhabit the healthy human gut. Personalized antibiograms might help but only on a case-by-case scenario.

Making the user interface more focused and clean by allowing the user to hide and collapse certain parameters or buttons would allow for a better user experience by reducing the amount of information on the screen.

Changes related to *Pyinstaller* would also be addressed. As of now there is only an executable for the Windows OS, meaning that for both Linux OS and Mac OS the user must go through the process of installing all the dependencies required to run the program. This is prone to errors and may be a difficult process for less computer literate users, even though the documentation available for *SimulATe* describes the installation process for all three OS's mentioned. With an executable for each OS this would not be a problem as the user would only be required to download and run an executable, specifically made for their OS. This would be done with the *Pyinstaller* python module the same way it was done for Windows, but further and better tested. Reducing the executable to a single file would also be a very good modification, as it would simplify the usability of the program.

Implementing a way for the user to give feedback would allow for better bug correction, feature implementation and testing.

Having a web version as well as a handheld version (Android and/or iOS) of the simulator would also be a good idea, as it would allow the program to reach a wider audience as well as eliminate most system related dependencies.

We would like to define some proper usability testing procedures to better test the user interface of the program and detect possible design issues. A survey would accompany these tests to collect more data on the overall experience the average user has with the program.

Testing the simulator in a school environment with the support of a science school teacher would help us collect data on the performance of the simulator in these conditions and would contribute to a better and more robust simulator.

8 Source Code and License

The *SimulATe* program is publicly accessible on GitHub *(*https://github.com/Kronopt/SimulATe*)* and is licensed under the GNU General Public License v3.0.

Bibliography

1. Sturgen, N. O. & Casida, L. E. Antibiotic production by anaerobic bacteria. *Appl. Microbiol.* **10,** 55–9 (1962).

2. van der Meij, A., Worsley, S. F., Hutchings, M. I. & van Wezel, G. P. Chemical ecology of antibiotic production by actinomycetes. *FEMS Microbiology Reviews* **41,** 392–416 (2017).

3. Cook, A. H. & Lacey, M. S. Production of Antibiotics by Fungi. *Br J Exp Pathol* **26,** 404–409 (1945).

4. Brian, P. W. Antibiotics Produced by Fungi. *Bot. Rev.* **27,** 357–430 (1951).

5. Cowan, M. M. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **12,** 564–82 (1999).

6. Gould, K. Antibiotics: from prehistory to the present day. *J. Antimicrob. Chemother.* **71,** 572–575 (2016).

7. Forrest MB, R. D. Early history of wound treatment. *J. R. Soc. Med.* **75,** 198–205 (1982).

8. Fleming, A. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of b. influenzae. *Br J Exp Pathol* **10,** 226–236 (1929).

9. Richards, A. N. Production of Penicillin in the United States (1941–1946). *Nature* **201,** 441–445 (1964).

10. Lesch, J. E. The First Miracle Drugs: How the Sulfa Drugs Transformed Medicine. in *The First Miracle Drugs: How the Sulfa Drugs Transformed Medicine* 51–67 (Oxford University Press, 2007).

11. Lewis, K. Platforms for antibiotic discovery. *Nat. Rev.* **12,** 371–387 (2013).

12. Wright, G. D. Q&A: Antibiotic resistance: where does it come from and what can we do about it? *BMC Biol.* **8,** 123 (2010).

13. Bessa, L. J. *et al.* Neofiscalin A and fiscalin C are potential novel indole alkaloid alternatives for the treatment of multidrug-resistant Gram-positive bacterial infections. *FEMS Microbiol. Lett.* **363,** fnw150 (2016).

14. Ling, L. L. *et al.* A new antibiotic kills pathogens without detectable resistance. *Nature* **517,** 455–459 (2015).

15. Higgins, P. G., Dammhayn, C., Hackel, M. & Seifert, H. Global spread of carbapenem-resistant Acinetobacter baumannii. *J. Antimicrob. Chemother.* **65,** 233–238 (2010).

16. Munita, J. M. & Arias, C. A. Mechanisms of Antibiotic Resistance. *Microbiol. Spectr.* **4,** (2016).

17. Furuya, E. Y. & Lowy, F. D. Antimicrobial-resistant bacteria in the community setting. *Nat. Rev. Microbiol.* **4,** 36–45 (2006).

18. McGowan, J. E. & Tenover, F. C. Confronting bacterial resistance in healthcare settings: a crucial

role for microbiologists. *Nat. Rev. Microbiol.* **2,** 251–258 (2004).

19. Ventola, C. L. The antibiotic resistance crisis: part 1: causes and threats. *P T* **40,** 277–83 (2015).

20. Grigoryan, L. *et al.* Attitudes, beliefs and knowledge concerning antibiotic use and self-medication: a comparative European study. *Pharmacoepidemiol. Drug Saf.* **16,** 1234–1243 (2007).

21. Jones, D. S., Podolsky, S. H. & Greene, J. a. The Burden of Disease and the Changing Task of Medicine. *N. Engl. J. Med.* **366,** 2333–2338 (2012).

22. Fleming, S. A. Nobel Lecture: Penicillin. *Nobel Lect. Physiol. or Med. 1942-1962* 83–93 (1945).

23. Keller, M. A. & Stiehm, E. R. Passive immunity in prevention and treatment of infectious diseases. *Clin. Microbiol. Rev.* **13,** 602–14 (2000).

24. Levin, B. R. & Bull, J. J. Population and evolutionary dynamics of phage therapy. *Nat. Rev. Microbiol.* **2,** 166–173 (2004).

25. Medzhitov, R. Recognition of microorganisms and activation of the immune response. *Nature* **449,** 819–826 (2007).

26. Gjini, E. & Brito, P. H. Integrating Antimicrobial Therapy with Host Immunity to Fight Drug-Resistant Infections: Classical vs. Adaptive Treatment. *PLOS Comput. Biol.* **12,** e1004857 (2016).

27. Handel, A., Margolis, E. & Levin, B. R. Exploring the role of the immune response in preventing antibiotic resistance. *J. Theor. Biol.* **256,** 655–62 (2009).

28. Geli, P., Laxminarayan, R., Dunne, M. & Smith, D. L. "One-Size-Fits-All"? Optimizing Treatment Duration for Bacterial Infections. *PLoS One* **7,** e29838 (2012).

29. Ankomah, P. & Levin, B. R. Exploring the collaboration between antibiotics and the immune response in the treatment of acute, self-limiting infections. *Proc. Natl. Acad. Sci.* **111,** 8331–8338 (2014).

30. Meidani, M., Naeini, A. E., Rostami, M., Sherkat, R. & Tayeri, K. Immunocompromised patients: Review of the most common infections happened in 446 hospitalized patients. *J. Res. Med. Sci.* **19,** S71-3 (2014).

31. Joos, L. & Tamm, M. Breakdown of Pulmonary Host Defense in the Immunocompromised Host: Cancer Chemotherapy. *Proc. Am. Thorac. Soc.* **2,** 445–448 (2005).

32. Martinez, J. L. General principles of antibiotic resistance in bacteria. *Drug Discov. Today Technol.* **11,** 33–39 (2014).

33. Ventola, C. L. The antibiotic resistance crisis: part 2: management strategies and new agents. *P T* **40,** 344–52 (2015).

34. Bonito, J. *et al.* Metas Curriculares Ensino Básico Ciências Naturais, 5º, 6º, 7º e 8º anos. *Ministério da Educ. e Ciência* 23 (2013).

35. Bonito, J. *et al.* Metas Curriculares Ensino Básico Ciências Naturais, 9º ano. *Ministério da Educ. e Ciência* 7 (2014).

36. Fonseca, M. J., Santos, C. L., Costa, P., Lencastre, L. & Tavares, F. Increasing Awareness about Antibiotic Use and Resistance: A Hands-On Project for High School Students. *PLoS One* **7,** (2012).

37. National Research Council. *A Framework for K-12 Science Education: Practices, Crosscutting Concepts, and Core Ideas*. (National Academies Press, 2012). doi:10.17226/13165

38. National Research Council. *Taking science to school. Learning and teaching science in grades K-8*. (National Academies Press, 2007). doi:10.17226/11625

39. Granado, A. & Malheiros, J. V. *CULTURA CIENTÍFICA EM PORTUGAL*. (Fundação Francisco Manuel dos Santos, 2015).

40. Paiva, J., Morais, C. & Moreira, L. *O MULTIMÉDIA NO ENSINO DAS CIÊNCIAS*. (Fundação Francisco Manuel dos Santos, 2015).

41. Martins, G. d'Oliveira *et al.* PERFIL DOS ALUNOS À SAÍDA DA ESCOLARIDADE OBRIGATÓRIA. *Editor. do Ministério da Educ. e da Ciência* (2017).

42. Etebu, E. & Arikekpar, I. Antibiotics: Classification and mechanisms of action with emphasis on molecular perspectives. *IJAMBR* **4,** 90–101 (2016).

43. Adzitey, F. Antibiotic Classes and Antibiotic Susceptibility of Bacterial Isolates from Selected Poultry; A Mini Review. *World's Vet. J. J. World Vet J* **5,** 36–41 (2015).

44. European Union. European Centre for Disease Prevention and Control. Available at: https://ecdc.europa.eu. (Accessed: 16th February 2018)

45. Llewelyn, M. J. *et al.* The antibiotic course has had its day. *BMJ* **358,** j3418 (2017).

46. ECDC. TESSy - The European Surveillance System. Available at: https://ecdc.europa.eu/en/publications-data/european-surveillance-system-tessy. (Accessed: 3rd June 2017)

47. Liu, B. & Pop, M. ARDB--Antibiotic Resistance Genes Database. *Nucleic Acids Res.* **37,** D443–D447 (2009).

48. Andrews, J. M. Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemother.* **48 Suppl 1,** 5–16 (2001).

49. Andersson, D. I. & Hughes, D. Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbiol.* **12,** 465–78 (2014).

50. Vogwill, T. & MacLean, R. C. The genetic basis of the fitness costs of antimicrobial resistance: a meta-analysis approach. *Evol. Appl.* **8,** 284–95 (2015).

51. Durão, P., Balbontín, R. & Gordo, I. Evolutionary Mechanisms Shaping the Maintenance of Antibiotic Resistance. *Trends Microbiol.* (2018). doi:10.1016/j.tim.2018.01.005

52. Morris, S. C. *Earth's earliest biosphere. Its origin and evolution*. *Geological Journal* (1985). doi:10.1002/gj.3350200107

53. Leadbetter, E. R. & Poindexter, J. S. (Jeanne S. *Bacteria in Nature: Volume 1: Bacterial activities in perspective*. (Plenum Press, 2013).

54. Pudlo, N. A. *et al.* Symbiotic Human Gut Bacteria with Variable Metabolic Priorities for Host Mucosal Glycans. *MBio* **6,** (2015).

55. Nikoopour, E. & Singh, B. Reciprocity in microbiome and immune system interactions and its implications in disease and health. *Inflamm. Allergy Drug Targets* **13,** 94–104 (2014).

56. Schmid-Hempel, P. & Frank, S. A. Pathogenesis, virulence, and infective dose. *PLoS Pathog.* **3,** 1372–3 (2007).

57. Lederberg, J. Ome Sweet 'Omics—a genealogical treasury of words. *Sci.* **15,** (2001).

58. NIH HMP Working Group, T. N. H. W. *et al.* The NIH Human Microbiome Project. *Genome Res.* **19,** 2317–23 (2009).

59. Costello, E. K. Bacterial Community Variation in Human Body Habitats Across Space and Time. *Princ. Relativ.* **326,** 75–91 (2013).

60. Morgan, X. C., Segata, N. & Huttenhower, C. Biodiversity and functional genomics in the human microbiome. *Trends Genet.* **29,** 51–58 (2013).

61. Hooper, L. V. *et al.* Molecular Analysis of Commensal Host-Microbial Relationships in the Intestine. *Science (80-.).* **291,** 881–884 (2001).

62. Schumann, A. *et al.* Neonatal antibiotic treatment alters gastrointestinal tract developmental gene expression and intestinal barrier transcriptome. *Physiol. Genomics* **23,** 235–245 (2005).

63. Fanaro, S., Chierici, R., Guerrini, P. & Vigi, V. Intestinal microflora in early infancy: composition and development. *Acta Paediatr. Suppl.* **91,** 48–55 (2003).

64. Silva, M. J. B. *et al.* The multifaceted role of commensal microbiota in homeostasis and gastrointestinal diseases. *J. Immunol. Res.* **2015,** (2015).

65. Vaishampayan, P. A. *et al.* Comparative metagenomics and population dynamics of the gut microbiota in mother and infant. *Genome Biol. Evol.* **2,** 53–66 (2010).

66. Dominguez-Bello, M. G. *et al.* Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc. Natl. Acad. Sci.* **107,** 11971–11975 (2010).

67. Yatsunenko, T. *et al.* Human gut microbiome viewed across age and geography. *Nature* **486,** 222–227 (2012).

68. Wu, G. D. *et al.* Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. *Science (80-.).* **334,** 105–108 (2012).

69. Voreades, N., Kozil, A. & Weir, T. L. Diet and the development of the human intestinal microbiome. *Front. Microbiol.* **5,** 1–9 (2014).

70. Fallani, M. *et al.* Intestinal Microbiota of 6-week-old Infants Across Europe: Geographic Influence

Beyond Delivery Mode, Breast-feeding, and Antibiotics. *J. Pediatr. Gastroenterol. Nutr.* **51,** 77–84 (2010).

71. Singh, R. K. *et al.* Influence of diet on the gut microbiome and implications for human health. *J. Transl. Med.* **15,** 73 (2017).

72. O'Mahony, C. *et al.* Commensal-Induced Regulatory T Cells Mediate Protection against Pathogen-Stimulated NF-κB Activation. *PLoS Pathog.* **4,** e1000112 (2008).

73. Holzapfel, W. H. & Schillinger, U. Introduction to pre- and probiotics. *Food Res. Int.* **35,** 109–116 (2002).

74. Wang, X. & Gibson, G. R. Effects of the in vitro fermentation of oligofructose and inulin by bacteria growing in the human large intestine. *J. Appl. Bacteriol.* **75,** 373–80 (1993).

75. Finegold, S. M. Interaction of antimicrobial therapy and intestinal flora. *Am. J. Clin. Nutr.* **23,** 1466–71 (1970).

76. Beaugerie, L. & Petit, J.-C. Microbial-gut interactions in health and disease. Antibiotic-associated diarrhoea. *Best Pract. Res. Clin. Gastroenterol.* **18,** 337–352 (2004).

77. Arumugam, M. *et al.* Enterotypes of the human gut microbiome. *Nature* **473,** 174–180 (2011).

78. Janeway, C., Travers, P., Walport, M. & Shlomchik, M. *Immunobiology: the immune system in health and disease*. (Garland Science Publishing, 2005).

79. Pulendran, B. & Ahmed, R. Immunological mechanisms of vaccination. *Nat. Immunol.* **12,** 509–17 (2011).

80. Levin, B. R., Baquero, F., Ankomah, P. (Pierre) & McCall, I. C. Phagocytes, Antibiotics, and Self-Limiting Bacterial Infections. *Trends Microbiol.* **25,** 878–892 (2017).

81. Novick, A. & Szilard, L. Description of the Chemostat. *Science (80-.).* **112,** 715–716 (1950).

82. Dionisio, F. *Quimiostato: Teoria e Aplicações (Personal Communication)*. (2009).

83. James, T. W. Continuous Culture of Microorganisms. *Annu. Rev. Microbiol.* **15,** 27–46 (1961).

84. Antibiotic Resistance Game. Available at: https://www.brainpop.com/games/antibioticresistancegame/. (Accessed: 9th February 2018)

85. Superbugs: The Game. Available at: https://longitudeprize.org/antimicrobial-resistance/superbugs. (Accessed: 9th February 2018)

86. Antibiotic Resistance Game. Available at: https://forio.com/simulate/busekirmaz92/antibiotic-resistance/simulation/. (Accessed: 9th February 2018)

87. 10-day Stochastic Simulation of E.Coli Antibiotic Resistance. Available at: https://www.linkedin.com/pulse/10-day-stochastic-simulation-ecoli-antibiotic-resistance-kathy-gao?articleId=7446650357141226306. (Accessed: 9th February 2018)

88. Antibiotic Resistance Simulation. Available at:

https://bl.ocks.org/tonyhschu/067d977d6d4ae7224dcebdf3775d9e7e. (Accessed: 9th February 2018)

89. Campos, M. *et al.* A membrane computing simulator of trans-hierarchical antibiotic resistance evolution dynamics in nested ecological compartments (ARES). *Biol. Direct* **10,** 41 (2015).

90. Python Software Foundation. Python Programming Language. Available at: http://www.python.org. (Accessed: 24th July 2017)

91. JetBrains s.r.o. PyCharm Community Edition. Available at: https://www.jetbrains.com/pycharm. (Accessed: 24th July 2017)

92. Kivy organization. Kivy Framework. Available at: https://kivy.org. (Accessed: 24th July 2017)

93. PyInstaller Development Team. Pyinstaller. Available at: http://www.pyinstaller.org/. (Accessed: 24th July 2017)

94. Shinners, P. & Pygame Community. Pygame. Available at: https://www.pygame.org. (Accessed: 25th July 2017)

95. Shipman, J. W. Tkinter 8.5 reference: a GUI for Python. Available at: http://infohost.nmt.edu/tcc/help/pubs/tkinter/web/index.html. (Accessed: 25th July 2017)

96. Kivy organization. garden.graph. Available at: https://github.com/kivy-garden/garden.graph. (Accessed: 25th July 2017)

97. Hunter, J. D. Matplotlib: A 2D graphics environment. *Comput. Sci. Eng.* **9,** 99–104 (2007).

98. Software Freedom Conservancy. Git. Available at: https://git-scm.com/. (Accessed: 16th February 2018)

99. DeAngelis, D. L. & Grimm, V. Individual-based models in ecology after four decades. *F1000Prime Rep.* **6,** 39 (2014).

100. Vieira-Silva, S. & Rocha, E. P. C. The systemic imprint of growth and its uses in ecological (meta)genomics. *PLoS Genet.* **6,** (2010).

101. Mendes, A. *et al.* Programa de Biologia e Geologia 11º ano, Curso Científico-Humanístico de Ciências e Tecnologias. *Ministério da Educ. Dep. do ensino secundário* (2003).

102. Mendes, A. M. P., Rebelo, D. H. V, Pinheiro, E. J. G., Sanches, ilda S. & Cunha, J. M. Biologia 12º ano Curso Científico-Humanístico de Ciências e Tecnologias. *Ministério da Educ. Direção Geral Inovação e Desenvolv. Curric.* (2004).

103. Tängdén, T. Combination antibiotic therapy for multidrug-resistant Gram-negative bacteria. *Ups. J. Med. Sci.* **119,** 149–53 (2014).

Appendix A

BioClub - Mutualistic symbiosis: implications and applications

"Who's in charge: you, or your bacteria? Mutualistic interactions with and between your microbes."

Pedro David[1], Xana Sá-Pinto[2,3], Teresa Nogueira[1]

Illustration (Fig. 2): Sara Algarvio

[1] cE3c – Centro de Ecologia, Evolução e Alterações Ambientais, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal

[2] CIDTFF.UA – Centro de Investigação em Didáctica e Tecnologia na Formação de Formadores, Universidade de Aveiro, Campus Universitário, 3810-193 Aveiro, Portugal
portugal

[3] P.Porto: ESE – Escola Seuperior de Educação, Politécnico do Porto, Rua Dr. Roberto Frias, 602 4200-465 Porto Portugal

Close your eyes. Imagine one ecosystem with soft and hard substrates, 100% moist, temperature between 34 and 37ºC all year around and abundant food resources. Imagine a second ecosystem: dry, with significant temperature fluctuations, high UV exposure and a relatively high substrate instability. Now open your eyes and closely observe these ecosystems. No, we are not describing tropical forests or sand deserts, we are talking about you! More specifically we are talking about your mouth {van Houte, 1972} and your hand's skin {Grice, 2011} {Cundell, 2016}, two of the several ecosystems present in your body that harbour a large and diverse community of microbes including bacterial, viral, archeal and fungal species {Lloyd-Price, 2016}. This community of microbes living in your body includes commensal, mutualistic and even a few (most probably opportunistic) pathogenic species {Consortium, 2012} which, as a whole, are referred to by microbiologists as a microbiome {Lederberg, 2001}. Telling you what microbes live within/on you is not an easy task, as the characterisation of the microbiomes of hundreds of people shows these communities are variable and dependent on factors such as body surface area, gender, age, diet, daily life habits, ethnicity and geography, health status and your own genome {Cundell, 2016} {Lloyd-Price, 2016} {Consortium, 201}. But whichever microbes you carry, they are for sure affecting your health. People tend to better know microbes by the diseases they cause, but human microbiomes have been shown to be essential for our health, ensuring certain functions like the ability to completely digest some nutrients like carbohydrates and providing metabolic pathways that complement those encoded by our genome (reviewed in {Lloyd-Price, 201}, {Kilian, 2016} and {Yong, 2017}). We feed them, and they work for us. In fact, at this moment, your microbes are actively

regulating the pH of some of your body areas, fighting other (possibly pathogenic) microbes, providing you with resistance to infections, educating and regulating your immune system to avoid or decrease autoimmune diseases and exaggerated immune responses, and digesting some of the food you ingested and producing nutrients that are essential to you {Lloyd-Price, 2016} {Kilian, 2016} and {Yong, 2017}. Other essential functions of a healthy human microbiome includes gene activity regulation, and the differentiation and maturation of some of our organs and tissues {Kilian, 2016} {Yong, 2017} {Brown, 1977}.

Taken together the genomes of the bacteria and viruses residing in the human gut encode 3.3 million genes. These are so essential in supplementing our genome {Konkel, 2013} that, in the Nature Magazine number 464 of march 2010, Liping Zhao called "Our Other Genome" to all the genes belonging to the microbes of our microbiome. So how much of you are actually you and how much of your body functions are ensured by your cells? According to Martin Blaser (a specialist in the human microbiome) from the nearly 30 trillion cells in our bodies, only less than a third is human, and the remaining 70 to 90% are microbial. And approximately 99% of the unique genes in your body are indeed bacterial {, 2017} and these are encoding functions that are essential for your health. As Ed Yong says "I contain multitudes" {Yong, 2017}. In fact, excluding the vaginal area, reduced species diversity in human microbiomes is usually associated with pathologies {Lloyd-Price, 2016}. The the lack of species that perform some of these essential functions in gut microbiome is associated with obesity, inflammatory bowel disease, types 1 and 2 diabetes, and in skin is associated with atopic dermatitis and psoriasis (reviewed in {Lloyd-Price, 2016}). But these can also be due to the reduction of species with similar functions in the community (functional redundancy) which may turn microbial communities more susceptible to changes in their environment (such as diet changes, pathogenic infections, medication, or others) and less able to recover from these {Bodelier, 2011} {Lloyd-Price, 2016}. Yet how have your microbiomes' diversity developed, how does it change over time and what factors affect it? We will now focus on the most studied microbiome, the human gut microbiome, as a model to answer these questions and understand the role and function of our microbiomes.

Your gut harbours a wide variety of microbes {Eckburg, 2005} {Hold, 2002} {Suau, 1999}, which most probably have been co-existing in a fairly stable equilibrium since your adulthood, and dominated by the phyla Firmicutes, Bacteroidetes and Actinobacteria. An equilibrium is usually defined by the existence and abundance of 3 types of bacteria: *Bacteroides*, *Prevotella* or *Ruminococcus.* Each of these bacteria genus defines a different microbiota group, known as an enterotype {Arumugam, 2011}, where the predominant above mentioned bacteria establish positive interactions with some other bacterial groups, and negative interactions with some others, which are thus not favoured and disappear. Although mostly stable, your enterotype can be disturbed by changes in your habits such as when you change your diet, take antibiotic, probiotics, prebiotics and other factors. The good new is it will recover most of the times if these unusual situations are not too prolonged or radical.

During the normal development of a human baby, several factors influence the maturation process of the gut microbiome before it finally settles on an enterotype, starting-off immediately after birth. It's believed that human babies have a sterile gut up until birth, and is then immediately colonized by

microorganisms originating from the mother. Babies born via a vaginal birth are first colonized by microbes originating from the mother's vaginal canal and intestines, while infants born via C-section are mainly colonized by microbes originating from the mother's skin and neighbouring environments. So, if you were born via vaginal birth, you were most probably a baby initially dominated by bacterias such as *Lactobacillus*, *Prevotella* and *Sneathia,* but if you were a caesarean baby, you were probably initially colonised by *Staphylococcus*, *Corynebacterium*, and *Propioni* having lower counts of *Bifdobacteria*, *Escherichia coli,* and *Bacteroides fragilis* and higher *Clostridia*, *Klebsiella* and *Clostridium difficile* counts {Vaishampayan, 2010} {Fitzpatrick, 2008} {Gerding, 1995} {Thomas, 2003} {Adlerberth, 2007} {Dominguez-Bello, 2010}. If you were a preterm baby, you most probably had a delayed development of your gut microbiota, and were initially colonized predominantly by *Coliforms*, *Enterococci* and *Bacteroides* {Blakey, 1982}. Most of your initial colonizers were facultative anaerobes, like *Streptococci* and *E. coli*, which were then succeeded by *Staphylococcus, Enterococcus* and *Lactobacillus* that contributed to develop an anaerobic environment making your gut available to more bacterial species {Fanaro, 2003} {Orrhage, 1999}. But your microbiome was also influenced by the type of feeding regime that you went through while baby: breast-feeding, infant milk formulas or a combination of both. These feeding regimes introduce and allow different species to develop thus shapping the microbiome {Collado, 2009} {Voreades, 2014}. The gradual introduction of solid foods in babies' diet further helps the gut microbiome to mature {Edwards, 200}. During this period of adaptation to the new diet the microbiome is still not stable enough, meaning its bacterial composition can easily change, taking up to 3 years to stabilize {Bergstrom, 2014} {Vaishampayan, 2010}. Other aspects can affect either the development or the already stabilized gut microbiome. An overweight mother most likely affects her baby microbiome, which ends up having a different bacterial composition when compared to babies of average weight mothers {Collado, 2010}. Prebiotics and probiotics both have similar effects on the gut microbiome, by allowing certain bacteria species to more easily grow or by introducing beneficial bacteria directly in the system {Holzapfel, 2002} {Wang, 1993}. Antibiotics, on the other hand, even though they are used to fight off harmful bacteria, they can also affect beneficial species, especially wide spectrum antibiotics {Finegold, 1983}. On adult gut microbiomes, the factor that more deeply impacts its composition is diet {Voreades, 2014} but, by simply living on different geographical locations, and/or in countries at different levels of development, different people can have different gut microbiome compositions, and extended migrations can permanently change the gut microbiota {Fallani, 2010}.

According to Martin Blaser, ancient transmission of microbes from mother to child would be: "oral (pre-mastication of food), mammary, through breastfeeding and cutaneous (contact with skin), vaginal (passage through birth channel)". However, modern human practices in industrialized countries - such as: "early-life antibiotics, dental amalgams, bottle feeding, early / extensive bathing and Cesarian section" - has been reducing microbe mother to child transmission of the indigenous microbiota, lowering microbial richness of the human microbiome from generation to generation {Cho, 2012} {Blaser, 2016} {Blaser, 2009}.

Antibiotics kill or stop the growth (proliferation) of microorganisms in a microbiome. Exposure to antibiotics affect different bacteria in different ways, and thus alter the composition of the microbiome

through time. In developed countries, humans are continuously exposed to antibiotics, either from medical prescriptions for infections treatment or prophylactic purposes, but also, passively, from the agricultural and livestock maneuvers {Larsson, 2014}. Antibiotics exert a differential selective pressure on bacteria that populate the intestine as each bacteria has a different susceptibility to antibiotics. Antibiotic exposure may lead to the extinction of the most susceptible species, and even if the treatment is prolonged or made up of a combination of different antibiotics, it may lead to the extinction of several species, with a concomitant a decrease in microbial diversity {Dethlefsen, 2008}.

Figure 1 represents the evolution of the relative frequencies of a hypothetic bacterial community composed of ten different types of bacteria (genus, species, strains…). While under antibiotic therapy, the entire bacterial community is exposed to the antibiotic; those bacteria that are susceptible will decrease in frequency and eventually disappear leading to a disruption of the microbial equilibrium – dysbiosis. After treatment, the microbiome tends to restore its equilibrium. However bacterial diversity can be impaired and unable to restore some physiologic functions of the healthy intestine, by function loss.

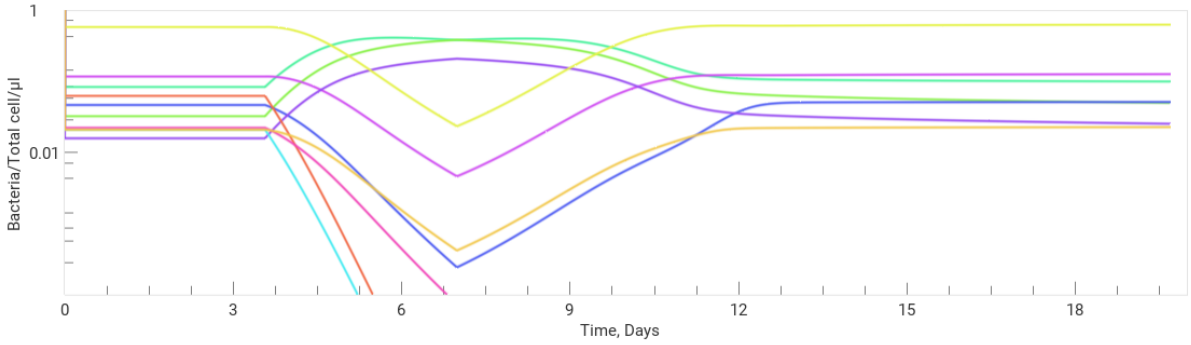

Fig. 1 Evolution of the relative frequencies of different types of bacteria of a microbiome. Shortly after day 3 the microbiome was exposed to an antibiotic for 3 days.

Since different microorganisms are associated with different metabolic functions, or production of compounds, intestinal physiology may be compromised or impaired. Some bacteria, due to their fitness or ability to produce natural protective antibiotics and stimulate immunity, plays a protective role in healthy gut by exerting a colonization resistance to pathogenic bacteria {Kristie, 2014} {Sophie, 2014}. Therefore, the decrease of the diversity and/or on the protective bacterial load in the microbiome allows opportunistic colonization of the intestinal lumen by harmful microorganisms that normally are unable to out compete the dominant organisms. In fact, microbiome dysbiosis increase the susceptibility to pathogens as it can be unable to restrict proliferation of opportunists (usually some less frequent bacterial pathogens) that are able to trigger an infection. It may also leads to other disease states such as: inflammatory bowel disease, ulcerative colitis, rheumatoid arthritis, type 1 diabetes, obesity, atopy and asthma {Yong, 2017} {Turnbaugh, 2009; Turnbaugh, 2009; Turnbaugh, 2007}.

In fact dysbiosis (due to antibiotherapy or other causes) is similar to equilibrium disruptions in other ecossystems. Figure 2 represents a forest ecosystem decimated by a fire (left side of the figure). In this environment, the lack of some key species - like the predator owl – will disrupt the ecossytem equilibrium allowing the proliferation of invading organisms – the rat prey. Reforestation with endogenous species (represented by the water can on the right-hand side of the figure) allows the restoration of the healthy ecosystem that keeps invasive (harmful) species at low, non-threatening levels.

Fig. 2 Representation of the effect of reforestation and repopulation of a burned forest (where there was extinction of species) that restore ecossystem equilibrium and prevents the colonization by harmful invasive species.

Clostridium sp. can be one of these harmfull opportunistic colonizers of the human gut. They are fastidious growing gram-positive spore-forming bacilli, mostly strict anaerobes. As they share a thick cell wall, they can persist as spores in a vegetative or dormant state when the environmental conditions are unfavourable. The spores are very resistant and thus very difficult to eliminate. They can be the etiological agents of nosocomial infections and are a concern in hospitals and health care facilities {Vincent, 2015} as many species of this genus are able to synthesise and release an arsenal of toxins

that are very harmful to the human host and can cause human disease such as: botulism, gas gangrene, sepsis or tetanus.

One example is *Clostridium difficile* that can naturally colonize the gut microbiome of healthy individuals, yet in very low densities. During the last few decades specimens of *C. difficile* has begun to be detected in stool cultures of patients with gastrointestinal disease that have underwent antibiotic therapy {Gerding, 1995}{Thomas, 2003}. Antibiotherapy, in particular with antibiotics such as: ampicillin, clindamycin, fluoroquinolones, and cephalosporins, have been associated with the microbiota disruption (dysbiosis), and overgrowth of *C. difficile*. *C. difficile* is able to generate an opportunistic infection by producing and releasing two similar toxins: enterotoxin (TcdA) and cytotoxin (TcdB) {Pérez-Cobas , 2015}. They are both responsible for triggering pseudomembranous colitis (PMC), an inflammatory disease that involves damage of the intestinal mucosa, a severe ulceration of the colon, haemorrhagic necrosis, and eventually septicaemia when bacteria enter the bloodstream, a situation that can cause septic shock and death {Theriot, 2016} {Theriot, 2016}. They can also code for an arsenal of other virulence factors, like adhesins, that allow them to stick to human cells, and hyaluronidase that dissolves tissues, allowing the progression of the bacteria.

Treating PMC may involve long-term antibiotic therapy. But a decade ago, The New York Times reported the case of a woman that was admitted to a Minnesota state hospital in 2008, with severe diarrhoea due to *C. difficile* infection, unresponsive to a cocktail of antibiotics, that had lost over 12 Kg weight in eight months. The physician Alexander Khoruts tried a non-canonical new procedure: to ask the husband of the patient to donate a stool sample to be transplanted into her intestine. Not only the woman survived the fatal infection, she had recovered overnight and got cured. Two weeks after transplantation a microbiological analysis showed clearly that her husband's bacteria had recolonized and replaced her abnormal gut microbiome. Faecal transplantation has been used for over 50 years, but now it is a very promising and very demanded medical procedure for a myriad of diseases linked to the gut microbiome {LeBeau, 2014} {Borody, 2012} {Kang, 2017}. Since then faecal microbiota transplant ("stool transplant") has been repeatadly used in recurrent debilitated patients {Gerding, 1995}{Cohen, 2017} {Fitzpatrick, 2008}{Rao, 2016}. During faecal microbiota transplantation (FMT) a healthy individual donates its intestinal microbiota to restore the intestinal environment of a diseased individual. A stool sample from a healthy person is blended in a saline solution and surgically injected into recipient patient, either through the nose or mouth into the small bowel, or into the colon by colonoscopy. The new colonizers composed of an healthy community will restore the protective effect against harmful bacteria like Clostridia. This effect is represented in the right part of the cartoon (Fig.2), by the water can (that symbolizes an enrichment with bacterial strains that restore an equilibrium in a microbiome), and the owl that represents the protective effect of the healthy microbiome against opportunistic pathogens like Clostridia. Recent studies confirmed that FMT is a useful and valuable tool to treat various chronic gastrointestinal diseases as it increases significantly the species richness {Gu, 2016}.

The impacts of microbiome dysbiosis and the effects of faecal transplantations clearly highlight that you are not the only one in charge of your body and your health. You count with the indispensable help of a

community of millions of microscopic helpers that complement your own genome and cells. Keeping these community healthy is fundamental for your own health!

ACKNOWLEDGEMENTS:

This work is financially supported by National Funds through FCT – Fundação para a Ciência e a Tecnologia, I.P., under the project UID/CED/00194/2013. XSP is supported by Programa Operacional Capital Humano, Portugal 2020, European Social Fund and National Funds FCT/MEC (PIDDAC), through the SFRH/BPD/103613/2014 research grant.

REFERENCES:

(2017). "Meet Your Microbiome." Retrieved 28-07-2017, from http://www.amnh.org/explore/science-topics/health-and-our-microbiome/meet-your-microbiome.

Adlerberth, I., D. P. Strachan, et al. (2007). "Gut microbiota and development of atopic eczema in 3 European birth cohorts." Journal of Allergy and Clinical Immunology 120(2): 343-350.

Arumugam, M., J. Raes, et al. (2011). "Enterotypes of the human gut microbiome." Nature 473(7346): 174-180.

Bergstrom, A., T. H. Skov, et al. (2014). "Establishment of Intestinal Microbiota during Early Life: a Longitudinal, Explorative Study of a Large Cohort of Danish Infants." Applied and Environmental Microbiology 80(9): 2889-2900.

Blakey, J. L., L. Lubitz, et al. (1982). "Development of Gut Colonisation in Pre-term Neonates." Journal of Medical Microbiology 15(4): 519-529.

Blaser, M. J. (2016). "Antibiotic use and its consequences for the normal microbiome." Science (New York, N.Y.) 352(6285): 544-545.

Blaser, M. J. and S. Falkow (2009). "What are the consequences of the disappearing human microbiota?" Nat Rev Microbiol 7.

Bodelier, P. (2011). "Toward Understanding, Managing, and Protecting Microbial Ecosystems." Frontiers in Microbiology 2(80).

Borody, T. J. and A. Khoruts (2012). "Fecal microbiota transplantation and emerging applications." Nat Rev Gastroenterol Hepatol 9(2): 88-96.

Brown, J. P. (1977). "Role of gut bacterial flora in nutrition and health: a review of recent advances in bacteriological techniques, metabolism, and factors affecting flora composition." CRC Crit Rev Food Sci Nutr 8.

Cho, I. and M. J. Blaser (2012). "The human microbiome: at the interface of health and disease." Nat Rev Genet 13(4): 260-270.

Cohen, N. A. and N. Maharshak (2017). "Novel Indications for Fecal Microbial Transplantation: Update and Review of the Literature." Digestive Diseases and Sciences 62(5): 1131-1145.

Collado, M. C., S. Delgado, et al. (2009). "Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR." Letters in Applied Microbiology 48(5): 523-528.

Collado, M. C., E. Isolauri, et al. (2010). "Effect of mother's weight on infant's microbiota acquisition, composition, and activity during early infancy: a prospective follow-up study initiated in early pregnancy." The American Journal of Clinical Nutrition 92(5): 1023-1030.

Consortium, T. H. M. P. (2012). "Structure, function and diversity of the healthy human microbiome." Nature 486(7402): 207-214.

Cundell, A. M. (2016). "Microbial Ecology of the Human Skin." Microbial Ecology.

Dethlefsen, L., S. Huse, et al. (2008). "The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing." PLoS Biol 6.

Dominguez-Bello, M. G., E. K. Costello, et al. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns.

Eckburg, P. B., E. M. Bik, et al. (2005). "Diversity of the human intestinal microbial flora." Science 308.

Edwards, C. A. and A. M. Parrett (2002). "Intestinal flora during the first months of life: new perspectives." British Journal of Nutrition 88(S1): s11-s18.

Fallani, M., D. Young, et al. (2010). "Intestinal Microbiota of 6-week-old Infants Across Europe: Geographic Influence Beyond Delivery Mode, Breast-feeding, and Antibiotics." Journal of Pediatric Gastroenterology and Nutrition 51(1): 77-84.

Fanaro, S., R. Chierici, et al. (2003). "Intestinal microflora in early infancy: composition and development." Acta Pædiatrica 92: 48-55.

Finegold, S. M., V. L. Sutter, et al. (1983). Normal indigenous intestinal flora. Human intestinal microflora in health and disease. D. J. Hentges. New York, Academic Press, Inc.

Fitzpatrick, F., A. Oza, et al. (2008). "Laboratory diagnosis of Clostridium difficile-associated disease in the Republic of Ireland: a survey of Irish microbiology laboratories." Journal of Hospital Infection 68(4): 315-321.

Gerding, D. N., S. Johnson, et al. (1995). "Clostridium Difficile-Associated Diarrhea and Colitis." Infection Control & Hospital Epidemiology 16(8): 459-477.

Grice, E. A., H. H. Kong, et al. (2009). "Topographical and temporal diversity of the human skin microbiome." Science 324.

Grice, E. A. and J. A. Segre (2011). "The skin microbiome." Nat Rev Micro 9(4): 244-253.

Gu, J.-L., Y.-Z. Wang, et al. (2016). "Gut microbiota community adaption during young children fecal microbiota transplantation by 16s rDNA sequencing." Neurocomputing 206: 66-72.

Higgins, M. W. (1984). "The Framingham Heart Study: review of epidemiological design and data, limitations and prospects." Prog Clin Biol Res 147.

Hold, G. L., S. E. Pryde, et al. (2002). "Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis." FEMS Microbiol Ecol 39.

Holzapfel, W. H. and U. Schillinger (2002). "Introduction to pre- and probiotics." Food Research International 35(2): 109-116.

Kang, D.-W., J. B. Adams, et al. (2017). "Microbiota Transfer Therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: an open-label study." Microbiome 5: 10.

Kilian, M., I. L. C. Chapple, et al. (2016). "The oral microbiome - an update for oral healthcare professionals." Br Dent J 221(10): 657-666.

Konkel, L. (2013). The Environment Within: Exploring the Role of the Gut Microbiome in Health and Disease. Environmental Health Perspective. 121.

Kristie, M. K., Y.-D. Sophie, et al. (2014). "Effects of Antibiotics on Human Microbiota and Subsequent Disease." Annual Review of Microbiology 68(1): 217-235.

Larsson, D. G. J. (2014). "Antibiotics in the environment." Upsala Journal of Medical Sciences 119(2): 108-112.

LeBeau, S. and A. Khoruts (2014). "Fecal Microbiota Transplantation: An Interview With Alexander Khoruts." Global Advances in Health and Medicine 3(3): 73-80.

Lederberg, J. (2001) "'Ome Sweet 'Omics-- A Genealogical Treasury of Words." The Scientist Volume, DOI:

Lloyd-Price, J., G. Abu-Ali, et al. (2016). "The healthy human microbiome." Genome Medicine 8(1): 51.

Orrhage, K. and C. E. Nord (1999). "Factors controlling the bacterial colonization of the intestine in breastfed infants." Acta Pædiatrica 88: 47-57.

Pérez-Cobas , A., A. Moya, et al. (2015). "Colonization Resistance of the Gut Microbiota against Clostridium difficile." Antibiotics 4(3): 337.

Rao, K. and N. Safdar (2016). "Fecal microbiota transplantation for the treatment of Clostridium difficile infection." Journal of Hospital Medicine 11(1): 56-61.

Shafquat, A., R. Joice, et al. (2014). "Functional and phylogenetic assembly of microbial communities in the human microbiome." Trends in Microbiology 22(5): 261-266.

Sophie, Y.-D., A. Marie-Claire, et al. (2014). "Gastrointestinal Microbiota-Mediated Control of Enteric Pathogens." Annual Review of Genetics 48(1): 361-382.

Suau, A., R. Bonnet, et al. (1999). "Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut." Appl Environ Microbiol 65.

Theriot, C. M., A. A. Bowman, et al. (2016). "Antibiotic-Induced Alterations of the Gut Microbiota Alter Secondary Bile Acid Production and Allow for Clostridium difficile Spore Germination and Outgrowth in the Large Intestine." mSphere 1(1).

Theriot, C. M. and V. B. Young (2016). "Interactions Between the Gastrointestinal Microbiome and Clostridium difficile." Annual review of microbiology 69: 445-461.

Thomas, C., M. Stevenson, et al. (2003). "Antibiotics and hospital-acquired Clostridium difficile-associated diarrhoea: a systematic review." Journal of Antimicrobial Chemotherapy 51(6): 1339-1350.

Turnbaugh, P. J. and J. I. Gordon (2009). "The core gut microbiome, energy balance and obesity." J Physiol 587.

Turnbaugh, P. J., M. Hamady, et al. (2009). "A core gut microbiome in obese and lean twins." Nature 457.

Turnbaugh, P. J., R. E. Ley, et al. (2007). "The human microbiome project." Nature 449.

Vaishampayan, P. A., J. V. Kuehl, et al. (2010). "Comparative Metagenomics and Population Dynamics of the Gut Microbiota in Mother and Infant." Genome Biology and Evolution 2: 53-66.

van Houte, J., R. J. Gibbons, et al. (1972). "Ecology of Human Oral Lactobacilli." Infection and Immunity 6(5): 723-729.

Vincent, C. and A. Manges (2015). "Antimicrobial Use, Human Gut Microbiota and Clostridium difficile Colonization and Infection." Antibiotics 4(3): 230.

Voreades, N., A. Kozil, et al. (2014). "Diet and the development of the human intestinal microbiome." Frontiers in Microbiology 5: 494.

Wang, X. and G. R. Gibson (1993). "Effects of the in vitro fermentation of oligofructose and inulin by bacteria growing in the human large intestine." Journal of Applied Bacteriology 75(4): 373-380.

Yong, E. (2017). Nós, os microbios e uma visão alargada da vida. Lisboa, Círculo de Leitores.

Appendix B

To take or not to take: what to do in antibiotic treatments.

Problem: **To take or not to take: what to do in antibiotic treatments.**

Curricular contextualisation in Portugal: 9th grade. *Individual and community health: 1.6- relate antibiotic misuse with frequency increase of antibiotic resistance.*

Predicted time needed: 120 minutes

Educational goals:

- Recognize the importance of keeping effective antibiotics
- Understand the effects of antibiotic misuse for individual health and for the evolution of antibiotic resistant bacteria.
- Understand the principles of natural selection and how this process leads to environmental adaptation
- Recognize how science knowledge can be used to inform our daily life choices and how it impacts individual and social well being
- Understand the applications of mathematical models in science and their limitations and be able to use these to test predictions and hypotheses.
- Develop scientific skills namely data analyses, data interpretation, scientific discussion based on scientific evidence.

To take or not to take: what to do in antibiotic treatments.

Case: *To take or not to take*: what to do in antibiotic treatments

Last Monday, João's classmate has coughed the entire day. On the following day, João started to feel sick and with fever. On Wednesday, three days after he was with his classmate, João went to the doctor who diagnosed him with a bacterial infection and prescribed him an antibiotic that he would had to take for 8 days. João immediately started the prescribed treatment and two days after he was already feeling perfectly well. He then started to wonder if he should still take the antibiotic. In fact, he had recently read an article in a magazine about scientific studies suggesting that people should interrupt antibiotic treatment as soon as they were no symptoms.

To help João decide about what to do, run the software SimulATe using the parameters that match his infection history (depicted above) and fill in Table 1 with the expected results for each possible alternative option.

Infection history parameters:

Default options for all parameters except

Symptoms at infectious level of 10^4

Death infectious level - 10^8

Antibiotic treatment starting three days after the infection

Discussion leading questions:

- What was the change observed in the frequency of resistant bacterial during the first and second day of infection and what was the cause for such change?
- What was the change observed in the frequency of resistant bacterial during the 3rd and 5th of infection and what was the cause for such changes?
- How do you explain the differences in outcomes between the distinct simulated scenarios in Table 1?
- During the simulation, what factor caused the decrease of antibiotic resistant bacteria?

- Based on these results what advises would you give to João?
- Would these results hold for other infections with distinct parameters? What would be your predictions and how can you test these?
- Will the simulator results always be true? What could be the limitations to the use of this simulator results? And how can we apply the results of this simulator to inform our choices?
- From your results what is the best procedure to treat the infection and avoid the frequency increase of resistant bacteria?

References and additional sources of information:

https://www.ted.com/talks/maryn_mckenna_what_do_we_do_when_antibiotics_don_t_work_any_more

https://www.youtube.com/watch?v=znnp-lvj2ek

Additional activities:

- ✓ Ask your students to make an educational campaign in school to promote the wise use of antibiotics by other students.

Appendix 1: Table1 – Please fill this table with SimulATe results

Scenario	Relative Frequency of bacteria resistant to antibiotic at the				Final result of João's choices
	Initial infection	3rd day	5th day	8th day	
No antibiotic treatment					
Prescribed antibiotic treatment					
Prescribed antibiotic treatment interrupted at the 5th day					
Prescribed antibiotic treatment interrupted at the 5th day and restarted at the 6th day					