UNIVERSITI
MALAYSIA
KELANTAN

DETERMINATION OF OCCURRENCE OF *ESCHERICHIA COLI* ATCC 25922 ANTIBIOTIC RESISTANCE MUTANTS AS A RESULT OF EXPOSURE TO SUBINHIBITORY CONCENTRATION OF β-LACTAM ANTIBIOTIC, AMPICILLIN.

Latha S Davandran

J20A0462

A thesis submitted in fulfilment of the requirements for the degree of Bachelor of Applied Science (Bioindustrial Technology) with Honours

FACULTY OF BIOENGINEERING AND TECHNOLOGY

UMK

2023

DECLARATION

I declare that this thesis entitled "Determination of Occurrence of *Escherichia Coli* ATCC 25922 Antibiotic Resistance Mutants as A Result of Exposure to Subinhibitory Concentration of β-lactam Antibiotic, Ampicillin." is the results of my own research except as cited in the references.

Signature : 

Student's Name : LATHA A/P S DAVANDRAN

Date : 7/03/2024

Verified by:

Signature : _____

Supervisor's Name : DR WEE SENG KEW

Stamp : _____

Date : _____

ACKNOWLEDGEMENT

I am deeply grateful to all those who have contributed to enhancing my knowledge and practical skills, particularly in the research area. Their guidance and encouragement have been invaluable, motivating me to explore new avenues and pursue excellence. I extend my deepest gratitude to Dr. Wee Seng Kew, my supervisor, whose expertise, and wisdom have been instrumental in shaping this project. His insightful feedback and constructive criticism have played crucial role in its development and refinement. I am truly grateful for his dedication, which is evident through his consistent availability, as exemplified by our weekly Tuesday group meetings where he ensures our progress is on track. I offer sincere thanks to Kak Ain Atiqah and Syafiqa for their invaluable contributions in teaching laboratory skills and their readiness to provide guidance whenever I needed clarification. I am deeply indebted to Yithenthrathevi Nair for her continuous encouragement and unwavering moral support, as well as her willingness to engage in discussions about my topic, enriching my understanding. I am also grateful to my friends, who have made this journey fulfilling and enjoyable. Together, we have navigated challenges, celebrated victories, and grown as scholars and individuals. Finally, I would like to express my heartfelt appreciation to my mother and my family for their unwavering support and understanding throughout this endeavour. Their encouragement and belief in me have served as a constant source of motivation, sustaining me through the inevitable ups and downs of the research process.

Penentuan Kejadian Mutan Perintang Antibiotik *Escherichia coli* ATCC 25922 sebagai Akibat Daripada Pendedahan kepada Konsentrasi Subinhibitory Antibiotik β-laktam, Ampicilin.

ABSTRAK

Kajian ini meneroka kemunculan mutan perintang antibiotik dalam *Escherichia coli* ATCC 25922 akibat daripada pendedahan kepada kepekatan subinhibitory antibiotik beta-laktam ampicilin. *Escherichia coli* ATCC 25922 telah terdedah kepada pelbagai kepekatan ampicilin, yang mendedahkan kesan bergantung kepada kepekatan terhadap pertumbuhan bakteria. Kepekatan subinhibitory menunjukkan kesan hormesis, meningkatkan pertumbuhan semasa fasa eksponensial. Ampicilin bawah MIC menginduksi mutagenesis, menyiratkan potensi bagi respon adaptif di bawah tekanan alam sekitar. Kultur yang terdedah kepada 5 µg/mL ampicilin menunjukkan tindak balas bergantung kepada kepekatan, menunjukkan kemunculan strain yang tahan. Sebaliknya, kepekatan yang lebih tinggi memberi kesan perintang. Siasatan ini menekankan keseimbangan yang rumit antara kepekatan perintang dan subinhibitory, menekankan keperluan untuk strategi dos yang disempurnakan dalam pentadbiran antibiotik. Kajian ini memberikan wawasan berharga tentang interaksi dinamik antara antibiotik dan populasi bakteria, menawarkan pandangan mengenai implikasi untuk strategi dos yang berkesan dalam memerangi perintang antibiotik.

Kata kunci: *Escherichia coli* ATCC 25922, Mutan perintang antibiotik, Antibiotik beta-laktam, Ampicillin.

Determination of Occurrence of *Escherichia coli* ATCC 25922 Antibiotic Resistance Mutants as a Result of Exposure to Subinhibitory Concentration of β-lactam Antibiotic, Ampicillin.

ABSTRACT

This study explores the emergence of antibiotic resistance mutants in *Escherichia coli* ATCC 25922 resulting from exposure to subinhibitory concentrations of the beta-lactam antibiotic ampicillin. *Escherichia coli* ATCC 25922 was exposed to various concentrations of ampicillin, revealing concentration-dependent effects on bacterial growth. Subinhibitory concentrations demonstrated a hormesis effect, enhancing growth during the exponential phase. Sub-MIC ampicillin induced mutagenesis, implying the potential for adaptive responses under environmental stress. Cultures exposed to 5 µg/mL ampicillin exhibited a concentration-dependent response, indicating the emergence of resistant strains. Conversely, higher concentrations exerted inhibitory effects. The investigation underscored the intricate balance between inhibitory and subinhibitory concentrations, emphasizing the necessity for refined dosing strategies in antibiotic administration. This study provides valuable insights into the dynamic interactions between antibiotics and bacterial populations, offering perspectives on the implications for effective dosing strategies in combating antibiotic resistance.

Keywords: *Escherichia coli* ATCC 25922, Antibiotic resistance mutants, Beta-lactam antibiotic, Ampicillin

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

MIC →	Minimum Inhibitory Concentration
E. coli →	*Escherichia Coli*
MGE →	Mobile Genetic Elements
MDR →	Multi-drug Resistance
MRSA →	Methicillin-resistant *Staphylococcus aureus*
RNA →	Ribonucleic Acid
DNA →	Deoxyribonucleic Acid
PBP →	Penicillin-binding proteins
DMSO →	Dimethyl sulfoxide
CFU →	Colony forming units.
+Amp →	With ampicillin
-Amp →	Without ampicillin

LIST OF SYMBOLS

℃	Degree Celsius
µg	Micro Gram
µL	Micro Litter
rpm	Rotation per minute
mL	Milliliter

CHAPTER 1

INTRODUCTION

1.0 Background of study

Antibiotics, which are common medicinal substances, are used to stop bacteria from multiplying by either destroying their cells or preventing their growth (Kümmerer, 2009). Among these antibiotics, beta-lactams such as penicillins, cephalosporins, monobactams, and carbapenems, are commonly prescribed for the management and treatment of bacterial infections. Their effectiveness stems from the presence of a beta-lactam ring, which plays a vital role in their antibacterial activity. Beta-lactam antibiotics have a wide range of clinical applications and have significantly transformed the approach to combating bacterial infectious diseases since their development in the 1930s. The annual expenditure on these antibiotics is approximately $15 billion USD, accounting for 65% of the total antibiotics market (Thakuria & Lahon, 2013). However, the widespread use of antibiotics has resulted in increased excretion and environmental release, contributing to the emergence of drug-resistant bacterial strains. This global issue poses a significant threat to human health.

Microorganisms are frequently exposed to antibacterial medication concentrations below the minimal inhibitory concentration (MIC), termed as sub-inhibitory concentration (sub-MIC) (Davies et al., 2006). According to research, antibacterial drugs at sub-MIC levels can cause bacterial genetic and phenotypic changes, such as the enrichment of already drug-resistant bacteria, the emergence of new drug-resistant strains, the development of persister cells, and an impact on the formation of bacterial biofilms, among other outcomes (Andersson & Hughes, 2014). Certain antibacterials have been discovered to activate the bacterial SOS response at sub-MIC concentrations, increasing mutation rates and lateral gene transfer (Baharoglu & Mazel, 2014). Furthermore, it has been suggested that antibacterial sub-MIC levels rather than fatal concentrations may be more pertinent to the problem of bacterial resistance (Laureti et al., 2013).

Microbial mutation refers to the genetic alterations that take place in microorganisms, such as bacteria or fungi, as a result of exposure to antibiotics. When these microorganisms come into contact with antibiotics, their genetic code may undergo changes known as mutations.

These mutations can bring about various modifications in the biology of the microorganism, including its ability to withstand the effects of antibiotics. Consequently, microbial mutations can lead to the development of antibiotic resistance, wherein microorganisms become less susceptible to the inhibitory or lethal actions of antibiotics. Antibiotic resistance can arise through different mechanisms, such as mutations that modify or deactivate the target of the antibiotic, enhance the activity of drug efflux pumps to expel the antibiotic from the cell, or alter metabolic pathways to bypass the antibiotic's mode of action. Mutation stands as one of the primary mechanisms contributing to antibiotic resistance in bacteria (Woodford & Ellington, 2007).

Escherichia coli (E.coli), a seemingly ubiquitous Gram-negative bacterium, is best known for its ability to cause food-borne outbreaks. The strain ATCC 25922 is a commonly used quality control strain, particularly in antibody sensitivity assays and was originally isolated from a human clinical sample collected in Seattle and WA (1946).

1.2 Problem statement

The emergence and persistence of antibiotic resistance poses a significant challenge to healthcare systems worldwide. Beta-lactam antibiotics, being one of the most prescribed drug classes, are crucial for treating bacterial infections. However, the increased prevalence of antibiotic resistance has led to adverse consequences, including higher morbidity and mortality rates, prolonged hospitalization periods, and limited treatment options. Understanding the factors contributing to the emergence of antibiotic resistance in beta-lactam antibiotics, such as ampicillin, is essential for developing effective strategies to mitigate its impact. Therefore, this study aims to investigate the occurrence and potential implications of antibiotic resistance mutants in *E. coli* strain ATCC 25922 resulting from exposure to subinhibitory concentrations of beta-lactam antibiotics, shedding light on the biosafety considerations associated with their use.

1.3 Objectives

1. To determine subinhibitory concentration of beta lactam, ampicillin to *E. coli* ATCC 25922
2. To evaluate Ampicillin resistant capability of antibiotic resistance mutant as a result of exposure to a subinhibitory concentration of beta lactam, Ampicillin.

1.4 Scope of study

The scope of this study is to assess the occurrence and persistence of antibiotic resistance mutants in *E. coli* ATCC 25922 exposed to a subinhibitory concentration of Ampicillin. The research is designed as a statistical study, allowing for quantitative analysis and reporting of findings. The limitation of the study is the exclusion of genotypic assays to identify specific genetic changes associated with antibiotic resistance.

1.5 Significance of study

The significance of this study lies in its contribution to the understanding of microbial mutation and antibiotic resistance, particularly in relation to beta-lactam antibiotics. By evaluating the occurrence and persistence of antibiotic resistance mutants in *E. coli* exposed to subinhibitory concentrations of ampicillin, this research provides valuable insights into the adaptive response of bacteria to antibiotic stress. Understanding the mechanisms and dynamics of antibiotic resistance development can aid in the development of more effective strategies for combating antibiotic resistance and improving biosafety practices. Furthermore, the study's focus on statistical analysis provides a quantitative approach to assess the impact of subinhibitory antibiotic exposure on resistance development, offering valuable data for future research and decision-making in the field of antimicrobial stewardship.

This study holds significant importance in understanding the occurrence and persistence of antibiotic resistance mutants in *E. coli* strain ATCC 25922. By evaluating the impact of subinhibitory concentrations of beta-lactam antibiotic, specifically ampicillin, the study aims to shed light on the development of antibiotic resistance. The findings will

contribute to antibiotic resistance surveillance, providing valuable insights for monitoring resistance patterns and optimizing antibiotic use. Additionally, the study will enhance biosafety practices by highlighting the need to manage subinhibitory concentrations to prevent inadvertent promotion of resistance. Furthermore, assessing the survival and growth of antibiotic-resistant mutants in the presence of diverse beta-lactam antibiotics will aid in identifying effective treatment options and guiding clinicians in selecting appropriate therapies.

CHAPTER 2

LITERATURE REVIEW

2.1 History of antibiotics

The discovery of antibiotics undoubtedly stands as one of the most significant advancements in the treatment of infectious diseases in human history. Prior to the existence of antibiotics, numerous individuals succumbed to bacterial infections before reaching adulthood (Guyer et al., 2000). The introduction of penicillin in the 1940s marked a ground-breaking milestone, as it was a remarkable medication with greater potency compared to the antiseptics available at the time, while also being relatively non-toxic to humans (Hewitt, 1967). Antibiotics not only provided a cure for many previously life-threatening diseases but also had a profound and indirect impact on society as a whole by significantly extending the average lifespan of the population (Lederberg, 2000). Between 1937 and 1952, the mortality rate in the United States attributable to infectious diseases experienced a steep decline of 8.2% annually, dropping from 283 deaths per 100,000 individuals to 75 (Armstrong et al., 1999).

During the "golden age" of antibiotics in the 1950s and 1960s, numerous new classes of antibiotics were introduced to clinical practice, resulting in a larger arsenal of antibiotics than ever before (Bérdy, 2012). This historical period was characterized by optimism as several infectious illnesses seemed to be on the approach of extinction. WHO scientists discussed the potential "eradication of infectious diseases," which would be equivalent to the successful eradication of smallpox by vaccination in 1979. (Snowden, 2008). Many prominent researchers in the field held the belief that humanity was nearing a solution to the problem of bacterial infections. Unfortunately, this viewpoint influenced the priorities of pharmaceutical research and development. With the perception that the issue of bacterial infections had been resolved, the focus shifted towards developing drugs targeting cancer, cardiovascular diseases, diabetes, and various other ailments. This shift resulted in an "innovation gap," a period spanning almost 40 years from 1962 to 2000 when no new classes of antibiotics were introduced to clinical practice (Fischbach & Walsh, 2009).

Regrettably, over the past 70 years, the extensive utilization and improper handling of antibiotics have subjected bacteria to significant selective pressure, driving the evolution of resistance. Consequently, a substantial number of pathogenic bacteria encountered in clinical

settings have developed resistance to multiple antibiotics. The majority of naturally occurring antibiotics that have been identified originate from soil microorganisms, particularly bacteria belonging to the genera Streptomyces and Actinomyces (Aminov, 2009).

2.2 Antibiotics

Antibiotics are a class of chemicals that stop the growth of bacteria by either killing them (bactericidal) or limiting their ability to divide (bacteriostatic). Although the phrases "antibiotic" and "antimicrobial" are frequently used interchangeably, they do not necessarily mean the same thing. While "antimicrobial" refers to any chemical, including synthetic compounds, that has the ability to kill germs, "antibiotics" particularly refer to drugs generated from microorganisms, for example penicillin (Guardabassi & Courvalin, 2005). Antibiotics are commonly employed to treat and prevent illnesses in both people and animals. Effective antibiotics have a very positive influence on lowering the number of deaths brought on by bacterial infections, including basic skin infections as well as bloodstream, lung, stomach, and even brain infections. It's critical to comprehend how antimicrobial agents operate in order to comprehend the mechanisms of antibiotic resistance. Targeting the bacterial cell wall, which is found in prokaryotic bacteria but missing in eukaryotic human cells, is one of the most popular strategies. Due to their selectivity, antimicrobial drugs can target vital bacteria processes while minimizing or avoiding negative effects on host processes. Different kinds of antibiotics have unique mechanisms of action that allow them to stop the growth of bacteria or kill existing ones (John & Sons, 2001).

2.3 Antibiotic resistance

Antibiotic resistance refers to the ability of bacteria or other germs to live and proliferate upon exposure of antibiotic doses that were previously thought to be successful in combating them (*Tackling antibiotic resistance from a food safety perspective in Europe / World Health Organization Regional Office for Europe*, 2011). Although studies on clinical isolates obtained before the introduction of antibiotics have shown susceptibility, even in the presence of conjugative plasmids, the precise cause of antibiotic resistance genes are not well understood (Stephen P. Denyer, 2011).

In a susceptible bacterial population, the majority of cells are normally vulnerable to a specific antibiotic when exposed to it. However, there is always a small sub-population of bacterial cells that possess resistance and are capable of multiplying at higher concentrations of the antibiotic, even when the concentration is insufficient to eliminate the sub-population completely. This phenomenon allows these microorganisms to survive in the environment (Smith et al., 2005). It has been proposed that limiting antibiotic use may exert selective pressure that advantages healthier, susceptible bacteria, allowing them to eventually outcompete resistant species. Resistance is frequently associated with a decline in bacterial fitness (Levin et al., 1997).

Because illnesses caused by antibiotic-resistant bacteria are more challenging and costly to treat, they are a serious public health problem. Several forms of Salmonella, for example, have evolved resistance to a number of medicines during the 1990s. The use of antibiotics in human and animal husbandry is hypothesized to have resulted in antibiotic resistance. The emergence of multidrug resistance, or resistance to a wide range of antimicrobial drugs, is posing an enormous obstacle in clinical practice today (Amenu, 2014).

The infecting bacterium may acquire antibiotic resistance or inherit it. Resistance can develop by mutation or the transfer of extrachromosomal genetic material, and then during treatment, resistant organisms are chosen (Courvalin, 2005). Genetic mutation is the most straightforward way for a microbe to gain resistance. When exposed to antibiotics, resistant mutants will have a large survival advantage. As a result, populations will utilize antibacterial drugs more frequently, and the proportion of isolates that are resistant to those agents will rise (Hegstad et al., 2010).

2.4 Mechanism of antibiotic resistance

Antibiotics can kill or inhibit the development and proliferation of bacteria in a variety of ways, and germs have developed a variety of resistance mechanisms as a result of antibiotic exposure. It is possible for an organism to develop resistance to a wide range of antibiotics via a single mechanism, particularly if the mechanisms of action are identical. Individual bacteria can occasionally share resistance by manufacturing "resistance plasmids," which are pieces of DNA that can be passed from one cell to another. (Clewell, 2014).

The transmission of resistance genes among organisms through mobile genetic elements (MGEs) is the primary and clinically significant cause of multi-drug resistance (MDR) in Gram-negative bacteria, surpassing resistance that arises from mutations. MGEs have the capability to transfer resistance mechanisms between different taxa, exemplified by the transfer of enterococci MGEs to *Staphylococcus aureus* (Hegstad et al., 2010).

A microbe is considered resistant when its susceptibility is significantly reduced compared to the original isolate or a group of susceptible strains. Resistance can occur due to mutations in essential structural or regulatory genes, or through the acquisition of genetic information horizontally (Courvalin, 2005).

Resistance can be classified into two types: intrinsic or natural resistance, which occurs when microorganisms lack target sites for specific drugs, resulting in their ineffectiveness (e.g., penicillin-resistant Mycoplasma species); or intrinsic resistance can arise due to low permeability of the drug across the microbial membrane, especially when there are differences in the chemical properties of the drug and the structure of the microbial membrane, particularly in the case of drugs that need to enter the microbial cell to exert their effects (e.g., many Enterobacteriaceae). The other type is acquired resistance, where a microorganism that is initially susceptible to a drug acquires mechanisms to evade its impact, enabling them to remain unaffected by the drug (Fluit et al., 2001).

Antibiotic inactivation can occur when a cell develops resistance by producing enzymes that render the medication ineffective or reduce its effectiveness. A prime example is the production of beta lactamases, which can break the beta-lactam rings found in antibiotics like penicillin. This breakdown of the beta-lactam ring prevents the antibiotic from binding to peptidoglycan precursors, thus hindering its action. However, as long as the organism continues to produce beta lactamases, the integrity of the cell wall is less likely to be compromised by penicillin or similar drugs (Sageman, 2015). The transfer of this resistance mechanism between bacteria can occur through the generation of R-plasmids and is commonly observed in strains of methicillin-resistant Staphylococcus aureus (MRSA) (Holcomb et al., 2008).

2.4.1 Reduced membrane permeability

Another way antibiotics can be rendered ineffective is by restricting the penetration of the drug into the bacterial cell. Gram-negative bacteria have an outer cell membrane, and the

drugs must pass through specialized channels known as cell pores. These pores span the outer membrane and facilitate the transport of substances in and out of the cell. In order for drugs to enter the cell or interact with the cell wall, they must effectively navigate through these pores (Joanne Willey, 2013). Mutation in a gene can alter the electrical charge or physical structure of pores, leading to a reduced ability of antibiotics to enter the bacterial cell. Although the antibiotic remains effective, it encounters difficulty reaching its intended target. This mechanism allows bacteria to develop resistance to multiple classes of medications simultaneously. It is worth noting that certain gram-negative bacteria are intrinsically resistant to certain drugs, such as vancomycin, which cannot enter the cell through the pores due to its large size, even without any mutations occurring.

2.4.2 Alteration of target site

Antibiotics often exert their action by interacting with specific molecular targets within bacteria. When the structure of the target molecule undergoes significant changes, the effectiveness of the antibiotic can be diminished as it loses its ability to bind to the altered target. For instance, tetracyclines bind to the transfer RNA access site and inhibit its function. Therefore, even minor modifications in the access site can result in microbial resistance to tetracyclines (Stephen P. Denyer, 2011).

2.4.3 Efflux or transportation of antibiotics

Microorganisms can develop antibiotic resistance through the action of efflux pumps. These biological pumps actively expel antibiotics out of the cell, preventing them from reaching or interacting with their target. This resistance mechanism often confers resistance to multiple classes of antibiotics, particularly macrolides, tetracyclines, and fluoroquinolones, as these antibiotics inhibit various aspects of protein and DNA synthesis and require intracellular presence to exert their effects (Joanne Willey, 2013). Genetic modifications in bacteria contribute to antibiotic resistance through four main mechanisms: structural alterations of target molecules to prevent antibiotic binding, reduction in membrane permeability to exclude antibiotics from cell entry, enzymatic degradation of antibiotics rendering them inactive, or efflux pumps pumping antibiotics out of the cell (Livermore, 2004).

2.5 β-lactams antibiotics

The class of antibiotics known as β-lactams is widely used in medical practice. These antibiotics share a common feature: a highly reactive four-membered β-lactam ring. This ring structure is crucial for their ability to kill bacteria. Typically, the β-lactam ring is combined with another type of ring called a heterocycle, resulting in a fused core structure. This bicyclic core undergoes modifications at specific positions, leading to various subclasses of β-lactam antibiotics, such as penicillins, cephalosporins, and carbapenems. The overall structures of β-lactam antibiotics, including their core scaffolds and substituents, play a vital role in determining their biological activities. These structural elements dictate how the antibiotics interact with target proteins and also influence the development of resistance mechanisms. Ampicillin belongs to the class of drugs called penicillins. Aminopenicillins, sometimes known as broad spectrum penicillins, include ampicillin. An oral active, broad-spectrum antibiotic, ampicillin is a semi-synthetic derivative of penicillin. (Lima et al., 2020).

2.6 Mechanism of Action

Ampicillin functions by inhibiting the final stage of peptidoglycan synthesis in the bacterial cell wall. It achieves this by specifically binding to one or more of the penicillin-binding proteins. This interaction prevents the biosynthesis of the cell wall, leading to the eventual lysis of the bacteria. Ampicillin demonstrates bactericidal effects against both gram-positive and gram-negative organisms. Its activity spectrum encompasses gram-positive bacteria like Streptococcus pneumoniae, various *streptococci*, and *Listeria monocytogenes*, as well as gram-negative bacteria such as *Moraxella catarrhalis*, *Neisseria meningitidis*, *Escherichia coli*, and *Salmonella*. The mode of action of ampicillin involves either inhibiting the formation of the bacterial cell wall or impeding its synthesis. It achieves this by binding to the enzymes responsible for constructing the cell wall structure, known as penicillin-binding proteins (PBPs). Ampicillin, like all penicillins, functions as a structural analogue of acyl-D-alanyl-D-alanine. By acylating the transpeptidase enzyme, which is crucial for the final step of peptidoglycan production, ampicillin interferes with the synthesis of the primary structural component of the cell wall (Baranowski et al., 2018).

2.7 Spectrum of resistance

The main pathway leading to ampicillin resistance involves the inactivation of the antibiotic by β-lactamases, which are hydrolytic enzymes that convert β-lactam compounds into inactive forms lacking antibacterial properties. Resistance to lactam antibiotics can also occur due to the absence of porin channels, proteins that regulate the permeability of the outer membrane in Gram-negative bacteria, thereby limiting the entry of antibacterial agents and their interaction with the bacterial environment. Another mechanism of resistance involves mutations in genes encoding penicillin-binding proteins (PBPs). Alterations in the structure of PBPs can significantly diminish their affinity for β-lactam antibiotics. It is worth noting that bacteria can possess multiple resistance mechanisms simultaneously. For instance, methicillin-resistant *Staphylococcus aureus* (MRSA) demonstrates resistance through the expression of the mecA gene, which leads to the production of an altered PBP2. Additionally, MRSA exhibits increased production of β-lactamases, contributing to its overall resistance to β-lactam antibiotics.

2.8 Antibiotic persistence

Antibiotic persistence also known as heterotolerance, refers to a bacterial subpopulation's ability to withstand large bactericidal drug concentrations to which the bacteria are totally sensitive (no change in MIC). One important feature of antibiotic persistence is biphasic death, with the susceptible population being killed quickly and the persister subpopulation being killed slowly. This phenomenon emphasizes phenotypic variation in bacterial cultures and the fact that not all bacteria in a clonal population get killed at the same pace. Unlike antibiotic resistance and heteroresistance, when a tiny subpopulation has a higher MIC for a short period of time, persistent bacteria cannot thrive in the presence of antibiotics. The terms antibiotic persistence and tolerance are frequently used interchangeably to describe an enhanced ability to survive in the presence of an antibiotic without a change in the minimum inhibitory concentration (MIC). However, there is a distinction between the two. Persistence refers to a specific subpopulation of bacteria that can survive antibiotic exposure, while tolerance refers to the ability of the entire bacterial population to withstand antibiotic effects. In other words, antibiotic persistence is a subset of tolerance, where a small fraction of persisters can endure antibiotic exposure, resulting in a biphasic killing curve.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Luria-Bertani agar (including NaCl, yeast extract, and tryptone, agar powder), LB broth (including NaCl, yeast extract, and tryptone), Ampicillin powder, Sterile distilled water, Ethanol or disinfectant, Distilled water, and *Escherichia coli* ATCC 25922.

3.2 Apparatus

Petri dish, Schott bottle, Dropper, Spatula, Falcon Tube, Beakers, Cuvette, Mask, Gloves, Eppendorf tubes, Sterile pipettes and pipette tips, Inoculation loop, Test tube, Micropipettes, Micropipette tips, Conical Flasks, Falcon Tube, 0.22 µm Nano Filter, parafilm, Syringe.

3.3 Methods

3.3.1 Bacterial Strain and Growth Condition

Escherichia coli ATCC 25922 was cultured at 37 °C in Luria-Bertani (LB) agar (10 g liter NaCl, 5 g liter^{1} yeast extract, 10 g liter^{1} tryptone, 16 g liter^{1} agar) for 2 days. A single colony of bacteria was inoculated into 5mL of LB broth (10 g liter^{1} NaCl, 5 g liter yeast extract, 10 g liter tryptone) and cultured in orbital shaker at 30 °C for 1 day to serve as seed culture.

3.3.2 Preparation of Ampicillin Stock Solution

To prepare the concentration of 40 mg/mL of ampicillin, 0.4 g of ampicillin powder was weighed out and added to the 10 ml of sterile distilled water. The ampicillin was filtered out in the sterile Falcon tube by using 0.22 µm of nano filter. Ampicillin stock solution was labelled and stored in 4 °C.

3.3.3 Antibiotic Mutagenesis

LB broths were supplemented with various ampicillin concentrations (80, 40, 20, 10, 5, 2.5, and 0 µg/mL). Cultures were incubated with *E. coli* at 30 °C for 2 days under aerobic conditions. Growth was monitored spectrophotometrically by measuring OD at 600 nm every 2 hours. Subsequently, cultures from the 0 µg/mL and 5 µg/mL conditions were sub-cultured into new LB broths containing 20 and 40 µg/mL ampicillin for further analysis. Each sub-culture was performed in duplicate.

3.3.4 Serial Dilution and Spread Plate

Serial dilution was performed to obtain countable single colonies for 0, 5, and 10 µg/mL concentrations. Diluted cell suspensions were spread on LB agar (control) and ampicillin agar (20 µg/mL). Plates were incubated at 37 °C for 48 hours, and bacterial growth was assessed by the appearance of single colonies.

3.3.5 Isolation and Characterization of Resistant Colonies

From the spread plates, one non-resistant and two resistant colonies were selected and inoculated into 5 mL LB broth. Each colony was grown separately in LB broth and LB broth with 20 µg/mL ampicillin. Spectrophotometric measurements were taken every 2 hours for 2 days. Additionally, the three colonies were streaked on normal LB agar and LB agar with 20 µg/mL ampicillin, followed by incubation at 37 °C for 48 hours. Bacterial growth was observed by the appearance of colonies.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Bacterial growth curve

Ampicillin, known for inducing mutagenesis in gram-negative bacteria, acts as a competitive inhibitor for transpeptidase during bacterial cell wall synthesis, leading to cell lysis (Sharma et al., 2013). This stress induction promotes mutagenesis in *E. coli* ATCC 25922. Aerobic growth in LB broth supplemented with ampicillin concentrations (80 µg/mL, 40 µg/mL, 20 µg/mL, 10 µg/mL, 5 µg/mL, 2.5 µg/mL, and 0 µg/mL) was monitored by measuring OD at 600 nm for 26 hours. Wild-type *E. coli* ATCC 25922 served as the positive control.

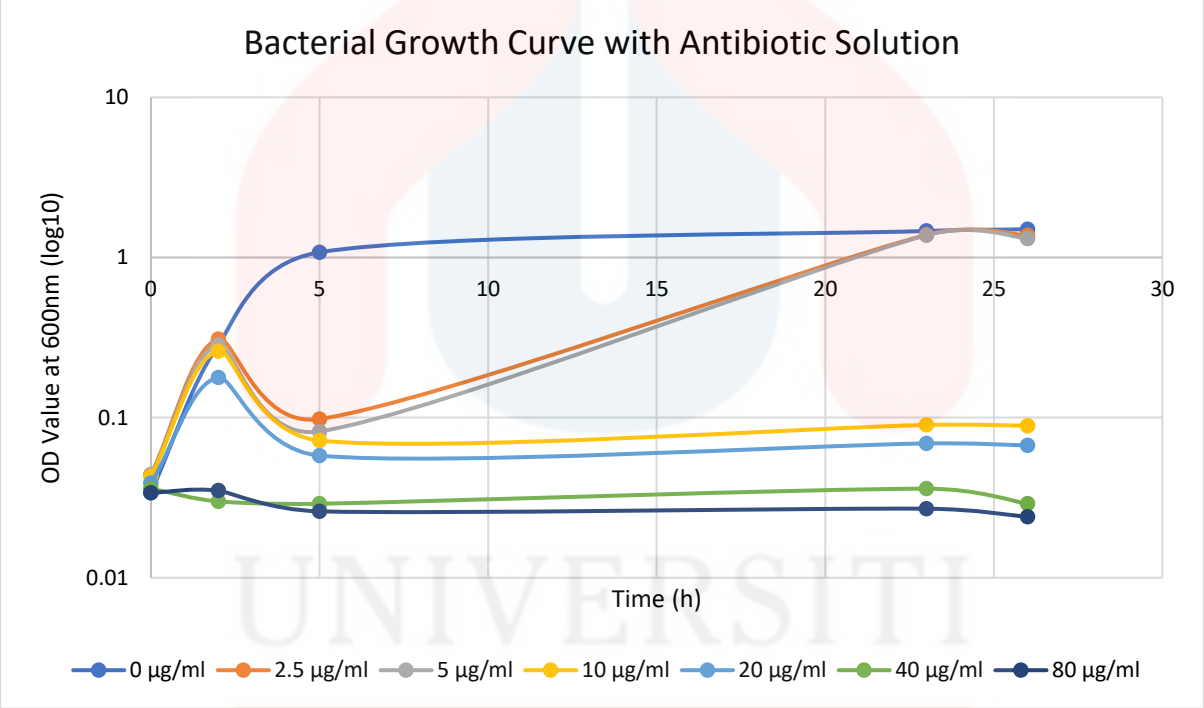


Figure 4.1 displays the *E. coli* ATCC 25922 growth curve in LB broth with varying ampicillin concentrations (0 µg/mL, 2.5 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL, and 80 µg/mL) over 0 to 26 hours.

The growth curve analysis depicted in Figure 4.1 illustrates the concentration-dependent effects of ampicillin on *E. coli* ATCC 25922. Surprisingly, subinhibitory concentrations of ampicillin (2.5 µg/ml and 5 µg/ml) exhibited a hormesis effect, stimulating bacterial growth during the exponential phase compared to the control (Calabrese & Baldwin, 2001). This unexpected phenomenon suggests a complex interplay between ampicillin-induced stress and bacterial adaptive mechanisms. Initially, the presence of ampicillin likely triggers

stress responses in the bacteria, leading to metabolic alterations and potentially increased growth rates during the early exponential phase. However, as the stress imposed by ampicillin intensifies, bacterial growth is temporarily inhibited, as evidenced by the decrease in optical density (OD) readings until approximately the 5th hour. Beyond this point, surviving bacteria may activate adaptive mechanisms to counteract the antibiotic's effects, resulting in a resurgence of growth. This adaptive response could involve the upregulation of stress response pathways, alterations in gene expression, or the acquisition of antibiotic resistance traits through mutagenesis. The subsequent stationary phase observed in these cultures suggests a balance between bacterial growth and the inhibitory effects of ampicillin.

Conversely, higher concentrations of ampicillin (10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml) induced a dose-dependent inhibitory effect on bacterial growth. As expected, the presence of these concentrations of ampicillin significantly reduced bacterial growth rates compared to the control throughout the experimental period. The decrease in OD for cultures with ampicillin suggests cell lysis (Yin et al., 2013). This inhibitory effect is likely due to the ampicillin's ability to interfere with essential cellular processes, such as cell wall synthesis, leading to cell lysis and a decrease in overall bacterial viability. The observed decrease in OD readings in cultures supplemented with higher concentrations of ampicillin further supports the notion of impaired bacterial growth and survival under these conditions.

Furthermore, the observed sub-MIC ampicillin-induced cell lysis and growth inhibition can be attributed to a complex interplay of bacterial adaptive responses. It is hypothesized that the downregulation of blaA expression, associated with slow antibiotic removal, contributes to the observed growth inhibition. Additionally, the theoretical framework suggests that bacteria adapt to antibiotics encountered in concentration gradients, as found in natural settings, by accelerating mutation rates through stress-induced mutagenesis (Hermsen et al., 2012). This adaptive response is exemplified by the emergence of antibiotic mutagenesis in *E. coli* ATCC 25922 when exposed to subinhibitory concentrations of ampicillin (2.5 µg/ml and 5 µg/ml), underscoring the bacteria's capacity to evolve under environmental stress.

4.2 Screening of antibiotic resistant mutants

Sub-culturing cultures from the 0 µg/mL and 5 µg/mL conditions into LB broths containing 20 and 40 µg/mL ampicillin unveiled distinct patterns elucidating the impact of

subinhibitory concentrations on bacterial behaviour. Cultures without ampicillin (0 µg/mL) showed decreasing OD readings over time at both 20 µg/mL and 40 µg/mL, indicating inhibited growth and a negative impact on cell viability, aligning with expectations for higher ampicillin concentrations. Conversely, cultures from the 5 µg/mL condition displayed a concentration-dependent response. When sub-cultured into LB broths with 20 µg/mL ampicillin, the bacterial population demonstrated substantial growth, possibly indicating the emergence of resistant strains in response to antibiotic stress. However, exposure to 40 µg/mL ampicillin led to a decline in OD readings, reinforcing the concentration-dependent impact of ampicillin on bacterial growth.

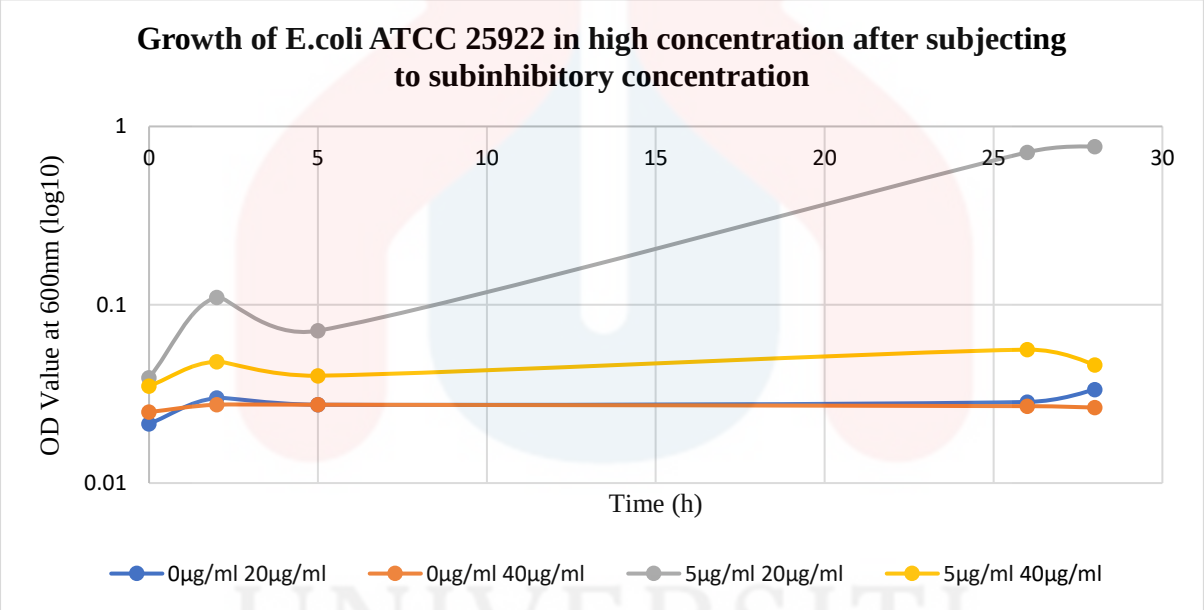


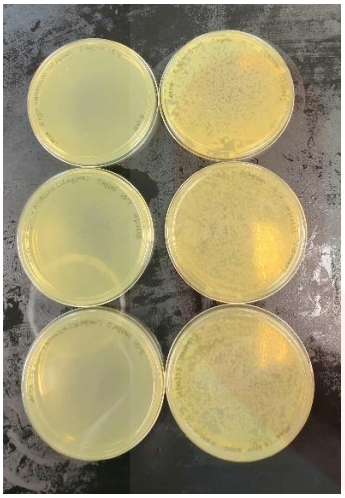
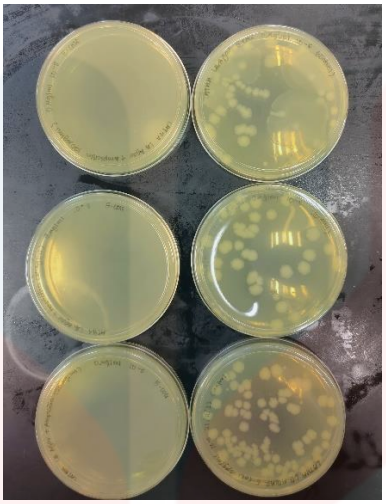
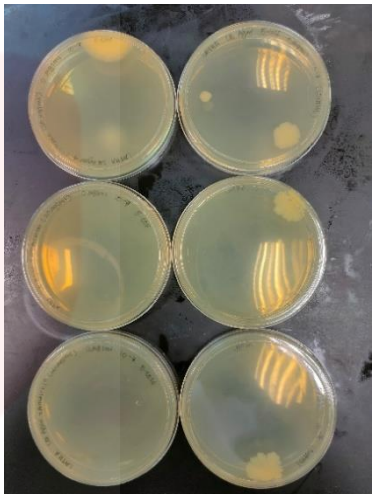
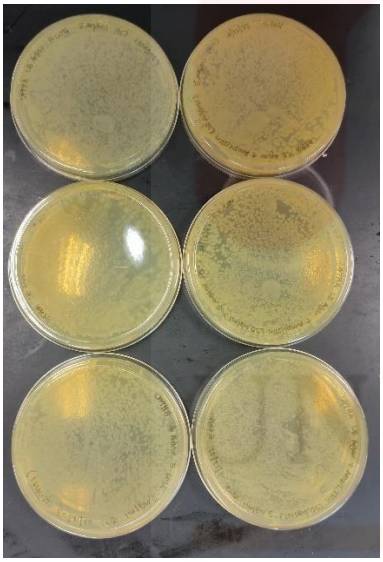
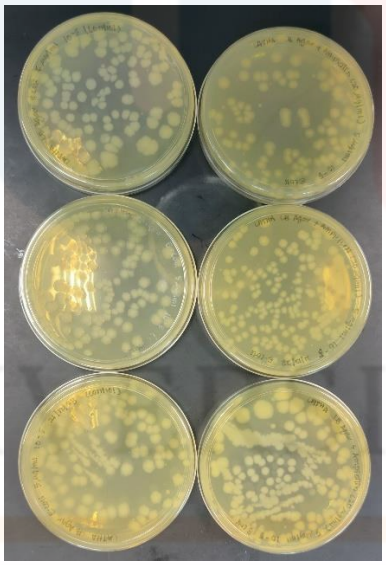
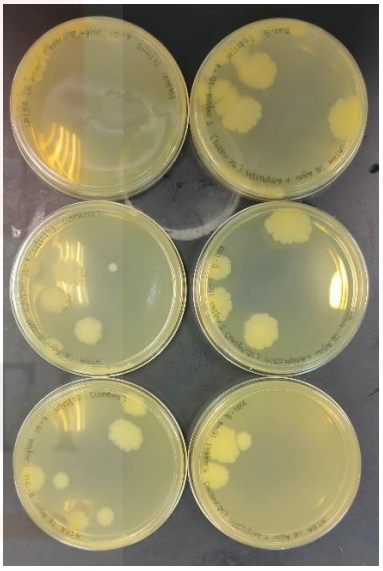
Figure 4.2 illustrates the growth curve of *E. coli* ATCC 25922, depicting the impact of high ampicillin concentrations (20 µg/mL and 40 µg/mL) following exposure to subinhibitory concentrations of 0 µg/mL and 5 µg/mL over a time span of 0 to 28 hours.

The observed resistance of the bacterial population initially exposed to 5 µg/mL ampicillin when sub-cultured into media with 20 µg/mL ampicillin suggests the emergence of adaptive responses leading to resistance, in line with previous studies highlighting subinhibitory concentrations as selective pressure for resistance mechanisms. Conversely, the lack of resistance development in cultures exposed to 40 µg/mL ampicillin implies that higher concentrations may exert a more immediate inhibitory effect, suppressing the emergence of resistant strains. This could be attributed to a threshold effect where the concentration surpasses

the capacity of the bacterial population to develop adaptive mechanisms within the observed timeframe.

The findings emphasize the importance of understanding the delicate balance between inhibitory and subinhibitory concentrations in dosing strategies. While subinhibitory concentrations may facilitate the emergence of resistance, higher concentrations might suppress adaptive responses. These nuances underscore the need for a nuanced dosing strategy that considers the potential selective pressures at various antibiotic concentrations. In the context of antibiotic stewardship, optimizing dosing regimens becomes crucial. Tailoring concentrations to avoid subinhibitory levels, such as 5 µg/mL, which may serve as a reservoir for resistance, becomes essential. However, simply escalating concentrations may not be a one-size-fits-all solution. Dosing strategies should aim for the right balance to ensure therapeutic efficacy while minimizing the risk of resistance development.

At 0 µg/mL ampicillin, the control condition without antibiotic, the bacterial population displayed robust growth at all dilutions (10^{-3}, 10^{-5}, and 10^{-7}) on LB agar. However, when exposed to ampicillin at a concentration of 20 µg/mL, the growth was completely inhibited across all dilutions, signifying the efficacy of ampicillin in preventing bacterial proliferation. When the antibiotic concentration was set at 5 µg/mL, a more nuanced response emerged. At the highest dilution (10^{-3}), equal growth was observed in both LB agar (control) and ampicillin agar, indicating a degree of resistance. At lower dilutions (10^{-5} and 10^{-7}), bacterial growth was evident not only in LB agar but also in ampicillin agar, suggesting the emergence of resistant colonies. Upon elevating the ampicillin concentration to 10 µg/mL, there was no growth observed in either LB agar or ampicillin agar at 20 µg/mL. This result implies the potent inhibitory action of ampicillin at this higher concentration, effectively suppressing bacterial growth even at lower dilutions. In summary, the findings highlight the concentration-dependent response of E. coli to ampicillin, ranging from effective inhibition at higher concentrations to the emergence of resistance at subinhibitory levels.

0 µg/ml LB Agar	+Amp -Amp  10^{-3}	+Amp -Amp  10^{-5}	+Amp -Amp  10^{-7}
5 µg/ml LB Agar	+Amp -Amp  10^{-3}	+Amp -Amp  10^{-5}	+Amp -Amp  10^{-7}

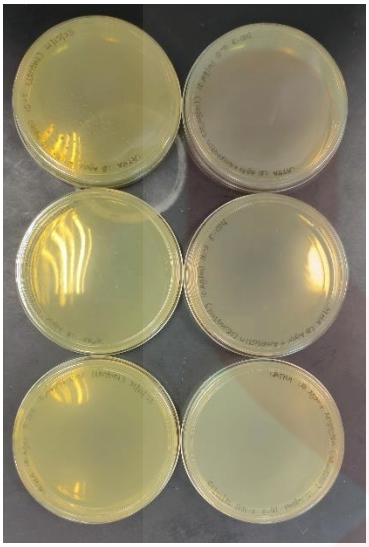
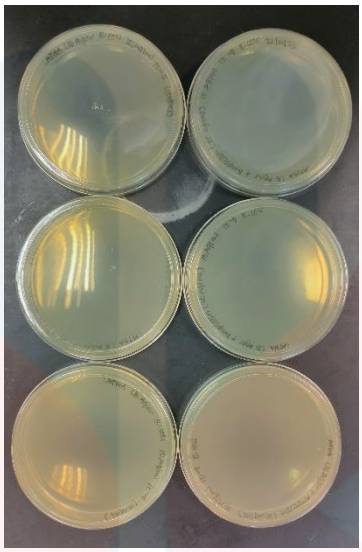
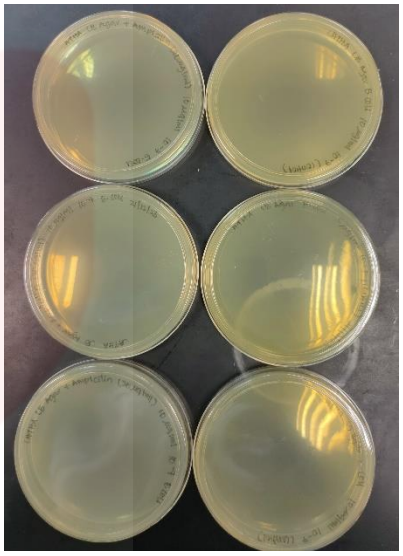
10 µg/ml LB Agar	+Amp -Amp  10^{-3}	+Amp -Amp  10^{-5}	+Amp -Amp  10^{-7}

Table 4.3 displays spread plate results from 10^{-3}, 10^{-5}, and 10^{-7} of 0, 5 and 10 µg/ml in LB and LB supplemented with 20 µg/ml ampicillin.

The investigation into the emergence of resistant colonies aimed to isolate and characterize bacterial strains obtained from spread plates, focusing on one non-resistant wildtype colony and two colonies exhibiting resistance. The spectrophotometric readings for resistant mutants 1 and 2 underscore their ability to sustain growth in the presence of 20 µg/mL ampicillin. Consistent OD readings over the incubation period indicate a lack of inhibitory effects compared to the wildtype. The wildtype, when exposed to 20 µg/mL ampicillin, exhibited a notable decrease in optical density (OD) readings, highlighting the inhibitory effect of the antibiotic on bacterial growth. In contrast, resistant mutants 1 and 2 displayed consistent growth patterns even in the presence of ampicillin, signifying their resistance to the antibiotic.

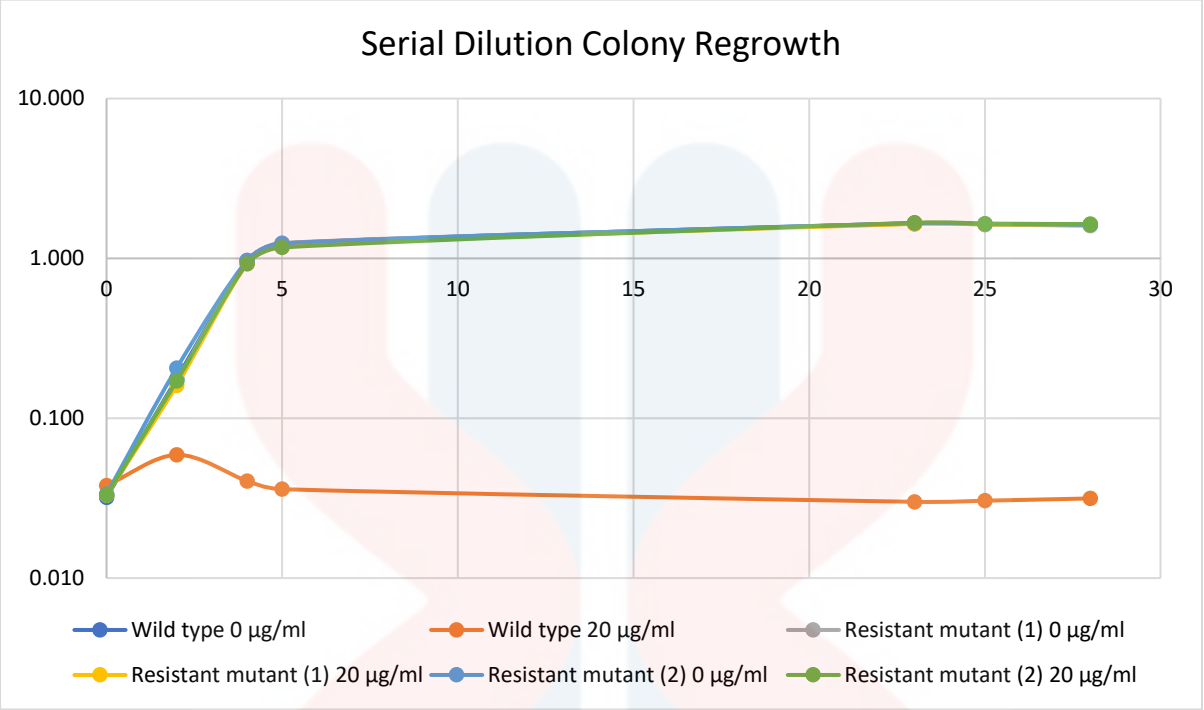


Figure 4.4 displays the *E. coli* ATCC 25922 wild type and resistant mutants' growth curve in LB broth supplemented with 0 µg/mL and 20 µg/mL ampicillin concentrations in over 0 to 28 hours.

Further characterization involved streaking the colonies on agar plates and incubating them under different conditions. The observed growth of resistant colonies on both standard agar and ampicillin-supplemented agar reaffirms their ability to thrive in the presence of the antibiotic. This suggests potential genetic adaptations or mechanisms that confer resistance. Streaking plate results revealed distinct behaviours of the wildtype and resistant colonies on LB agar and LB agar with 20 µg/mL ampicillin. The wildtype exhibited growth on standard LB agar but no growth on ampicillin-supplemented agar, confirming its susceptibility to the antibiotic. In contrast, both resistant colonies displayed growth on both types of agars, indicating their ability to withstand the inhibitory effects of ampicillin.

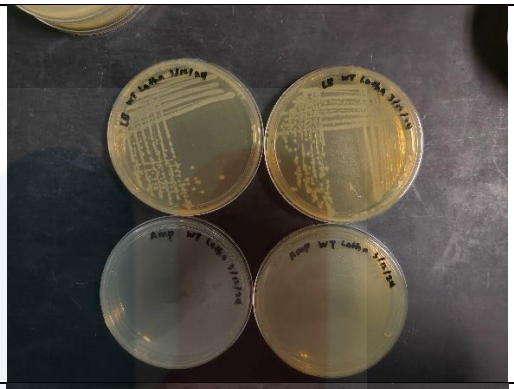
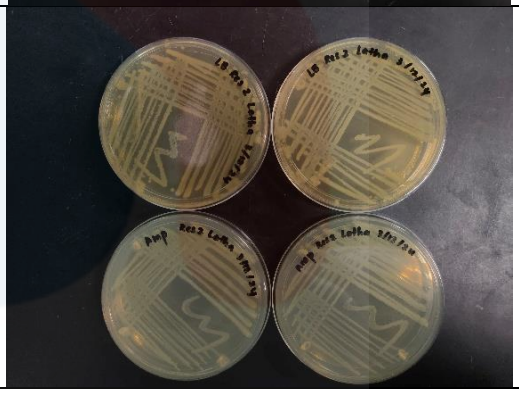
Wild Type		
Resistant Mutant (1)		
Resistant Mutant (2)		

Table 4.4 Figure displays the *E. coli* ATCC 25922 wild type and resistant mutants' growth curve in LB Agar supplemented with 0 µg/mL and 20 µg/mL ampicillin concentrations.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

In conclusion, this research has thoroughly examined the occurrence of antibiotic resistance mutants in *E. coli* ATCC 25922 exposed to subinhibitory concentrations of the beta-lactam antibiotic, ampicillin. The study has clarified how the concentration of ampicillin influences bacterial growth, demonstrating a unique phenomenon known as hormesis at subinhibitory levels. This highlights the subtle and varied responses of bacteria to different ampicillin concentrations. The emergence of resistant colonies, particularly under the influence of 5 µg/mL ampicillin, highlights the significance of subinhibitory concentrations as selective pressure for adaptive responses and resistance mechanisms. These findings underscore the importance of a well-balanced and adaptive approach to antibiotic therapy, considering the diverse impacts of both inhibitory and subinhibitory concentrations. To optimize therapeutic effectiveness while mitigating the risk of resistance, a nuanced dosing strategy should be employed. This study not only contributes to our understanding of antibiotic resistance dynamics but also advocates for a strategic and forward-thinking approach to antibiotic administration in the realm of public health.

5.2 RECCOMENDATION

To enhance the comprehensiveness of this study, future investigations should consider delving into the molecular mechanisms governing antibiotic resistance in *E. coli* under subinhibitory concentrations. Employing advanced molecular techniques, such as whole-genome sequencing or transcriptomic analyses, could unveil the specific genetic adaptations responsible for resistance emergence. Additionally, exploring a broader range of beta-lactam antibiotics and extending the study to diverse bacterial strains would contribute to a more comprehensive understanding of antibiotic resistance dynamics. Furthermore, incorporating real-world scenarios, such as conditions mimicking environmental niches, could provide insights into the ecological relevance of these findings. Lastly, considering the potential influence of bacterial biofilms on resistance development would enrich the study, as biofilm formation is known to

influence antibiotic susceptibility. Integrating these aspects into future research endeavours would refine our understanding of antibiotic resistance and contribute valuable insights to the ongoing efforts in antibiotic stewardship and public health.

REFERENCES

Amenu, D. (2014). Antimicrobial resistance for enteric pathogens isolated from acute gastroenteritis patients. *World J Nat Appl Sci, 1*(1), 1-14.

Aminov, R. I. (2009, Dec). The role of antibiotics and antibiotic resistance in nature. *Environ Microbiol, 11*(12), 2970-2988. https://doi.org/10.1111/j.1462-2920.2009.01972.x

Andersson, D. I., & Hughes, D. (2014). Microbiological effects of sublethal levels of antibiotics. *Nature Reviews Microbiology, 12*(7), 465-478.

Armstrong, G. L., Conn, L. A., & Pinner, R. W. (1999, Jan 6). Trends in infectious disease mortality in the United States during the 20th century. *Jama, 281*(1), 61-66. https://doi.org/10.1001/jama.281.1.61

Baharoglu, Z., & Mazel, D. (2014). SOS, the formidable strategy of bacteria against aggressions. *FEMS microbiology reviews, 38*(6), 1126-1145.

Baranowski, C., Welsh, M. A., Sham, L. T., Eskandarian, H. A., Lim, H. C., Kieser, K. J., Wagner, J. C., McKinney, J. D., Fantner, G. E., Ioerger, T. R., Walker, S., Bernhardt, T. G., Rubin, E. J., & Rego, E. H. (2018, Oct 16). Maturing Mycobacterium smegmatis peptidoglycan requires non-canonical crosslinks to maintain shape. *Elife, 7*. https://doi.org/10.7554/eLife.37516

Bérdy, J. (2012, Aug). Thoughts and facts about antibiotics: where we are now and where we are heading. *J Antibiot (Tokyo), 65*(8), 385-395. https://doi.org/10.1038/ja.2012.27

Calabrese, E. J., & Baldwin, L. A. (2001). Hormesis: U-shaped dose responses and their centrality in toxicology. *Trends in pharmacological sciences, 22*(6), 285-291.

Clewell, D. B. (2014). Antibiotic Resistance Plasmids in Bacteria. https://doi.org/10.1002/9780470015902.a0001491.pub3

Courvalin, P. (2005, Oct). Antimicrobial drug resistance: "Prediction is very difficult, especially about the future". *Emerg Infect Dis, 11*(10), 1503-1506. https://doi.org/10.3201/eid1110.051014

Davies, J., Spiegelman, G. B., & Yim, G. (2006). The world of subinhibitory antibiotic concentrations. *Current opinion in microbiology, 9*(5), 445-453.

Fischbach, M. A., & Walsh, C. T. (2009, Aug 28). Antibiotics for emerging pathogens. *Science, 325*(5944), 1089-1093. https://doi.org/10.1126/science.1176667

Fluit, A. C., Visser, M. R., & Schmitz, F. J. (2001, Oct). Molecular detection of antimicrobial resistance. *Clin Microbiol Rev, 14*(4), 836-871, table of contents. https://doi.org/10.1128/CMR.14.4.836-871.2001

Guardabassi, L., & Courvalin, P. (2005). Modes of Antimicrobial Action and Mechanisms of Bacterial Resistance. In *Antimicrobial Resistance in Bacteria of Animal Origin* (pp. 1-18). https://doi.org/https://doi.org/10.1128/9781555817534.ch1

Guyer, B., Freedman, M. A., Strobino, D. M., & Sondik, E. J. (2000, Dec). Annual summary of vital statistics: trends in the health of Americans during the 20th century. *Pediatrics, 106*(6), 1307-1317. https://doi.org/10.1542/peds.106.6.1307

Hegstad, K., Mikalsen, T., Coque, T. M., Werner, G., & Sundsfjord, A. (2010, Jun). Mobile genetic elements and their contribution to the emergence of antimicrobial resistant Enterococcus faecalis and Enterococcus faecium. *Clin Microbiol Infect, 16*(6), 541-554. https://doi.org/10.1111/j.1469-0691.2010.03226.x

Hermsen, R., Deris, J. B., & Hwa, T. (2012). On the rapidity of antibiotic resistance evolution facilitated by a concentration gradient. *Proceedings of the National Academy of Sciences, 109*(27), 10775-10780.

Hewitt, W. L. (1967, Sep 27). Penicillin-historical impact on infection control. *Ann N Y Acad Sci, 145*(2), 212-215. https://doi.org/10.1111/j.1749-6632.1967.tb50219.x

Holcomb, H. G., Durbin, K. J., Cho, M., Choi, K.-j., & Darling, N. (2008). Methicillin-Resistant Staphylococcus aureus as a Threat to Public Health: a Cellular Approach.

Joanne Willey, L. S., Christopher J. Woolverton (2013). *Prescott's Microbiology* (Vol. 9th). McGraw-Hill Education.

John, W., & Sons, L. (2001). *eLS*. https://doi.org/10.1002/047001590x

Kümmerer, K. (2009, Apr). Antibiotics in the aquatic environment--a review--part I. *Chemosphere, 75*(4), 417-434. https://doi.org/10.1016/j.chemosphere.2008.11.086

Laureti, L., Matic, I., & Gutierrez, A. (2013). Bacterial responses and genome instability induced by subinhibitory concentrations of antibiotics. *Antibiotics, 2*(1), 100-114.

Lederberg, J. (2000, Apr 14). Infectious history. *Science, 288*(5464), 287-293. https://doi.org/10.1126/science.288.5464.287

Levin, B. R., Lipsitch, M., Perrot, V., Schrag, S., Antia, R., Simonsen, L., Walker, N. M., & Stewart, F. M. (1997, Jan). The population genetics of antibiotic resistance. *Clin Infect Dis, 24 Suppl 1*, S9-16. https://doi.org/10.1093/clinids/24.supplement_1.s9

Lima, L. M., Silva, B., Barbosa, G., & Barreiro, E. J. (2020, Dec 15). β-lactam antibiotics: An overview from a medicinal chemistry perspective. *Eur J Med Chem, 208*, 112829. https://doi.org/10.1016/j.ejmech.2020.112829

Livermore, D. (2004, Jan). Can better prescribing turn the tide of resistance? *Nat Rev Microbiol, 2*(1), 73-78. https://doi.org/10.1038/nrmicro798

Sageman, A. (2015). Antibiotic Resistance Mechanisms, Problems, and Solutions.

Sharma, S., Singh, L., & Singh, S. (2013). Comparative study between penicillin and ampicillin. *Scholars Journal of Applied Medical Sciences, 1*(4), 291-294.

Smith, D. L., Dushoff, J., & Morris, J. G. (2005, Aug). Agricultural antibiotics and human health. *PLoS Med, 2*(8), e232. https://doi.org/10.1371/journal.pmed.0020232

Snowden, F. M. (2008, Oct). Emerging and reemerging diseases: a historical perspective. *Immunol Rev, 225*(1), 9-26. https://doi.org/10.1111/j.1600-065X.2008.00677.x

Stephen P. Denyer, N. A. H., Sean P. Gorman, Brendan F. Gilmore (2011). *Hugo and Russell's Pharmaceutical Microbiology, 8th Edition* (November 2011 ed., Vol. 8th).

Tackling antibiotic resistance from a food safety perspective in Europe / World Health Organization Regional Office for Europe. (2011). World Health Organization Regional Office for Europe. http://www.euro.who.int/__data/assets/pdf_file/0005/136454/e94889.pdf

Thakuria, B., & Lahon, K. (2013, Jun). The Beta Lactam Antibiotics as an Empirical Therapy in a Developing Country: An Update on Their Current Status and Recommendations to Counter the Resistance against Them. *J Clin Diagn Res, 7*(6), 1207-1214. https://doi.org/10.7860/jcdr/2013/5239.3052

Woodford, N., & Ellington, M. J. (2007, Jan). The emergence of antibiotic resistance by mutation. *Clin Microbiol Infect, 13*(1), 5-18. https://doi.org/10.1111/j.1469-0691.2006.01492.x

Yin, J., Sun, L., Dong, Y., Chi, X., Zhu, W., Qi, S.-h., & Gao, H. (2013). Expression of blaA underlies unexpected ampicillin-induced cell lysis of Shewanella oneidensis. *PLoS One, 8*(3), e60460.

APPENDIX A

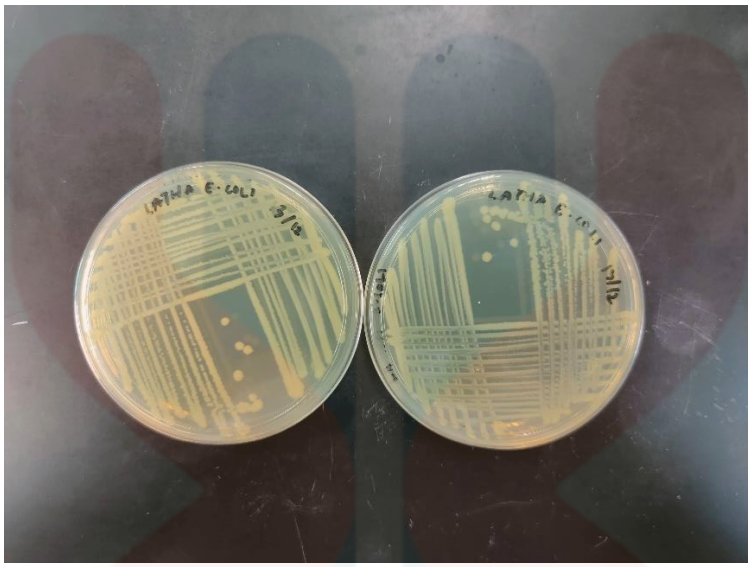


Figure A.1: Growth of *E. coli* ATCC 25922 on LB agar

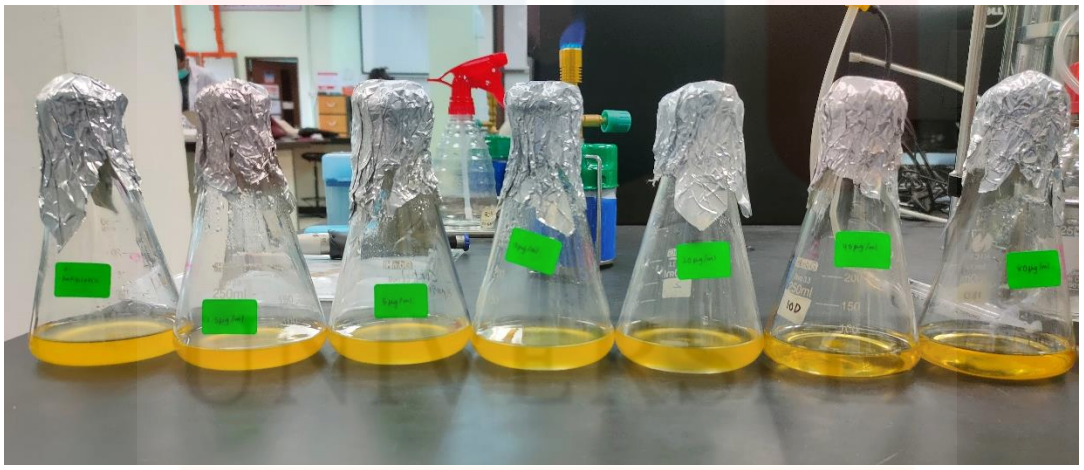


Figure A.2: Growth of *E. coli* in 0, 2.5, 5, 10, 20, 40, 80 µg/mL of ampicillin

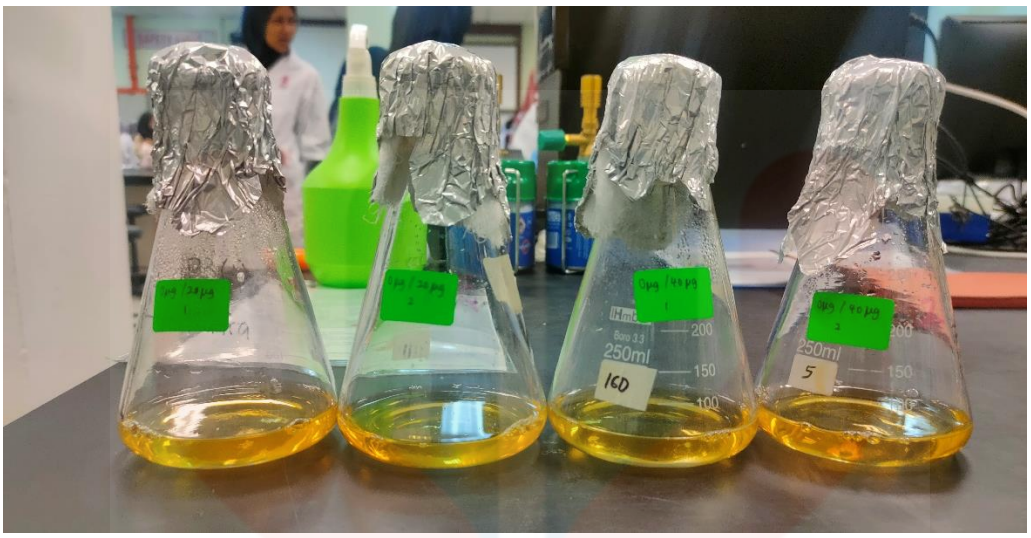


Figure A.3: Growth of *E. coli* previously cultured at 0 µg/mL, on 20 and 40 µg/mL concentrations.

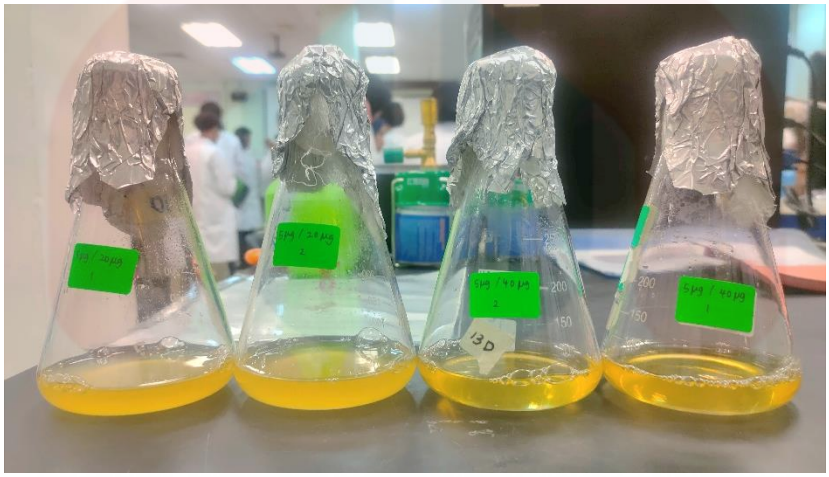


Figure A.4: Growth of *E. coli* previously cultured at 5 µg/mL, on 20 and 40 µg/mL concentrations.

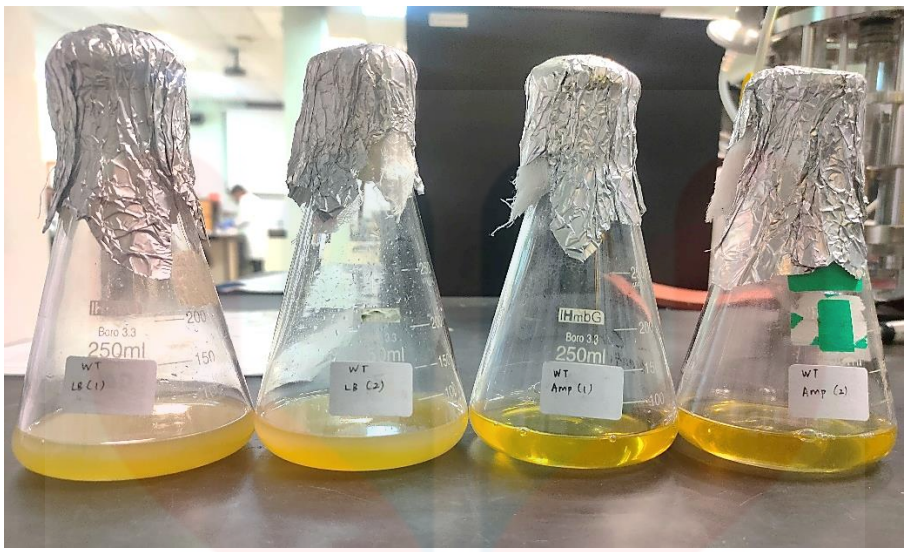


Figure A.5: Growth of wild type *E. coli* in 0 and 20 µg/mL concentrations.

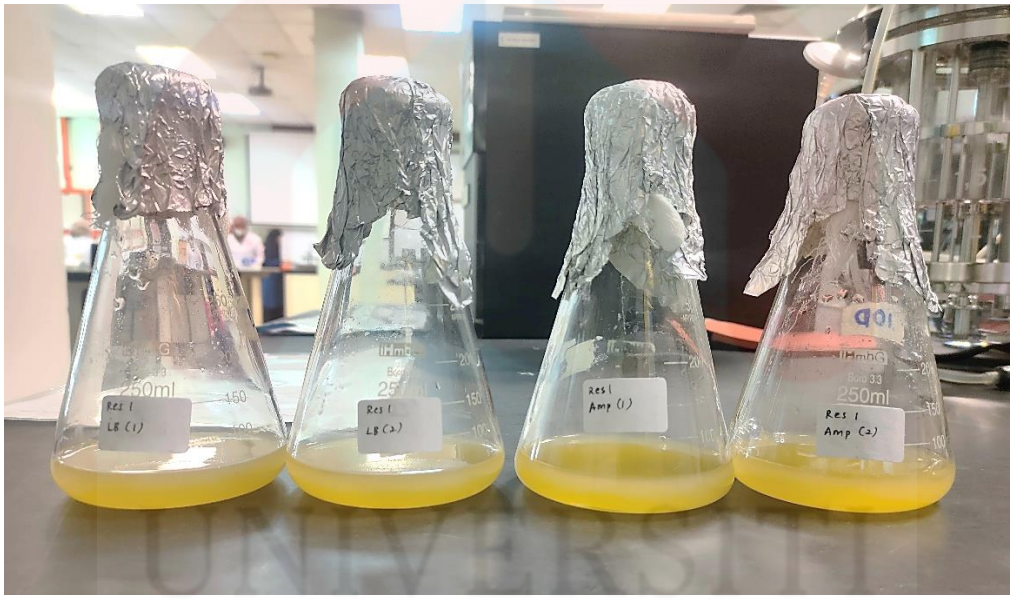


Figure A.6: Growth of resistant *E. coli* in 0 and 20 µg/mL concentrations.

APPENDIX B

Table B.1: *E. coli* ATCC 25922 growth curve in LB broth with varying ampicillin concentrations over 0 to 26 hours.

Time (hour)	0 µg/ml	2.5 µg/ml	5 µg/ml	10 µg/ml	20 µg/ml	40 µg/ml	80 µg/ml
0	0.034	0.044	0.044	0.043	0.039	0.036	0.034
2	0.284	0.310	0.284	0.260	0.178	0.030	0.035
5	1.078	0.098	0.082	0.072	0.058	0.029	0.026
23	1.464	1.384	1.379	0.090	0.069	0.036	0.027
26	1.500	1.373	1.314	0.089	0.067	0.029	0.024

Table B.2: Growth curve of *E. coli* ATCC 25922 at 20 and 40 µg/mL following exposure to subinhibitory concentrations of 0 µg/mL and 5 µg/mL over a time span of 0 to 28 hours.

OD Reading	Ampicillin Concentration	0	2	5	26	28
0µg/ml	20µg/ml	0.022	0.030	0.028	0.029	0.034
	40µg/ml	0.025	0.028	0.028	0.027	0.027
5µg/ml	20µg/ml	0.039	0.110	0.072	0.717	0.772
	40µg/ml	0.035	0.048	0.040	0.056	0.046

Table B.3: Growth curve of *E. coli* ATCC 25922 wild type and resistant mutants in LB broth with 0 µg/mL and 20 µg/mL ampicillin concentrations over 0 to 28 hours.

OD Reading	Ampicillin Concentration	0	2	4	5	23	25	28
Wild type	0 µg/ml	0.032	0.177	0.969	1.242	1.659	1.638	1.622
	20 µg/ml	0.038	0.059	0.041	0.036	0.030	0.031	0.032
Resistant mutant (1)	0 µg/ml	0.033	0.172	0.958	1.247	1.649	1.628	1.605
	20 µg/ml	0.033	0.161	0.926	1.194	1.645	1.632	1.610
Resistant mutant (2)	0 µg/ml	0.034	0.207	0.970	1.240	1.662	1.643	1.619
	20 µg/ml	0.033	0.172	0.928	1.171	1.672	1.651	1.639