Ph.D. thesis prepared within the Warsaw-4-PhD doctoral school at the

Institute of Physical Chemistry

Polish Academy of Sciences

Kasprzaka 44/52, 01-224, Warszawa

Deciphering the role of antibiotic exposure at the sub-Minimum inhibitory concentration on bacterial heterogeneity at single-cell level

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Warsaw, May 2025

To live only for some unknown future is superficial. It is like climbing a mountain to reach the peak without experiencing its sides. The sides of the mountain sustain life, not the peak. This is where the things grow, experience is gained and technologies are mastered. The importance of the peak lies only in the fact that it defines the sides.

Avul Pakir Jainulabdeen Abdul Kalam

केवल कुछ अनजान भविष्य के लिए जीना सतही है। यह ऐसा है जैसे बिना पहाड़ के किनारों का अनुभव किए केवल शिखर तक पहुंचने के लिए चढ़ाई करना। पहाड़ के किनारे जीवन को बनाए रखते हैं, ना कि शिखर। यहीं चीजें बढ़ती हैं, अनुभव मिलता है और तकनीकों में महारत हासिल की जाती है। शिखर का महत्व केवल इस तथ्य में निहित है कि यह किनारों को परिभाषित करता है।

अवुल पकिर जैनुलाब्दीन अब्दुल कलाम

صرف چند نامعلوم مستقبل کے لئے جینا سطحی ہے۔ یہ ایسے ہے جیسے کسی پہاڑ پر چڑھنا تاکہ چوٹی تک پہنچا جا سکے بغیر اس کی جانبوں کے تجربے کے۔ پہاڑ کی جانبیں زندگی کو برقرار رکھتی ہیں، نہ کہ چوٹی۔ یہی وہ مقام ہے جہاں چیزیں بڑھتی ہیں، تجربہ حاصل ہوتا ہے اور ٹیکنالوجیز کی مہارت حاصل کی جاتی ہے۔ چوٹی کی اہمیت صرف اس حقیقت میں ہے کہ یہ جانبوں کی تعریف کرتی ہے۔

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Declaration

The thesis presented here entitle **"Deciphering the role of antibiotic exposure at the sub-Minimum inhibitory concentration on bacterial heterogeneity at single-cell level"** is a result of my original work and does not contain any material previously published or written by another person except where the reference has been made in the text as required. The contributions of other people have been clearly stated including for data analysis, writing scripts etc. The contents of this doctoral thesis do not violate any of the existing copyright laws in Poland or internationally to the best of my knowledge.

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Acknowledgement

The PhD journey and the completion of this thesis has been a very fulfilling experience for me comprising of many challenges, struggles, rejections, realizations, self-discovery, triumphs and infinite moments of joy throughout this odyssey, especially this moment- the submission, where I feel happy towards a very important milestone in my life but also a bit sad for the fact that such a beautiful journey at the institute is ending. I will forever be indebted to all the individuals who have been around me during this academic journey, their unwavering support and motivations every now and kept me going.

First of all, I would like to extend my heartful gratitude to my supervisors, Prof. Piotr Garstecki and Dr Ilona P. Foik, who had constant belief in me through this endeavor with their able guidance. Piotr has always been a source of motivation and inspiration for me with his scientific and non-scientific discussions. Ilona, thank you for coming by at the best moment when I needed to design a clear research plan, and for letting me explore my own ideas in all directions I ever wanted to. I am highly grateful to you both for dedicating your precious time in all these years and helping me transform this PhD to reality.

I would also like to thank Dr Ladislav Derzsi for his guidance in the early years of my PhD and Dr Marco Constantini for letting me be a part of his group in the last year. Artur, Witold, Marcin and Natalia, thank you for passing on the skills which I absolutely needed to start this journey. I would also like to mention the invaluable support and guidance of each and every member of Team 11, you guys were extremely needed for all the lab run and off the lab fun. Your contribution during the starting years of my journey is highly appreciated. I am highly thankful to Ms Justyna Gruszka for all the off-science support and information through all these years.

Nelam, Hari, Abhishek, Swaraj, Vineeta and Aurabinda- thank you guys for all the very essential distractions from work, I found home in you. Qamar Bhai, Sourav Da, Arif Bhai- thank you for being a family away from family. I extend my sincere thanks to each and every person in Poland who have been associated with me scientifically and non-scientifically for being very the important characters during this expedition.

Last but not the least, I owe eternal gratitude to my parents, who constantly supported me with everything they could, since I came into this world. Always thankful to my sister,

brothers and cousins with whom I share a very special bond. Many thanks to Imteyaz Bhai for constantly teaching me the skills of patience, perseverance and hard work, I learnt both science and life from you. Raj, Vijeta, Qaisar, Kasif- thanking you for all the long talks, stupid jokes and unrealistic backup plans beyond PhD.

You all hold a very special place in my heart, and you have not only helped me in my personal growth but also enriched my mind and soul with ways I cannot explain.

Funding acknowledgement

I would like to thank the National Science Center (NCN), Poland grant Maestro 10 (2018/30/A/ST4/00036) for the research funding and salary.

I would also like to thank Warsaw-4-PhD Doctoral School for the doctoral scholarship.

Finally, I would like to extend my gratitude to Institute of Physical Chemistry PAS for necessary lab space and funding as required.

Publications and presentations

Publications related to the thesis:

Ahmad, S., Foik, I. P., Jankowski, P., Samborski, A., Vasantham S.K., & Garstecki, P. Ciprofloxacin pre-exposure influences individual cell MIC and heteroresistance of bacteria inside microfluidic droplets (Submitted)

Ahmad, S., Foik, I. P., Jankowski, P., Samborski, A., Vasantham S.K., & Garstecki, P. Ciprofloxacin and streptomycin influence the heteroresistance of *Pseudomonas aeruginosa* PA14 cells inside microfluidic droplets (Under preparation)

Other publications:

Rangam, N. V.‡, Ahmad, S.‡, Koronkiewicz, R., Foik, I. P., Borowicz, P., Wisniewska, A., Hołdyński, M., Tóth, J., Kövér, L., Roszko, M. Ł., Lesiak-Orłowska, B., & Sudagar, A. J. (2024). Bactericidal efficiency of silver nanocomposites obtained using Brewer's spent grains. *Applied Surface Science*, *661*, 159958. https://doi.org/10.1016/J.APSUSC.2024.159958
‡ Equal contribution

Shahryari, S., Ahmad, S., Foik, I. P., Jankowski, P., Samborski, A., Równicki, M., Vasantham S.K., & Garstecki, P.
Bacterial strain type and TEM-1 enzyme allele impact antibiotic susceptibility distribution in monoclonal populations: a single-cell droplet approach (Submitted)

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Conference presentations:

Ahmad, S., Vasantham, S., Promovych, Y., & Garstecki, P. (2022). High-throughput microfluidic toolbox to study monoclonal bacterial population from single-cell inside nanolitre droplets. *NanoBioTech*, Switzerland. Poster presentation and flash talk

Ahmad, S., Vasantham, S., Promovych, Y., & Garstecki, P. (2023). High-throughput microfluidic toolbox to sort and study single-cells inside droplets from monoclonal bacterial populations. *IPC PAS Microsymposium*, Poland. Poster presentation

Shahryari, S., Ahmad, S., Foik, I., Jankowski, P., Samborski, A., Równicki, M., Vasantham, S., & Garstecki P. (2023). Mutations increasing catalytic activity of TEM-1 promote hetero-resistance in *E. coli* at single-cell level. *FEMS*, Germany. Poster

Ahmad, S., Foik, I., Jankowski, P., Samborski, A., Vasantham, S., & Garstecki P. (2023). Exploring the role of sub-MIC antibiotic exposure on bacterial hetero-resistance at single-cell level. *ASM/ESMID*, USA. Poster presentation

Ahmad, S., Foik, I., Jankowski, P., Samborski, A., Vasantham, S., & Garstecki P. (2024). Deciphering the effects of sub-MIC antibiotic pre-exposure on bacterial heteroresistance at single-cell level. *ESCMID*, Spain. Poster presentation

Ahmad, S., Foik, I., Jankowski, P., Samborski, A., Vasantham, S., & Garstecki P. (2025). Pre-exposure with Ciprofloxacin at sub-MIC levels alters the heteroresistance of bacterial cells in microfluidic droplets. *AoM*, Poland. Oral talk

Ahmad, S., Foik, I., Jankowski, P., Samborski, A., Vasantham, S., & Garstecki P. (2025). Sub-inhibitory antibiotic concentration pre-exposures alter the heteroresistance of bacteria inside microfluidic droplets. *Symbioza*, Poland. Oral talk

Abbreviations and symbols

c	Concentration
$f_+(0)$	Fraction of non-empty droplets without antibiotic treatment
$f_+(c)$	Fraction of non-empty droplets at antibiotic concentration c
$F_R(c)$	Fraction of droplet with bacteria that were able to proliferate
$N_+(c)$	Number of non-empty droplets
$N(c)$	Total number of droplets
AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
Ca	Capillary number
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CPR	Ciprofloxacin
DNA	Deoxyribonucleic acid
DoH	Degree of heteroresistance
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FISH	Fluorescence In-Situ Hybridization
GLCM	Grey-level co-occurrence matrices
iMIC	Individual cell minimum inhibitory concentration
$(iMIC)_{all}$	Concentration needed to completely inhibit growth in all droplets
$(iMIC)_{exp}$	Concentration stopping growth in all droplets seen with microscope
$(iMIC)_{mode}$	Most frequently occurring *iMIC* value
$(iMIC)_{start}$	Concentration where the first drop observed on Gompertz fitting
$(iMIC)_{total}$	Concentration inhibiting growth >95% with respect to control
LAMP	Loop-mediated isothermal amplification
LPS	Lipopolysaccharides
LTA	Lipoteichoic acid
MDR	Multidrug resistant
MH	Mueller-Hinton

MEMS	Micro-electromechanical systems
MIC	Minimum inhibitory concentration
NAATs	Nucleic acid amplification tests
NAG	N-acetyl glucosamine
NAM	N-acetyl muramic acid
OD	Optical density
PAP	Population analysis profile
PBP	Penicillin binding protein
PC	Polycarbonate
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
p(iMIC)	Probability density distribution of iMIC
Re	Reynolds number
RMSD	Root mean square deviation
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
scMIC	Single-cell minimum inhibitory concentration
scRNA-seq	single cell RNA sequencing
SD	Standard deviation
SERS	surface-enhanced Raman spectroscopy
scAST	single-cell antibiotic susceptibility testing
sub-MIC	Sub-minimum inhibitory concentration
TA	Teichoic acid
YFP	Yellow fluorescent protein
WGS	Whole genome sequencing
XDR	Extremely drugs resistant

Abstract

Bacterial cells can exhibit a variety of phenotypes with respect to growth, multiplication, gene expression, metabolism and more. This phenotypic diversity becomes extremely significant under stress conditions, such as exposure to an antibiotic. Within a seemingly isogenic bacterial population, the presence of diverse subpopulations is referred to as heterogeneity. A specific form of heterogeneity, known as heteroresistance, is characterized by a wide range of susceptibilities to an antibiotic within a clonal population. Importantly, this allows a subset of population to temporarily survive antibiotic exposure, potentially acting as a reservoir for the development of stable resistance. Unlike conventional resistance, heteroresistance often goes undetectable by standard susceptibility tests, leading to its underestimation during the clinical diagnostics. However, it is emerging as an underrecognized but pivotal phenomena with potential to complicate treatment strategies and diagnostics, ultimately contributing to the global threat of antimicrobial resistance.

One of the key drivers of heteroresistance is the inefficient and inappropriate use of antibiotics, including subtherapeutic dosing, incomplete treatment courses and overuse. Despite its significance, the impact of subtherapeutic dosing on individual bacterial cell responses has not been thoroughly explored. This thesis addresses an important question in this domain- how does brief exposures to sub-MIC levels of antibiotic alter the distribution of single-cell susceptibilities upon subsequent exposure to same or a different antibiotic. To investigate this, three different concentrations of two bactericidal antibiotics, ciprofloxacin and streptomycin, were used to pre-expose *Escherichia coli MG1655* and *Pseudomonas aeruginosa* PA14 cells in independent experiments. The susceptibility of individual cells was then analyzed using droplet microfluidics upon re-exposure to either of the antibiotics.

Experimental results reveal that pre-exposures to sub-lethal doses of antibiotics can increase the MICs of individual cell growing inside microfluidic droplets, as well as alter the degree of heteroresistance. Ciprofloxacin, a DNA damaging antibiotic, was found to be a stronger inducer of heteroresistance at sub-MIC pre-exposures, both when cells were subsequently treated with ciprofloxacin and streptomycin independently. Moreover, the obtained results were also dependent on the strain, the antibiotic type and the concentration of pre-exposure.

In MG1655 cells, pre-exposures with quarter and half MIC of ciprofloxacin in separate experiments increased the heteroresistance of cells by more than 20-folds during subsequent treatment with ciprofloxacin. Not only that, half MIC ciprofloxacin pre-exposure also increased the heteroresistance of cells by 15-folds when susceptibilities were performed with streptomycin, highlighting the possibility of cross antibiotic effect.

In PA14 cells, half MIC streptomycin pre-exposure increased the heteroresistance of cells by 4-fold on subsequent streptomycin exposure and 3-fold on subsequent ciprofloxacin exposure. Surprisingly, one eighth MIC ciprofloxacin pre-exposure, was more potent in generating heteroresistance than the higher concentrations, but only for susceptibility testing with ciprofloxacin. Some pre-exposures also led to reduced heteroresistance, highlighting the complexity of these interactions.

The findings presented in this thesis offer a novel perspective on the consequences of suboptimal antibiotic use, particularly regarding how it influences heteroresistance in bacteria at the single-cell level during subsequent treatments. These insights may prove valuable for designing more effective antibiotic regimens, developing new antibacterial agents and also addressing the broader issue of antibiotic resistance.

Streszczenie

Komórki bakteryjne mogą wykazywać różnorodne fenotypy pod względem wzrostu, namnażania, ekspresji genów, metabolizmu i innych. Ta różnorodność fenotypowa staje się niezwykle istotna w warunkach stresowych, takich jak ekspozycja na antybiotyk. W pozornie izogenicznej populacji bakteryjnej obecność zróżnicowanych subpopulacji jest określana jako heterogeniczność. Specyficzna forma heterogeniczności, znana jako heteroooporność, charakteryzuje się szerokim zakresem podatności na antybiotyk w obrębie pojedynczej populacji klonalnej. Co ważne, pozwala to podgrupie populacji na tymczasowe przetrwanie ekspozycji na antybiotyk, potencjalnie działając jako rezerwuar dla rozwoju stabilnej oporności. W przeciwieństwie do konwencjonalnej oporności, heteroooporność często pozostaje niewykrywalna za pomocą standardowych metod badania wrażliwości na antybiotyki, co prowadzi do jej niedoszacowania w diagnostyce klinicznej. Jednak wyłania się jako niedoceniane, ale kluczowe zjawisko, które może skomplikować strategie leczenia i diagnostykę, ostatecznie przyczyniając się do globalnego zagrożenia opornością na środki przeciwdrobnoustrojowe.

Jednym z głównych czynników heteroooporności jest nieefektywne i niewłaściwe stosowanie antybiotyków, w tym dawkowanie subterapeutyczne, niekompletne cykle leczenia i nadużywanie antybiotyków. Pomimo swojego znaczenia, wpływ dawkowania subterapeutycznego na reakcje poszczególnych komórek bakteryjnych nie został dokładnie zbadany. Niniejsza rozprawa zajmuje się ważnym pytaniem w tej dziedzinie - w jaki sposób krótkie ekspozycje na antybiotyki w stężeniach poniżej wartości MIC zmieniają rozkład wrażliwości pojedynczych komórek po kolejnym eksponowaniu na ten sam lub inny antybiotyk. Aby to zbadać, trzy różne stężenia dwóch antybiotyków bakteriobójczych, cyprofloksacyny i streptomycyny, zostały użyte do wstępnej ekspozycji komórek *Escherichia coli* MG1655 i *Pseudomonas aeruginosa* PA14 w niezależnych eksperymentach. Następnie, analizowano wrażliwość poszczególnych komórek przy użyciu mikroprzepływów kropelkowych po ponownym eksponowaniu na którykolwiek z antybiotyków. Wyniki eksperymentów ujawniają, że wstępna ekspozycja na subletalne dawki antybiotyków może zwiększyć MIC pojedynczych komórek rosnących wewnątrz mikroprzepływowych kropelek, a także zmienić stopień heteroooporności. Stwierdzono, że cyprofloksacyna, antybiotyk uszkadzający DNA, jest silniejszym induktorem heteroooporności przy wstępnych espozycjach poniżej wartości MIC, zarówno gdy komórki były następnie niezależnie leczone cyprofloksacyną, jak i streptomycyną. Ponadto, uzyskane wyniki zależały również od szczepu bakteryjnego, rodzaju antybiotyku i stężenia wstępnej ekspozycji.

W komórkach MG1655 wstępne narażenie na dawkę równą jeden i półwartości MIC cyprofloksacyny w oddzielnych eksperymentach zwiększyło heteroooporność komórek ponad 20-

krotnie podczas późniejszego leczenia cyprofloksacyną. Co więcej, dawka równa połowie wartości MIC przed ekspozycją na cyprofloksacynę zwiększyła również heteroooporność komórek o 15-krotnie, gdy wykonano badania wrażliwości ze streptomycyną, co wskazuje na możliwość krzyżowego efektu antybiotykowego.

W komórkach PA14, dawka równa połowę wartości MIC przed ekspozycją na streptomycynę zwiększyło heteroooporność komórek 4-krotnie po kolejnej ekspozycji na streptomycynę i 3-krotnie po kolejnej ekspozycji na cyprofloksacynę. Co zaskakujące, dawka wynosząca jedną ósmą wartości MIC przed ekspozycją na cyprofloksacynę była bardziej skuteczna w generowaniu heteroooporności niż wyższe stężenia, ale tylko w przypadku badania wrażliwości z cyprofloksacyną. Niektóre wstępne ekspozycje doprowadziły również do zmniejszenia heteroooporności, co wskazuje na złożoność tych interakcji.

Odkrycia przedstawione w niniejszej rozprawie oferują nową perspektywę dotyczącą konsekwencji suboptymalnego stosowania antybiotyków, w szczególności w odniesieniu do tego, jak wpływa to na heteroooporność u bakterii na poziomie pojedynczej komórki podczas kolejnych terapii. Wnioski te mogą okazać się cenne przy projektowaniu skuteczniejszych schematów leczenia antybiotykami, opracowywaniu nowych środków przeciwbakteryjnych, a także przy rozwiązywaniu szerszego problemu oporności na antybiotyki.

1 Introduction

Chapter 1 *sheds light on bacterial habitat, structure and pathogenicity. It also discusses the methods to quantify bacteria in lab and susceptibility testing for antibiotics. Introductions on phenotypic and genotypic heterogeneity of bacterial populations are also made, with transition to antibiotic resistance, heteroresistance of population to an antibiotic with special focus on effects of low-level antibiotic pre-exposure. The chapter is concluded on droplet microfluidics as a very potential tool used in this thesis to study the heteroresistance in bacteria at single-cell level.*

1.1 Bacteria

Bacteria are a vast and diverse group of single-celled prokaryotic microorganisms belonging to the domain Bacteria. They are among the earliest known life forms on earth, with fossil evidence dating back to more than 3 billion years[1]. Typically, bacteria are around 0.5-1.0 µm in diameter and 2.0-5.0 µm in length, although exceptions do exist[2]. They also exhibit a wide variety of shapes including spherical, rod-shaped, spiral-shaped, and comma-shaped forms.

1.1.1 Habitat

Bacteria are ubiquitous and inhabit a wide range of environments across the Earth including soil, water, plants and animals. It is estimated that there are approximately 2×10^{30} bacteria on the planet, with a total biomass second only to that of the plants[3]. Despite their immense numbers and global distribution, only about 2% of bacterial species have been studied in detail[4].

1.1.2 Cellular structure

Bacterial cells have been extensively studied, and although they are structurally very simple, they do possess well-developed features, some of which are unique to bacteria that support their survival in diverse and often extreme environments. Typically, a bacterial cell consists of a cell envelope, cytoplasm with genetic material in the form of DNA and/or plasmids, ribosomes and protein complexes. Additionally, some species exhibit extracellular structures, such as flagella, fimbriae, and pili which play roles in motility, adhesion, and genetic exchange.

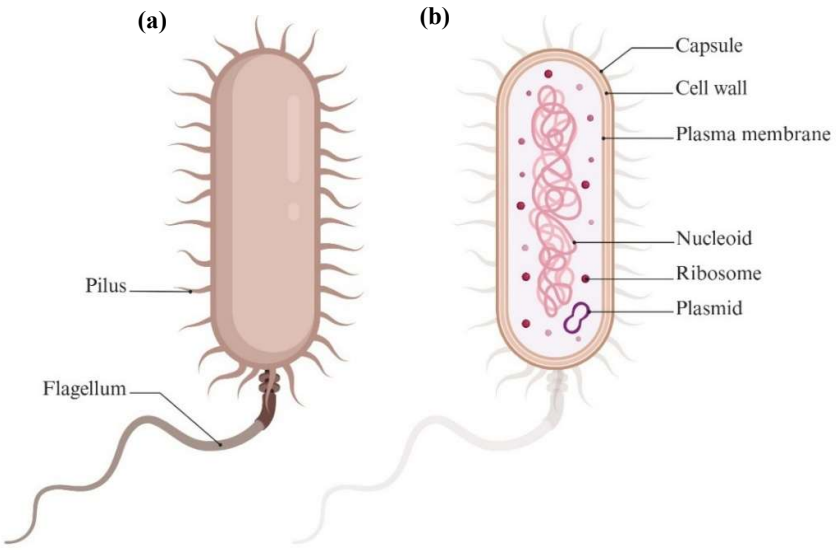

Figure IF1: Structure of a bacterial cell
(a) shows the external structure and morphology; **(b)** shows the detailed internal structure and organelles

*Created in https://BioRender.com

The cell envelope can be 2-3 layered depending on species of bacteria:

- **Capsule:** Capsule is an outermost layer made up of polysaccharide found in some bacterial species. Its main role is protection especially the evasion during phagocytosis by the host cell. Moreover, it can also carry virulence factors needed to cause infection[5].
- **Cell wall:** Most bacterial species have a cell wall and based on the composition and structure of the cell wall bacteria can be placed into two distinct groups- gram-negative (e.g. *Escherichia coli*) and gram-positive (e.g. *Staphylococcus aureus*). Due to the differences in physical and chemical structures of the cell wall, gram-positive bacteria stain purple and gram-negative bacteria stain pink with the Gram's

stain, a method of staining developed by Hans Christian Gram in 1884[6]. The cell wall of gram-positive bacteria is stiff due to presence of a very thick peptidoglycan layer, along with Teichoic Acid (TA) and Lipoteichoic Acid (LTA) which provide additional rigidity the cell. These unique properties of gram-positive cell wall give the bacteria resistance to high salt concentration and temperature, and to β-lactam antibiotics. Many gram-positive bacteria can also have a proteinaceous surface layer, that assists in surface attachment and biofilm formation.

On the contrary, the cell wall of gram-negative bacteria has very less concentration of peptidoglycan as compared to gram-positive but have an additional membrane made up of lipopolysaccharide and phospholipids, outside the peptidoglycan layer. The surface layer is also present in many wild gram-negative bacteria, although this generally disappears in laboratory cultures.

Worth mentioning are the bacteria which do not fall in either gram-positive or gram-negative category, as they do not respond to Gram stain including *Mycoplasma*, *Mycobacterium tuberculosis* and *Mycobacterium leprae*.

- **Plasma membrane:** Bacterial plasma membrane is a phospholipid bilayer acting as selective barrier to molecules in and out of the cell. Additionally, it also acts as an energy generation site and helps in cell-to-cell communication. Structurally the phospholipid bilayer, arranged in a way that have the hydrophilic polar heads are in contact with cytoplasm and with the aqueous environment outside the cell, while the hydrophobic non-polar head remains buried inside. The entire bilayer is spanned by embedded integral proteins which are involved in the transport, signal transduction, and energy production, while the membrane's surface has loosely attached peripheral proteins that provide structural support and play role signaling[7].

When it comes to the internal structure of bacterial cells, they are significantly simpler than their eukaryotic counterparts. While membrane-bound organelles are absent, certain distinct and observable structures can still be found within the cytoplasmic matrix.

- **Nucleoid:** It is an irregularly shaped region where bacterial chromosome is located, and is not enclosed by any membrane. The bacterial DNA is just a highly compact supercoiled structure lacking any histones. The chromosomes in most bacterial species are circular, with some exception of it being linear as in *Borrelia burgdorferi*. Moreover, typically only one chromosome is present in most species, although species with multiple chromosomes have also been found[8].

- **Plasmids:** Besides the chromosomal DNA, most bacteria also carry some small independently replicating pieces of DNA termed as plasmids. Plasmids can be present in varied number in the bacterial cell and in general are not essential to the bacteria itself, but can code for traits which can be advantageous in conditions of stress. Frequently occurring genes on the plasmids can be those coding for antibiotic resistance or certain virulence factors needed to cause infection[9]. These plasmids (or its parts) can get transferred from one bacterium to another during processes, such as conjugation, transduction and transformation.
- **Ribosomes:** Ribosomes are the most abundant structures inside a bacterial cell, which is the protein synthesis machinery for all living cells. The ribosome in bacteria is 70S type and made up of two fragments- 50S and 30S.
- **Other structures:** Additionally certain bacterial species also possess some special internal structures. Intracellular membranes are additional membranes (or an extensions of cytoplasmic membrane) found in nutrifying and phototropic bacterial species. Prokaryotic cytoskeleton, a collective name for structural filaments, have also been observed using advanced microscopic techniques, which are believed to play a critical role in protection, cell shape maintenance and cell division[10]. Cyanobacteria species have been found to possess membrane-bound spindle-shaped gas vacuoles, which provide buoyancy to the cell. Finally, certain nutrient storage bodies have also been found in bacteria, as these bodies are useful for survival during harsh times.

On the outer surface of bacterial cell, certain appendages can also be found. These structures are not typical for all species, and are bound to cell wall or/and membrane.

- **Pili and fimbriae:** Fimbriae are short protein tube like structures extending from the outer membrane, and are present in huge numbers all along the bacterial surface. *Escherichia coli* is one such commonly existing bacteria known to exhibit fimbriae on their surface. The main function of fimbriae is to facilitate attachment of bacteria- to surfaces during biofilm formation or to host cell during infection[11]. Pili are similar structures, just much longer in size and present in fewer numbers. Bacterial conjugation for exchange of plasmids occurs with the help of these structures where they are designated as sex pili[12].
- **Flagella:** These are possibly the most recognizable structures on the bacterial surface. They are whip-shaped structures attached at one end or both polar ends

or peritrichously arranged all over the cell. The main function of the flagella is to provide motility to the bacterial cell, and may assist in surface colonization[12].

In response to stress, some bacteria, such as members of *Bacillus* and *Clostridium* species can also form non vegetative structures known as endospores. Endospores are highly resistant to various kinds of chemical or environmental stress conditions and help in survival of bacteria in these conditions, which otherwise are lethal to vegetative forms[13].

1.1.3 Beneficial roles of bacteria

Bacteria are a very essential part of our ecosystem, continuously affecting various processes which makes them indispensable in maintaining the life-support systems of the planet. We humans have also learned to use bacteria for our own benefits in day-to-day life.

- **Organic matter and nutrient recycling:** Bacteria play very important role in nutrient recycling and maintain the organic balance in the ecosystem.
  Bacteria species from several genera can decompose complex organic matter to simpler compounds, releasing all nutrients back into the environment while some species (e.g. *Cyanobacteria*) fix the carbon dioxide from atmosphere generating organic matter and increasing biomass[14].
  Nitrogen fixing bacteria (e.g. *Rhizobium, Azotobacter*) convert atmospheric nitrogen into ammonia which increases its availability in soil while the nitrifying bacteria (e.g. *Nitrosomonas, Nitrobacter*) convert ammonia into nitrates, providing nutrients to plants. Denitrifying bacteria (e.g. *Pseudomonas*) send back the nitrogen to atmosphere as nitrogen gas which completes the cycle. There are certain sulfur-oxidizing bacteria (e.g. *Thiobacillus*) in the environment that actively participate in in the sulfur cycle[15].
- **Symbiotic associations:** Species from various bacterial genera can form mutually symbiotic relationship with other organisms, providing ecological benefits. The nutrient fixing bacteria, can increase availability of usable compounds, while living symbiotically with the plants. Endophytic bacteria produce antimicrobial compounds that protect plants against pathogens, assist in nutrient uptake and modulation of phytohormones during growth and stress[16]. Symbiotic bacteria (e.g. *Ruminococcus*) living in gut of ruminants help in digestion of cellulose and nutrient absorption[17]. Human gut also harbors a

plethora of bacteria that is beneficial to human body in a number of ways, such as providing ability to harvest nutrients which are otherwise not accessible[18].

- **Bioremediation:** Some bacteria are used to clean up pollutants like plastics, oil spills and heavy metals. Hydrocarbon-degrading (e.g. *Pseudomonas*) bacteria has a huge potential in tackling the problem of oil spillage in marine water[19].
- **Food and beverage industry:** *Lactobacillus* and *Streptococcus* is widely used to produce yoghurt, kefir and cheese, and *Acetobacte*r helps in production of vinegar through oxidation of ethanol. Various enzymes, such as lipases, proteases and cellulases used in food industry are also extracted from bacteria.
- **Scientific and medical research:** *Escherichia coli* is widely used as model system in research for genetic and molecular studies. Recombinant bacteria are used to produce macromolecules such as nucleic acids and proteins. Others are used in medicine to produce antibiotics to be used for treating infections.

In addition to the beneficial roles of bacteria, they also pose many threats to the well-being of living organisms bringing significant economic, social and medical challenges. Some bacteria can be pathogenic (e.g. *Xanthomonas, Erwinia*) to food plants, causing species extinction and affecting food security. The pathogenic bacteria are responsible for plethora of disease in birds, animals and fishes, particularly affecting dairy and meat industry. From food spoilage (e.g. *Pseudomonas*) to food poisoning (e.g. *Salmonella*), the list of harmful bacteria just goes on, however, the interactions between bacteria and human health have always been of particular importance. Continuous efforts are going on to better understand this relationship to tackle diseases caused by bacteria.

1.1.4 Problems caused by bacteria with respect to human health

In general, human body is colonized by hundreds of bacterial strains and there can be as many bacterial cells as cells in our body[20]. They peacefully exist on the skin, airways, mouth, gastrointestinal, urogenital tract and the gut[21], and cause no harm to the host. However, there exists a group of pathogenic bacteria that are responsible for a range of serious infections in humas, such as tetanus, tuberculosis, pneumonia, various urinary and genital tract infections and severe sepsis that can lead to death[22]. These bacterial infections cause huge losses financially, mentally and physically to individuals as well as to society as a whole.

1.2 Antibiotics

Antibiotics are compounds that inhibit bacteria growth or division and thereby used very often in modern healthcare to prevent or treat bacterial infections. However, this was not the case always and this miracle drug is only a century old. Nevertheless, antibiotics did revolutionize the way bacterial infections are treated since their inception in early 1930s.

The history of relationship between bacteria and human society is ancient, multifaceted, and covers both the positive and negative interactions. It is marked by humanity's evolving understanding of bacteria and their role in various diseases. However, in early days, poor understanding of bacterial infection and transmission led to unsanitary practices, often causing severe disease spread and huge number of deaths. In 1676, Antonie van Leeuwenhoek observed the first bacteria using a microscope, and called them as animalcules[23]. After over a century, the works of Louis Pasteur and Robert Koch between 1860 and 1880 demonstrated that specific bacteria cause specific diseases, laying the foundation of germ theory. The discovery of *Anthrax* in 1876 by Koch launched the field of "medical bacteriology" and a 'golden age' of scientific discovery ushered[24]. He even tried to develop vaccines against certain diseases, such as tuberculosis[25].

1.2.1 Discovery of antibiotics: historical perspective

A lot of advancements took place from the beginning of 20th century and for convenience, and important events can be put into broadly divided timescale.

1910s: The use of microbes producing some compounds to prevent diseases stretches back millennia, however development of first anti-infective drugs is widely accredited to Paul Ehrlich for the discovery of antibiotic salvarsan[26].

1920s to 1960s: The discovery of penicillin by Alexander Flemming in 1928 became a gamechanger in the fight against pathogenic bacteria, and it revolutionized the treatment of bacterial infections. Initially, a lucky accidental finding, Flemming managed to show that fungus *Penicillium notatum* inhibits *in vitro* growth of bacteria, however he was unable to isolate or characterize penicillin[27,28]. But his article became the basis for a team lead by Howard Florey and Ernest Chain to start research on new antimicrobials. In 1940, they published paper on penicillin purification in quantities enough for clinical testing[26,28]. Tests were conducted on wounded World War II soldiers and came out as highly successful. Further improvements in the protocol paved for penicillin's mass production in 1945 and

by 1946 was widely available for prescription[27,28]. This discovery changed the course of medical history, saving thousands of wounded people, further laying the foundations for the "golden era of antibiotics".

Few other important milestones during this time period were discovery of prontosil in 1935, which was clinically available even before penicillin as "miracle" cures against pneumonia and child fever[29]. René Dubos is credited for the discovery of gramicidin and tyrocidine, with the help of Rollin Hotchkiss[30]. Selman Waksman discovered streptomycin and neomycin produced by soil actinomycetes[26]. Chloramphenicol, colistin, vancomycin, erythromycin, tetracycline were few more important antibiotics discovered[31]; expanding the antibiotic arsenal with multiple novel discoveries.

It is also worth mentioning that even before the wide spread use of antibiotics, bacteria were already finding ways to evade conditions threatening their survival, including from exposure to natural antibacterials. Ancient resistance genes against β-lactam antibiotics have already been found in 30,000-year-old permafrost samples[32]. Reports as early as from 1940, suggest that bacteria were able to destroy penicillin enzymatically[33]. As the use of antibiotics became more frequent, so was the observation of species that can evade the treatment- a phenomenon commonly referred to as antibiotic resistance[34]. This led to rise in studies for synthetically modified penicillin to protect its enzymatic degradation[35].

1970s to 1980s: This period was mostly dominated by substantial innovation in the antibiotic discovery with several important antibiotics being added to combat bacterial infections, as well as production of semi-synthetic antibiotics. Fosfomycin, linezolid and daptomycin were important antibiotics popularized during this time, to cope up with the decreasing effectiveness of the existing antibiotics.

1990s to today: By the 1970s the antibiotic pipeline already got slowed down dramatically and the discovery of novel antibiotics has become very slow. One possible proposed reason can be that pharmaceutical companies shifted to chronic disease treatment research which are more lucrative financially, and offered long-term stable revenue than antibiotics[36]. Moreover, failure of antibiotics over time is of great concern with bacteria evolving ways to circumvent the drug effects and is greatest challenge to new antibiotic discovery[37]. However, the good news is that the research is progressing well towards handling bacterial infections using alternative strategies, such as antimicrobial peptides, bacteriophage therapy, antibacterial nanoparticles, immunomodulators and monoclonal antibodies[38].

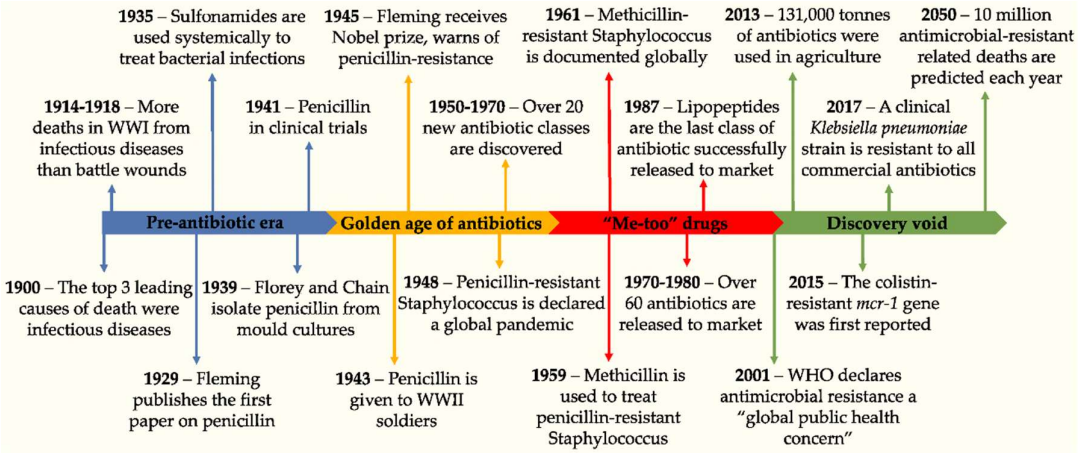

Figure IF2: Important events in timeline of antibiotic discovery and resistance development

*Figure reproduced from the article published by Browne et al[39].

1.2.2 Types of antibiotics

Antibiotics can be broadly classified into two categories based on their mode of action on bacteria- bacteriostatic and bactericidal[40].

Bacteriostatic antibiotics manage the infections by inhibiting the bacterial growth and reproduction without actively killing them, thereby giving host's immune system enough time to eliminate the pathogens. They are typically used where the host's immune response is robust enough to clear the pathogens once bacterial growth is kept under check. These antibiotics primarily target cellular processes that are essential for growth and division, although the effects are reversible and cells generally continue their normal growth once the antibiotic pressure is removed. Examples include tetracycline that binds to bacterial ribosomes inhibiting the translation of mRNA into protein, or trimethoprim activity that binds to dihydrofolate reductase blocking the bacterial DNA synthesis[41]. However, higher concentration of bacteriostatic may show bactericidal activity[42].

Bactericidal antibiotics on the other hand directly kill the bacteria by targeting and damaging bacterial cell structures, such as cell wall, membrane or DNA. These antibiotics are indispensable in treating infections, particularly those requiring immediate bacterial elimination. The action of the antibiotics is irreversible as the damage caused to the cell components is beyond repair. Prominent example is gentamicin which irreversibly binds to ribosome leading to disruption of essential proteins[43], and ciprofloxacin which target DNA gyrase leading to DNA damage[44].

1.3 Quantification of bacteria

The constant efforts by the researchers to understand bacteria got a major push after it was understood that specific bacteria cause specific behavior. With the advancements in microbiology, technology and scientific understanding, the methods to identify and quantify bacteria also evolved. Initially, the studies were mostly limited to the indirect observations of bacterial presence, and over time various techniques were developed to enumerate and analyze the bacterial populations. Since then, the quantification of bacteria has been a cornerstone in microbiology, medicine and environmental science. Today it plays a very crucial role in defining the dosage of antibiotics in both research and clinical studies. The standard ways for bacteria counting are plate count method, direct microscopic count, measurement using spectrophotometry and flow cytometry. Each of these methods carry their own set of advantages and disadvantages making their use situational.

- **Plate counting method:** A traditional but very commonly used method to enumerate viable bacteria is the plate counting method. In this method a liquid culture containing unknown concentration of bacteria is serially diluted, and aliquots are spread on solid agar plates. Generally, serial dilutions are prepared from original sample and a small fixed volume from each concentration is plated on independent agar plates. After incubation, the observed bacterial colonies are counted, and colony forming units/ml (CFU/ml) of the original sample is established taking into consideration the plated volume and dilution factor. The typical counting ranges are 25-300 colonies per standard plate, so the dilution having colony count in this range is to be used. Plate counting is often used in food safety, water quality testing, microbiological research and clinical microbiology[45]. The method is inexpensive and simple, but can be labor intensive, time consuming and inapplicable for unculturable bacteria.
- **Direct microscopic count:** This is a simple method that employs a specialized ruled slide, a coverslip and a microscope. One such specialized device is Petroff-Hausser counting chamber constructed in a way so that the coverslip, slide and the lines delimit a known volume of liquid. The prepared liquid sample with bacteria is carefully loaded and directly counted under a microscope. The bacterial concentration in the original sample can be determined by extrapolation, adjusting with any dilutions. It is rapid and easy method for counting both live and dead cells, although precision is hard to achieve.

- **Spectrophotometry:** Measurement of optical density is a common method employed to determine the concentration of bacteria in cultures. The method directly relies on the correlation between observed turbidity of the sample and changes in the bacterial cell numbers. Simple spectrophotometer consists of a light source, a sample holder and a detector. Higher the cell density, higher is the absorption and scattering of the light by cells and less light reaches the detector. Continuous reading over fixed time interval for growing cultures can be recorded to establish growth kinetics. The measurements are usually taken 600 nm due to the ease of generating and measuring this wavelength[46]. Spectrophotometric methods are fast, accurate and can be used for large number of samples at once. Drawbacks with this method include inability to distinguish between live or dead cells, the results can be affected by clumping of culture, and the sensitivity is limited[47].
- **Flow cytometry:** Flow cytometry-based methods are relatively novel and more advanced, offer much more information, can distinguish live vs dead cells, are high-throughput and very sensitive. The bacterial cells are labelled with relevant dyes depending on the kind of information one wants to extract. The prepared bacterial sample is injected into the flow cytometer, which then gets focused into a narrow buffer stream to flow one cell at a time cutting a laser beam. The bacterial cells scatter light, and the interaction of the laser beam with labelled dye emit characteristic wavelength which are recorded by the detector[48,49]. Obvious disadvantages include need to label the bacteria, expensive equipment and little bit sophisticated handling of the setup.

There are more methods available and their applications depend on the specific context, however, the above-mentioned methods remain most widely used in research and medicine.

1.4 Antimicrobial susceptibility testing (AST)

Discovery of antibiotics remains a very important milestone of scientific discovery in the fight against bacterial infections, and so was the ability to isolate and culture bacteria. The next important task for scientists was to determine the sensitivity of the antibiotics to specific pathogens by methods, such as antimicrobial susceptibility testing (AST), so as the clinicians can define proper treatment regimen. AST is a laboratory procedure routinely done to identify which antibiotic can be most effective for the patient[50,51]. It is also

performed by researchers to establish the susceptibility pattern of a specific strain to one or more antibiotics. The gold-standard AST techniques are based on the detection of phenotypic bacterial growth in presence of antibiotic under consideration[52]. A parameter called minimum inhibitory concentration (MIC) is very commonly used in AST and which corresponds to the lowest concentration at which an antibiotic inhibits the visible growth of bacteria under consideration[53]. Important international organizations (Clinical and Laboratory Standards Institute, CLSI, and the European Committee on Antimicrobial Susceptibility Testing, EUCAST) annually give out breakpoint concentrations and they can be used as reference to find if a tested strain is susceptible to the antibiotic. Today there are various methods employed for AST and the choice of method is generally based on time, skills, sample type, cost, equipment available and the accuracy required[54].

1.4.1 Broth dilution method

Broth dilution method is a gold-standard technique and is highly in use even today, based on observation of bacterial growth over a range of antibiotic concentrations. Depending on the initial sample volume, the method can be classified into two: micro-dilution (0.05 - 0.2 ml) or macro-dilution (1.0 mL) type. First a serial two-fold dilutions of the antibiotics are prepared in the culture media, and then a known concentration of bacteria is added to each of the tubes. After appropriate incubation temperature and duration, the turbidity measurements are done to find MIC[54,55]. The broth dilution method is easy to perform and a very well standardized method, however it can be time consuming and is also prone to individual mistakes.

1.4.2 Disk diffusion method

Disk diffusion method was first developed in 1940 and since then it has remained the most widely used AST method in clinical microbiological laboratories[56]. In this method, pre-soaked antibiotic disks are placed on previously inoculated agar with bacterial suspension. The antibiotic then immediately starts to diffuse outward from the disks, forming a gradient of antibiotic concentrations. The plates are then incubated at proper temperature for desired duration, and the inhibition zone diameters around the disks are recorded[57]. Advantages of this method include the ease of usage and being relatively inexpensive, however the method is time consuming and cannot provide any quantitative measurement, so the MIC cannot be established[55].

1.4.3 E-test

E-test is a commercially available paper strip-based test, impregnated with the particular antibiotic in decreasing concentration along the strip. A printed numerical scale on the strip denotes the antibiotic concentrations. It is basically a combination of disk diffusion and broth dilution methods, offering advantages of both the methods i.e. it allows MIC to be determined while being simple and is easy to use. The E-test strip is placed on a freshly inoculated agar plate and incubated at required temperature for required duration. The growth of bacteria around the strip is noted and MIC is determined according to the numerical scale of antibiotic concentrations[52]. It is a very simple and convenient method to perform however takes long time depending on observable bacterial growth.

1.4.4 Automated antimicrobial susceptibility testing systems

There is immense pressure on clinical microbiology laboratories to offer fast and reliable microbial identification and AST methods[52]. As a result of the demand, automated and semi-automated methods started to appear which nowadays are widely used offering great efficiency, low cost and fast speed[58,59]. These instruments use advanced optical systems for measuring subtle changes in the bacterial growth and can quickly establish the antibiotic susceptibility[60]. Few popular examples include VITEK 2 (bioMérieux, France) and MicroScan Walkaway (Dade-Behring Microscan, USA), which of course come with their unique set of advantages, such as handling large number of samples, being fast and accurate however the instruments themselves are expensive and require technical expertise[54].

1.4.5 Genotypic methods

Most of the times the antibiotic resistance is genetically encoded and this can be determined by detection of the specific genes involved[61]. Genotypic methods can directly detect these resistant genes as well as relevant mutations and gene expression levels. These methods are generally in complementation to conventional AST and are very fast, typically just one to few hours[62]. Some of the most commonly used techniques in genotypic methods are polymerase chain reaction (PCR)[63], fluorescence in-situ hybridization (FISH)[64], loop mediated isothermal amplification assay (LAMP)[65] and Whole genome sequencing (WGS)[66]. These methods are fast, highly sensitive and can be used even for slow growing bacteria. Major disadvantage is the cost associated with these methods and the technical expertise required.

1.4.6 Mass spectrometry

Mass spectrometry-based methods are only recent introduction for the rapid detection of antimicrobial resistance. These methods are simple, fast, need low sample volume however does not allow the establishment of MIC value and can be quite expensive. Several mass spectrometry-based strategies have been used for detection of resistance including by monitoring the modification of enzyme in the growing bacteria[67], direct detection of proteins conferring antibiotic resistance[68] and fluoroquinolone acetylation[69].

1.5 Interactions between antibiotics and bacteria

The events that occur during the interaction between bacteria and antibiotics are very important in the context of microbiology, pharmacology and medicine. Antibiotics have revolutionized the healthcare by offering a reliable treatment for bacterial infections; however, bacteria do try to evade the antibiotic effects, making the understanding of these interactions very crucial.

1.5.1 Mechanism of antibiotic action

One way to classify antibiotic is based on their mechanism of action on the bacterial cell.

- **Cell wall inhibitors:** As discussed earlier, a very important constituent of cell wall is peptidoglycan, which consists of long sugar polymers- N-acetyl muramic acid (NAM) alternating with N-acetyl glucosamine (NAG), connected by chains of amino acids[70]. NAM and NAG form the peptidoglycan precursor which later undergoes a lot of cross-linking with the help of two enzymes- carboxy peptidase and trans peptidase[71]. The β-lactam antibiotics, such as ampicillin, cefixime, meropenem etc. target the enzymes for peptidoglycan cross linking, eventually leading to inhibition of cell wall synthesis and cell death[72]. Another class of antibiotics glycopeptides, such as vancomycin make non-covalent bonding with terminal carbohydrates, inhibiting the cross linking[73].
- **Cytoplasmic membrane inhibitors:** The integrity of cell membrane is very important for the bacteria, as it helps in selective transport of molecules and any damage to it can be fatal to the cell. Antibiotic polymyxin has a very strong affinity with phospholipids and disrupts the membrane[74]. It is very effective against gram negative bacteria and are is last resort antibiotic against certain bacteria[75]. Gramicidin is another antibiotic used against gram positive bacteria that works by

disrupting cell membrane and increasing its permeability[76]. However, these antibiotics are also toxic to eukaryotic cell, and so their usage is only recommended in controlled doses and as last resort.

- **Nucleic acid synthesis inhibitors:** Bacteria generally undergo binary fission to give rise to two new daughter cells[77]. However, before the separation of the two cells, an exact copy of the circular DNA is created by replication[78]. Fluoroquinolone antibiotics, such as ciprofloxacin, levofloxacin inhibit a crucial enzyme DNA gyrase required for DNA replication process, thereby causing the cell death[79].
- **Protein synthesis inhibitors:** The information on bacterial DNA is the basis to make RNA molecules through a process called transcription, and then the ribosomes in the cell synthesize proteins from the RNA by a process called translation. Some antibiotics act by targeting the protein synthesizing machinery of bacteria, specifically the 30S or 50S subunit of the ribosomes[80]. Tetracyclines and aminoglycoside antibiotics, such as amikacin, gentamicin interact with the 30S subunit of the ribosome causing misreading and premature termination of translation[81,82]. Others, such as chloramphenicol and macrolides affect the 50S subunit, leading to inhibition of protein synthesis[83].
- **Folate synthesis inhibitors:** Folic acid is necessary for synthesis of nucleic acids, growth and multiplication of the cell. Bacteria have the ability to synthesize folic acid and the enzymes of the pathway are a vital target for antibiotics. Sulfonamides affect enzyme dihydropteroate synthase, while trimethoprim affects dihydrofolate reductase- the enzymes essential for synthesis of folic acid[84].

In majority of antibiotics and most of the times there is cumulative effect of direct antibiotic action and the stress created in bacteria that leads growth inhibition and cell death.

1.5.2 Antimicrobial resistance

Antimicrobial Resistance (AMR) occurs when microbes no longer respond to antimicrobial compounds which previously used to inhibit them. It is an umbrella term used in the context of all microbes and related antimicrobial drugs; however, it generally refers to bacterial AMR as resistance of bacteria to antibiotics is the most critical one. As previously discussed, the discovery and subsequent developments of antibiotics have been pivotal milestones in the history of medicine, revolutionizing the management of bacterial infections. However, their large-scale uncontrolled use over time has led to the emergence

of antibiotic resistance, bringing significant challenges to public health. In fact, the phenomenon of antibiotic resistance was observed as early as in 1930s, immediately following the introduction of first antimicrobial agents[35]. A transformative shift occurred in the treatment of bacterial infections with introduction of penicillin in clinical practice during World War II. However, shortly after, the enzyme penicillinase was discovered in naturally occurring bacteria, indicating that the resistance mechanisms were already present in environment even before the antibiotics became widely used[85,86]. The pattern continued even with the semi-synthetic penicillin antibiotic, methicillin, as in methicillin resistant *Staphylococcus aureus* or MRSA[87]. These examples underscore the rapid emergence of resistance, emphasizing the inherent plasticity of bacterial genomes and their ability to evolve in response to selective pressures exerted by antibiotics[85,86].

In 2021, it was estimated that 4.71 million deaths were associated with the antibiotic resistance problem, including 1.14 million deaths attributable directly. It is also predicted that by 2050 it could directly cause 1.91 million deaths annually, while contributing to a whopping total of 8.22 million deaths per year[88]. This makes AMR as one of the biggest threats to human health and needs urgent attention for new antibiotic discovery and improving stewardship practices.

Resistance mechanisms have evolved in response to selective pressures from antibiotic use, making previously treatable infections increasingly difficult to manage. The emergence of extremely drug-resistant (XDR) and multidrug-resistant (MDR) strains bring further challenges, complicating treatment options and leading to increased medical costs, severe disease spread and increased mortality rates[85,89,90]. The multifactorial nature of antibiotic resistance includes various contributing factors, such as non-compliance with treatment protocols, inadequate infection control measures in healthcare units and improper use of antibiotics in livestock farming. These faulty practices accelerated the spread of resistant bacteria, which can reach to humans through the food supply or direct contact, perpetuating the cycle of resistance development[91–93]. Additionally poor sanitation and non-hygienic infrastructure further exaggerates the situation, particularly in developing countries[93].

However, some bacteria as just naturally resistant to certain antibiotics due to the inherent structure of the cell or functional characteristics. The natural resistance can be intrinsic (always expressed in species, such as reduced permeability of the outer membrane in gram

negative bacteria and natural activity of efflux pumps) or can be induced (the genes for resistance occur naturally, but expressed only on antibiotic exposure)[89,94].

Bacteria can also acquire resistance over time through spontaneous mutations[61] or they can obtain resistance genes from other bacteria horizontal gene transfer i.e. conjugation, transduction, and transformation[85]. I conjugation, plasmids containing resistance genes are transferred between bacteria through direct contact. Transduction involves bacteriophages mediating the transfer of resistance genes between bacterial cells, while transformation allows bacteria to uptake free DNA from their environment.

The whole process of resistance can be complex phenomenon, evolving from various mechanisms that enable bacteria survive the antibiotic treatment, however it can be divided into few major types based on the strategies used by bacteria,

- **Enzymatic degradation or modification of the antibiotic**

  This is one of the most common mechanisms of acquired resistance where certain bacteria can produce enzymes, such as beta-lactamases, that can hydrolyze the beta-lactam ring of penicillin and cephalosporins, rendering these drugs ineffective. This allows bacteria like *Escherichia coli* and *Klebsiella pneumoniae* survive the exposure of antibiotic[61]. Antibiotic molecules can also be chemically modified by transferase enzymes through addition of functional groups on antibiotics, rendering them ineffective[89].
- **Changes in the antibiotic target site**

  Another frequent strategy used by bacteria to gain resistance is to avoid the antibiotic action by interfering with the antibiotic target, such as their protection or modifications. For example, the penicillin binding proteins (PBPs) in the bacteria can get altered structurally, affecting the activity of penicillin[95]. Resistance to antibiotics that target ribosomal subunits have been observed to occur through subunit methylation (oxazolidinones) or protection of ribosomes (tetracycline) and thereby hindering the binding of the antibiotic to ribosomes[89]. Modification in DNA gyrase or topoisomerase IV have led to decreased binding of fluoroquinolones, leading to resistance[96]. Another strategy used by bacteria is to switch to an alternative metabolic pathway, helping bacteria to flourish normally even if the original metabolic pathway was hampered by the antibiotic, as seen in resistance against trimethoprim-sulfamethoxazole[61].

- **Decreasing drug uptake or increasing active efflux**

  Some bacteria have developed mechanisms to prevent the antibiotic from reaching its target inside the cell by decreasing the uptake of antibiotic. Hydrophilic antibiotics, such as β-lactams and tetracyclines are particularly blocked from entering the cell due to changes in permeability of the outer membrane[97]. The active efflux pump mechanism to pump antibiotics out from the cell thereby decreasing the antibiotic concentration in the cytosol, was first described in *Escherichia coli*[98]. Since then, many such efflux pumps have been discovered in both gram negative and gram-positive strains.

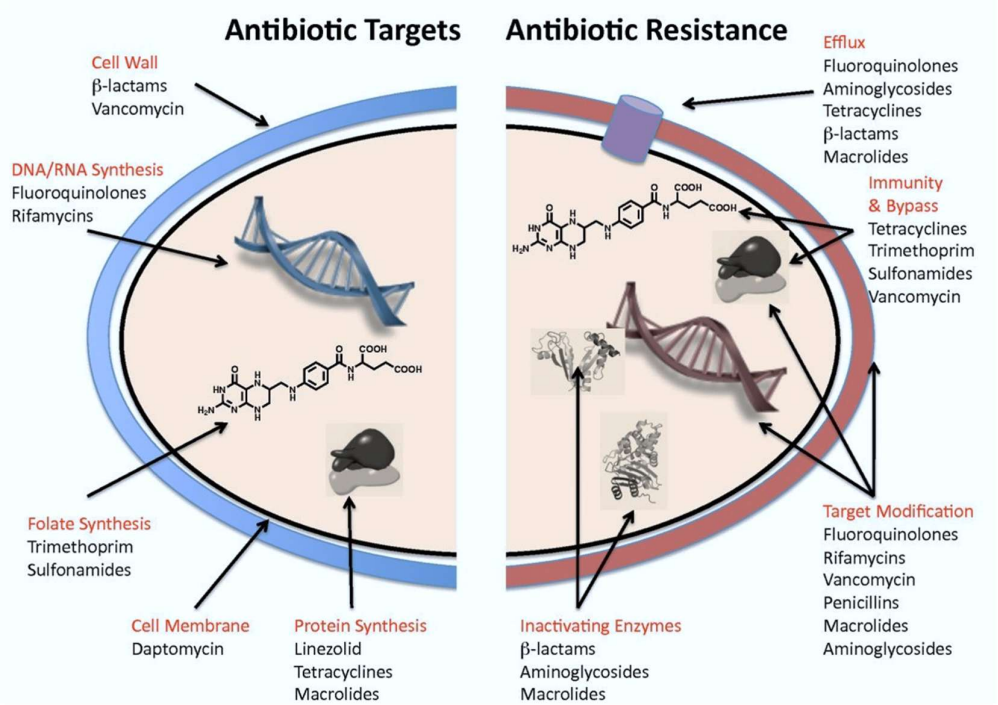

Figure IF3: Antibiotic mechanism of action, their target and mechanism of resistance
A model of bacterial cell shows the various targets for different antibiotic types and the strategies resistant bacteria use to counter the antibiotic action. Few important examples of antibiotics from each mechanism of action class as well as type of resistance is also given.

*The figure is reproduced from article published by Wright[99].

1.5.3 Role of Reactive oxygen species in antibiotic action

Reactive oxygen species (ROS) are chemically reactive molecules that exist independently, and contain one or more unpaired electrons and at least one oxygen atom[100]. Examples of ROS include superoxide hydroxyl radicals, superoxide anion radicals, hydroperoxyl radicals and singlet oxygen. ROS produced by all aerobic cells as a result of metabolic

processes which is tolerable in small quantities, however, it is also produced during cellular stress[101]. Bacteria harbor protective proteins that can help detoxify the ROS and mitigate associated damage, however, during severe stress bacteria might use ROS as a self-destruction mechanism, which, contributes to lethal action of many antibiotics[102].

Multiple studies have shown that bactericidal antibiotics including aminoglycosides, β-lactams and quinolones can induce oxidative stress in bacteria[102–104]. Antibiotics trigger a cascade of events leading to ROS generation, metabolic disruptions and ultimately cell death[105]. The antibiotic-induced changes in the electron transport chain and other metabolic pathways increase the intracellular ROS levels, overwhelming bacterial defense systems[105]. Further studies validated that ROS production promotes DNA, protein and lipid damage, worsening the lethality effects prompted by antibiotics[106]. The strains with better antioxidant defenses have been reported to possess greater tolerance for antibiotics; and this strengthens the link between ROS and the death caused by antibiotics[106].

There are multiple ways through which ROS contributes to bacterial killing:

- **DNA damage:** ROS can cause DNA strand breaks, base alterations and lesions causing genomic instability and bacterial stress response activation. Ultimately, the irreparable DNA damage stalls replication and leads to cell death[107].
- **Protein oxidation:** Oxidative stress causes irreversible damage to the amino acids, misfolding and degradation proteins and impairment of essential enzymes[108].
- **Iron-sulfur cluster disruption:** A number of bacterial enzymes possess iron-sulfur clusters that are prone to ROS; leading to metabolic dysfunction or cell death[105,109].
- **Lipid Peroxidation:** ROS damages lipids in the bacterial membranes, which increase their permeability and cause leakage of essential intracellular components, affecting membrane integrity and cellular homeostasis[110,111].

The involvement of ROS in antibiotic efficacy has led to significant research interest in their role during bacterial killing and antibiotic resistance. Many bacteria have already been reported to have evolved defense mechanisms against ROS, such as superoxide dismutase and catalases to better handle the oxidative stress caused by the antibiotics. Targeting these defense mechanisms can make bacteria vulnerable to ROS and thereby more susceptible to antibiotics. Novel ROS based therapies to treat antibiotic infections are being proposed to tackle increasing antibiotic resistance[112]. ROS generation is also critical in the context of

antibiotic exposure as non-lethal level of H_2O_2 is reported to enhance bacterial survival[113] and ROS can also cause the de novo acquisition of resistance to bactericidal antibiotics[114].

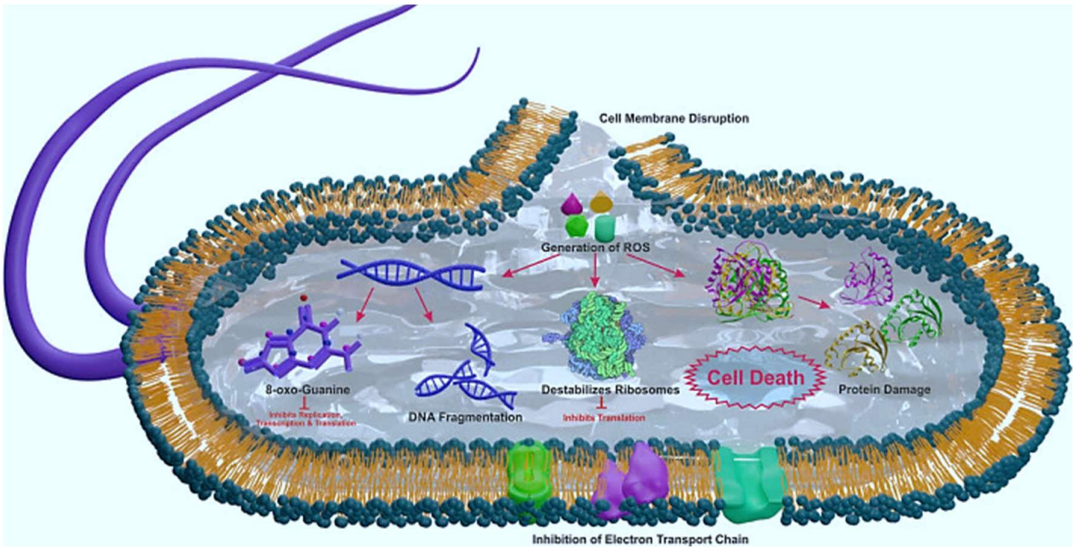

Figure IF4: Figure shows the model of ROS induced damage in bacterial cell
The induced oxidative stress causes a plethora of harmful effects in the bacterial cell including the nucleotide damage, ribosomal destabilization, protein denaturation which can eventually lead to cell death.

*The figure is reproduced from the article published by Bhatt et al[115].

1.5.4 Inefficient antibiotic exposure

The treatment regime by an antibiotic plays a pivotal role in determining how effective the treatment is in the context bacterial clearance, and preventing the emergence of resistance. However, inefficient antibiotic usage, such as suboptimal dosing, incomplete treatment course, or inappropriate antibiotic selection by both the medical professionals and the patient pose a significant challenge in global healthcare. An inappropriate dosing concentration, especially less than MIC, exerts low level selective pressure on bacteria without effectively eradicating them, allowing the selected strains to thrive and propagate[116]. Furthermore, empirical treatment regimes, without proper pathogen identification or AST increases the likelihood of choosing wrong antibiotic, allowing the infections to linger and leading to resistance development[117]. The wrong practices not only foster the development of antibiotic resistance but also adversely affects patient's well-being and increases healthcare costs[118]. Although this is a worldwide problem, the issue is more prevalent in underdeveloped or developing nations with inadequate healthcare access[119] or lack proper regulations for antibiotic prescription and usage[120].

Several other factors also lead to continuous low level antibiotic exposure of bacteria including the use of antibiotics in agriculture and aquaculture which can cause their leaching in nearby areas including soil, water and air. The inefficient management of hospital waste can is also responsible for unwanted presence of antibiotics in the environment around, and even to far-away places through waterways. Improper implementation of regulations at industry, government or at personal can further accelerate the process.

Whatever be the reason of inefficient antibiotic exposure, it has significant implications on the bacterial physiology, metabolism and evolution of antibiotic resistance. Some effects of sub-minimum inhibitory concentrations (sub-MICs) on bacterial culture include- increased phenotypic variability, promotion of mutagenesis and upregulation of virulence factors.

- **Promotion of phenotypic variability:** Sub-MIC levels of antibiotics can induce the formation of persisters- very slow or non-growing variants that can tolerate antibiotic exposure. A significant increase in the proportion of persister cells was seen during the sub-lethal exposure of *Pseudomonas* to aminoglycosides and fluoroquinolones[121]. The sub-MIC concentrations are also reported to alter biofilm formation and adherence properties in *Staphylococcus aureus*[122]. Biofilms are adherent aggregates of bacterial cells occurring on both biotic and abiotic surfaces, capable to resist antibiotic treatment and contribute to bacterial persistence[123].
- **Induction of mutagenesis and resistance:** Sub-MIC concentrations can also induce genetic mutations and promotes horizontal gene transfer, and lead to development of antibiotic resistance. Studies have documented that exposure to low doses of bactericidal antibiotics increased mutation rates in *Escherichia coli*, and resistance development against multiple drugs[124]. It can also invoke stress-based SOS response, cause mutations, promote genetic recombination and facilitate the acquisition of resistance genes[125–128].
- **Upregulation of virulence factors and improved pathogenicity:** Sub-MIC antibiotic can increase bacterial virulence by upregulating genes responsible for encoding toxins, adhesins, and secretion systems. Increased exoproteins levels were observed during clindamycin sub-MIC exposure in *Streptococcus* while induction of hemolysins by β-lactams was seen in *Staphylococcus*[116]. In another study, Sub-MIC β-lactam treatment enhanced the MRSA pathogenicity by upregulating the expression of a cluster of lipoproteins like genes[129].

The impact of low-level antibiotic exposure can be broadly distributed into three distinct categories: (1) resistance selection- allowing for the existing resistant cells to proliferate or selection for de novo resistance, (2) creating genetic or phenotypic heterogeneity- affecting the rate of adaptive evolution as well as promotion of resistance development, and (3) signaling molecules- acting as mediators for the physiological activities, for example gene expression and virulence[116]. In summary, the sub-lethal antibiotic exposure is believed to significantly accelerate the evolution of resistance, enhances virulence, and promotes the biofilm formation[124]. Understanding the mechanisms behind this selection[130] due to low-level antibiotic exposure is therefore crucial for optimizing the antibiotic therapy and implementing stewardship programs.

1.5.5 Heterogeneity in bacterial populations

Bacterial populations are heterogeneous, which in many cases can confer them a selective advantage during environmental changes, particularly at times of stress[131]. Heterogeneity, in broad context, refers to phenotypic and genotypic variations that occur within the bacterial populations, and therefore implying existence of distinct subpopulations. These variations enable bacteria to adapt to environmental changes, evade host immune responses and develop resistance to antibiotics[131,132]. The differences can be pre-existing in the population ensuring some individuals will survive any conditions, or the heterogeneity can be driven by environmental changes[133]. The presence of these distinct subpopulations growing in an environment is commonly attributed to a combination of stochastic processes and genetic architecture of the cells[131,134]. With growing cases of antibiotic resistance due to single-cell variations gathered the attention of scientists and clinicians in the domain was drawn, as this surviving subpopulation was typically under or over-represented in traditional antibiotic susceptibility studies[135].

- **Persistence**: Persisters refer to a very small fraction of the susceptible cell population, which are non-growing or slow-growing bacteria able to survive stress conditions, such as starvation or antibiotic exposure, and the phenomenon is called persistence[133,136]. These cells resume normal growth once stress is removed, but still susceptible to the antibiotic[136]. Persisters significantly contribute to persistence of chronic infections and recurrence of diseases following antibiotic treatment[137]. They are formed stochastically in the population in response to stress and are only a transient state, which is non-genetic and non-heritable[138].

- **Bio-films:** Biofilms are structured communities of bacteria that adhere to surfaces and are encased in a self-produced extracellular matrix composed of polymeric substances, providing structural integrity and protection to the microbial community[139]. Biofilms play a critical role in the survival of bacterial populations within the stress environment and contribute to severe disease states during infection with certain bacteria[140]. Inside the biofilm, cells exist in remarkably distinct physiological states, probably due to differential gene expression and metabolism[141]. They are implicated in various chronic infections, such as those associated with medical devices, periodontal disease, and cystic fibrosis. The protective nature of the biofilm matrix contributes to persistence of these infections, making conventional antibiotic therapies challenging[142,143].
- **Heteroresistance:** As mentioned earlier, multiple methods are used to assess the effectiveness of antibiotics on bacteria. However, these methods rely on average population data, and do not consider the response of individual cells, which is critical to elucidate heteroresistance within the population at smallest scale[144,145]. Seemingly isogeneic bacterial subpopulations can show a wide range of susceptibilities to an antibiotic, and the phenomenon is called heteroresistance[146]. This variability among cells can lead to incorrect AST results, improper antibiotic use and treatment failures, as the less resistant subpopulations may survive the antibiotic therapy, potentially leading to the emergence of fully resistant strains[146]. The frequency of heteroresistance differs greatly amongst species, but is believed to be equal to normal mutation rate, around one sub-clone per 10^4–10^6 colonies[147]. The mechanisms underlying the heteroresistance are multifaceted, complex, and not fully understood but studies in past have identified mutations, gene expression levels and regulatory changes as key contributors. Depending on the fitness cost of mutations, either give rise either to an unstable (cells revert to susceptibility) or a stable (cells do not revert to susceptibility) heteroresistance phenotype[148].

  One of the important mechanisms is amplification of genes which encode for antibiotic resistance. This results in increased gene dosage, leading to higher levels antibiotic tolerance in certain subpopulations. However, these amplifications are often unstable and resistant phenotype may revert to susceptibility in the absence of the selective pressure[135]. An increase in the copy number of plasmids carrying resistance genes is also known to contribute to heteroresistance. This state again is typically very unstable, with the resistance phenotype potentially reverting to

susceptible type when the selective pressure is removed[148]. The transposition of resistance genes onto cryptic plasmids, which then increase in copy number can also be a causative factor for heteroresistance, which again stays highly unstable[148]. Regardless of the source and stability of heteroresistance in the population, its detection remains highly crucial to establish correct susceptibility profile, to devise proper treatment regimen and to stop development of antibiotic resistance.

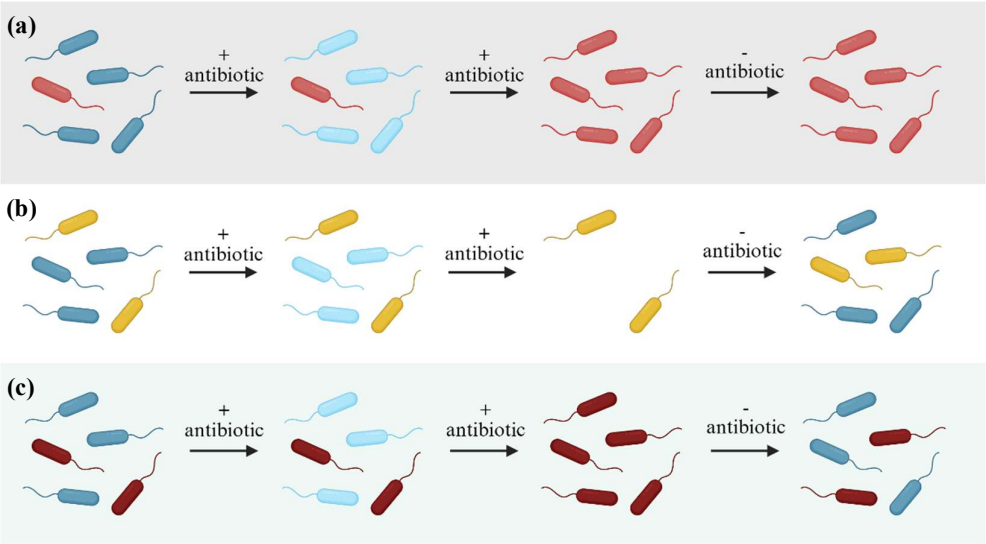

Figure IF5: Figure shows the difference between resistance, persistence and heteroresistance
(a) Resistance occurs mostly due to a stable genetic mutation that allows the bacteria to grow in presence of antibiotic, and finally this genetically novel mutant resistant cells take over the whole population; **(b)** Persisters are phenotypic variants that can survive antibiotics, although they stay metabolically inactive when antibiotic is present and do not divide. When antibiotic stress is removed, they can switch back to the sensitive phenotype and the new population is genetically identical to original; and **(c)** heteroresistance is much like resistance, and these cells can survive as well as grow in presence of antibiotic. However, the phenotype is generally unstable, and cells revert to the sensitive type when the antibiotic pressure is removed[131].

*Created in https://BioRender.com

1.5.6 Detection of heteroresistance

Heterogeneous antibiotic resistance was first described as early as in 1947[149]. However, there is still a lack of laboratory standards and recommendations to study heteroresistance. This complicates treatment regimens and presents significant challenges in clinical settings, as standard susceptibility tests may fail to detect resistant subpopulations, potentially leading to treatment failures and prolonged infections[150]. The ability to accurately detect heteroresistance is crucial for effective disease management, as antibiotic-resistant infections continue to pose a major health threat, with millions of cases and deaths

attributed to such pathogens annually[144]. Increased scientific interests and technological advancements led to the evolution of detection methods over the years, utilizing various approaches ranging from simple culture-based techniques to advanced genomic analyses.

The most common methods currently employed to detect bacterial heteroresistance are PAP (population analysis profile) test, E-test, disc diffusion and broth dilution assay[146]. PAP, one of the most reliable methods for detecting heteroresistance, involves exposing bacterial population to a gradient of antibiotic concentrations and observing the number of colonies growing at each concentration. A population exhibiting heteroresistance shows a bimodal or multimodal distribution of colony growth, with a population that can tolerate high antibiotic concentrations while the majority of cells remain susceptible. Although PAP is considered the gold standard, it is time-consuming, requires precise techniques for interpreting the data and needs high bacterial cell numbers. E-test can also be used for the detection of heteroresistance, by observing if colonies appear within the zone of inhibition, indicating that some bacteria can grow at higher concentrations of the antibiotic. It is relatively rapid and less labor-intensive method compared to PAP, though its sensitivity in detecting low-frequency heteroresistance is limited. The disk diffusion method employs the same strategy and heteroresistant colonies may appear inside the inhibition zone. This method is not very sensitive leaving minor resistant subpopulations undetectable, and generally requires extended incubation times. In broth dilution method, heteroresistance is indicated by the presence of a broad MIC distribution or the detection of growth in cultures that appear to be susceptible at lower concentrations. This method offers more precision in determining the resistance profile, but it does require a longer incubation time.

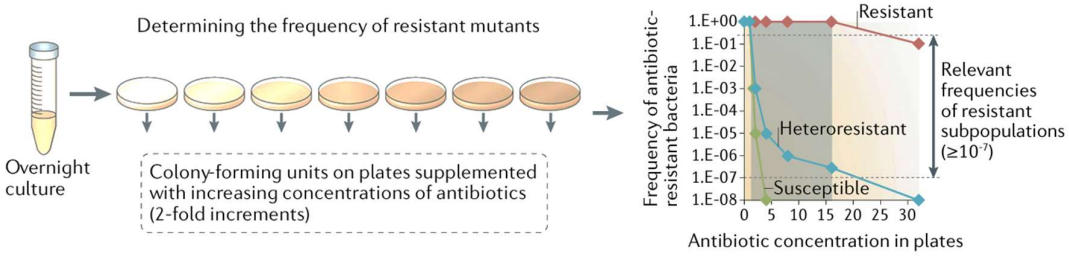

Figure IF6: Heteroresistance detection using population analysis profile (PAP) test
Left panel shows the schematics of whole test performed where bacteria are spread on agar plates with different concentration of antibiotics, colonies are counted after incubation. The panel on the right shows the presence of resistant, heteroresistant and susceptible variants.

*Figure reproduced from the article published by Andersson et al[135].

Some other advanced methods to detect heteroresistance include whole genome sequencing (WGS), nucleic acid amplification tests (NAATs) and automated systems[135]. WGS has become an increasingly valuable tool in identifying genetic mutations associated with resistance and understanding the molecular mechanisms underpinning heteroresistance. WGS can also be used to identify the low-frequency resistant subpopulations that may not be detectable using traditional phenotypic methods. However, the cost and the computational requirements of WGS remain barriers to its widespread use in routine diagnostics. Additionally, the ability of WGS to directly detect heteroresistance depends on the resolution of the sequencing platform, as detecting low-frequency mutations can be challenging. NAATs are PCR-based methods allowing the detection of known resistance genes by their amplification. It can simultaneously test for multiple resistance genes, reducing costs and turnaround times, however, they can be expensive and require strict quality control measures. Automated systems, such as the VITEK and BD Phoenix systems, are also capable of detecting heteroresistance by identifying unusual growth patterns or discrepancies in expected MIC results. These systems offer faster and more efficient resistance testing, but may not always detect heteroresistance, especially if the resistant subpopulations are present at very low frequencies.

Most of the techniques mentioned above carry the disadvantage of either population-level studies or turning out to be too slow when it comes to studying the resistance of individual cells. With the evolution of more single-cell study techniques, detecting heteroresistance at single-cell level started gaining attention due to their ability to identify resistant subpopulations within genetically homogeneous populations. These techniques provide high sensitivity by analyzing individual cells rather than bulk populations, making them particularly effective at detecting low-frequency resistant subpopulations that might be missed by traditional method. One method utilizes fluorescence microscopy, particularly fluorescent in situ hybridization technique, where fluorescent probes are used to detect specific rRNA sequences[151]. The sequence differences can help to identify the heteroresistance in individual cells. Another approach to identify antibiotic-tolerant subpopulation is the utilization of fluorescent reporter constructs and analysis of cells by flow cytometry[133]. The accumulated fluorescent signal provides information about cells that are sensitive or resistant to the antibiotic in the picture. Single-cell sequencing technologies, such as scRNA-seq combined with DNA sequencing and proteomics remain another powerful tool to study single-cell heteroresistance[152]. Last but not least,

microfluidics remains a powerful technology to study single-cells as it allows for the manipulation and analysis of small volumes of fluids, making it a promising tool for detecting heteroresistance in bacterial populations.

1.6 Microfluidics

Microfluidics is the science and technology of manipulating fluids at the microscale, typically using microchannels, allowing for handling nanoliter to picolitre volumes[153,154]. It gained significant attention due to its revolutionary applications in various fields, including chemistry, biology, physics, and engineering. By confining fluids to miniaturized channels, microfluidic systems enable precise control over fluid behavior, leading to enhanced mixing, rapid reaction rates, and analysis capabilities.

The origin of microfluidics can be seen in the basic sciences of chemistry and physics. However, the advancement in engineering methods significantly shaped its evolution. A group of researchers in 1990 first introduced the idea of miniature total chemical analysis systems, which led to a significant boost in the development of microfluidics[155]. The concept was inspired by the evolving techniques like chromatography, soft lithography, capillary electrophoresis and inkjet printing during the 1970s and 1980s[156,157]. Over the years, the technology just flourished with the developments of pumps and valves, allowing for more precise control in liquid flow handling. The ability to generate tiny aqueous droplets with the help of droplet microfluidics served as another milestone in the growth of microfluidics and indicated the possibility of using these droplets as nanoliter reactors in the future[158]. Today, microfluidic systems are revolutionizing many aspects of microbiology in labs, reducing manual labor and opening new perspectives in research[159,160]. Microfluidics has great applications in biomedical diagnostics, cell culture, tissue engineering, environmental monitoring and chemical synthesis[160,161].

At the microscale level, fluids possess very different characteristics than at the macroscale level and these characteristics are defined by factors, such as Reynolds number (Re), capillary forces and surface tension.

- **Reynolds number:** It is a dimensionless quantity describing the ratio of inertial to the viscous forces within a fluid. Re is proportional to flow speed and the length of the system, while inversely proportional to fluid viscosity. It can be used to describe the type of flow occurring in a fluid. A high Re is indicative of a turbulent flow,

dominated by inertial forces creating chaotic eddies, vortices, or other instabilities. At low Re, laminar flow tends to occurs where the viscous forces are dominant making the fluid motion constant and smooth. Laminar flow is a characteristic of microfluidic system, allowing for highly predictable fluid dynamics.

- **Capillary action:** Capillary action is the process in which a liquid can flow in narrow spaces without the assistance of any external forces, such as gravity. The capillary forces allow for the flow of liquid in the microfluidic system.
- **Surface and interfacial tension:** Surface tension is the property of a liquid that allows it to resist an external force due to cohesive nature of its molecules, causing shrinkage of liquid-air surfaces at rest to reduce free energy. Interfacial tension is a similar phenomenon, but it occurs between two immiscible fluids. These forces dominate the characteristics of a liquid at the microscale, even more than gravity.

1.6.1 Droplet microfluidics

Based on the number of fluid phases used, microfluidic setups can be classified into two types: single-phase continuous-flow system or a multiple immiscible fluids-based system[162]. Droplet microfluidics, a latter type system, enables the generation and manipulation of droplets through immiscible multiphase flows within microchannels. The size and uniformity of these droplets can be precisely controlled by factors, such as flow rate ratios, interfacial tension, and channel geometry. Over the past 25 years, droplet microfluidics has made significant progress in both theoretical and technical aspects. Due to its efficiency and reproducibility, it has found applications in a wide variety of domains, including single-cell studies[154,163], medical diagnostics[164,165], environmental monitoring[166], drug research[167] and material synthesis[168]. The potential of droplet microfluidics is considerable, as it facilitates enhanced mixing and mass transfer over short diffusion distances, eliminates boundary effects and allows for precise control over experimental time scales- from milliseconds to months[169,170]. Additionally, it enables single-cell and molecular analysis, compartmentalization into independent miniature reactors, and parallelization, making it a highly suitable platform for a variety of high-throughput applications. In 2008, a significant advancement was made when microfluidic droplets were successfully used as distinct compartments for microbial growth[171], opening up new possibilities for their use in a wide array of microbiological applications.

1.6.2 Droplet generation methods

Droplet generation in microfluidics can be achieved through either passive or active methods[172]. In passive droplet generation, the flow of two immiscible fluids is driven by an external pressure source, such as syringe pumps, pressure regulators or gravity-based pressure units, which are positioned away from the droplet formation site. In contrast, active methods employ external forces to locally control the droplet formation process, enabling faster and more precise manipulation. Depending on the type of energy applied, active droplet generation can be classified into thermal, electrical, magnetic or mechanical[173]; however, passive droplet generation methods remain the most widely used.

Recent advancements in passive droplet generation techniques have significantly enhanced their capabilities Some parallelized microfluidic systems can now produce picolitre and nanoliter sized droplets at frequencies reaching hundreds of kilohertz[174–176]. There are four main microfluidic designs for droplet formation: T-junction[158], flow-focusing[177], co-flowing[178] and step emulsification[179]. Although these designs differ structurally, they all operate on a shared principle: two immiscible fluids, a continuous phase and a dispersed phase converge at a junction, where interfacial deformation leads to droplet breakup. Whether a fluid acts as the dispersed or continuous phase depends on modifications to the surface of the microfluidic channels[180]. In most cases, hydrophobic channels are used, causing the aqueous phase to form droplets while a thin layer of oil phase surrounds them.

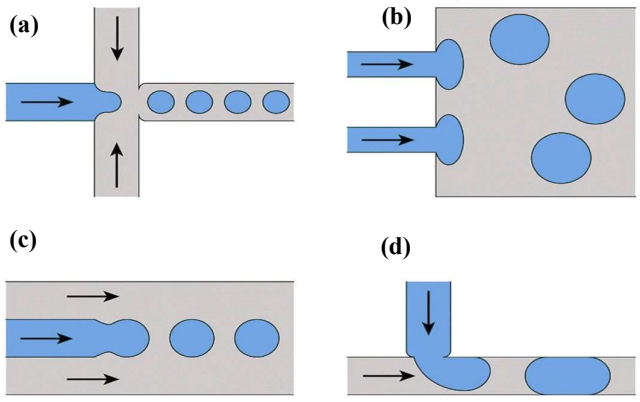

Figure IF7: Various types of channel geometries for passive droplet generation
(a) shows a flow focusing geometry where dispersed and continuous phases flow coaxially in a structured region leading to elongation of fluid and formation of droplet; **(b)** in step emulsification method the dispersed phase exits through a wide and shallow nozzle to a large reservoir of continuous phase. **(c)** in co-flow design dispersed phase is inside an inner channel, while continuous phase flows in an outer channel; and **(d)** in T-junction type dispersed and continuous phases run through orthogonal channels and meet at a cross-junction

*The figures are reproduced from article published by Hess et al[181].

In the T-junction type geometry, the dispersed and continuous phases run through orthogonal channels and then meet at a cross-junction. A shear gradient forms, the dispersed phase elongates and finally breaks into tiny droplets. In flow-focusing design, the two phases flow coaxially through a narrow constriction; the shear forces elongate the dispersed phase, resulting in droplet formation. Droplet size in such systems can be tuned by adjusting channel geometry, flow rates, fluid viscosity, and use of surfactants. In co-flow design, both the phases flow through a set of coaxial microchannels, with the dispersed phase flowing through the inner channel and the continuous phase through the outer one. Here, droplet size is determined by the flow rates and fluid properties. In step emulsification, the dispersed phase exits through a shallow rectangular nozzle into a large reservoir of immiscible fluid. The sudden confinement relief changes the tip of the to-be-dispersed phase to become spherical, leading to formation of a "neck" at the tip of the nozzle that eventually ultimately pinches off as a droplet.

Generally, the fluid flow in all designs can be characterized by using few dimensionless numbers calculated by fluid properties, flow conditions and geometry of the microfluidic device[173]. These include the Reynolds number (Re), which represents the ratio of inertial to viscous forces; the capillary number (Ca), which measures the balance between viscous shear and interfacial tension forces often used to predict droplet formation and size [182–184]; and Weber number (We), which quantifies the ratio of inertial forces to interfacial tension.

1.6.3 Single-cell encapsulation

The method for encapsulation of cells within monodisperse droplets has provided a novel means to perform single-cell studies in biology for large cell populations. The encapsulation is performed in a random process which is statistically defined by the Poisson distribution[185,186]:

$$p(k,\lambda) = \frac{\lambda^k e^{-\lambda}}{k!}$$

In the above equation, k represents the number of cells in a droplet, and λ is the average number of cells per droplet volume. The cell suspension is directly emulsified into droplets, and the encapsulation of cells in the droplets happens stochastically. To ensure that only

single-cell encapsulations occur, the cell suspension is highly diluted, however, it also results in a very high number of empty droplets.

1.6.4 Droplet stability

Droplet microfluidics undoubtedly remains a very popular and attractive platform for single-cell and high-throughput assays due to the fact that vast number of droplets can be generated very quickly which serve as separate bioreactors. These droplets are formed by making use of two immiscible fluids (most often water in oil), inside microfluidic channels etched on silicon, glass or other polymers. Polymers are the most widely used materials in microfluidic devices and Polydimethylsiloxane (PDMS) is quite popular[187]. The generation of stable water in oil droplets needs the channel surface to be hydrophobic and it is generally performed with the use of fluorosilane polymers[188]. An amphibolic substance, surfactant, is added to oil phase providing stability to the droplet interface by adsorbing at the oil water interface[189]. In addition to stabilization, surfactants also play role in molecular exchanges between droplets and in the biocompatibility of the system, which is very important for biochemical applications[190,191]. For fluorinated oil, the choice relies on low solubility of organic compounds and high solubility of gases, which are essential for cell growth.

Some issues which are encountered in the context of droplet stability are droplet coalescence and Ostwald ripening. During coalescence, the thin oil film between two adjacent droplets gets ruptured and the droplets fuse. In Ostwald ripening, there is a diffusion of aqueous phase from smaller to large droplets, and this causes the larger droplets to swell and ultimately rupturing.

1.6.5 Droplet microfluidics for microbiology

Droplet microfluidics has now emerged as a transformative technology in microbiology, offering high-throughput, miniaturized and simple platforms for the culture, analysis and manipulation of microorganisms at the single-cell level. Droplets provide the advantage of bacterial cells compartmentalization in tiny volume of liquid, favoring faster accumulation of growing cells or secreted molecules allowing faster detection when compared to bulk culture. It also allows for performance of iterative operations on droplets, such as their formation, splitting, merging, sorting or injecting additional reagents. Another significant advantage is the possibility of generating a huge number of separate bioreactors for single-

cell and high-throughput applications. Some documented and potential uses of droplet microfluidics in the studies around microbial organisms are listed below.

- Droplet microfluidics has revolutionized the cultivation of microorganisms, specially of rare and unculturable species. By encapsulating single cells in droplets, scientists can check interspecies competition and growth under controlled conditions[192].
- High-throughput AST by encapsulating bacteria with antibiotics can be performed in droplets enabling growth inhibition analysis at the single-cell level[160].
- Droplet based system also allow to study the microbial metabolism, gene expression, and other interactions by offering isolated environments where specific parameters can be altered and effects can be monitored[193].
- Droplet microfluidics also aids in dissecting complex microbial communities by allowing for the possibility of single-cell sequencing and functional analysis, which in turn helps uncovering the microbial diversity and their interactions[194].

1.6.6 Detection of droplets

Efficient and accurate detection of droplets remains very critical for real-time monitoring, sorting or data acquisition. Fluorescent labelling methods offer high sensitivity and is widely used for detecting specific biomolecules within the microfluidic droplets. They provide excellent signal-to-noise ratios and are suitable for detecting molecules at picomolar concentrations. An example of external label is resazurin, which is a redox indicator and gets metabolically converted to resorufin by active cells[171,195]. However, the dyes can leak out of the droplets making their detection difficult and can limit the incubation time[196]. Bacteria can also be genetically modified to express fluorescent proteins during active growth for easy detection, making it a popular choice[197,198].

Many label-free methods for droplets detection have also been developed, such as laser-based detection, impedance-based detection, surface-enhanced Raman spectroscopy (SERS) and a machine learning based approach. Laser based detection method usually rely on optical scattering of light by bacteria inside the droplet and this information is read by a detector [185]. Impedance detection method captures the changes in the electrical impedance of droplets as they pass through microchannels and remains very sensitive to presence of any particles inside the droplets [199]. Raman spectroscopy provides a molecular fingerprint

of analytes without requiring labels and SERS significantly boosts signal intensity, allowing for the detection of trace analytes within droplets [200].

The emergence of deep machine learning has greatly enhanced the droplet detection capabilities and is now employed for automated droplet counting and segmentation [201]. These models outperform traditional methods by learning complex droplet features from large image datasets, enabling real-time and accurate detection.

1.6.7 AST using microfluidics

Multiple microfluidic platforms with different approaches have been developed for testing bacterial susceptibility to antibiotics based on phenotypic/genotypic signatures of bacteria.

Phenotypic AST methods can be of different types based on their liquid handling approach: static chamber arrays, chamber with flow, droplet arrays and flowing droplets. In the static chamber systems, there is a central channel with multiple chambers on sides along. The sample solution fills the entire device and then the chambers are separated by oil, creating discrete bioreactors[202]. It is quick, efficient and easy-to-use device but can be hard to scale up and this limits its usability in heteroresistance studies. Another approach combined with microscopy is the flow chamber array where growth of many individual cells can be observed over time[203], such as in mother machine[204]. Mother machine consists of numerous channels on a chip where the width of the channels matches the width of a bacterial, trapping of just one mother bacterial cell per channel. The cell continuously divides, and the excess cells get flushed away at the end of the channel by continuous media supply. Mother machine allows for long term experiments with continuous monitoring of growth over time under precisely controlled conditions. The droplet array method uses arrays of droplets placed in microwells which is very versatile and allows for large number of time dependent observations to be made[205,206]. Another popular system relies on system relies on flowing droplets[207,208] where bacteria and antibiotic containing droplets are generated in flow and can be used as separate bioreactors to observe bacterial growth at specific antibiotic concentrations. Genotype based methods like digital PCR and digital lamp are more advance and rapid, however they can only be used in specific cases, such as when the resistance genes are known, which makes them very useful for confirmatory studies[209].

1.6.8 Droplet microfluidics for AST and detection of heteroresistance

Among all the microfluidic based antimicrobial susceptibility testing approaches, the droplet-microfluidics offers the greatest scalability and maximum throughput. This becomes critically essential when a huge number of single-cells need to be analyzed, such as in studies elucidating heteroresistance of the population. Researcher have earlier demonstrated various droplet-based systems for determining the susceptibility of individual cells to antibiotics. A plug-based platform for sensitivity testing of *Staphylococcus* to different antibiotics was used, where individual bacterial cells and indicator resazurin were encapsulated together inside droplets[171] followed by incubation to achieve the sufficient number of bacterial cells for reliable detection. Another similar fluorescence-based AST assay, dropFAST, was used to test the effect of gramicidin on growth of *Escherichia coli* using 20 pl droplets[210]. The system allowed for the generation, incubation, and screening of droplets one single platform. A system[208] with improved throughput was also presented where droplet libraries of 1900 compartments each, were semi-automatically generated, each library corresponding to one antibiotic concentration.

As discussed previously that traditional determination of MIC is not always informative enough to give accurate picture of bacterial susceptibility to an antibiotic as different cells in a population can show wide range of susceptibilities. Moreover, the measured MIC value can strongly depend on the size of initial cell density, a phenomenon known as inoculum effect[211]. A superior and excellent approach, however, to study heteroresistance is to measure the minimum inhibitory concentrations for single-cells. Previously, single-cell analysis of the bacteria population has been performed by encapsulating individual cells of *Escherichia coli* in agarose nanoparticles with different antibiotic concentrations[212]. Another group presented a digital droplet PCR to detect resistance genes and point mutations in *Heliobacter* cells isolated from stool samples[213]. An excellent demonstration for digital quantification of heteroresistance was performed based on the MIC determination of single cells using a fluorescent probe[214,215].

The measurement of MIC of individual cells using droplet microfluidics is an excellent way to quantify the distribution of each cell's phenotypic responses to antibiotics in the bacterial populations. Compared to the genetic basis of antibiotic resistance, phenotypic variations in isogenic cells remains poorly studied[35,216], where the cells differ from each other with respect to their gene expression levels or certain phenotypic traits[217,218].

A very convenient, reproducible, high-throughput and efficient AST method (Figure IF8) using droplet microfluidics was demonstrated by a group of researchers[214]. Using the platform, they quantified the distribution of single-cell phenotypic response to antibiotics. *Escherichia coli* DH5α cells carrying a yellow fluorescent protein (YFP) were encapsulated in water-in-oil droplets with different concentrations of antibiotic. Poisson's statistics was used to ensure that each droplet contains just one bacterium. Post incubation, for all concentrations, the total number of droplets was determined, along with the number of droplets giving a positive YFP signal indicating bacterial growth. The results of single-cell antibiotic susceptibility testing were displayed in the form of fraction of individual cells growth as a function of antibiotic concentration as well as a probability distribution density of cells having a given MIC was also generated.

The method employed in the above study forms the basis of the experimental protocol used in this thesis and will be elaborated in further sections.

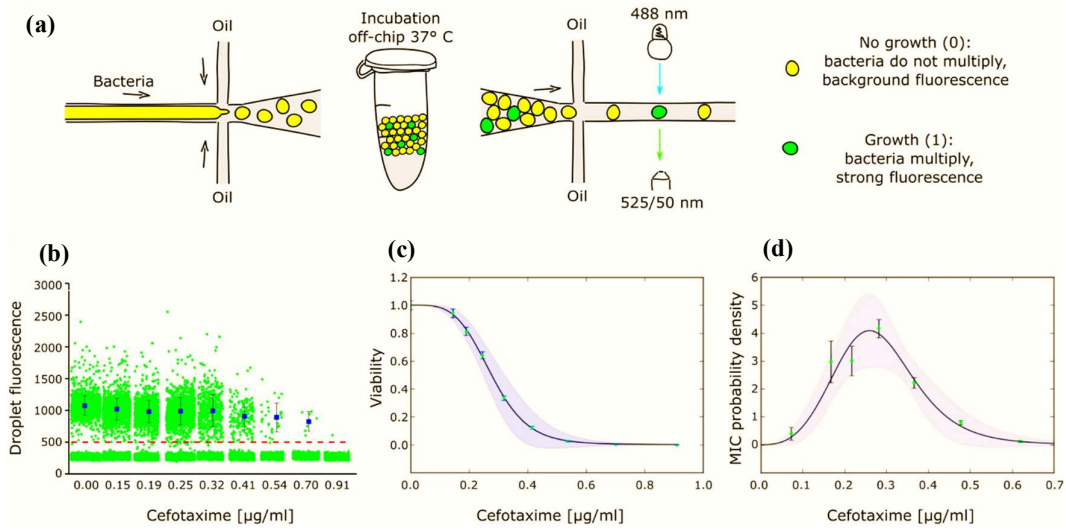

Figure IF8: Detection of heteroresistance using droplet microfluidics
(a) microfluidic workflow for the fluorescently tagged single-cell encapsulated droplet generation, encapsulation and fluorescence-based detection. Multiple droplet libraries are generated with each library having different antibiotic concentration and incubated at 37 ^{0}C. On the following day, droplets screened using fluorescence microscope one by one, strong fluorescent signal means growth of bacteria inside droplet; **(b)** signal intensities of individual droplets droplet in the experiment. Red dashed line represents the threshold for positive droplets and blue rectangles show the average signal from the droplets containing bacteria positive, while error bars represent the standard deviation; **(c)** viability of cells (fraction of positive droplets for each concentration is normalized with the positive fraction in the sample without antibiotic) as a function of antibiotic concentration; and **(d)** probability distribution of individual MICs in the population is calculated from **(c)** as the numerical derivative of the fitting line.

*Figure reproduced from the article published by Scheler et al[214].

2 Goal of the thesis

Chapter 2 *describes the objective of the study performed, the need and potential future applications in terms of antibiotic susceptibility testing or treatment regimen. A brief overview is given about how low-level antibiotic stress is generated in the environment or in an organism and how it leads to the development of resistance.*

Antibiotics have been widely used since their discovery in the 1930s and for good reason, as they have saved millions of lives and significantly improved the management of bacterial infections. Their usage in livestock farming and agriculture has also greatly enhanced the production of meat, dairy, and crops by minimizing losses caused by infectious diseases.

However, the use of antibiotics is not without consequences. When misused, antibiotics can exert selective pressure on bacterial populations, accelerating the emergence and evolution of resistant strains. Suboptimal dosing, for instance, may fail to eliminate the entire bacterial population, allowing some cells to persist under continuous low-level antibiotic stress- suitable conditions that can drive resistance development. This phenomenon is also observed in the environment, such as in agriculture or in cases of improper disposal of hospital or industrial waste. In such scenarios, residual antibiotics can enter surrounding soil and water systems, exerting similar selective pressures on bacterial communities in the environment.

Most studies for understanding the effect of antibiotic on bacteria rely on susceptibility testing, where cells are exposed to antibiotics to determine the minimum inhibitory concentration (MIC) value which is the lowest concentration required to prevent visible growth. These tests, typically performed on bulk populations, provide an average susceptibility value but overlook variations among the susceptibilities of individual cells.

This variability, known as heteroresistance, is often missed in standard AST and diagnostic practices, however, can significantly impact outcomes of treatment.

This thesis aims to explore how sub-MIC antibiotic exposure influences the heteroresistance at single-cell level. To address this, droplet microfluidics was used to analyze susceptibilities of individual cells following brief pre-exposure to sub-MIC levels of antibiotics. Two widely used antibiotics with distinct mechanisms of action were selected: ciprofloxacin, which targets DNA replication, and streptomycin, which disrupts protein synthesis. Since both targets are critical to bacterial survival, alterations in these pathways may significantly influence the cell's future responses to future antibiotic treatments. Bacterial cells were pre-exposed to three sub-minimum inhibitory concentrations (0.125X MIC, 0.25X MIC, 0.5X MIC), after which individual cells were encapsulated into microdroplets along with a range of antibiotic concentrations for susceptibility testing.

The results of the study are very crucial in understanding response of individual cells to antibiotics and how low-level antibiotic alters the heteroresistance of the population during future antibiotic exposures. The data presented here stresses on the need to consider each and every cell for susceptibility testing and also make judicial use of antibiotics to reduce the risk of resistance development.

3 Materials

Chapter 3 *presents in detail the information about all the bacterial strains, materials, reagents, substance, kits and every related equipment used for bacterial culture, microfluidic chip fabrication, droplet generation and imaging.*

3.1 Biological materials

Escherichia coli MG1655

Pseudomonas aeruginosa PA14, with integrated *yfp* gene and a constitutively-expressed *mCherry* gene, named PA14

3.2 Media & Chemicals

Mueller-Hinton Broth and Agar (DifcoTM, Becton, Dickinson and Company, USA)

Glycerol (Thermo Fisher Scientific, USA)

Ciprofloxacin ((TOKU-E, Japan)

Streptomycin (Alfa Aesar, USA)

Polydimethylsiloxane (Sylgard 184, Dow Corning, USA)

Tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (United Chemicals, USA)

Novec 7500 fluorocarbon oil (3M, USA)

Novec 1720 (3M, USA)

SU-8 (Kayaku Advanced Materials, USA)

FluoSurf-O™ (Emusleo, France)

3.3 Consumables

Petri and 96-well plates (Wuxi NEST Biotechnology Company, China)

Glass slides (Fisherbrand™, Thermo Fisher Scientific, USA)

Sterile loop (Wuxi NEST Biotechnology Company, China)

3.4 Instruments & Software

Varioklav steam sterilizer (HP Medizintechnik GmbH, Germany)

DiluPhotometer™ (Implen GmbH, Germany)

Incubator (Heratherm, Thermo Scientific, USA)

Shaker incubator (Biogenet, Sp. z o.o., Poland)

BioTek Synergy HTX plate reader (Agilent Technologies, USA)

CNC machine (MFG 4025, Ergwind, Poland)

PC 3001 VARIOpro vacuum pump (Brand Group, Germany)

Plasma cleaner (Harrick Plasma, USA).

Syringe pumps NemesyS and QmixElements software (Cetoni GmBH, Germany)

Nikon Ti2 microscope and NIS-elements AR software (Nikon Corporation, USA)

Camera (Andor Zyla sCMOS, Oxford Instruments, Ireland)

AutoCAD (Autodesk, USA)

Python (Python Software Foundation, USA)

3.5 Miscellaneous

Purified water (Hydrolab Smart SLP, Hydrolab Sp. z o.o, Poland)

Polycarbonate plate (Macroclear, Bayer, Germany)

Polytetrafluoroethylene tubing (Adtech AG, Germany)

Syringe filter (Googlab Scientific Sp. z o.o, Poland)

4 Methodology

Chapter 4 *contains detailed protocol followed for the stocking and culturing of bacteria as well as calculations of minimum inhibitory concentrations. Procedure for microfluidic chip fabrication, droplet generation and droplet image acquisition by microscope is also given.*

4.1 Bacterial culture

All the operations on bacteria are performed under sterile environment with sterile consumables, media and glassware. Autoclaving of the glassware, purified water and reagents are performed for sterilization wherever required.

4.1.1 Banking of stocks

A master agar plate streaked with each bacterial strain was used to inoculate fresh 10 ml MH and incubated for overnight at 37 ° C with 225 rpm shaking. 600 µl of culture is taken in a cryovial and 400 µl of 50% glycerol is added drop by drop with gentle mixing in between. After sufficient mixing and proper labelling, the cryovial is transferred to - 80 °C deep freezer for long term storage.

4.1.2 Preparation of overnight cultures

A freshly prepared MH agar plate is first streaked from the frozen glycerol stock using a sterile loop. The plate is incubated at 37 °C overnight in hanging agar position. The overnight cultures can be prepared in fresh MH by inoculating with the required number of colonies.

4.1.3 Optical density of culture vs CFU calculation

The whole experimental protocol for determining colony forming units (CFU) vs optical density at 600 nm can be performed in 3 days.

1. On day 1, 10 ml MH is inoculated with 3 colonies from different areas of a plate and incubated overnight at 37 °C with shaking at 225 rpm.
2. On day 2, the OD_{600} of the culture is read and diluted to: $OD_{600} = 0.5$ and $OD_{600} = 0.1$.
3. Further dilutions of both cultures are done in the range 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}.
4. 20 µL of each dilution is then plated in freshly prepared sterile individual agar plates.
5. Plates are incubated overnight at 37 °C in hanging agar position.
6. On day 3, the number of colonies are counted for the plates in countable range.
7. CFU/ml at 0.1 OD_{600} and 0.5 OD_{600} are calculated taking into consideration the dilution factor and the plated volume.

4.1.4 Determination of MIC by microdilution method

Determination of MIC takes around 24 h and can be performed as follows:

A. Preparation of the antibiotic dilutions
    1. Antibiotic stock is freshly prepared by dissolving required amount in autoclaved purified water and filtered using a 0.22 µm sterile filter.
    2. The antibiotic range for MIC determination is initially chosen (For example: 1, 2, 4, 8, 16 µg/ml)
    3. Antibiotic dilutions are made from the main stock, and each dilution is prepared in double the concentration required for MIC (For above example it will be: 2, 4, 8, 16, 32 µg/ml).
    4. 100 µL of each antibiotic concentration from step 3 is pipetted in triplicates to a 96 sterile well plate.
B. Preparation of bacterial culture
    5. Few independent colonies are picked up from a freshly streaked culture plate using a sterile loop and resuspended in MH.
    6. The suspension is thoroughly mixed with pipette so as it becomes homogenous and cells are not clumping with each other.

7. OD_{600} of the prepared suspension is taken and is further diluted with MH to bring the OD_{600} to 0.1.
8. Further dilutions are performed to get concentration of $1X10^6$ CFU/ml.

C. Seeding bacteria and incubation

9. To the 96-well plated containing the antibiotic dilutions, 100 µl of the culture from step 8 is added to every dilution. Each well now has $5X10^5$ CFU/ml of bacteria and appropriate antibiotic concentrations as chosen for susceptibility testing.
10. Three controls in triplicates are also set: 1) Growth control: only bacteria in MH; 2) positive control: bacteria treated with very high antibiotic concentration for complete growth inhibition; 3) media control: only MH for sterility testing.
11. The plate is incubated in plate reader for 18 h at 37 °C with shaking (180 rpm, orbital) for 18 h. OD_{600} for each recorded every half hour.

Figure MF1: Bacterial growth kinetics and MIC determination setup
Picture shows BioTek Synergy HTX plate reader (Agilent Technologies, USA) with a 96-well plate inside for growth kinetics measurements. The plate is incubated at 37 °C with 180 rpm orbital shaking for 18 hours and OD_{600} is taken every 30 minutes using the supplied software

4.2 Fabrication of microfluidic chips

For making the droplet generator master chip, the channels and the flow focusing geometry was itched on a polycarbonate plate (PC) using a CNC milling machine (done by Partyk Adamczuk, IPC PAS). The master chip was placed in a casting tray and 1:10 ratio of curing agent and polydimethylsiloxane (PDMS) was thoroughly mixed, degassed in vacuum chamber and poured into the tray. The casting tray was kept in oven at a temperature of 75 °C for 2.5 hours. The PC chip was carefully removed, and the mold surface was thoroughly cleaned. The mold was then placed in a silanization chamber filled with vapors of tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane for 1 hour at 15 mbar. The leftover PDMS and curing agent mixture was poured in the mold cavity and baked in as earlier, followed by precisely cutting out the chip along its edges. The new chip, which is now PDMS replica of master PC chip was then plasma bonded to a 1 mm glass using plasma chamber. The chip was flushed with Novec 1720 and the liquid was allowed to evaporate, repeating the process three times.

The droplet imaging chip was fabricated (lithography done by Shreyas K. Vasantham IPC PAS) using standard photolithography method on a 4-inch Silicon wafer. The design and geometry of the photomask was made using AutoCAD. A negative photoresist SU-8 was spin coated at 3000 rpm as per the desired channel specifications and further steps as per standard procedures. The baking of the spin coated wafer was done according to manufactures recommendations, at 65 °C and 95 °C. UV exposure was done on the silicon master mold and processed for further development. PDMS replica of this master was prepared similar to the droplet generation chip.

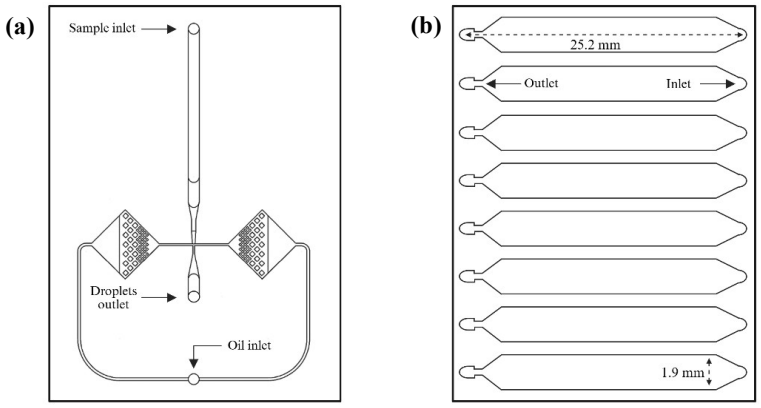

Figure MF2: Microfluidic chips used in the study
(a) shows a schematic of droplet generator chip having a flow focusing geometry. The oil phase cuts the sample flow from two sides generating droplets of desired volume which is optimized by the flow rates. A

connected tubing then transfers the droplets to Eppendorf tubes; and **(b)** shows a schematic of the droplet imaging chip with a total of 8 chambers which can be reused multiple times. The droplets after desired incubation are pushed from the inlet and the chamber gets filled. The microscope automatically images all fields of the chamber as defined and the droplets are then pushed out to make space for next set of imaging.

* Figure **(a)** is reproduced from an article published by Pacocha et al.

* Created in https://BioRender.com

4.3 Pre-exposure protocol

An overnight culture diluted 1:100 in MH and OD_{600} was measured every 30 minutes to obtain an OD_{600} of 0.1-0.2. The antibiotic pre-exposure was done in a conical flask containing 20 ml MH with desired antibiotic concentration, followed by addition of bacteria so as to get a final concentration of $5X10^5$ CFU/ml. The shaking incubation was done at standard conditions and OD_{600} taken every 30 minutes till 0.1-0.2.

In the meantime, a range of antibiotic concentration (2X of each required concentration) for AST was prepared in a 96 well plate. The culture of bacteria washed three times using centrifugation to get rid of any residual antibiotics and resuspended in fresh MH. The bacteria were then added to each well to get a final concentration of $1X10^5$ CFU/ml. The sample from each well will then be used to generate droplets using already fabricated microfluidic chips.

4.4 Droplet generation, image acquisition and analysis

4.4.1 Generation of droplets

Droplets of 1 nl were generated from all wells of the 96-well plate, with sample being the aqueous phase while Novec 7500 fluorocarbon with 2% FluoSurf-O™ surfactant served as the oil phase. The flow rates were predetermined for required droplet size and controlled using syringe pumps operated by the software QmixElements. Generated droplets from all samples were separately collected in 200 µl microtubes and incubated at 37 ^{0}C for 18 hours.

4.4.2 Image acquisition

Each sample of droplets post incubation was taken up in a tubing using the same reverse flow in syringe pump and pushed in the chamber of droplet imaging chip. Using an inverted microscope equipped with camera, the bright field images were acquired. The droplets were flushed after one set of imaging and imaging was continued. Acquired images were extracted to grey tag image format using the NIS-elements software for further analysis.

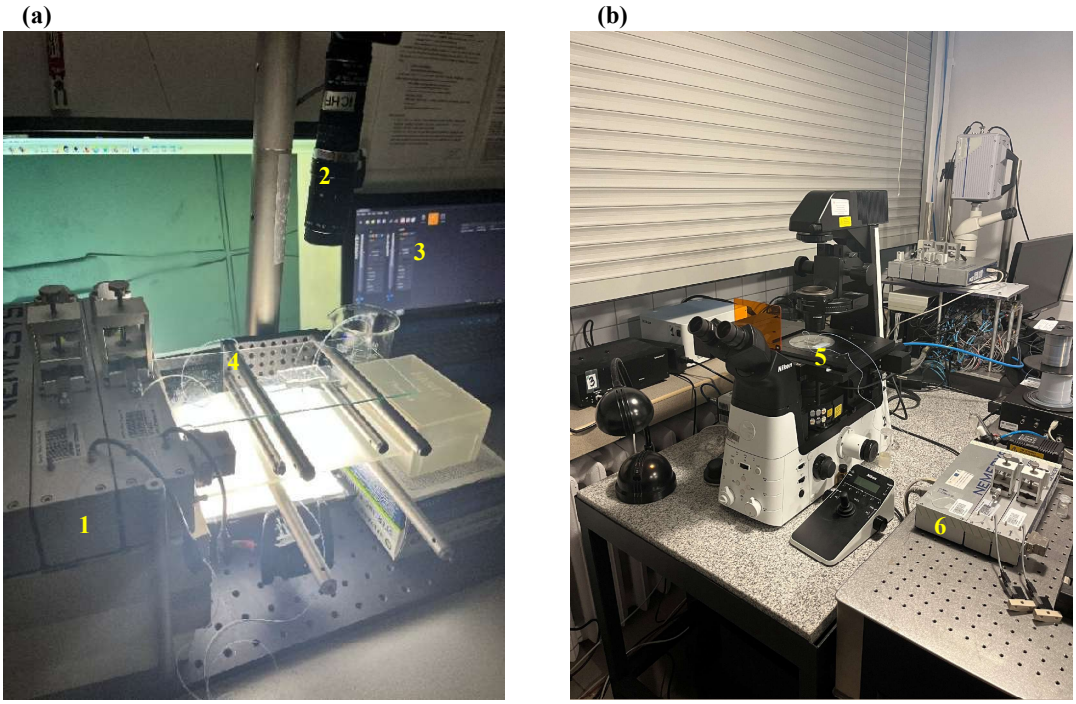

Figure MF3: Droplet generation and imaging setup used for the experiments
(a) a photo of droplet generation setup with (1) syringe pumps, (2) camera for real time observation of droplet generation, (3) computer program to control pumps, (4) is a glass stage with generator; and **(b)** a photo of inverted microscope along with (5) droplet imaging chip on stage, (6) syringe pumps to push droplets in chip.

4.4.3 Image analysis

A python-based computer code was prepared (code developed and written by Paweł Jankowski, IPC PAS) to analyze the images of droplets acquired using the microscope. The code uses publicly and freely available libraries such as Scikit-image and OpenCV at its core for analysis of images[219–221]. A novel approach based on the analysis of pixel texture through grey-level co-occurrence matrices (GLCMs) of the images was utilized, as applied previously during aerial photography images for earth surface analysis[222]. The code first recognizes all the individual droplets in the images using OpenCV methods, followed by selecting small patches and calculating the GLCM properties for each patch. The script finally generates a plot showing the correlation between each patch's GLCM properties (dissimilarity, homogeneity). The center point for each droplet data (average on the axes) is found out and the distribution of these points for all droplets can be displayed on a chart. Eventually, using Sklearn library a k-means clustering algorithm is implemented and the grouping for droplets can be done by thresholding.

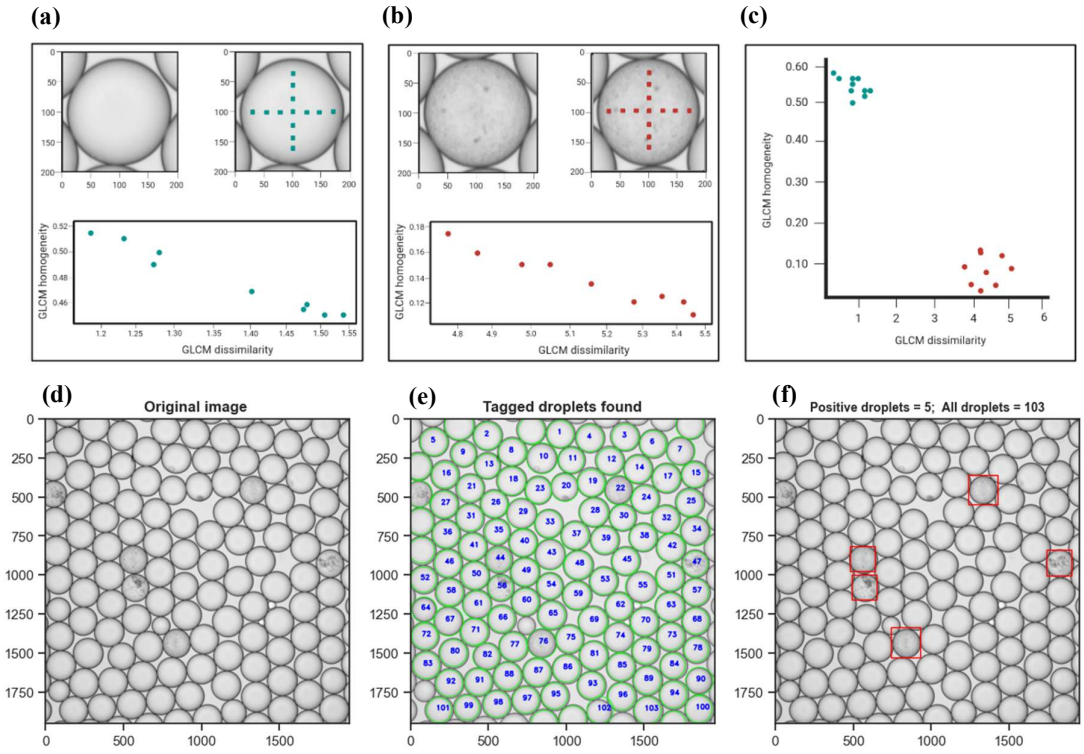

Figure MF4: Droplet analysis and enumeration using python script
(a) depicts analysis of pixels in and empty droplet through grey-level co-occurrence matrices (GLCMs)**; (b)** depicts the same in a droplet with bacteria; **(c)** shows a plot with grouped droplets based on then GLCM properties with green dots showing population of empty droplets while red shows the droplet population with bacteria in them; **(d)** is an image of droplets as acquired by bright field microscopy; **(e)** the image with all identified droplets; and **(f)** is the same image with only droplets containing bacteria marked.

4.5 Data analysis and parameters used in the study

Multiple studies have been performed for AST of individual cells using different methods, describing single-cell MIC differently, and no standardized definition of it exists. In one publication[211] authors define single-cell MIC (scMIC) as the measured MIC values at very small initial cell densities, as low as 500 CFU/ml. Another definition of scMIC is proposed as the average value of the MICs measured for individual cells encapsulated in droplets[185]. To avoid confusion and misinterpretation, a new term individual MIC, *iMIC*, is used and defined as the minimum antibiotic concentration needed to completely inhibit the growth of individual bacterium inside a microfluidic droplet.

The image analysis script generated the information about total number of droplets, number of empty droplets and number of droplets containing bacteria. Further analysis was performed using another python code (development of code and analysis performed by Adam Samborski, IPC PAS) which takes the above information as input data. The whole

population of droplets (total number of droplets) in each sample was denoted by, *N(c),* and the number of droplets showing detectable bacterial growth (positive droplets) was represented by $N_+(c)$. The droplet library of the control sample should theoretically show bacterial growth in all successfully encapsulated droplets and gives maximum possible number of positive droplets, $N+(0)$. The positive fraction of the droplets is denoted as $f+(c) = N+(c)/N(c)$. For each experimental set, to accurately analyze the bacterial growth under every antibiotic concentration of AST, the number of positive droplets for each concentration, $f+(c)$, was normalized with the number of positive droplets in control sample i.e. the sample without any antibiotic, $f+(0)$. This gave the fraction of individual bacterial cells inside droplets which were able to proliferate successfully as a function of antibiotic concentration: $F_R(c) = f+(c)/f+(0)$.

The experimental data points for each fraction of individual cells that were able to proliferate, $F_R(c)$, were plotted against their respective concentrations and a Gompertz fitting [214,223,224] was performed using the function: $\varphi(c) = exp\{-(\frac{c}{p_1})^{p_2}\}$, where c refers is antibiotic concentration, p_1 is the concentration at maximum slope, and p_2 is slope parameter at $c=p_1$.

Furthermore, the inflection point, on the Gompertz fitting curve was also extracted and mathematically it represents the second derivative of $F_R(c)$ equals zero for $c=c_{(iMIC)}$. The solution of equation $d^2F_R(c)/dc^2 = 0$ gives $c_{(iMIC)mode} = p_1\left\{1 - \frac{1}{p_2}\right\}^{\frac{1}{p_2}}$.

To calculate $p(iMIC)$, the probability density distribution of *iMIC* which represents the likelihood of a specific antibiotic concentration inhibiting the growth of a bacterium, the expression $p(c) = -dF_R(c)/dc$ was utilized. The $p(iMIC)$ is derived from the Gompertz function $\varphi(c)$ and is calculated by: $p(c) = \frac{p_2 c^{(p_2-1)}}{p_1 p_2} exp\{-(\frac{c}{p_1})^{p_2}\}$.

The probability density distribution curve of *iMIC* shows the spread of individual MICs on concentration scale and the maximal point of the curve is denote by $(iMIC)_{mode}$. $(iMIC)_{mode}$ also corresponds to inflection point on Gompertz fitting and is the antibiotic concentration which is likely to be *iMIC* for major number encapsulated bacteria.

For the parameter *p(c),* average value μ, variance σ and skewness γ is also calculated utilizing the gamma function **Γ**(x): $\mu = \int_0^\infty dc\ c\ p(c)$, $\sigma^2 = \int_0^\infty dc\ (c-\mu)^2 p(c)$ and $\gamma = \frac{\mu_3}{\sigma^3}$ where $\sigma^3 = \int_0^\infty dc\ (c-\mu)^3 p(c)$ and $\mu_3 = \int_0^\infty dc\ (c-\mu)^3 p(c)$:

$$\mu = \left(p_1/p_2\right)\Gamma\left(1/p_2\right);\ \sigma = \sqrt{\left(2p_1^2/p_2\right)\Gamma\left(2/p_2\right) - \left(p_1^2/p_2^2\right)\Gamma^2\left(1/p_2\right)};$$

$$\gamma = \frac{\left[\left(3p_1^3/p_2\right)\Gamma\left(3/p_2\right) - \mu(3\sigma^2+\mu^2)\right]}{\sigma^3}.$$

From the Gompertz fitting curve various informative values were extracted, such as $(iMIC)_{total}$ which is the value of antibiotic concentration inhibiting the bacterial growth in >95% droplets as compared to control sample while lowest concentration needed to completely stop the growth of bacteria in all the droplets is represented by $(iMIC)_{all}$. An experimentally determined parameter, $(iMIC)_{exp}$, refers to the minimum antibiotic concentration that completely inhibited the growth of bacteria in all droplets as observed under microscope. $(iMIC)_{start}$ was deduced from Gompertz fit values and is the minimum antibiotic concentration where the first drop in growth is observed as seen in the Gompertz fitting curve. Collectively, all these parameters provide a comprehensive framework for single cell data analysis and characterization of individual cell antibiotic susceptibilities, allowing for accurate assessment of heteroresistance within bacterial populations.

5 Results

Chapter 5 *presents the results of various experiments performed such as, calculation of MICs, CFU vs* OD_{600}*, droplet generation, image acquisition, and data analysis. Final results are shown using parameters such as, degree of heteroresistance, distribution of iMIC, skewness, tailing of the distribution etc. categorically displayed for both the strains.*

5.1 *Escherichia coli* MG1655

5.1.1 Calculation of MICs and sub-MICs used

The MICs of ciprofloxacin and streptomycin tested for MG1655 were found to be 12 ng/ml and 3 µg/ml respectively (Figure RF1). The concentrations for pre-exposure were decided based on MIC values (Table RT1).

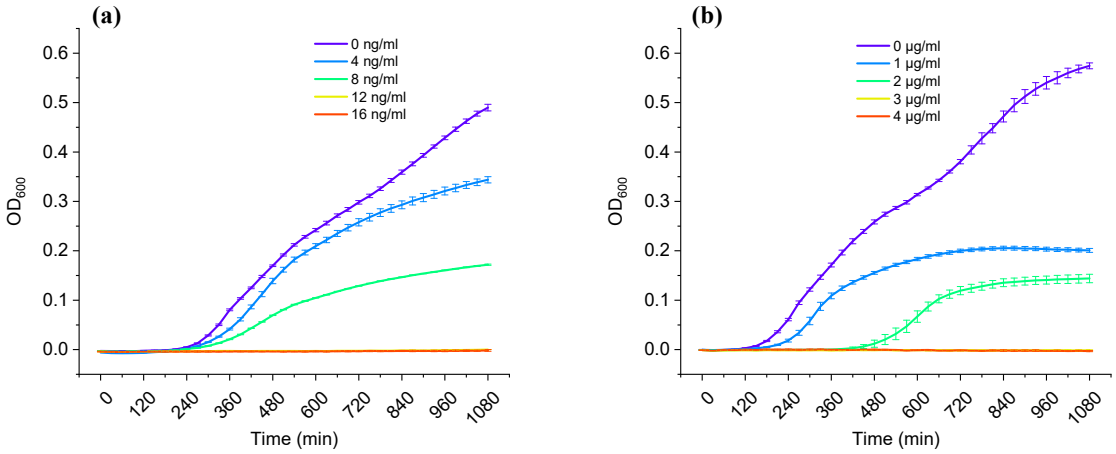

Figure RF1: The kinetics of MG1655 growth at different concentrations of antibiotic
(a) and **(b)** show MG1655 growth of at different concentrations of ciprofloxacin and streptomycin respectively. Each data point denotes average of three independent experiments; error bars show SD.

Antibiotic	MIC	0.5X MIC	0.25X MIC	0.125X MIC
Ciprofloxacin	12 ng/ml	6 ng/ml	3 ng/ml	1.5 ng/ml
Streptomycin	3 µg/ml	1.5 µg/ml	0.75 µg/ml	0.375 µg/ml

Table RT1: MICs as determined and sub-MICs used for pre-exposure

			CIP pre-exposure			STR pre-exposure		
		Unexposed	*0.125X MIC*	*0.25X MIC*	*0.5X MIC*	*0.125X MIC*	*0.25X MIC*	*0.5X MIC*
CIP susceptibility testing	$(iMIC)_{mode}$	9.2	9.9	2.4	2.2	9.5	10	9.5
	$(iMIC)_{mean}$	8.9	9.6	6.8	5.1	9.3	9.8	9.3
	SD	2.3	4.3	5.3	3.7	2.7	3	2.6
	CoV	0.26	0.27	0.78	0.73	0.29	0.31	0.28
	RMSD	0.25	0.26	2.20	1.68	0.28	0.3	0.27
	$(iMIC)_{start}$	3.09	3.09	0.13	0.13	2.73	2.57	2.9
	$(iMIC)_{total}$	12.5	14.11	15.94	14.5	13.7	14.56	13.28
	$(iMIC)_{all}$	15	15.94	ND	ND	15.94	18	15.9
	$(iMIC)_{exp}$	14	16	16	16	16	18	16
	DoH	4.86	5.16	122.62	111.54	5.84	7	5.48
	Skewness	-0.17 (±9.7e-09)	-0.11 (±7.3e-09)	1.3 (±4.8e-07)	1.2 (±3.2e-10)	-0.07 (±4.3e-09)	-0.01 (±3.2e-09)	-0.1 (±6.2e-09)
	Kurtosis	2.8 (±3e-07)	2.76 (±3.7-07)	5.43 (±2.3e-05)	4.88 (±7e-06)	2.74 (±1.3e-08)	2.72 (±1.7e-07)	2.76 (±1.7e-06)
STR susceptibility testing	$(iMIC)_{mode}$	1.1	1.5	0.74	0.33	1.1	0.93	0.88
	$(iMIC)_{mean}$	1.1	1.4	0.77	0.47	1.0	0.9	0.86
	SD	0.2	0.25	0.31	0.28	0.27	0.19	0.25
	RMSD	0.18	0.17	0.42	0.85	0.25	0.20	0.28
	CoV	0.18	0.18	0.40	0.60	0.27	0.21	0.29
	$(iMIC)_{start}$	0.50	0.67	0.13	0.03	0.34	0.38	0.25
	$(iMIC)_{total}$	1.36	1.76	1.3	1	1.48	1.19	1.3
	$(iMIC)_{all}$	1.55	1.93	1.62	1.42	1.69	1.36	1.48
	$(iMIC)_{exp}$	2	2	1.5	2	2	2	1.5
	DoH	3.1	2.88	12.46	47.32	4.97	3.58	5.92
	Skewness	-0.39 (±6.6e-09)	-0.43 (±6.7e-09)	0.27 (±1.3e-08)	0.82 (±4.4e-12)	-0.14 (±1.5e-10)	-0.32 (±3.4e-09)	-0.07 (±9.6e-11)
	Kurtosis	3.06 (±8.4e-09)	3.13 (±1.3e-09)	2.78 (±6.8e-09)	3.65 (±6.5e-09)	2.78 (±6.5e-09)	2.96 (±2.7e-09)	2.74 (±1e-10)

Table RT2: The values of various parameters derived from the Gompertz fitting curve are listed above, these parameters are further used to assess the heteroresistance profile of MG1655 cells

SD: Standard deviation; RMSD: Root-mean square deviation; CoV: Coefficient of variance; ND: Not determinable; CIP: Ciprofloxacin; STR: Streptomycin. The concentration units, wherever applicable, are in ng/ml and μg/ml for ciprofloxacin and streptomycin, respectively.

5.1.2 Resistance profile and Gompertz fitting

According to methodology discussed in section 4.5, for each pre-exposure, the positive fractions of droplets were plotted with respect to the concentrations and the Gompertz fitting (Figure RF2, RF3, RF4, RF5) was done. Various parameters (Table RT2) used in the study were then derived from the Gompertz fit line.

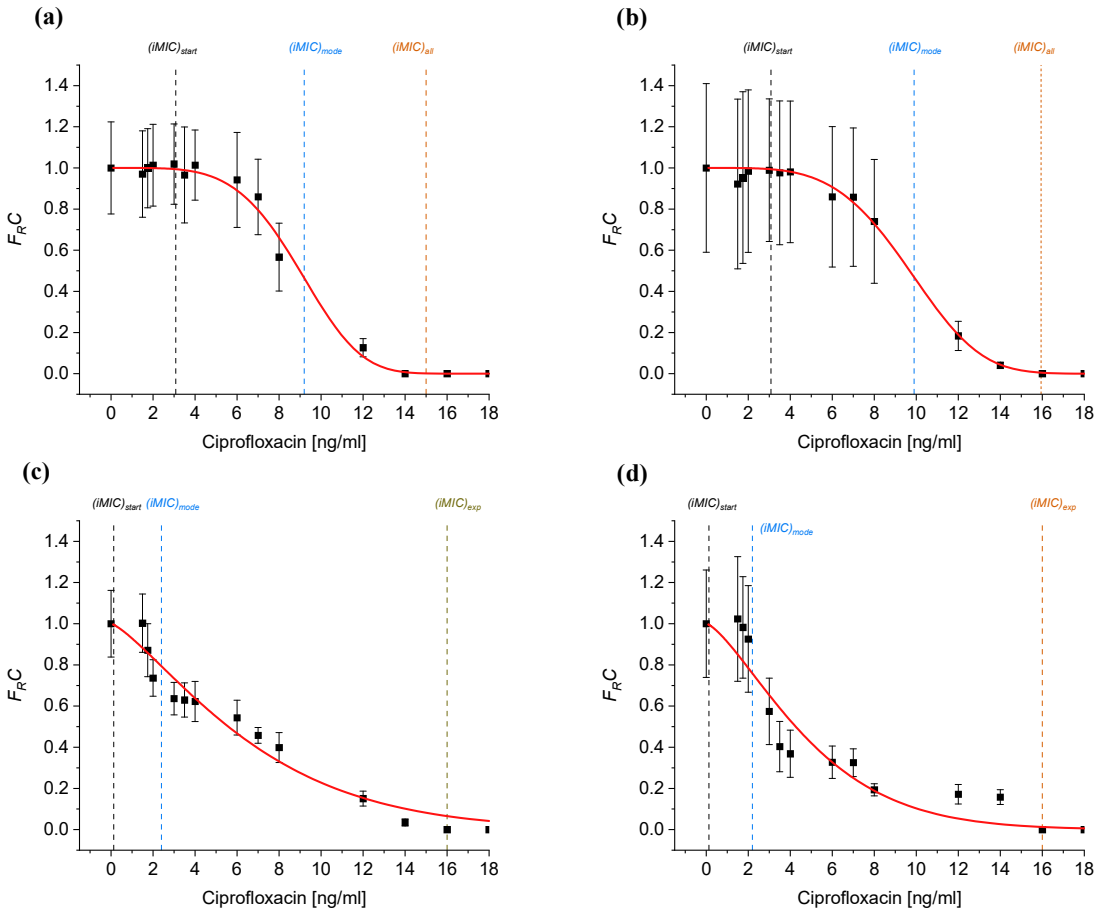

Figure RF2: plot depicting the resistance profile and the Gompertz fitting for positive fraction of droplets, F_RC, as a function of antibiotic concentration, c for experiments performed on MG1655. The susceptibility tests were done independently on MG1655 against ciprofloxacin on **(a)** unexposed sample and **(b)** bacteria pre-exposed with 0.125X MIC of ciprofloxacin, **(c)** 0.25X MIC of ciprofloxacin, **(d)** 0.5X MIC of ciprofloxacin. Each black solid square data point is the average of three independently performed experiments and error bars are representing the standard deviations. Red line is derived after performing the Gompertz fitting of the experimentally determined values. It was impossible to determine $(iMIC)_{all}$ for (c) and (d) from Gompertz fitting, hence the experimentally determined concentration for inhibition of all cells, *(iMIC)exp,* is represented instead. More information is described in the manuscript text and in the Table T2.

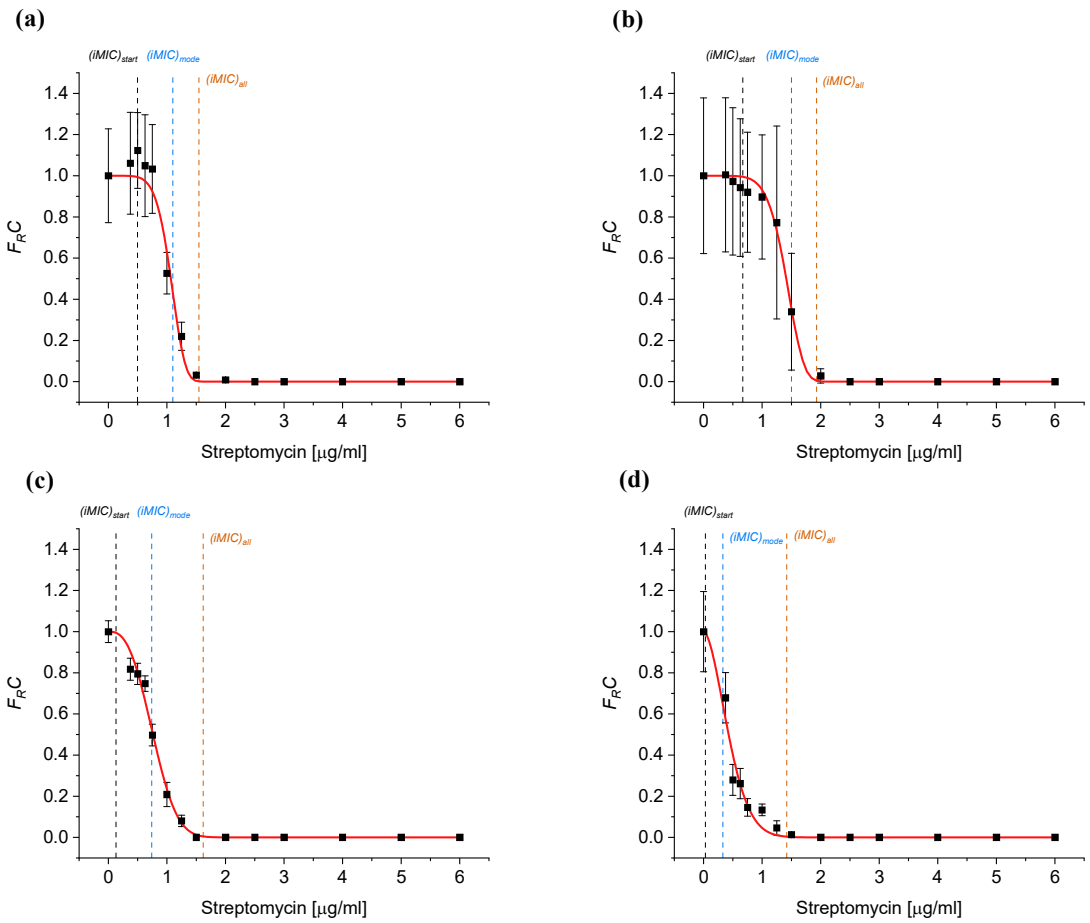

Figure RF3: plot depicts the resistance profile and the Gompertz fitting for positive fraction of droplets, F_RC, as a function of antibiotic concentration, c, from lowest to highest.
The susceptibility tests were done independently on MG1655 against streptomycin on **(a)** unexposed sample and **(b)** bacteria pre-exposed with 0.125X MIC of ciprofloxacin, **(c)** 0.25X MIC of ciprofloxacin, **(d)** 0.5X MIC of ciprofloxacin. Each black solid square data point is the average of three independently performed experiments and error bars are representing the standard deviations. Red line is derived after performing the Gompertz fitting of the experimentally determined values.

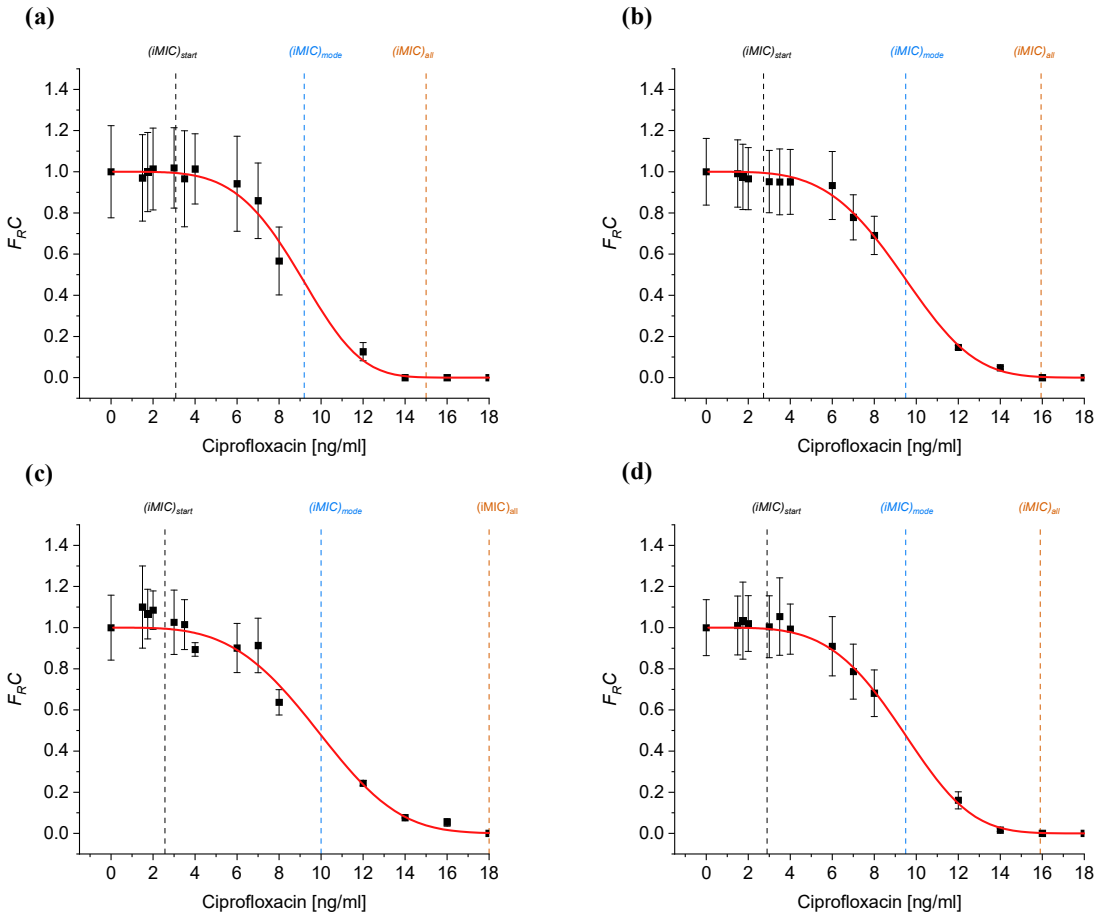

Figure RF4: plot depicting the resistance profile and the Gompertz fitting for positive fraction of droplets, F_RC, as a function of antibiotic concentration, c for experiments on MG1655.
The susceptibility tests were done independently on MG1655 against ciprofloxacin on **(a)** unexposed sample and **(b)** bacterial sample pre-exposed with 0.125X MIC of streptomycin, **(c)** 0.25X MIC of streptomycin, **(d)** 0.5X MIC of streptomycin. Each black solid square data point is the average of three independently performed experiments and error bars are representing the standard deviations. Red line is derived after performing the Gompertz fitting of the experimentally determined values.

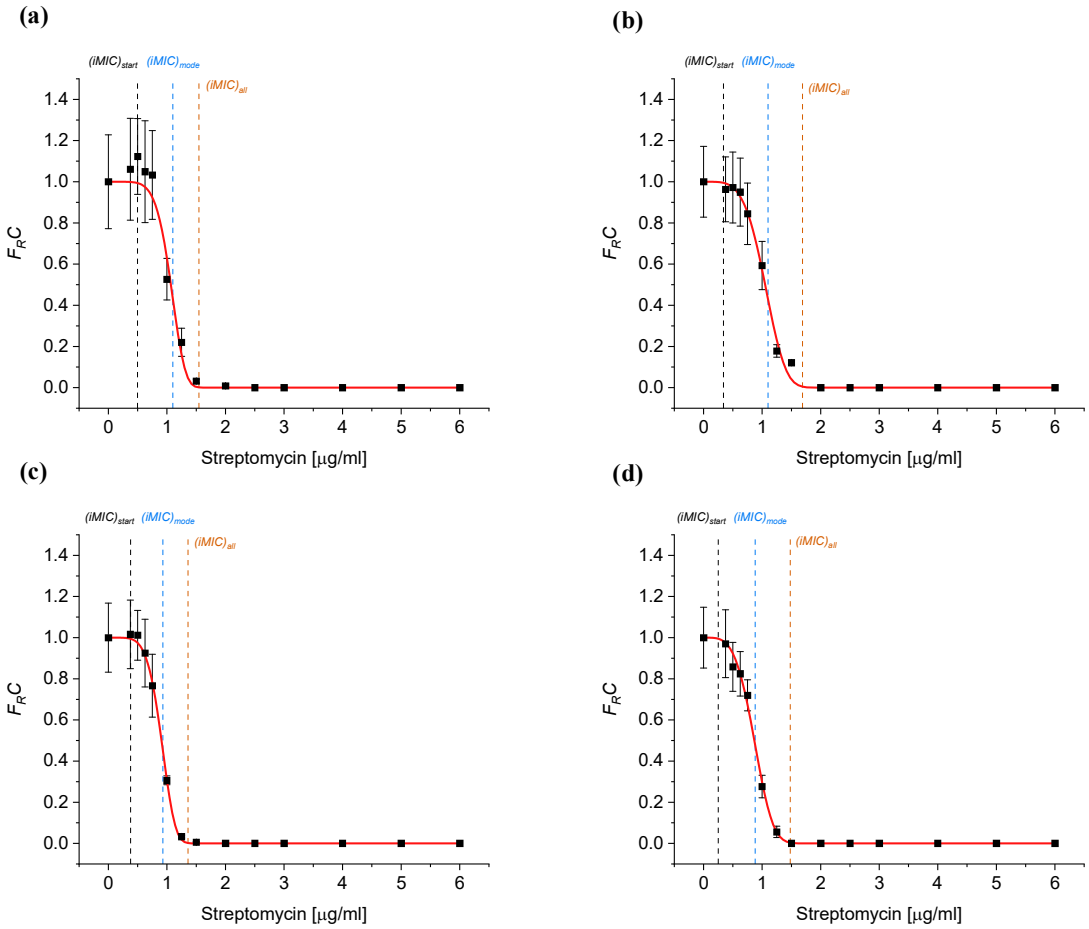

Figure RF5: plot depicts the resistance profile and the Gompertz fitting for positive fraction of droplets, F_RC, as a function of antibiotic concentration, c, from lowest to highest.
The susceptibility tests were done independently on MG1655 against streptomycin on **(a)** unexposed sample and **(b)** bacteria pre-exposed with 0.125X MIC of streptomycin, **(c)** 0.25X MIC of streptomycin, **(d)** 0.5X MIC of streptomycin. Each black solid square data point is the average of three independently performed experiments and error bars are representing the standard deviations. Red line is derived after performing the Gompertz fitting of the experimentally determined values.

5.1.3 Effects of pre-exposure on $(iMIC)_{mode}$, $(iMIC)_{start}$, $(iMIC)_{all}$ and $p(iMIC)$

To understand how pre-exposures with sub-MIC levels of antibiotics affects the AST during subsequent antibiotic exposures $(iMIC)_{mode}$, $(iMIC)_{start}$, $(iMIC)_{all}$ were calculated.

As mentioned earlier, $(iMIC)_{mode}$ denotes the most probable antibiotic concentration and at this concentration the greatest number of positively encapsulated droplets show growth inhibition. $(iMIC)_{mode}$ of unexposed sample against ciprofloxacin was found to be 9.2 µg/ml, while after the pre-exposures with streptomycin it was 9.5 ng/ml, 10 ng/ml and 9.5 ng/ml for the 0.125X, 0.25X and 0.5X MIC respectively. A decrease of 4-folds in $(iMIC)_{mode}$ value was seen in the samples pre-exposed with 0.25X MIC and 0.5X MIC ciprofloxacin,

as compared to unexposed, implying that the majority of cells were either already in the dying stage because of ciprofloxacin pre-exposure when the encapsulation was performed or they were in a state where even a very low ciprofloxacin concentration was enough to arrest their growth. The only elevated $(iMIC)_{mode}$ value for susceptibility testing against streptomycin when compared to unexposed was with 0.125X MIC ciprofloxacin pre-exposure i.e. 1.1 µg/ml in unexposed and 1.5 µg/ml after pre-exposure. Pre-exposures with all other concentrations of both antibiotics decreased the $(iMIC)_{mode}$ in comparison to respective unexposed samples (Table RT2).

For $(iMIC)_{start}$ the most important change was seen with the ciprofloxacin AST in the samples pre-exposed with 0.25X MIC and 0.5X MIC ciprofloxacin, both showing around a 24-fold decrease as compared to unexposed. During the ASTs against streptomycin, 0.5X MIC ciprofloxacin pre-exposure yielded a 17-fold decrease in the $(iMIC)_{start}$, 0.03 µg/ml, in comparison to 0.5 µg/ml for the unexposed sample. The only elevated $(iMIC)_{start}$ value was 0.67 µg/ml and was observed during 0.125X MIC ciprofloxacin pre-exposure.

In the study $(iMIC)_{all}$ is used as a clear indicator of resistance for subset of population against high antibiotic concentrations. Higher the $(iMIC)_{all}$ value is, the greater is the ability of bacteria to survive antibiotic treatment at a specific concentration. For the susceptibility against ciprofloxacin, after 0.25X MIC streptomycin pre-exposure, the $(iMIC)_{all}$ was found to be 18 ng/ml as compared to 15 ng/ml of the unexposed sample. For the tests against streptomycin, $(iMIC)_{all}$ in pre-exposure with 0.125X MIC ciprofloxacin was highest i.e. 1.93 µg/ml, when compared to 1.55 µg/ml of the unexposed sample. Moreover, the lowest $(iMIC)_{all}$ value of 1.36 µg/ml was seen in the sample pre-exposed with 0.25X MIC streptomycin. In some experiments, such as during the AST against ciprofloxacin after the pre-exposures with 0.25X MIC and 0.5X MIC ciprofloxacin, because of unpredictable bacterial growth in response to antibiotic and due to the nature of the Gompertz fit function, it was not possible to determine $(iMIC)_{all}$ values. The Gompertz fitting line was infinitely long, although stays almost parallel and very close to the concentration axis. In such cases the experimentally determined value, $(iMIC)_{exp}$, was taken as the concentration for complete growth inhibition, as both the values are numerically very close (Table RT2 and Figure RF2)

The probability distribution density of *iMIC*, gives a rough idea about heterogeneity of cells in terms of their response to antibiotic. More the width of the curve on X-axis, higher heterogeneous response individual cells display and vice versa (Figure RF6).

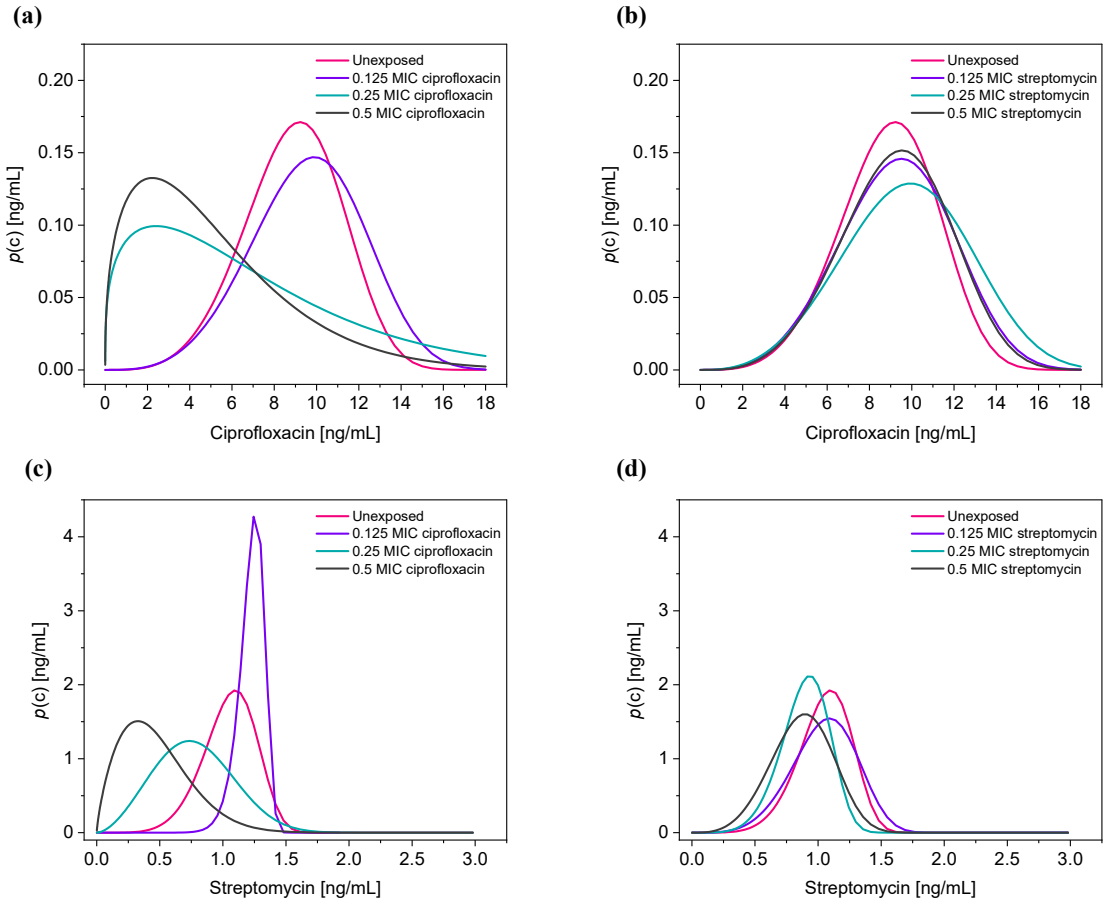

Figure RF6: Probability density distribution of *iMIC*.
(a) and **(b)** represent the distributions of *p*(*iMIC)* during AST with ciprofloxacin in cells pre-exposed with different independent concentrations of ciprofloxacin and streptomycin respectively. Similarly, **(c)** and **(d)** show the distribution of *p*(*iMIC)* for AST with streptomycin in cells pre-exposed with different ciprofloxacin and streptomycin concentrations respectively. The peak of each curve corresponds to $(iMIC)_{mode}$ and the width of each distribution is directly proportional to heteroresistance in the population. Each curve is derived from its respective resistance profile, and the resistance profile was plotted earlier based on average values of three independent experiments (Figure F2, F3, F4, F5).

5.1.4 Effect of pre-exposure on heteroresistance

An established term, degree of heteroresistance (DoH), calculated as the ratio between $(iMIC)_{all}$ and $(iMIC)_{start}$, is used in the study to assess the distribution pattern of *iMICs*, allowing for direct comparison of heteroresistance between pre-exposure samples. DoH shows how spread the distribution of iMIC is within an antibiotic concentration range, or simply speaking, how diverse the iMIC values are for bacteria inside individual droplets.

The analysis of data showed a >20-fold increase in DoH during AST against ciprofloxacin for samples pre-exposed with 0.25X MIC and 0.5X MIC ciprofloxacin, as compared to unexposed sample (Figure RF7 and Table RT2). Similarly, during AST with streptomycin, 0.5X MIC ciprofloxacin pre-exposure caused a 15-fold increase while 0.25X MIC ciprofloxacin pre-exposure led to a 4-fold increase in DoH.

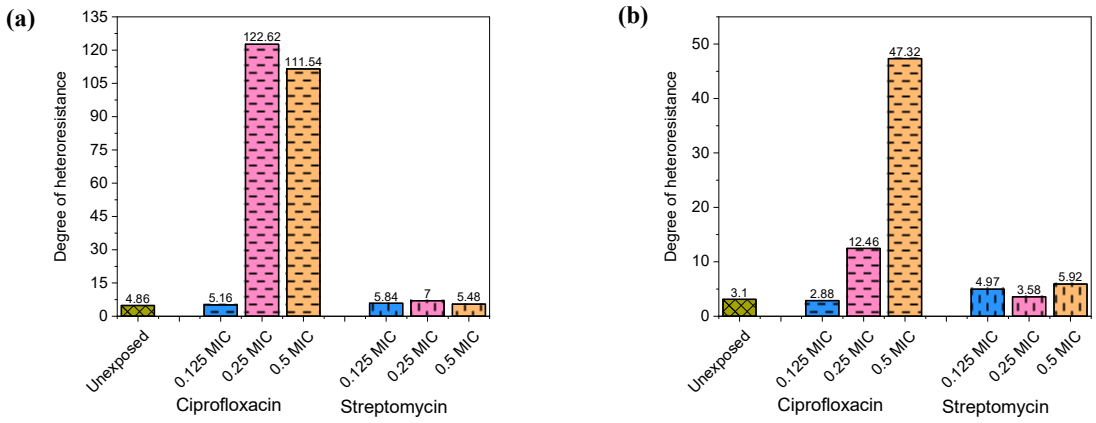

Figure RF7: Bar chart showing the degree of heteroresistance (DoH).
(a) shows DoH during susceptibility testing with ciprofloxacin for populations independently pre-exposed with different concentrations of ciprofloxacin or streptomycin. As evident from the graph, a >20-fold increase in DoH can be seen samples pre-exposed with 0.25X and 0.5X MIC ciprofloxacin as compared to unexposed sample. **(b)** depicts the DoH during AST with streptomycin in the samples independently pre-exposure with different concentrations of ciprofloxacin or streptomycin. Here, a 15-fold increase in DoH was observed for sample pre-exposed with 0.5X MIC ciprofloxacin, hinting that low level antibiotic pre-exposures can alter the sensitivity of cells even against an antibiotic from a different class with different mechanism of action.

5.1.5 Effect on SD, skewness and root mean square deviation

Skewness (S), simply defining is a measure for degree of asymmetry, and here quantifies the asymmetry in the *p(iMIC)* distribution. This is useful in analyzing if the pre-exposure causes a shift in distribution *iMIC* towards concentrations > or < than $(iMIC)_{mode.}$

In the AST experiments with ciprofloxacin, all pre-exposures displayed a negatively skewed value similar to unexposed sample, except for 0.25X MIC and 0.5X MIC ciprofloxacin where skewness was +1.3 and +1.2 respectively. These two concentrations also showed tailing on the right side of the peak, implying presence of cells with very high iMIC values. Similar results were also observed for AST with streptomycin with these two pre-exposures leading to a positive skewness, +0.27 and +0.82 for 0.125X MIC and 0.5X MIC ciprofloxacin respectively.

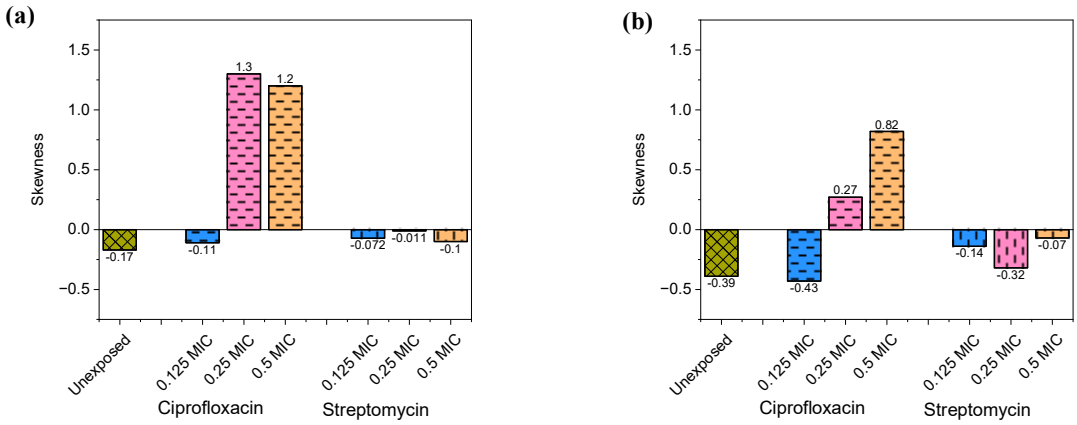

Figure RF8: Bar chart representing the skewness in *p(iMIC)* distribution.

Skewness measures the degree of asymmetry in the *p(iMIC)* distribution. **(a)** for AST with ciprofloxacin in samples independently pre-exposed with different concentrations of ciprofloxacin or streptomycin. **(b)** the skewness when AST was done with streptomycin, in the samples independently pre-exposed with same antibiotic concentrations. Positive skewness, as in 0.25X MIC and 0.5X MIC ciprofloxacin pre-exposures hint that response was more heterogeneous at concentrations above $(iMIC)_{mode}$ than the concentrations lower.

The standard deviation (SD) values (Figure RF9) remained always high in all pre-exposures as compared to unexposed sample during the ASTs against ciprofloxacin. A similar pattern was observed in SD values of *p(iMIC)* distribution during susceptibility testing with streptomycin and all pre-exposure resulted in higher SD values than the unexposed sample.

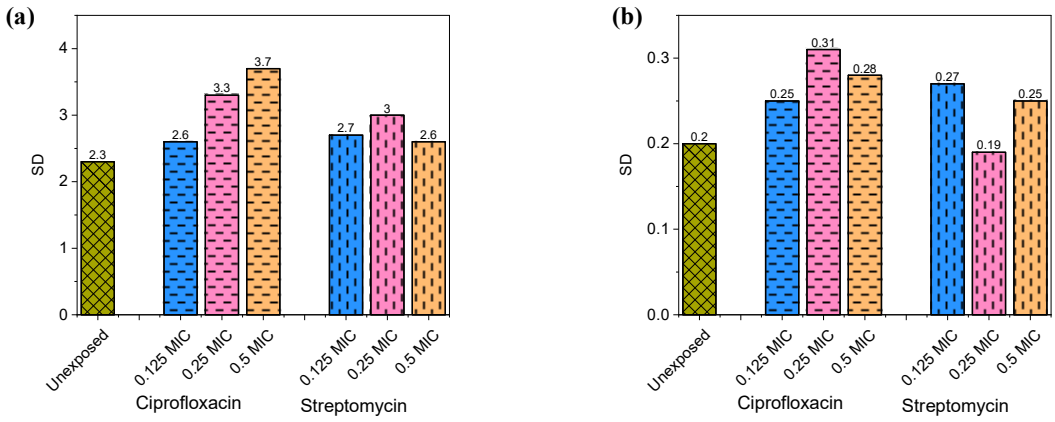

Figure RF9: Plot represents the standard deviation of *p(iMIC)*.

(a) shows the SD during AST with ciprofloxacin in samples independently pre-exposed with different antibiotic concentrations. (**b**) shows SD when AST was done for streptomycin on the pre-exposed samples.

Pre-exposures influenced the individual cells and their *iMIC* to different extents, and the root mean square deviation (RMSD) serves as a more standardized measure of dispersion unifying the comparison. Samples pre-exposed with 0.25X MIC and 0.5X MIC ciprofloxacin showed highest RMSD values on AST with ciprofloxacin and also against streptomycin (Figure RF10).

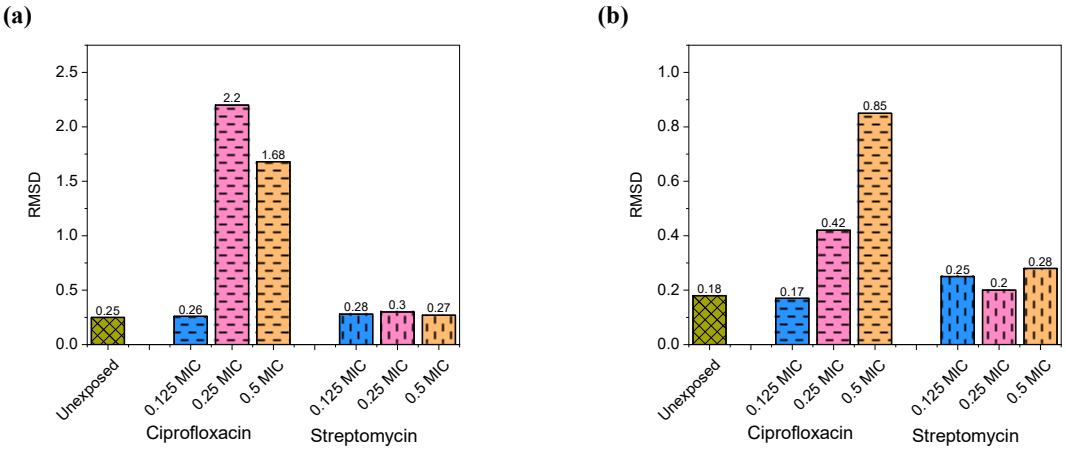

Figure RF10: Bar chart showing root mean square deviation (RMSD). RMSD is ratio between SD and $(iMIC)_{mode}$ and offers a more standardized measure of dispersion of the probability distribution. **(a)** RMSD for AST with ciprofloxacin in samples pre-exposed with different concentrations. (**b**) RMSD for the AST with streptomycin, in the samples pre-exposed with the same antibiotic concentrations.

5.2 *Pseudomonas aeruginosa* PA14

5.2.1 Calculation of MICs and sub-MICs used

The minimum inhibitory concentrations against ciprofloxacin and streptomycin tested for PA14 were found to be 250 ng/ml and 8 µg/ml respectively (Figure RF11). Also, the concentrations for pre-exposure of each antibiotic were decided based on calculated MIC values (Table RT3).

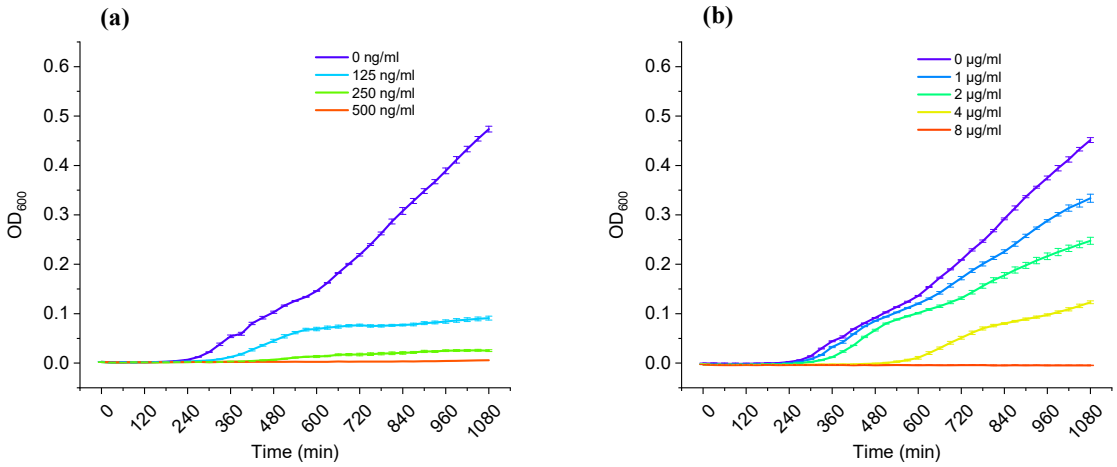

Figure RF11: The kinetics of PA14 growth at different concentrations of antibiotic
(a) and **(b)** show PA14 growth at different concentrations of ciprofloxacin and streptomycin respectively. Each data point denotes the average for three independent experiments and error bars show the SD.

Antibiotic	MIC	0.5X MIC	0.25X MIC	0.125X MIC
Ciprofloxacin	250 ng/ml	125 ng/ml	62.5 ng/ml	31.25 ng/ml
Streptomycin	8 µg/ml	4 µg/ml	2 µg/ml	1 µg/ml

Table RT3: MICs and sub-MICs used for pre-exposure
Table shows the MICs of ciprofloxacin and streptomycin against PA14 as elucidated using the microdilution method. The details about different concentrations used for sub-MIC exposure is also given.

5.2.2 Resistance profile and Gompertz fitting

As per the methodology discussed in section 4.5, for each pre-exposure, the positive fractions of droplets were plotted with respect to the concentrations and the Gompertz fitting (Figure RF12, RF13, RF14, RF15) was done. Various parameters (Table RT4) to be used in the study were then derived from the Gompertz fit line for further calculations.

			CIP pre-exposure			STR pre-exposure		
		Unexposed	*0.125X MIC*	*0.25X MIC*	*0.5X MIC*	*0.125X MIC*	*0.25X MIC*	*0.5X MIC*
CIP susceptibility testing	$(iMIC)_{mode}$	94	70	85	110	190	170	90
	$(iMIC)_{mean}$	91	89	98	100	180	180	86
	SD	25	47	47	21	27	69	20
	CoV	0.27	0.53	0.48	0.21	0.15	0.38	0.23
	RMSD	0.27	0.67	0.55	0.19	0.14	0.41	0.22
	$(iMIC)_{start}$	28.17	6.87	10.99	45.10	106.86	32.95	30.47
	$(iMIC)_{total}$	130.10	178.05	185.03	135.20	225.28	296.21	115.58
	$(iMIC)_{all}$	158.17	253.21	253.21	158.17	253.21	374.79	135.2
	$(iMIC)_{exp}$	250	200	300	200	300	400	150
	DoH	5.61	36.86	23.04	3.5	2.4	11.37	4.46
	S	-0.12 (±1.8e-05)	0.65 (±3.5e-04)	0.5 (±2.1e-04)	-0.33 (±9.2e-06)	-0.53 (±7.2e-05)	0.23 (±8.2e-01)	-0.2 (±2.3e-05)
	K	2.77 (±8e-03)	3.28 (±3.4e-03)	3.03 (±6.9e-02)	2.97 (±1.1e-03)	3.31 (±9.2e-03)	2.76 (±1.3e-05)	2.83 (±2.2e-04)
STR susceptibility testing	$(iMIC)_{mode}$	2.7	4.4	2.2	2.4	3.6	3.2	2.4
	$(iMIC)_{mean}$	3	4.3	2.6	3.5	3.6	3.7	4
	SD	1.4	1.3	1.3	2	1.3	1.8	2.6
	CoV	0.47	0.3	0.5	0.57	0.36	0.49	0.65
	RMSD	0.52	0.3	0.59	0.83	0.36	0.56	1.08
	$(iMIC)_{start}$	0.37	1.13	0.24	0.19	0.74	0.38	0.16
	$(iMIC)_{total}$	5.38	6.5	5.01	7.14	5.78	6.81	9.04
	$(iMIC)_{all}$	7.14	7.85	6.81	10.42	7.14	9.04	ND
	$(iMIC)_{exp}$	8	8	8	8	8	12	12
	DoH	19.3	6.95	28.38	54.84	9.65	23.79	75
	S	0.43 (±4.1e-09)	-0.01 (±2e-09)	0.58 (±1e-08)	0.82 (±3.3e-08)	0.15 (±1.3e-08)	0.51 (±4.1e-08)	0.98 (±1.1e-07)
	K	2.94 (±5.5e-08)	2.72 (±6.2e-08)	3.15 (±1.1e-07)	3.67 (±4.5e-07)	2.72 (±6.4e-08)	3.04 (±1.8e-07)	4.09 (±1.7e-07)

Table RT4: The values of various parameters derived from the Gompertz fitting curve are mentioned above, these parameters are further used to assess the heteroresistance profile of PA14 cells

SD: Standard deviation; RMSD: Root-mean square deviation; CoV: Coefficient of variance; ND: Not determinable; CIP: Ciprofloxacin; STR: Streptomycin. The concentrations, wherever applicable, are in ng/ml and µg/ml units for ciprofloxacin and streptomycin respectively.

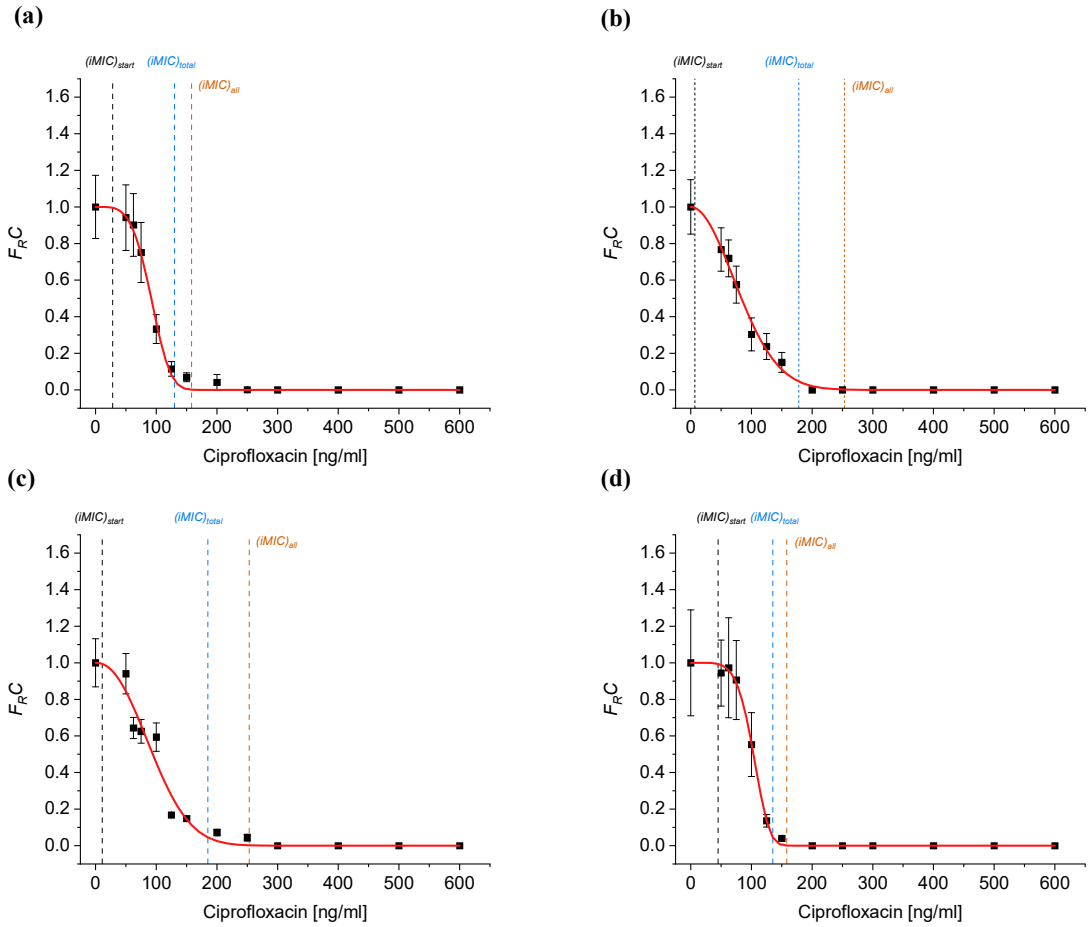

Figure RF12: plot represents the resistance profile and the Gompertz fitting for the positive fraction of droplets, F_RC, as a function of antibiotic concentration, c.

The susceptibility tests were done independently with PA14 culture against ciprofloxacin on **(a)** unexposed sample and **(b)** cells pre-exposed with 0.125X MIC of ciprofloxacin, **(c)** 0.25X MIC of ciprofloxacin, **(d)** 0.5X MIC of ciprofloxacin. Each black solid square data point is the average of three independently performed experiments and error bars represent the standard deviations. Red line represents the Gompertz fitting from the experimentally determined values.

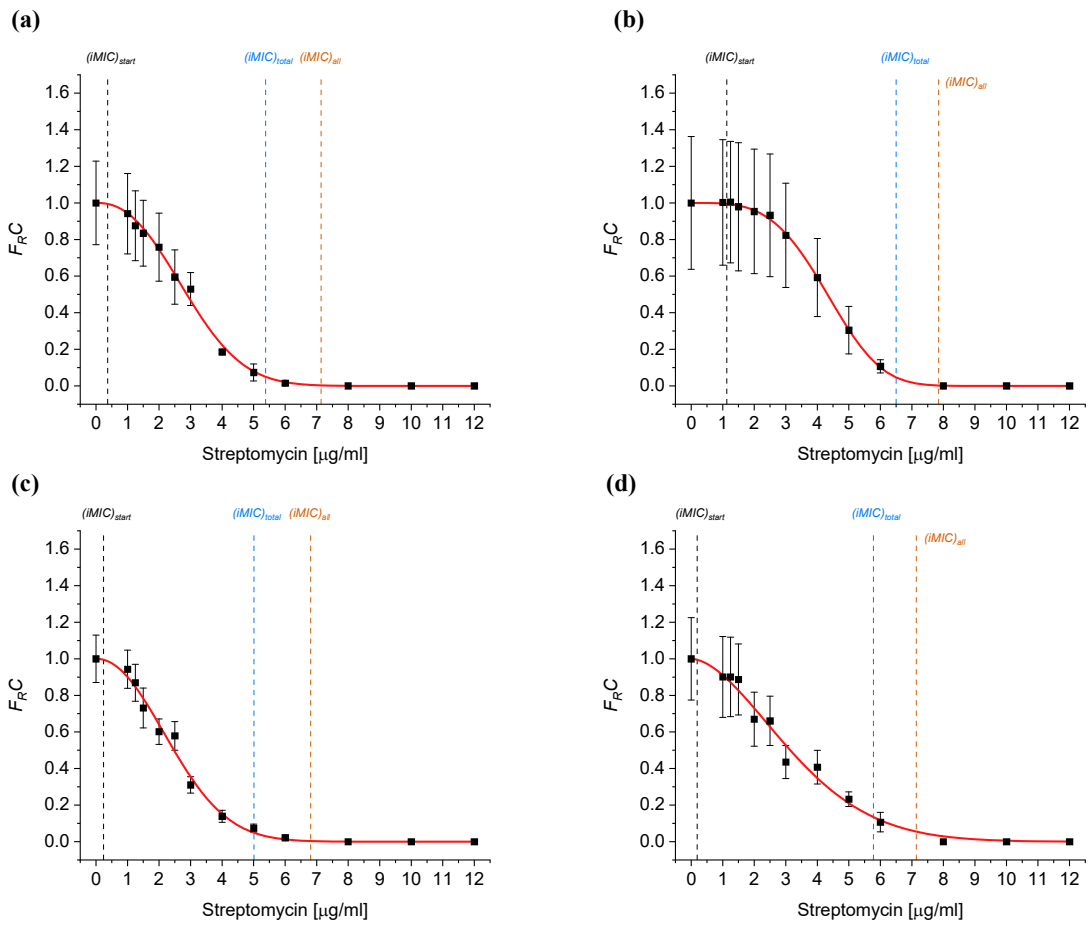

Figure RF13: the plot depicts the resistance profile and the Gompertz fitting for positive fraction of droplets, F_RC, as a function of antibiotic concentration, c, from lowest to highest.
The ASTs were performed independently for PA14 against streptomycin on **(a)** unexposed sample and **(b)** bacteria pre-exposed with 0.125X MIC of ciprofloxacin, **(c)** 0.25X MIC of ciprofloxacin, **(d)** 0.5X MIC of ciprofloxacin. Each black solid square data point is the average value of three independently performed experiments and error bars are represent the standard deviations. Red line is derived after performing the Gompertz fitting of the experimentally determined values.

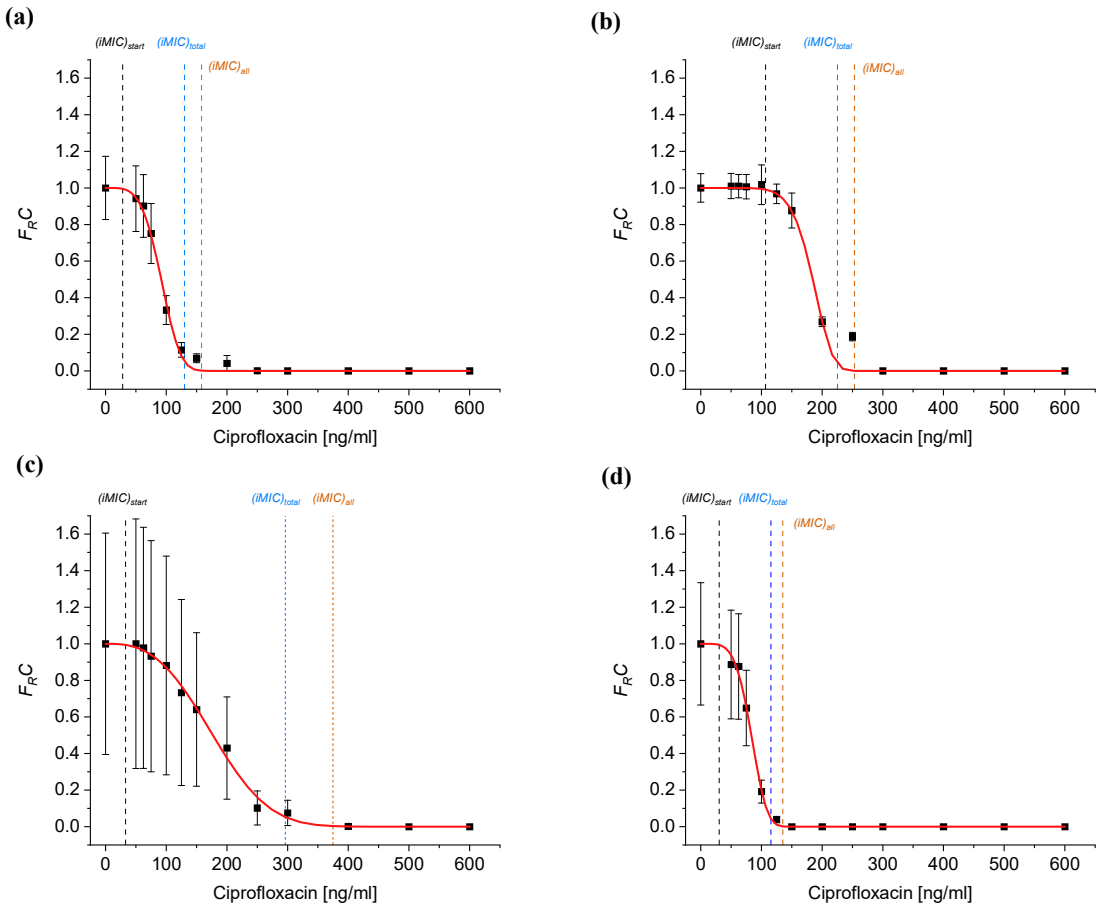

Figure RF14: graph depicts the resistance profile and the Gompertz fitting for positive fraction of droplets, F_RC, as a function of antibiotic concentration, c, for experiments performed on PA14.
The susceptibility tests were done independently for PA14 against ciprofloxacin on **(a)** unexposed sample and **(b)** bacterial sample pre-exposed with 0.125X MIC of streptomycin, **(c)** 0.25X MIC of streptomycin, **(d)** 0.5X MIC of streptomycin. Each black solid square data point is the average value of three independently performed experiments and error bars represent the standard deviations. Red line is derived the Gompertz fitting from the experimentally determined values.

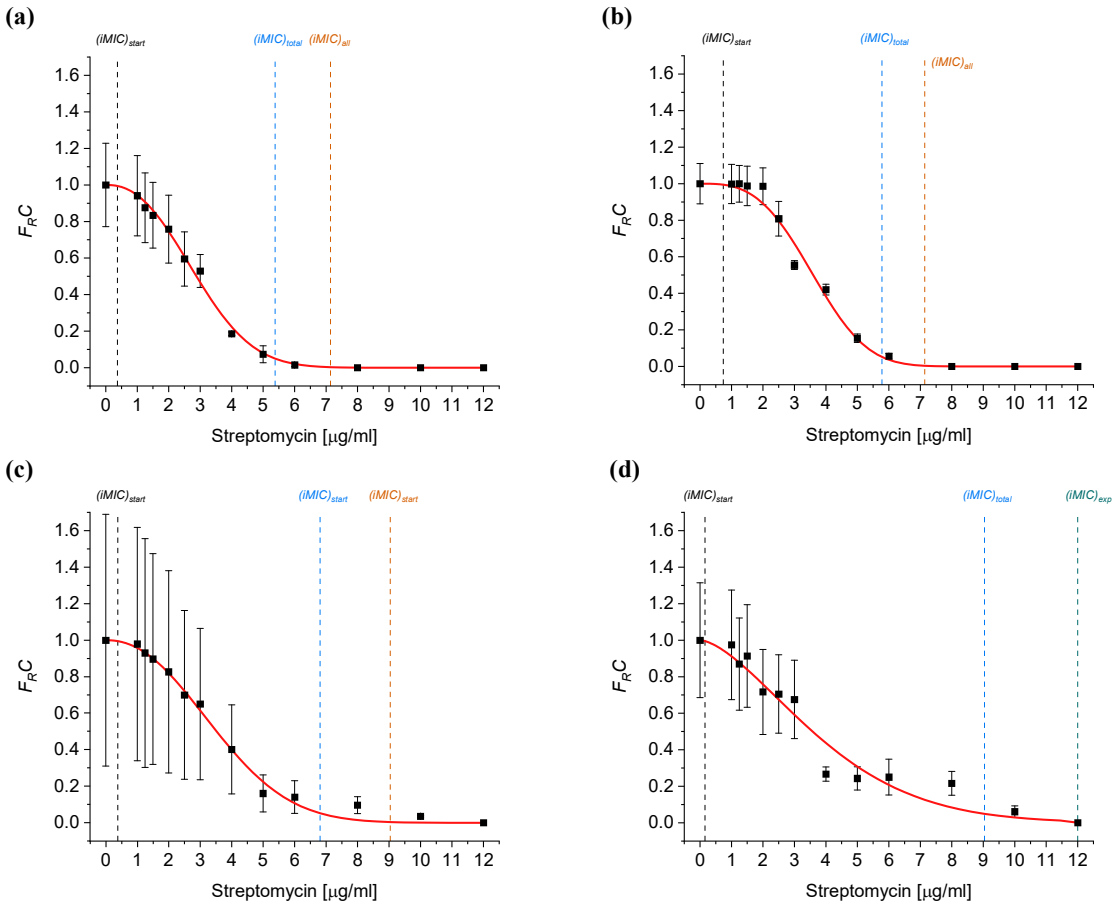

Figure RF15: plot shows the resistance profile and the Gompertz fitting for positive fraction of droplets, F_RC, as a function of antibiotic concentration, c, from lowest to highest.
The streptomycin susceptibility tests were done independently with PA14 culture on **(a)** unexposed sample and **(b)** bacteria pre-exposed with 0.125X MIC of streptomycin, **(c)** 0.25X MIC of streptomycin, **(d)** 0.5X MIC of streptomycin. Each black solid square data point is the average of three independently performed experiments and error bars are representing the standard deviations. Red line is derived and shows the Gompertz fitting from the experimentally determined values. It was impossible to determine $(iMIC)_{all}$ for (d) from Gompertz fitting, hence the experimentally determined concentration for inhibition of all cells, *(iMIC)exp,* is shown instead. More information about this is given in the manuscript text and in Table RT4.

5.2.3 Effects of pre-exposure on $(iMIC)_{mode}$, $(iMIC)_{start}$, $(iMIC)_{all}$ and $p(iMIC)$

As discussed, $(iMIC)_{mode}$ is the mode commonly occurring *iMIC* value for the bacteria growing inside droplets. For the unexposed sample, $(iMIC)_{mode}$ during susceptibility testing with ciprofloxacin was found to be 94 ng/ml, while in 0.125X MIC and 0.25X MIC streptomycin exposed samples it was 190 ng/ml and 170 ng/ml respectively, almost twice with respect to unexposed sample. During susceptibility tests against streptomycin, the $(iMIC)_{mode}$ values for all pre-exposed samples remained fairly similar to unexposed, except with 0.125X MIC of ciprofloxacin where a 1.6-fold elevation was noted (Table RT4).

For $(iMIC)_{start}$ the most noticeable change with the ciprofloxacin AST was observed in the sample pre-exposed with 0.125X MIC streptomycin with a value of 106.86 ng/ml as compared to 28.17 ng/ml of control, around 4-fold increase. This increase in $(iMIC)_{start}$ value imply that the above pre-exposure may impart some sort of protective mechanism to most cells and the concentration at which the growth inhibition started was increased, as compared to unexposed control sample. Interestingly, pre-exposures with 0.125X MIC and 0.25X MIC ciprofloxacin led to a reduction in the $(iMIC)_{start}$ values to 6.87 ng/ml and 10.99 ng/ml respectively. Concerning the ASTs against streptomycin, the highest $(iMIC)_{start}$ was seen in the 0.125X MIC ciprofloxacin and 0.125X MIC streptomycin pre-exposed samples with concentrations of 1.13 µg/ml and 0.74 µg/ml respectively. Moreover, the noticeable reductions occurred in 0.5 MIC ciprofloxacin and 0.5 MIC streptomycin pre-exposures to 0.19 µg/ml and 0.16 µg/ml.

As previously mentioned, $(iMIC)_{all}$ is the indicator about bacteria's ability to survive high antibiotic concentrations. However, $(iMIC)_{all}$ values for most pre-exposed samples were comparable to unexposed control, except in pre-exposure with 0.5X MIC ciprofloxacin where it was found to be 10.42 µg/ml as compared to 7.14 µg/ml of unexposed sample. For the sample pre-exposed with 0.5X MIC streptomycin, $(iMIC)_{all}$ was not determinable due to the nature of Gompertz fitting function and unpredictable response of bacterial cells to antibiotics. Here the Gompertz fit line stayed infinitely long and very close to the concentration axis (Table RT4 and Figure RF15). In this case, the experimentally determined value, $(iMIC)_{exp}$, was taken as the concentration for complete growth inhibition, as both the values are numerically very close.

The distribution density of *p(iMIC),* presents the picture of heterogeneous response of individual cells in terms of response to antibiotic. Wider the distribution of *iMIC* on the X-axis, greater the heterogeneity in response and vice versa (Figure RF16). However, as mentioned earlier, quantification of heteroresistance in the population can be done by calculating DoH.

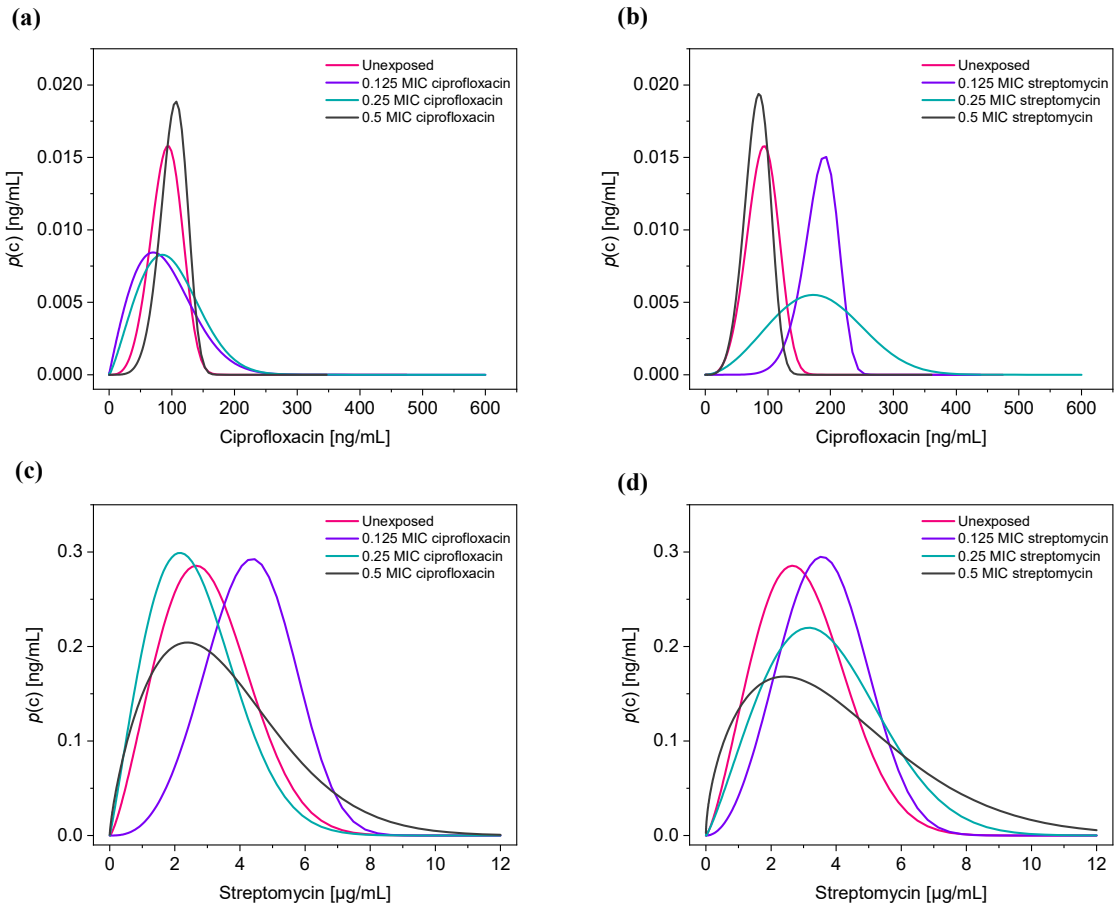

Figure RF6: Probability density distribution of *iMIC*.
(a) and **(b)** represent the distributions of *p*(*iMIC)* during AST with ciprofloxacin in PA14 pre-exposed cells with different concentrations of ciprofloxacin and streptomycin respectively. Similarly, **(c)** and **(d)** show the distribution of *p*(*iMIC)* for susceptibility against streptomycin in PA14 cells pre-exposed with different concentrations of ciprofloxacin and streptomycin respectively. The peak in each curve represents the $(iMIC)_{mode}$ value and the width of each distribution is directly proportional to heteroresistance. Skewness, as the degree of curve asymmetry, can also be identified by the tailings on left and right side $(iMIC)_{mode.}$ Each curve is derived from its respective resistance profile, and the resistance profile was plotted earlier based on the average values from three independent experiments (Figure F12, F13, F14, F15).

5.2.4 Effect of pre-exposure on heteroresistance

DoH is directly proportional to $(iMIC)_{all}$ or $(iMIC)_{exp}$ while inversely proportional to $(iMIC)_{start}$ and is used to find the diversity in individual cell response for a particular bacterial population. A 6-fold, 4-fold and 2-fold increase in DoH was observed in the samples pre-exposed with 0.125X MIC ciprofloxacin, 0.25X MIC ciprofloxacin and 0.25X MIC streptomycin as compared to unexposed sample when the AST was done against ciprofloxacin (Figure RF17). Similarly, for streptomycin AST, a 3-fold and 4-fold increase in DoH was seen compared to unexposed, in pre-exposures with 0.5X MIC ciprofloxacin

and 0.5X MIC streptomycin respectively. Interestingly, 0.5X MIC streptomycin pre-exposure also elevated the DoH by 3-fold and 4-fold during AST with ciprofloxacin and streptomycin respectively.

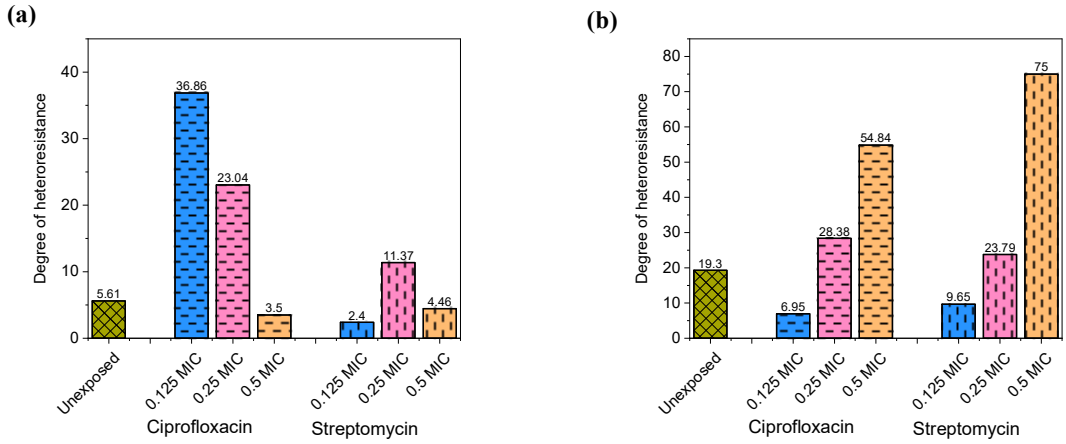

Figure RF17: Bar graph showing the degree of heteroresistance (DoH).
(a) represent DoH observed in PA14 samples during susceptibility testing with ciprofloxacin that were independently pre-exposed with different concentrations of ciprofloxacin or streptomycin. The chart shows 6-fold and 4-fold increase in DoH for the samples pre-exposed 0.125X MIC and 0.25X MIC ciprofloxacin respectively when compared to the unexposed sample. **(b)** shows the DoH during AST with streptomycin in the samples independently pre-exposure with different concentrations of ciprofloxacin or streptomycin. Here, a 3-fold and 4-fold increase in DoH was observed for the samples pre-exposed with 0.5X MIC ciprofloxacin and 0.5X MIC streptomycin respectively. The observations imply that sub-MIC antibiotic pre-exposures can alter the sensitivity of individual cells even against an antibiotic from a different class with different mechanism of action.

5.2.5 Effect on SD, skewness and root mean square deviation

Skewness here is used to find the degree of asymmetry in the *iMIC* distribution and tailing of the curve around the $(iMIC)_{mode}$ is a good indicator for the response being more heterogeneous on the tailed side. During AST with ciprofloxacin, 0.15X MIC ciprofloxacin, 0.125X MIC streptomycin and 0.5X MIC streptomycin pre-exposed samples produced a negative skewness along with the unexposed sample. This implies that there was tailing on the left side of $(iMIC)_{mode}$ value and *iMIC* was more diverse on left side as compared to right. On the other hand, positive skewness was observed in the samples pre-exposed with 0.125X MIC ciprofloxacin, 0.5X MIC ciprofloxacin and 0.25X MIC streptomycin. For the susceptibility testing against streptomycin, all the pre-exposure concentrations as well as the unexposed sample showed positively skewed curves for the *iMIC* distributions, except for the pre-exposure with 0.125X ciprofloxacin with a value of -0.01 (Figure RF18).

Positive skewness indicates that individual cell response to antibiotic was more diverse on the right side of $(iMIC)_{mode}$ value than on the left side.

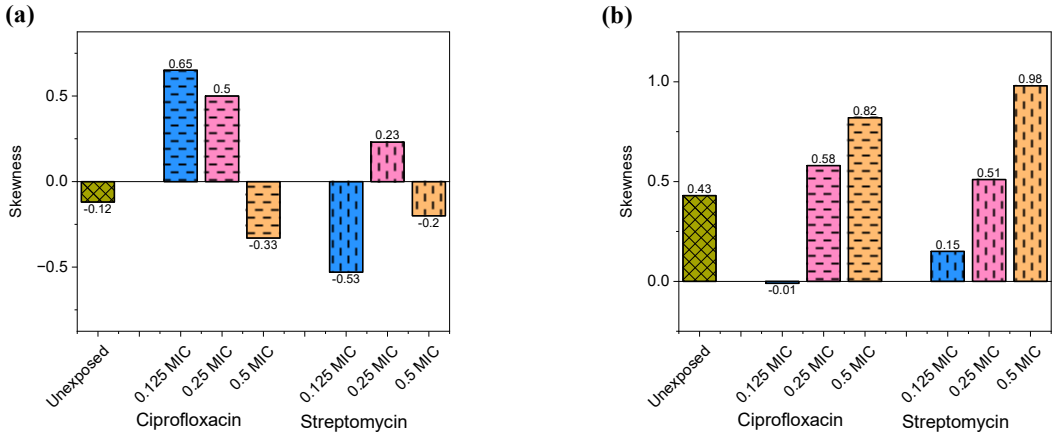

Figure RF18: Bar graph representing the skewness in *p(iMIC)* distributions of PA14 cells.
Skewness measures the degree of asymmetry of the curve, and here the asymmetry in the *p(iMIC)* distribution. **(a)** shows skewness during AST with ciprofloxacin in samples independently pre-exposed with different concentrations of ciprofloxacin or streptomycin. **(b)** displays the skewness when AST was done with streptomycin, in the samples independently pre-exposed with the same concentrations of antibiotics. A positive skewness, hints that the bacterial response was more heterogeneous at the concentrations greater than $(iMIC)_{mode}$ than the concentrations less than the $(iMIC)_{mode}$ value and vice versa.

The standard deviations (SD) (Figure RF19) during AST with ciprofloxacin showed variations based on the pre-exposures. Pre-exposures with all concentrations showed higher SD values as compared to control, except for 0.5X MIC ciprofloxacin and 0.5X MIC streptomycin. However, for susceptibility against streptomycin, all pre-exposures showed either higher or similar SD values to unexposed, with 0.5X MIC streptomycin resulting in a 2-fold increase.

To make the comparison more unified, RMSD is again used as a more standardized parameter to measure dispersion in *iMIC* distributions. Samples having high heteroresistance generally showed higher RMSD values, as seen in pre-exposures with 0.125X MIC ciprofloxacin, 0.25X MIC ciprofloxacin and 0.25X MIC streptomycin for ciprofloxacin AST (Figure RF20). AST for streptomycin gave similar results with 0.5X MIC ciprofloxacin and 0.5X MIC streptomycin pre-exposed samples displaying high RMSD values than unexposed and in turn higher DoH.

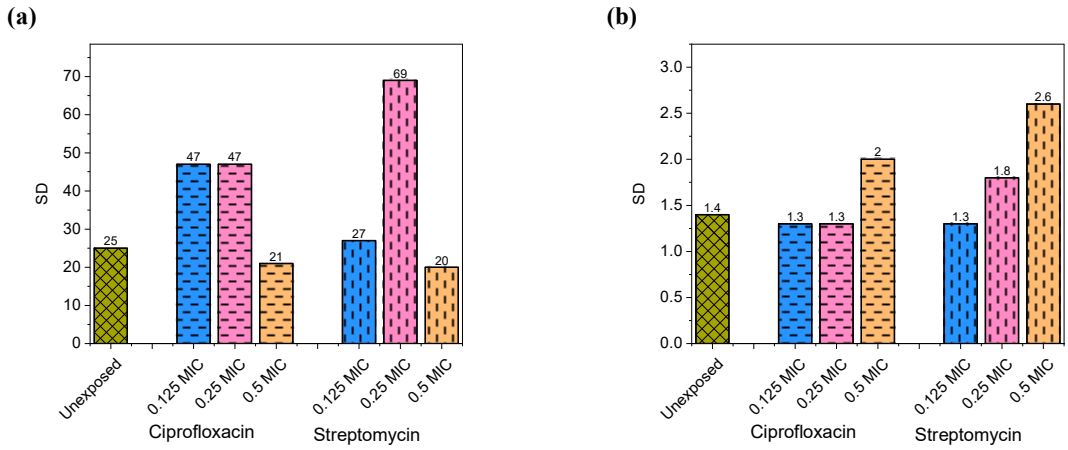

Figure RF19: Plot represents standard deviation of *p(iMIC)*. **(a)** shows the standard deviations during AST with ciprofloxacin in samples independently pre-exposed with different concentrations of ciprofloxacin or streptomycin (**b**) shows SD when AST was performed for streptomycin on independently pre-exposed samples in a similar manner.

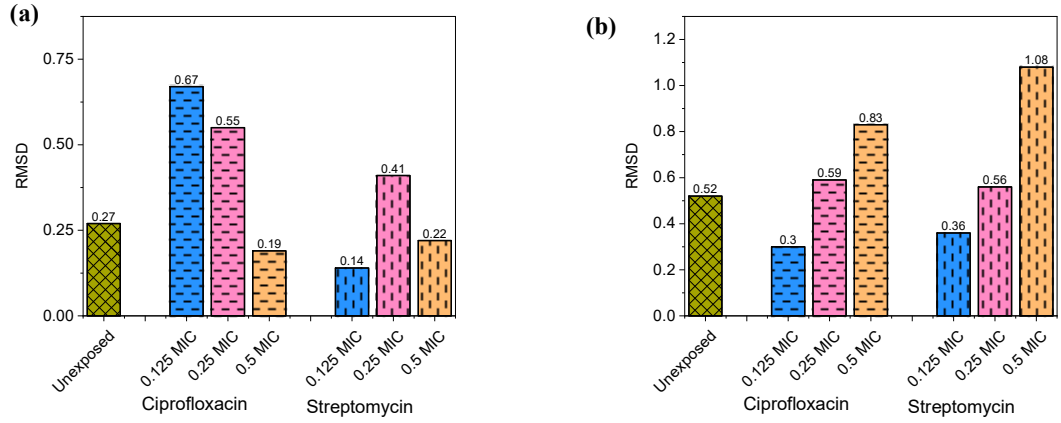

Figure RF20: Bar chart representing root mean square deviation (RMSD).

(a) shows RMSD for AST with ciprofloxacin in the bacterial populations independently pre-exposed with different concentrations of ciprofloxacin or streptomycin. Similarly, (**b**) shows the RMSD values for AST with streptomycin, in the samples independently pre-exposed with the same antibiotic concentrations. RMSD is an important indicator for heterogeneous antibiotic response of individual cells in the population and the results coincides with DoH calculations in the samples.

6 Discussion and conclusion

Chapter 6 *covers the discussion and conclusion about various results obtained regarding the effects of pre-exposures including why single-cell AST is crucial and why is droplet microfluidics is an excellent tool to study heteroresistance. The section is split between the two strains i.e. MG1655 and PA14. Discussions are made about the potential reasons for any change in the parameters used in the study and eventually why a change in heteroresistance was seen, if any.*

Finally, a brief general and combined conclusion is given in the end.

AST provides the basis for clinical microbiology and infection treatment. Susceptibility has traditionally been established by bulk population tests such as broth dilution or disk diffusion that measure the population average response of a bacterial population to an antimicrobial agent. These tests unfortunately ignore one of the most critical features of bacteria: the heterogeneity of response between individual cells[218]. Single-cell antibiotic susceptibility testing (scAST) has emerged as a groundbreaking method that overcomes this limitation, yielding profound insights into bacterial heterogeneity, treatment failure, and antibiotic resistance.

One of the greatest advantages of scAST lies in its ability to reveal phenotypic heterogeneity of isogenic bacterial populations usually not detectable by standard assays. Genetically equivalent bacteria may be profoundly disparate in their sensitivity to antibiotics owing to differences in metabolic state, growth rate, or environmental exposure[145] and some cells can survive antibiotic treatment due to transient phenotypic

states[218,225]. Importantly, even a single surviving cell may have the ability to proliferate into whole population and can lead to potential future infection[226].

Additionally, scAST enables a more nuanced understanding of the actions of antibiotics at the cellular level. Time-lapse microscopy, microfluidics, and fluorescent labels enable scientists to monitor the timing, mechanism, and course of drug action in real time[227,228]. It can resolve bacteriostatic and bactericidal antibiotic actions and determine how rapidly and efficiently an antibiotic enters bacterial cells. It also gives a chance to analyze how bacteria growing in mixed environments, such as biofilms or tissue in a host, react to antibiotics.

Clinically, scAST holds huge potential for precision antimicrobial therapy. With the ability to identify resistant or tolerant subpopulations early on, AST can help in devising better treatment plan, especially in infections involving heterogeneous or slow growing bacteria. This is particularly useful in chronic infections, such as those seen in cystic fibrosis or implanted medical devices, where conventional testing may underestimate the presence of hard-to-kill cells. Further, single-cell techniques are also being extremely helpful in drug discovery. They are more sensitive in detecting antimicrobial activity and can detect failures that bulk methods were unable to detect. This enhances new antibiotic screening and helps in designing better treatment protocols to minimize the emergence of resistance.

In summary, single-cell antibiotic susceptibility testing represents a paradigm shift in how bacterial drug responses are understood and evaluated. By moving beyond population averages and into the realm of single-cell dynamics, it provides critical insights into bacterial survival strategies, informs personalized treatment strategies, and enhances the development of next-generation antibiotics.

Microfluidics emerged as one of the best tools to perform scAST and multiple different variations of it have been developed over time. The approach used in this study i.e. droplet microfluidics-based AST is binary in nature, meaning it primarily classified cells as either growing or non-growing. While this method does not provide information about metabolically inactive or dormant cells, it still proved highly valuable for determining the MIC at the single-cell level and for assessing population heteroresistance[131].

In past, multiple studies have been performed about mutant selection at concentrations greater than the MIC values, however, limited literature is available about the effects of antibiotic pre-exposure at sub-MIC level, particularly in the context of heteroresistance. Moreover, majority of these studies were based on bulk population analysis[229–231] and the

response of individual cells to antibiotic was not taken into consideration. To address this gap, the current study investigated single-cell responses to short-term sub-MIC antibiotic exposures of two bactericidal antibiotics: ciprofloxacin and streptomycin. Ciprofloxacin, a fluoroquinolone, targets DNA replication and is known to induce the production of ROS[106], promote DNA mutations[232], and facilitate resistance evolution[233]. Streptomycin, on the other hand is an aminoglycoside, targeting the protein synthesis with potential to generate ROS[234], promote DNA mutations[235], cause protein aggregation[106] and selection of resistant cells[130]. The central hypothesis is that sub-MIC exposure to antibiotics induces ROS production, causing damage cellular components such as DNA, membrane lipids, and proteins which in turn triggers SOS response[105,124,236]. The response is also heterogeneous leading to a variety of physiological, morphological and metabolic changes in cells, and thus, they present a diverse response to subsequent antibiotic exposure[124,130].

6.1 Droplet microfluidics as an excellent tool single-cell study

Microfluidics offers a powerful and efficient approach to antibiotic susceptibility testing, addressing many of the limitations of traditional methods. By miniaturizing and automating experiments, microfluidics enables faster, more precise, and more sensitive assessment of microbial response to antibiotics. Droplet microfluidics in particular proves to be an exceptionally effective tool as it combines the advantages of microfluidics with the compartmentalization power of tiny droplets, essentially creating thousands to millions of miniature reaction chambers, each capable of housing and analyzing individual bacterium.

First and foremost, advantage of droplet microfluidics is providing the single cell resolution of AST, which is the basis of whole study discussed in this thesis. This provided the ability to capture minute differences in the response of individual cells and in turn quantification of heteroresistance. The cells were growing in stochastic confinement, which means the *iMIC* obtained was only the characteristic response of that particular cell. Over that, it was possible to generate thousands of droplets semi-automatically using very little sample volume and in a very short time at a very fast pace, essentially eliminating the major portion of manual labor, the need to have multiple plates and big sample volumes.

The detection of droplets can be a bit challenging when there is a prerequisite of it being label-free. However, the use of GLCMs for the identification of pixels turned out to be an excellent method for the automated analysis of bright field droplet images. Further analysis of the data obtained using programming was based on pre-standardized approaches. It was

also possible to sort and collect the droplets with bacteria into individual compartments for further molecular and genetic studies. However, it is beyond the scope of this thesis, but such studies in future can help to elucidate the molecular and genetic signatures implicated in heteroresistance.

6.2 *Escherichia coli* MG1655

By using the classic AST method of broth dilution, the growth kinetics of *Escherichia coli* MG1655 at different antibiotic concentrations were recorded by measuring the optical density at 600 nm. The MIC was established based on the lowest antibiotic concentration that inhibited the visible growth. Using this method, the MIC values for ciprofloxacin and streptomycin were determined to be 12 ng/ml and 3 µg/ml respectively.

As per the Gompertz fitting, the $(iMIC)_{all}$ for ciprofloxacin and streptomycin was calculated to be 15 ng/ml and 1.55 µg/ml respectively. For all the sub-MIC pre-exposure of ciprofloxacin, elevated $(iMIC)_{all}$ values can be attributed to the earlier discussed mechanisms such as DNA mutations, ROS generation and resistance selections[106,232,233]. However, the pre-exposure with 0.5X MIC ciprofloxacin could have caused enough damage to a large fraction of cells, resulting in a decreased $(iMIC)_{all}$ of 1.42 µg/ml on their AST with streptomycin. The highest $(iMIC)_{all}$ value for ciprofloxacin AST was calculated to be 18 ng/ml for the pre-exposure with 0.25X MIC streptomycin, though the specific reason for this observation remains unclear.

As previously mentioned, the most frequently occurring *iMIC* value during single cell AST is denoted by $(iMIC)_{mode}$. An increase in this value following pre-exposure suggests that the majority of cells in the population developed a higher tolerance or adaptive response to antibiotic. The 0.5X MIC ciprofloxacin pre-exposure considerably reduced the $(iMIC)_{mode}$ for both ciprofloxacin and streptomycin ASTs, probably due to residual inhibitory effects, especially from toxic ROS generated during the pre-exposure. The subsequent exposure might have acted synergistically with residual ciprofloxacin-induced stress. This is supported by previous reports of synergistic interactions between ciprofloxacin and aminoglycosides, where ciprofloxacin-induced DNA damage increases bacterial vulnerability, thereby enhancing the efficacy of protein synthesis inhibitors[233,237]. However,

very low levels of ciprofloxacin pre-exposures appeared to provide a protective advantage to most cells upon subsequent exposures to both ciprofloxacin and streptomycin.

Based on the Gompertz curves (Figure RF2, RF3, RF4, RF5), the bacterial cells that were proliferating between the concentration at which first growth inhibition is observed, $(iMIC)_{start}$, and the lowest concentration that stopped the growth of all tested bacteria, $(iMIC)_{all}$, are most important, as the ratio of $(iMIC)_{all}$ to $(iMIC)_{start}$ gives the degree of heteroresistance. A key observation was that the pre-exposures with 0.25X MIC and 0.5X MIC of ciprofloxacin significantly decreased the $(iMIC)_{start}$ value (Table RT1), suggesting that subsequent exposure to antibiotic showed synergistic effect with pre-exposure antibiotic concentration on a subset of population. However, due to the heterogeneous response of individual cells to antibiotics, some cells were still able to tolerate higher concentration of pre-exposure antibiotic and subsequently displayed reduced sensitivity to subsequent antibiotic treatments. The heterogeneous response of cells during the pre-exposure and then to the follow up antibiotic exposure together defined the overall heteroresistance in the tested population. The reduction of $(iMIC)_{start}$ value and ability to promote heteroresistance was more pronounced for ciprofloxacin pre-exposures compared to streptomycin. It is hypothesized that the ROS generation and DNA mutagenic potential of ciprofloxacin is more effective at inducing heteroresistance in MG1655, probably because aminoglycosides are less likely to invoke SOS response in *Escherichia coli* cells[238]. Moreover, ciprofloxacin-induced heteroresistance was more evident during subsequent ciprofloxacin treatment than with streptomycin.

Experiments also revealed some correlations between the degree of heteroresistance and the skewness of *iMIC* distribution. Positively skewed samples tended to exhibit higher degrees of heteroresistance and vice versa (Table RT2). A negative skewness indicates that more variability occurred below $(iMIC)_{mode}$, resulting in a left-tailed distribution of *iMIC* values (Figure RF6). On the other hand, a more positive skewness and a tailing on the right side of the curve indicates greater heterogeneous response on the right side, indicating the presence of small bacterial subpopulations exhibiting substantially higher resistance than majority of cells. The 0.25X MIC and 0.5X MIC ciprofloxacin gave positively skewed values for AST with ciprofloxacin and streptomycin in the current study, reinforcing the hypothesis that these doses of ciprofloxacin are especially potent in generating heteroresistance in MG1655. Additionally, the potential of ciprofloxacin in generating

heteroresistance for subsequent streptomycin exposure is dose dependent, which is evident in comparisons between 0.25X MIC and 0.5X MIC ciprofloxacin pre-exposures.

6.3 *Pseudomonas* aeruginosa PA14

Using the conventional AST method of broth dilution in a 96-well plate, the growth kinetics of *Pseudomonas aeruginosa* PA14 culture at different antibiotic concentrations was recorded by measuring the optical density at 600 nm over time. The MIC was established based on the lowest concentration of antibiotic that was able to inhibit the visible growth of cells. By this method, the MIC of ciprofloxacin was calculated to be 250 ng/ml and that of streptomycin was found to be 8 µg/ml. Streptomycin is not generally used for *Pseudomona*s infections as reports of high-level resistance against aminoglycosides have existed since 1970s[239], it was included in the study to stimulate scenarios where inappropriate antibiotic administration occurs due to pathogen misidentification.

From Gompertz fitting curve, the $(iMIC)_{all}$ for ciprofloxacin and streptomycin in unexposed cultures was elucidated and it was found to be 158.17 ng/ml and 7.14 µg/ml, respectively. For all sub-MIC pre-exposure of ciprofloxacin on PA14 cells, the observed elevated $(iMIC)_{all}$ values may be attributed to pre-exposure induced activity of efflux pumps, expression of quorum sensing genes and other metabolic alterations[240–242]. Furthermore, the $(iMIC)_{all}$ for ciprofloxacin AST against high pre-exposure concentrations, that is with 0.5X MIC ciprofloxacin and 0.5X MIC streptomycin remained comparable to the unexposed sample. In the case of streptomycin AST, 0.25X MIC and 0.5X MIC streptomycin pre-exposures resulted in high $(iMIC)_{all}$ as compared to the unexposed control. This could be attributed to adaptive resistance[243], where a reduced accumulation of antibiotic is seen due to the activity of efflux pumps during initial aminoglycoside antibiotic exposure[244]. A similar mechanism may explain the elevated $(iMIC)_{all}$ during ciprofloxacin AST after 0.25X MIC streptomycin pre-exposure, however why the same effect was not seen in cells pre-exposed with 0.5X MIC streptomycin remains unclear (Table RT4).

The most frequently occurring *iMIC* value, $(iMIC)_{mode}$, was again assessed for pre-exposures, and the only significant change during ciprofloxacin AST was observed for pre-exposures with 0.125X MIC and 0.25X MIC streptomycin. It is impossible to say with certainty what is the exact reason to such an observation but plausible is, that the

streptomycin pre-exposure induced efflux pump activity thereby conferring low level resistance to ciprofloxacin[237,243] and increasing the $(iMIC)_{mode}$ value.

In comparison to MG1655, on the PA14 cells, the effects of pre-exposure on $(iMIC)_{start}$ were found to be very diverse and it was impossible to establish any kind of pattern based on pre-exposure antibiotic or the pre-exposure concentration. There can be multiple reasons to it and for good, as this study is all about heterogeneous behavior of individual cells. The unpredictable diverse response may be attributed to relatively larger genome[245] of *Pseudomonas aeruginosa* compared to *Escherichia coli*. The larger genome offers enhanced coding capabilities allowing for greater metabolic versatility and provides higher adaptability to environmental changes[246]. Since the $(iMIC)_{start}$ affects the degree of heteroresistance calculation, no fixed pattern or direction can be established for degree of heteroresistance observations too (Table RT4 and Figure RF17).

However, correlations were also observed between heteroresistance and skewness in *iMIC* distribution, where a more positive skewness was associated with greater heteroresistance and vice versa (Table RT4). Tailing of curve on the right and a positive skewness implied higher heterogeneity in *iMIC* distributions. This also indicates presence of subpopulations having high *iMIC* values, as in the ASTs against streptomycin with 0.5X MIC ciprofloxacin and 0.5X MIC streptomycin pre-exposures.

6.4 General discussion and conclusion

Heteroresistance is a phenomenon in which a bacterial culture contains subpopulations that exhibit significantly higher minimum inhibitory concentration values than the main susceptible population. Clinically, this is a critical issue, as such subpopulations can survive the standard antimicrobial therapies, potentially leading to treatment failure. Moreover, bacterial population with heteroresistance to an antibiotic have the potential to readily evolve into fully resistant cells at later stages. In simple words, heteroresistance can be considered as an intermediate stage in the progression from sensitivity to resistance.

Most of the traditional methods for antibiotic susceptibility testing fail to differentiate between the cells showing heteroresistance, and single cell approaches such as droplet microfluidics became more popular to study individual cell response to antibiotic. With increasing interest in the phenomenon of heteroresistance, a central question has emerged:

how does improper antibiotic use influence existing heteroresistance within a population? Well, the results presented in this thesis provide clear evidence that sub-inhibitory antibiotic exposure can both enhance and suppress heteroresistance, depending on multiple factors. The outcomes were found to vary not only with the class of antibiotic but also with the concentration of the pre-exposure and the bacterial strain involved. Importantly, the extent and direction of these changes in heteroresistance are not universally predictable and are shaped by the complex interplay of bacterial physiology, genetic background, and antibiotic mechanisms.

In *Escherichia coli* MG1655 cells, only the sub-inhibitory pre-exposures of ciprofloxacin led to a significant increase in heteroresistance during subsequent antibiotic exposures to both ciprofloxacin and streptomycin. Moreover, a concentration dependent pre-exposure effect was observed. This underlines the crucial importance of correct antibiotic selection, dosing and the whole treatment plan during antibiotic therapy. Moreover, quarter and half levels of ciprofloxacin inhibitory concentrations were more potent in increasing the heteroresistance as compared to an eighth of inhibitory concentration.

In contrast, the responses observed in *Pseudomonas aeruginosa* PA14 were more varied and less predictable. No consistent pattern could be established in the relationship between antibiotic type, pre-exposure concentration, and heteroresistance change. This could possibly be because of PA14's larger genome having enhanced coding capabilities for metabolites, enzymes etc. as compared to MG1655 or just intrinsic resistance capabilities of *Pseudomonas* against a variety of antibiotics. Nevertheless, unlike in MG1655, streptomycin pre-exposures especially at high concentrations was able to increase the heteroresistance of the population on subsequent exposure against both ciprofloxacin and streptomycin. However, ciprofloxacin remained the more potent inducer of heteroresistance, although its effect was predominantly observed during AST with ciprofloxacin itself. A few pre-exposures even led to a decrease in heteroresistance compared to unexposed sample, highlighting the unpredictable and complex nature of these effects.

Taken together, these results demonstrate that indeed ciprofloxacin is a more potent inducer of heteroresistance than streptomycin, likely due to its DNA damaging mechanism of action and greater ability to generate ROS and to invoke SOS. However, the extent and the pattern of change in heteroresistance is also decided by the strain under study and concentration of

antibiotic. These findings underscore the urgent need for further investigations across diverse strains and antibiotic classes to develop a comprehensive understanding of how sub-MIC antibiotic exposures shape the heteroresistance. This will help to establish what treatment options, at least in setting, are the most profitable, most effective to eliminate bacteria and least contributing to development of resistance.

Although the results presented in this thesis were from short-term exposure studies, it is believed that the consistent presence of low-level selection pressure due to remanent sub-inhibitory level antibiotics in the environment can contribute to the evolution of resistance. The risk is amplified by the misuse of antibiotics, both through improper prescription practices and non-compliance by patients. Results presented here reinforce the necessity to incorporate single-cell techniques into antimicrobial research, as bulk population analyses are insufficient to capture the full complexity of bacterial responses. These individual-level responses may critically influence infection persistence and treatment outcomes. Overall, this thesis offers valuable insights into the dynamic landscape of heteroresistance. It highlights the risks posed by sub-optimal antibiotic use and emphasizes the potential of droplet microfluidics as powerful tool for unraveling bacterial heterogeneity. Future studies incorporating genomic, transcriptomic and proteomic analyses will be essential to decode the underlying mechanisms and to guide the development of more effective, resistance-conscious therapeutic strategies.

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