Xenobiotic Stress-Driven Adaptation and Antibiotic Cross-Tolerance in Bacteria: Insights from Experimental Evolution and Multi-Omics Analyses

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Declaration of Authorship / Eidesstattliche Erklärung

I hereby declare that I have written this dissertation independently and that the work presented here is the result of my own research. All sources of information, data, ideas, and quotations taken from the work of others have been appropriately acknowledged and cited. I affirm that no part of this thesis has been submitted in any previous application for a degree. In preparing this thesis, I used digital writing assistants (e.g., ChatGPT 5) solely for language refinement, clarity, and editing. These tools were not used to generate scientific ideas, results, or interpretations. All scientific concepts, analyses, and conclusions in this dissertation originate from my own work and from collaborations explicitly described in the Author Contributions section. I have endeavored to provide accurate references and follow good scientific practice to the best of my knowledge.

Hiermit erkläre ich, dass ich diese Dissertation selbstständig verfasst habe und dass die hierin präsentierten Arbeiten das Ergebnis meiner eigenen Forschung sind. Alle Informationen, Daten, Ideen und Zitate, die aus den Arbeiten anderer übernommen wurden, sind ordnungsgemäß angegeben und zitiert. Ich versichere, dass kein Teil dieser Dissertation bereits in einem früherenden Antrag auf einen akademischen Grad eingereicht wurde.

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Looking back, this PhD was not a straightforward or predictable process. It came with uncertainty, setbacks, and periods of doubt, but also growth in ways I did not expect both personally and professionally. I am grateful to everyone who was part of that.

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List of Abbreviations

AMR	Antimicrobial Resistance
ABC	ATP-Binding Cassette
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
CFU	Colony Forming Units
DNA	Deoxyribonucleic Acid
E. coli	Escherichia coli
FDR	False Discovery Rate
HPLC	High-Performance Liquid Chromatography
LC-MS/MS	Liquid Chromatography–Tandem Mass Spectrometry
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometry

NADH	Nicotinamide Adenine Dinucleotide
OD	Optical Density
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
PISA	Proteome Integral Solubility Alteration
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
TPP	Thermal Proteome Profiling
UPLC	Ultra-Performance Liquid Chromatography
FDR	False Discovery Rate
GNPS	Global Natural Products Social Molecular Networking
OmpF	Outer membrane porin F
MalE	Maltose-binding periplasmic protein
OxyR	Oxidative stress response transcriptional regulator

SlyA	Transcriptional regulator SlyA

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Summary

Antibiotic resistance has become one of the most urgent health crises of the twenty-first century, with resistant infections now responsible for millions of deaths. Understanding how and why bacteria develop resistance, and what determines the rate at which they do, is not only important but also urgent.

Bacterial exposure to diverse chemical stressors, including environmental xenobiotics and antibiotics, can significantly reshape cellular physiology and influence the evolution of antibiotic resistance. While antibiotic-driven resistance has been extensively studied, growing evidence suggests that non-antibiotic xenobiotics can modulate bacterial physiology and influence bacterial adaptation. However, the extent to which such exposures drive cross-resistance and reshape cellular pathways remains poorly understood. This thesis investigates how xenobiotics affect bacterial physiology and tolerance phenotypes using experimental evolution, phenotypic characterization and LC-MS/MS-based untargeted metabolomics and proteomics in *Escherichia coli K-12* with a focus on identifying shared molecular and phenotypic responses associated with xenobiotic and antibiotic exposure.

Chapter 2 presents a perspective review that establishes the conceptual framework of this thesis. Here, I synthesize current knowledge on xenobiotic-driven bacterial adaptations, integrating mechanistic insights, including alterations in membrane permeability, shared efflux responses, and global stress regulatory pathways. The broader ecological impact includes co-exposure scenarios and shifts in microbial community composition. This chapter highlights the need to move beyond antibiotic-centric models and proposes that xenobiotics represent a significant and less explored selective pressure in microbial ecosystems.

Chapter 3 presents the investigation of the adaptation of *E. coli* K-12 under xenobiotic exposure using laboratory evolution combined with proteomics,

metabolomics and phenotypic assays. The results reveal coordinated changes in metabolic pathways, membrane remodeling and redox homeostasis. The results also reveal that cellular responses extend beyond direct target pathways and contribute to cross tolerance to other compounds. Our data shows that even in cases where phenotypic resistance is limited, significant molecular reprogramming occurs, suggesting early-stage adaptation and initial cellular effects. This chapter also examines how exposure to a target specific antibiotic Fosfomycin can induce broader cellular responses beyond its primary mode of action.

Chapter 4 explores bacterial evolution under multiple classes of antibiotics over 16 days, examining whether resistance evolution rates vary across compounds. It will further explore whether the breadth of drug-protein interactions may explain variation in resistance evolution. At this stage, the analysis focuses primarily on evolutionary outcomes. The integration with proteome-wide analyses to investigate off-target effects remains part of ongoing and future work. This combined framework will contribute to understanding how off-target effects of antibiotics may influence resistance evolution with potential implications for the development of effective combination therapies.

Together, this thesis demonstrates that bacterial adaptation to xenobiotics is driven by interconnected physiological pathways rather than isolated molecular targets. This work supports the concept that xenobiotic exposure can contribute to increased antibiotic tolerance. By integrating conceptual and experimental approaches, this thesis provides mechanistic insights into the relationship between xenobiotic adaptation, antibiotic tolerance and resistance evolution. Collectively, these findings emphasize the importance of considering chemical complexity in resistance research and identify future directions for investigating the emergence and spread of antimicrobial resistance in both environmental and clinical settings.

Zusammenfassung

Antibiotikaresistenz zählt zu den gravierendsten Gesundheitskrisen des 21. Jahrhunderts. Resistente Infektionen fordern inzwischen jährlich Millionen von Menschenleben. Die Frage, wie und warum Bakterien Resistenzen entwickeln und welche Faktoren die Geschwindigkeit dieser Entwicklung bestimmen, ist daher von großer Wichtigkeit.

Die Exposition von Bakterien gegenüber unterschiedlichen chemischen Stoffen, darunter Xenobiotika und Antibiotika, kann die zelluläre Physiologie erheblich verändern und die Evolution von Antibiotikaresistenzen beeinflussen. Während antibiotikainduzierte Resistenzmechanismen intensiv untersucht wurden, mehren sich die Hinweise darauf, dass auch Nicht-Antibiotika-Xenobiotika die bakterielle Physiologie modulieren und bakterielle Anpassungsprozesse beeinflussen können. Dennoch ist bislang nur unzureichend verstanden, in welchem Ausmaß solche Expositionen Kreuzresistenzen fördern und zelluläre Signalwege umstrukturieren, insbesondere aus einer integrativen Perspektive, die proteomische und metabolomische Veränderungen mit phänotypischen Auswirkungen verknüpft.

Die vorliegende Arbeit untersucht, wie Xenobiotika die bakterielle Physiologie und Toleranzphänotypen von Bakterien beeinflussen. Hierzu wurden experimentelle Evolution, phänotypische Charakterisierung sowie LC-MS/MS-basierte untargeted Metabolomik und Proteomik in *Escherichia coli K-12* eingesetzt. Im Mittelpunkt steht die Identifizierung gemeinsamer molekularer und phänotypischer Reaktionsmuster, die unter Xenobiotika- und Antibiotikaexposition auftreten.

Kapitel 2 präsentiert eine perspektivische Übersicht, die den konzeptionellen Rahmen dieser Arbeit etabliert. Auf Grundlage des aktuellen Forschungsstands werden xenobiotikainduzierte bakterielle Anpassungen zusammengeführt und mechanistische Einblicke herausgerbeitet, darunter Veränderungen der

Membranpermeabilität, gemeinsame Effluxreaktionen sowie globale Stressregulationswege. Darüber hinaus werden breitere ökologische Auswirkungen diskutiert, darunter Ko-Expositionsszenarien und Verschiebungen mikrobieller Gemeinschaften. Das Kapitel verdeutlicht die Notwendigkeit, antibiotikazentrierte Modelle zu überwinden, und zeigt, dass Xenobiotika einen bedeutenden und bislang wenig untersuchten Selektionsdruck in mikrobiellen Ökosystemen darstellen.

Kapitel 3 untersucht experimentell die Adaptation von *E. coli K-12* unter Xenobiotikaexposition mittels Laborevolution in Kombination mit Massenspektrometrie bassierter Proteomik und Metabolomik sowie phänotypischen Assays. Die Ergebnisse zeigen koordinierte Veränderungen in Stoffwechselwegen, Membran-Remodelling und Redoxhomöostase. Darüber hinaus zeigen die Befunde, dass zelluläre Reaktionen über direkte Zielstrukturen hinausgehen und zur Kreuztoleranz gegenüber anderen Verbindungen beitragen. Selbst in Fällen begrenzter phänotypischer Resistenz lässt sich eine ausgeprägte molekulare Reprogrammierung beobachten, was auf frühe Adaptationsprozesse und initiale zelluläre Effekte hinweist. Zudem untersucht dieses Kapitel, wie die Exposition gegenüber dem zielspezifischen Antibiotikum Fosfomycin breitere zelluläre Reaktionen über dessen primären Wirkmechanismus hinaus auslösen kann.

Kapitel 4 analysiert die bakterielle Evolution unter verschiedenen Antibiotikaklassen über einen Zeitraum von 16 Tagen und untersucht, ob sich die Geschwindigkeit der Resistenzentwicklung zwischen einzelnen Verbindungen unterscheidet. Darüber hinaus soll untersucht werden, ob die Breite von Wirkstoff-Protein-Interaktionen Unterschiede in der Resistenzentwicklung erklären kann. Der Schwerpunkt dieser Analysen liegt derzeit auf evolutionären Endpunkten, während die Integration proteomweiter Analysen zur Untersuchung von Off-Target-Effekten Gegenstand laufender und zukünftiger Arbeiten ist. Dieses kombinierte Analyseframework soll dazu beitragen zu verstehen, wie Off-Target-Effekte von Antibiotika die Resistenzentwicklung beeinflussen können,

mit möglichen Implikationen für die Entwicklung wirksamer Kombinationstherapien.

In ihrer Gesamtheit zeigt diese Arbeit, dass die bakterielle Adaptation an Xenobiotika durch miteinander verknüpfte physiologische Prozesse gesteuert wird und nicht ausschließlich auf isolierte molekulare Zielstrukturen zurückzuführen ist. Die Ergebnisse unterstützen das Konzept, dass Xenobiotikaexposition zur erhöhten Antibiotikatoleranz beitragen kann. Durch die Verbindung konzeptioneller und experimenteller Ansätze liefert diese Arbeit mechanistische Einblicke in die Beziehung zwischen Xenobiotikaadaptation, Antibiotikatoleranz und Resistenzevolution. Insgesamt unterstreichen die Ergebnisse die Bedeutung chemischer Komplexität in der Resistenzforschung und identifizieren zukünftige Forschungsrichtungen zur Untersuchung der Entstehung und Ausbreitung antimikrobieller Resistenzen in Umwelt und Klinik.

Chapter 1

Introduction

1.1 Antibiotic resistance as a global and biological problem

Antibiotic resistance can compromise therapy in hospitals and communities worldwide, and the burden is already measurable at the global scale (Naghavi, et al. 2024). A landmark report by Murrey et al. estimated that bacterial antimicrobial resistance was directly responsible for 1.27 million deaths in 2019 and associated with 4.95 million deaths worldwide(Murray, et al. 2022). The updated 2024 Global Burden of Disease analysis extended these findings and forecast a continued rise in deaths associated with resistance without effective intervention (Murray, et al. 2022; Naghavi, et al. 2024). Antibiotic resistance is therefore a global problem that needs mechanistic understanding and policy action.

The classic framework of resistance evolution remains fundamental, where antibiotic exposure selects for heritable traits that reduce drug efficacy, such as enzymatic degradation, reduced permeability, target protection or modification, and active efflux. These mechanisms are genetically and biochemically well characterized (Blair, et al. 2015). The shift from single-gene studies to systems microbiology has broadened our understanding of antibiotic responses (Davies and Davies 2010). It is now clear that antibiotic activity is not determined by target engagement alone, but physiological state also strongly influences drug activity (Stokes, et al. 2019; Caño Muñiz, et al. 2025). Factors such as envelope composition, transport capacity, growth rate, redox status, metabolic flux, and stress signaling can shape a bacterium's response to a drug once it enters the cell (Stokes, et al. 2019; Morrison, et al. 2024). This broader view is central to the work presented in this thesis, which focuses not only on antibiotics but also on bacterial adaptation to diverse xenobiotic compounds.

1.2 Chemical Complexities of Microbial Environments

Microbes are rarely exposed to antibiotics alone. In natural and human-impacted environments, including soil, aquatic systems, wastewater, and host-associated environments, they are exposed to a complex and chemically diverse mixture of compounds (Berendonk, et al. 2015; Bengtsson-Palme, et al. 2018; Larsson and Flach 2022). These include a wide range of xenobiotics defined as foreign chemical compounds not naturally produced within biological systems, such as pesticides, industrial chemicals, pharmaceuticals, and disinfectants (Singh, et al. 2023).

Environmental studies have shown that xenobiotics such as pharmaceutical residues, pesticides, and industrial chemicals are widely detected in rivers, soils, and wastewater systems, often at low and fluctuating concentrations (Nishmitha, et al. 2025; Köninger, et al. 2026).

Importantly, these xenobiotics are not present in isolation, but mostly as persistent background contaminants resulting from continuous inputs associated with human activities (Wilkinson, et al. 2022; Mohapatra, et al. 2025; Köninger, et al. 2026). Microbial communities are consistently exposed to low but biologically relevant concentrations of these xenobiotics across different environments (Hou, et al. 2025; Köninger, et al. 2026) Such exposures are increasingly recognized as ecologically relevant as they can alter microbial physiology, reshape microbial communities and impose selective pressures over extended periods of time (Bengtsson-Palme, et al. 2018; De Filippis, et al. 2024; de la Cuesta-Zuluaga, et al. 2024; Hou, et al. 2025; Köninger, et al. 2026). For example, Industrial chemicals and human-targeted drugs have been shown to inhibit the growth of members of the gut microbiota (Maier, et al. 2018; Roux, et al. 2025). Similarly, pesticides have been shown to alter soil microbial community composition and reduce microbial diversity (Köninger, et al. 2026). These observations highlight that environmental chemical exposure is not incidental but a persistent factor shaping microbial communities.

1.3 How xenobiotics shape bacterial adaptation

At the cellular level, continuous exposure to low concentrations of xenobiotics imposes a persistent physiological stress on bacteria, triggering adaptive responses that allow bacteria to persist under sustained xenobiotic pressure. (De Filippis, et al. 2024; Noto Guillen, et al. 2024; Roux, et al. 2025) . These responses involve coordinated adjustments in key cellular processes. Experimental studies have shown that adaptation to xenobiotic stress can arise through reduced uptake, enhanced efflux activity, and broader changes in transport and membrane-associated functions (Noto Guillen, et al. 2024; Roux, et al. 2025). In some cases, microbial communities can also adapt by transforming and metabolizing xenobiotic compounds, further contributing to their persistence under xenobiotic stress (De Filippis, et al. 2024).

Despite their chemical diversity, disruption of shared mechanisms and pathways like efflux systems and global regulatory networks led to changes in susceptibility to both xenobiotics and antibiotics (Noto Guillen, et al. 2024; Binsfeld, et al. 2025; Roux, et al. 2025). Such convergence indicates that adaptation to xenobiotics may often be mediated through general cellular strategies rather than compound-specific mechanisms. These processes are discussed in Chapter 2 in detail.

1.4 From Xenobiotic adaptation to Antibiotic Cross-resistance.

Adaptation to xenobiotic stress has important implications for antibiotic cross-resistance. Continuous exposure to xenobiotics can induce persistent physiological in bacterial including alterations in membrane permeability, transport activity, metabolic state, and stress-associated pathways (Noto Guillen, et al. 2024). Such responses can influence bacterial susceptibility to antibiotics and promote cross-resistance across multiple antibiotics (Noto Guillen, et al. 2024). For example, sub-lethal concentrations of environmental xenobiotics like pesticides and pharmaceuticals have been shown to promote cross-resistance to different classes of antibiotics (Kurenbach, et al. 2015; Noto Guillen, et al. 2024). This provides the basis for Chapter 2, which examines how xenobiotic exposure drives bacterial adaptation and contributes to antibiotic cross-resistance.

1.5 Antibiotic-Specific Adaptive Responses in Bacteria

Antibiotics are commonly classified based on their cellular targets, including processes such as cell wall synthesis, protein translation and DNA replication (Kapoor, et al. 2017; Baran, et al. 2023). This target centric framework has traditionally guided our understanding of antibiotic mode of action and resistance development (Kapoor, et al. 2017; Darby, et al. 2023). But as discussed before, exposure to xenobiotics can induce broader physiological responses in bacteria (de la Cuesta-Zuluaga, et al. 2024; Noto Guillen, et al. 2024). Although antibiotics have defined primary targets, they may also influence bacterial physiology and potentially engage in off-target interactions. Several studies have shown that antibiotic exposure can trigger multiple cellular responses (Kohanski, et al. 2007; Kohanski, et al. 2010; Dwyer, et al. 2014). Sub-lethal concentrations of antibiotics have been shown to alter gene expression, affect metabolic pathways, and activate stress response systems (Andersson and Hughes 2014) .

The concept of polypharmacology further challenges the traditional single-target view of antibiotic action, suggesting drugs can interact with multiple targets (Kabir and Muth 2022). Proteome-wide interaction studies support this idea by showing that drugs can engage multiple proteins within the cell (Savitski, et al. 2014; George, et al. 2024). Many recent antibiotics appear to interact with multiple targets contributing to a broader physiological impact (Wetzel, et al. 2021). Such multi-target interactions may shape bacterial adaptive responses and influence resistance evolution.(Yeh, et al. 2009; Wetzel, et al. 2021; Feng, et al. 2023; Lozano-Huntelman, et al. 2023)

Understanding these interactions are important not only for moving beyond a strictly target-based view of antibiotic action but for clarifying how broader physiological responses influence antibiotic resistance. Such effects may shape the rate at which resistance evolves as well as the combined or synergistic outcomes of antibiotic exposure.

Building on these concepts, my third chapter examines how different classes of antibiotics influence bacterial adaptation during experimental evolution. The

study focuses on the rate and patterns of resistance in *E. coli* under different antibiotic pressures. It is based on the idea that antibiotics with narrower target specificity or fewer off-target effects may enable more rapid resistance acquisition, whereas broader cellular perturbation could delay or limit adaptive responses. While the present work primarily focuses on evolutionary outcomes, it provides a framework for an ongoing project investigating antibiotic off-target effects. In this context, approaches such as Thermal protein profiling and PISA are being used to identify protein-antibiotic interactions beyond primary targets and to characterize off-target effects and linked to observed adaptive responses.

1.6. Experimental evolution

Experimental evolution is a useful method to study how bacteria adapt to stress over time. Exposing bacteria to selective pressure under controlled conditions helps to monitor adaptation over generations and directly observe the emergence of adaptive traits (Barrick and Lenski 2013). This approach captures the constant changes in bacterial phenotype and physiology during adaptation (Hindré, et al. 2012; Barrick and Lenski 2013). This approach is particularly useful for investigating bacterial responses to antibiotics and other xenobiotic stressors, as prolonged exposure can gradually lead to the acquisition of traits that improve survival under stress (Jansen, et al. 2013; Oz, et al. 2014). It also makes it possible to compare similar or distinct evolutionary responses that arise under different compound exposures (Lenski 2017; Yen and Papin 2017). In this thesis, controlled experimental evolution frameworks were applied in Chapters 3 and 4 to investigate bacterial adaptation to xenobiotics and antibiotics. In Chapter 3, *E. coli* populations were exposed to sustained low dose xenobiotic exposure under reproducible conditions to examine adaptation over time. Populations were maintained under sub-lethal concentrations of three xenobiotics and one antibiotic for 24 days. In Chapter 4 *E. coli* was exposed to a sustained low dose of different classes of antibiotics for 16 days.

1.7. Omics Approaches as an important tool to interpret bacterial adaptation

Understanding complex cellular responses requires approaches that can capture functional changes at a system level. Different omics technologies provide complementary insights into these responses: genomics captures the genetic potential of the cell, transcriptomics reflects regulatory activity, proteomics characterizes the functional machinery responsible for executing cellular processes, and metabolomics profiles small molecules representing the biochemical outputs of cellular activity (Dettmer, et al. 2007; Gowda and Djukovic 2014; Johnson, et al. 2016; Hasin, et al. 2017; Acierno, et al. 2025) . Advances in omics technologies, particularly proteomics and metabolomics, have enabled detailed characterization of cellular responses through the analysis of protein expression and metabolic activity under different stress conditions (Aebersold and Mann 2016; Bian, et al. 2020; Aboagye, et al. 2023).

Among these approaches, proteomics is particularly well suited for exploring bacterial responses to xenobiotic stress as proteins directly mediate key cellular functions involved in survival (Aebersold and Mann 2016). Changes at the proteome level can provide insights into how different xenobiotic exposures reshape cellular functions and whether they engage shared pathways or give rise to distinct adaptive responses (Soufi, et al. 2015; Aebersold and Mann 2016).

Metabolomics provides complementary insights by capturing the small molecules that reflect ongoing cellular activity. As downstream products of biochemical reactions, metabolites provide insights into how cellular functions are altered under stress conditions (Patti, et al. 2012; Johnson, et al. 2016). Unlike other omics layers, metabolite levels can respond rapidly to perturbations making metabolomics particularly sensitive to xenobiotic exposure (Patti, et al. 2012). This rapid responsiveness allows early detection of stress associated responses including shifts in metabolic flux and pathway activity which may precede detectable transcriptional or proteomic changes (Mashego, et al. 2007; Patti, et al. 2012).

Changes in metabolite abundance can reflect shifts across multiple metabolic processes, including carbon metabolism, lipid metabolism, energy production, and biosynthetic pathways. These changes provide a functional readout of how bacteria adjust their physiology in response to xenobiotic exposure (Johnson, et al. 2016). Both targeted and untargeted metabolomics approaches are commonly used to study cellular responses (Dettmer, et al. 2007; Patti, et al. 2012). Targeted approaches focus on the quantification of specific metabolites or pathways while untargeted strategies enable a broader assessment by capturing a wide range of metabolites simultaneously (Dührkop, et al. 2021). This makes untargeted metabolomics particularly valuable for exploring global physiological changes under xenobiotic stress. In this thesis liquid chromatography-tandem mass spectrometry (LC-MS/MS) based approaches are employed to characterize changes in both protein and metabolite levels in bacteria under xenobiotic exposure. These omics layers enable adaptation to be interpreted not simply as isolated changes in proteins or metabolites but as coordinated cellular responses to xenobiotic stress. Emerging approaches such as thermal proteome profiling and PISA may further support the investigation of antibiotic-protein interactions and the characterization of off-target effects.

1.8 *E. coli* as a model organism

*E. coli i*s a gram-negative facultatively anaerobic gamma proteobacterium that functions both as a commensal organism in the vertebrate gastrointestinal tract and as a versatile pathogen capable of causing a wide range of intestinal infections (Kaper, et al. 2004; Riley, et al. 2006) . This dual role reflects its biological diversity and has made it one of the most extensively studied model organisms in microbiology. It has been widely used to investigate bacterial physiology, adaptation, and genetic manipulation, as well as antibiotic resistance (Blount 2015; Lenski 2017). Its genome is well characterized, and the availability of genetic and molecular tools makes it particularly suitable for exploring cellular responses under controlled conditions (Blount 2015; Zhang, et al. 2025). In addition, its rapid growth, high biomass yield, cost effectiveness and genetic

accessibility enable reproducible experimental designs which are especially important for long-term evolution studies.

E. coli has been used in experimental evolution research for quite a long time, providing detailed insights into how bacteria adapt to different external stimuli over time and their impact on fitness and stability (Lenski 2017) . This makes it a suitable organism for examining the influence of xenobiotics and antibiotics on adaptation at both phenotypic and omics levels. *E. coli* is not just used in laboratory research but it is prevalent across different environments, including the gut (Blount 2015).

E. coli K-12 is used as a model organism to assess how bacteria respond to continuous exposure to xenobiotics and antibiotics in a controlled environment with a focus on understanding how environmental xenobiotic exposure affects adaptive and evolutionary outcomes. In this thesis, *E. coli* K-12 will be referred to as *E. coli*.

1.9 Aim of thesis

This thesis aims to understand how bacteria adapt when exposed to xenobiotics and antibiotics. Traditionally, antibiotic resistance is often studied in the context of direct exposure to antibiotics, but bacteria in real environments are exposed to a much broader range of compounds. This work takes a broader perspective by examining how different xenobiotic pressures contribute to bacterial adaptation.

To explore this, experimental evolution is used to examine how *E. coli* responds to sustained exposure to xenobiotics and antibiotics. By comparing these responses across different compounds, the study examines whether similar or distinct adaptive patterns emerge.

In addition, proteomics and metabolomic approaches are used to investigate how these adaptations are reflected at the cellular level. This helps to link observed phenotypic changes in growth and resistance with underlying shifts in cellular function.

Overall, this work explores how exposure to different chemical stressors shapes bacterial adaptation and how this may contribute to the development of antibiotic resistance. It also provides a basis for future work investigating antibiotic off target effects through protein-wide assessment of changes in thermal stability and antibiotic-protein interactions.

Chapter 2

Xenobiotic abundance in the environment contributes to antibiotic resistance

2.1 Abstract

Traditionally, antibiotic resistance has been linked to antibiotic use and misuse. But, in natural and human impacted environments microbes are continuously exposed to a diverse range of xenobiotics along with antibiotics, many of which are widely detected across environments. Recent advances have shifted the traditional perspective, drawing attention to the possible role of xenobiotics, including pharmaceuticals, industrial chemicals and pesticides in contributing to antibiotic resistance. This review synthesizes current findings on mechanistic and ecological pathways through which xenobiotics influence bacterial adaptation, including unintended antimicrobial effects, changes in membrane permeability, efflux activity and stress response systems. It also discusses the impact of xenobiotic exposure and co-exposures on reshaping microbial community composition, biodiversity and emergence of resistance populations in different environments. While xenobiotics are increasingly recognized as contributors to antibiotic resistance, their relative impact compared to antibiotics themselves remains insufficiently quantified.

2.2 Introduction

Antimicrobial resistance (AMR) is a critical public health issue, leading to reduced treatment options for infectious diseases and potentially causing ten million deaths by 2050 (Price 2016).The rate of new antibiotic discovery is experiencing a decline, while the emergence of antibiotic resistance is increasing, primarily attributed to the overuse of antibiotics for both agricultural and medical purposes (Price 2016). Efforts to control antimicrobial resistance have primarily

focused on the cautious use of prescription antibiotics and maintenance of good hygiene practices (Weinstein 2001; Price 2016). However, bacteria do not only evolve in clinical settings but in natural environments, such as soil, water systems, animal microbiome, and human gut microbiome (Berendonk, et al. 2015; Bengtsson-Palme, et al. 2018). They encounter a large number of non-antibiotic stressors, like pesticides, herbicides, pharmaceuticals, heavy metals, biocides, and industrial chemicals (Murray, et al. 2024). Recent studies have shown that non-antibiotic compounds can also play a role in the development and spread of resistance (Malagón-Rojas, et al. 2020; Liao, et al. 2021). Natural processes and anthropogenic activities cause the accumulation of a wide range of xenobiotic compounds in the environment, which leads to a global health concern (Gianfreda and Rao 2008). Xenobiotics are compounds that are not naturally produced or expected within biological systems organism including pharmaceutical drugs, pesticides, cosmetics, food additives, and fragrances (Singh, et al. 2023). These compounds are often present in sub-lethal concentrations in the environment (Maggi, et al. 2023; Ehalt Macedo, et al. 2025). Studies have shown that exposure to xenobiotics can promote cross-resistance to antibiotics (Kurenbach, et al. 2015).

Bacteria respond to such chemical stresses through a variety of resistance associated, including efflux activity, enzymatic detoxification, target site modification, stress response regulation and broader physiological adaptation (Darby, et al. 2023). As a result, chronic exposure to environmental xenobiotics may shape bacterial physiology in ways that overlap with classic antibiotic resistance pathways.

Xenobiotics are continuously released into the environment through wastewater, manure, and agricultural runoff, where they persist and cycle across soil and aquatic systems depending on their physicochemical properties (Wellington, et al. 2013). These interconnected dissemination routes result in sustained, low level exposure of environmental and host-associated microbial communities beyond clinical settings (Wellington, et al. 2013; Larsson and Flach 2022; Wilkinson, et al. 2022; Köninger, et al. 2026) (Fig1).

Environmental frameworks for antimicrobial resistance have traditionally emphasized reservoirs of resistance genes and their dissemination across ecosystems (Larsson and Flach 2022). This perspective focuses on how chronic exposure to diverse non-antibiotic xenobiotics can shape bacterial physiology and impose selection pressures that overlap with classical resistance pathways. In this review, we synthesize current knowledge on how xenobiotics influence environmental and host-associated microbial communities and discuss ecological implications of xenobiotics exposure in antimicrobial resistance development. We highlight recent studies demonstrating xenobiotic exposures affect clinically and environmentally relevant bacteria. This raises the possibility that resistance selection may occur beyond antibiotic use. Finally, we identify current knowledge gaps and outline priorities for future research. Throughout this review the term **xenobiotics** is used broadly to refer to non-antibiotic environmental chemicals and related anthropogenic stressors.

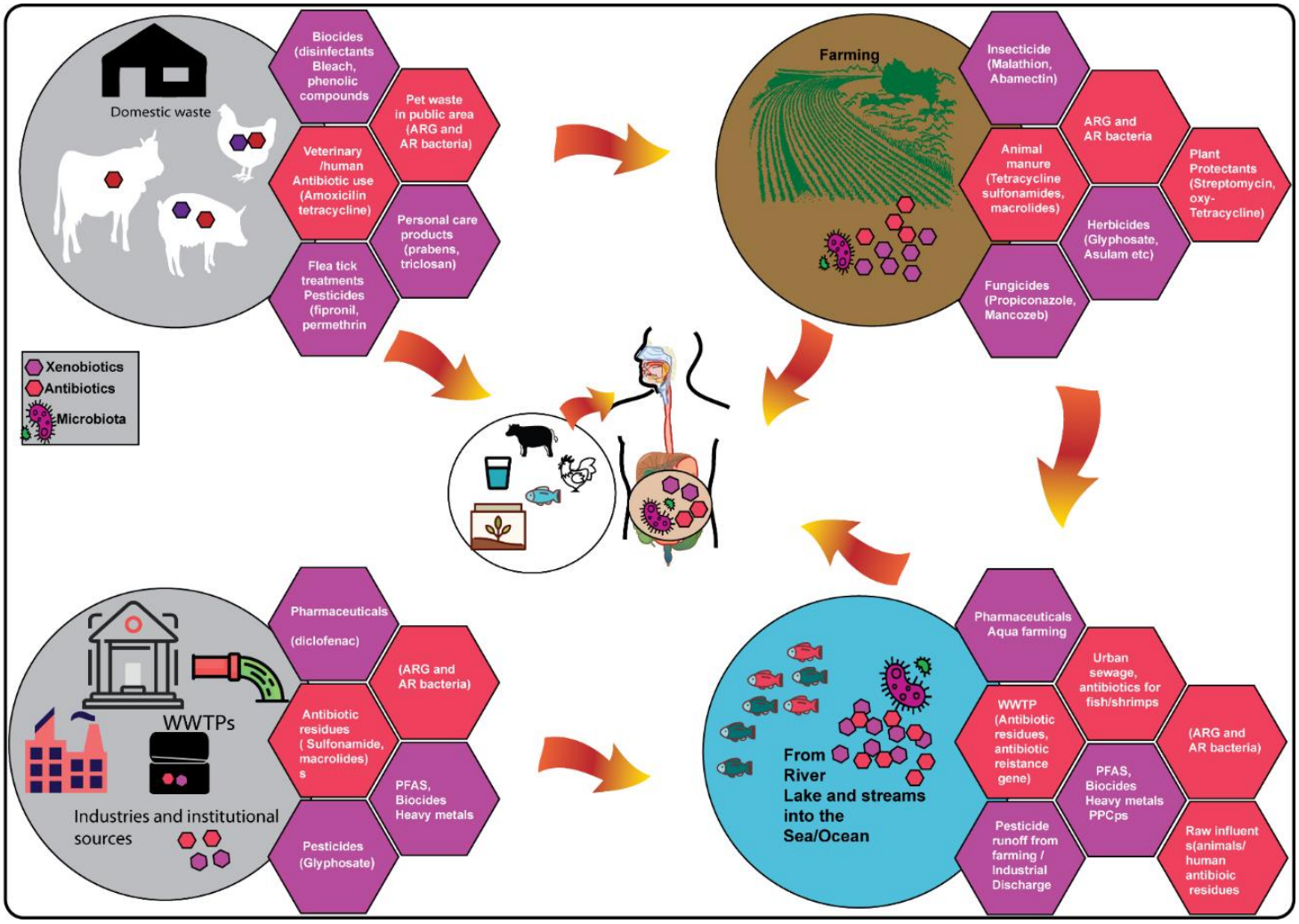


Figure 1. *Conceptual overview of the environmental circulation of antibiotics and other xenobiotics across domestic, agricultural, industrial, and aquatic systems, illustrating how these interconnected pathways expose microbial communities to mixed chemical pressures and contribute to environmental dissemination of antibiotic-resistant bacteria and resistance*

2.3 Xenobiotics as Hidden Selective Pressure across Microbial Ecosystems

Xenobiotics are increasingly recognized as a hidden but powerful driver of antimicrobial cross-resistance. These compounds are present in soils, rivers, oceans, and have even been detected in human serum. (Lamichhane, et al. 2023; Maggi, et al. 2023; Schultz, et al. 2023; Lindell, et al. 2025; Köninger, et al. 2026). The extensive global use of pesticides results in persistent exposure of soil and the aquatic microbiome to xenobiotics (Maggi, et al. 2023). While most compounds are degraded in soils, persistent residues, predominantly those associated with herbicides are transported into river networks (Maggi, et al. 2023).

Such persistent low level exposure is more likely to promote microbial stress adaptation rather than acute toxicity, potentially reshaping community structure and metabolic function over time (Roux, et al. 2025). Continuous environmental release of xenobiotics can generate sustained low level selective pressures that enable microbial populations to adapt and evolve tolerance to multiple stressors including antibiotics.(Feng, et al. 2023; Noto Guillen, et al. 2024).

Importantly the dissemination of these xenobiotics is not limited to the environmental ecosystems as xenobiotic accumulation can also occur in the human gut microbiome. Experimental evidence have shown that diverse gut bacteria are capable of bioaccumulating per- and polyfluoroalkyl substances (PFAS) (Lindell, et al. 2025). Xenobiotics may not only bioaccumulate but can alter metabolism and can reshape gut microbial community composition (Mesnage, et al. 2021; Grießhammer, et al. 2025). These observations suggest that environmental pollutant can host-associated microbial communities

potentially effect disease severity in vivo (Maier, et al. 2018; Xue, et al. 2020; Mesnage, et al. 2021).

Even dietary xenobiotics such as caffeine, which are repeatedly encountered by the gut and environment, are able to influence the *E.coli K12* regulatory pathways despite having little or no intrinsic antibacterial activity (Binsfeld, et al. 2025). Unlike antibiotics, many xenobiotics are continuously introduced into environmental systems through industrial discharge, agricultural runoff and consumer waste resulting in prolonged low level exposure across microbial communities (Wilkinson, et al. 2022; Ehalt Macedo, et al. 2025; Köninger, et al. 2026).

These observations suggest that xenobiotics such as pesticides, herbicides, fungicides, biocides, pharmaceuticals and other substances act as a hidden but powerful selective pressure contributing to antibiotic resistance.

2.4 Ecological Co-Exposure and Co-Selection in Xenobiotic-Driven Resistance

Microorganisms in natural and host-associated environments are rarely exposed to antibiotic and non-antibiotic xenobiotics in isolation but instead encounter complex chemical mixtures composed of overlapping environmental contaminants (Fig 1). Large-scale monitoring demonstrates widespread occurrence of antibiotics across aquatic ecosystems, with an estimated 8,500 tons of antibiotics entering river systems globally each year (Ehalt Macedo, et al. 2025). Complementing these observation a pan-European field survey detected pesticide residues in 70% of soils across 26 countries, with 63 different compounds identified (Köninger, et al. 2026).In addition global monitoring of river systems has detected a wide array of pharmaceutical compounds, including antibiotics and other compound residues, across 258 rivers in 104 countries, with many cities showing concentrations of ecological concerns (Wilkinson, et al. 2022). These observations highlight that microbes across environmental ecosystems are frequently co-exposed to diverse chemical mixtures including antibiotics and xenobiotics.

A recent large-scale screen demonstrated that numerous industrial and agricultural chemicals exhibit direct antimicrobial activity against prevalent human gut bacteria, indicating that environmentally abundant xenobiotics can impose selective or growth-modulating pressure on microbial populations even in the absence of antibiotics (Roux, et al. 2025). This suggests that xenobiotics may influence stress responses and antibiotic susceptibility within microbial communities. Experimental evidence from a defined system has reported that *E. coli* exposed to the antidepressant duloxetine together with chloramphenicol evolved resistance faster than those exposed to chloramphenicol alone (Shi, et al. 2022). Large-scale profiling of human-targeted drugs revealed that some of these compounds exert direct inhibitory effects on gut bacteria. Maier et.al screened more than 1000 pharmaceuticals, and 24% of them impaired bacterial growth, often at concentrations relevant to intestinal exposure (Maier, et al. 2018). These effects were widespread across diverse taxa and were sufficient to alter community composition in *vitro* and *vivo*, indicating that many xenobiotics unintentionally reshape gut microbiome ecosystems (Maier, et al. 2018; Grießhammer, et al. 2025). Exposure to non-antibiotics has also been associated with reduced colonization resistance against enteric pathogens through changes in microbial biomass, species richness and community interaction (Grießhammer, et al. 2025). These effects are not solely explained by direct toxicity toward invading organisms but also by xenobiotic induced restructuring of resident microbial communities. Such ecological perturbation may favor the expansion of opportunistic or resistant taxa within host associated (Maier, et al. 2018; Grießhammer, et al. 2025).

A large-scale survey of European agricultural soils reported that sites with higher multi-residue pesticide burden exhibit reduced soil biodiversity and measurable alteration in microbial community structure (Köninger, et al. 2026). In parallel wastewater and sediments, where antibiotics and xenobiotics frequently co-occur, act as major reservoirs that expose for microbial communities to complex chemical mixtures and an enriched pool of resistance genes (Lee, et al. 2023).

Large scale analysis of European waste water treatment plant effluents further revealed that treated discharges contain hundreds of biologically active chemicals, many exceeding ecological risk thresholds when evaluated as mixtures (Finckh, et al. 2022). Waste water treatment plants integrate inputs from hospitals, households, agriculture and industries and have been identified as hotspots for the accumulation and downstream dissemination of antibiotic resistance genes and associated mobile elements into river systems (Marti, et al. 2013; Munoz-Escudero, et al. 2023).

Metagenomic surveys further show that although the total abundance of some ARGs declines after treatment, the composition of ARGs in effluent and receiving waters differs from the influent's profile and includes genes conferring resistance to sulfonamides, β-lactams and aminoglycosides that remain detectable 20 km downstream (Sabri, et al. 2020; Lee, et al. 2023).

Collectively, these observations suggest that xenobiotic exposure across environmental and host associated ecosystems can alter microbial community composition, disrupt colonization resistance and facilitate the expansion of tolerant or opportunistic taxa as summarized in Figure 3.

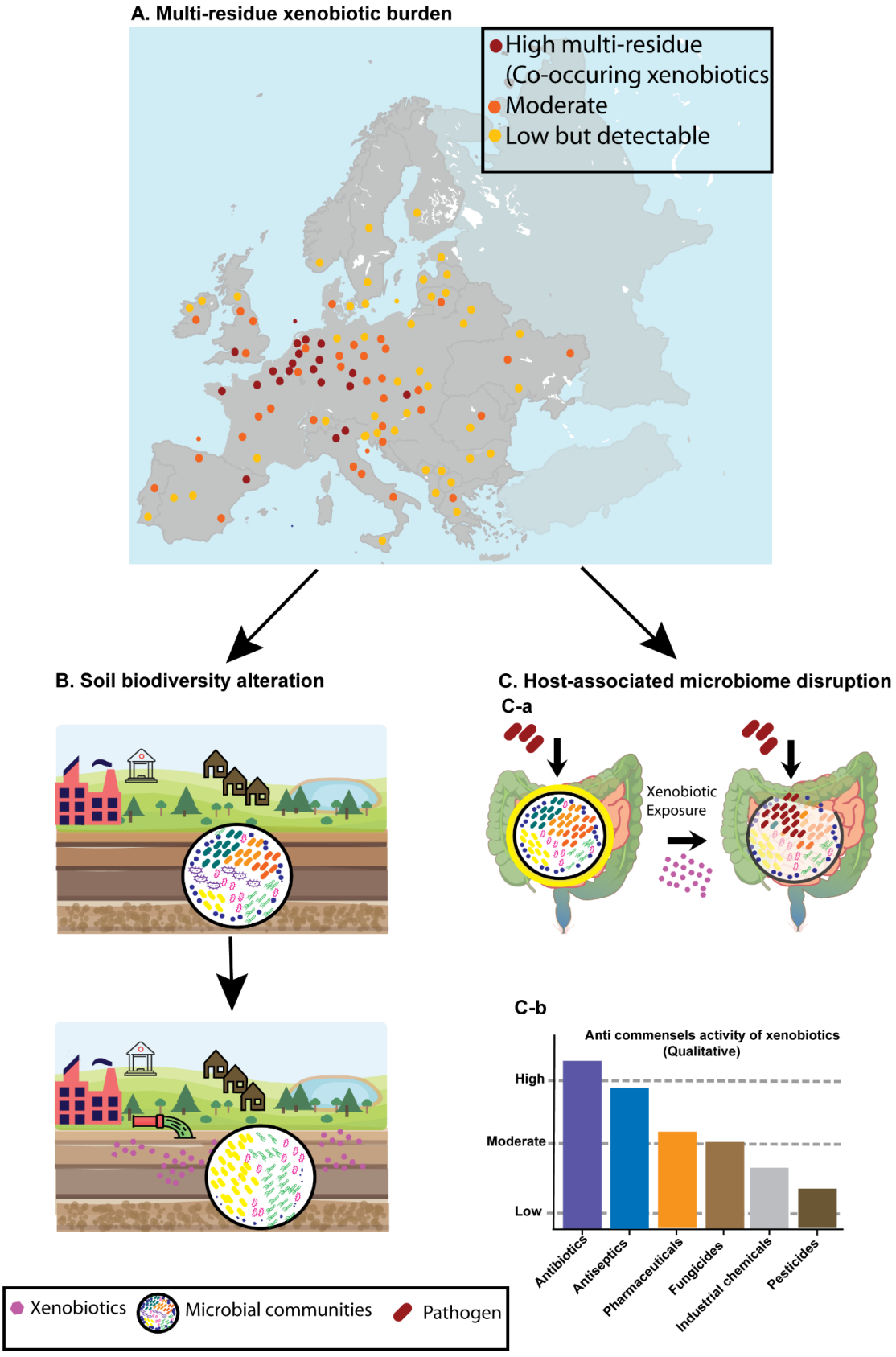

A. Multi-residue xenobiotic burden
High multi-residue
(Co-occuring xenobiotics
Moderate
Low but detectable
B. Soil biodiversity alteration
C. Host-associated microbiome disruption
C-a
Xenobiotic
Exposure
C-b
Anti commensels activity of xenobiotics
(Qualitative)
High
Moderate
Low
Antibiotics
Antiseptics
Pharmaceuticals
Fungicides
Industrial chemicals
Pesticides
Xenobiotics
Microbial communities
Pathogen

Figure:3 Ecological and Host-associated consequences of widespread xenobiotic exposure.

A: Conceptual representation of the heterogeneous geographic distribution of co-occurrence of xenobiotics across European landscapes. Colored markers indicate regions with different levels of multi-residue xenobiotic burden (high, moderate, or low but detectable xenobiotic burden arising from agricultural, industrial, and urban resources. B: Soil biodiversity alteration associated with environmental exposure to xenobiotic mixtures. Pesticide residues and other contaminants have been shown to affect taxonomic and functional diversity of soil biota and microbial communities, potentially suppressing beneficial, such changes may favor tolerant taxa and modify ecosystem function. C: Host-associated microbiome disruption following xenobiotic exposure. C-a: non-antibiotic xenobiotics can inhibit commensal bacteria, causing a change in gut microbial community composition, including weakening of colonization resistance and expansion of opportunistic or pathogen taxa. C-b: Qualitative overview of reported anti-commensal activity of antibiotics and xenobiotics across major compound classes, indicating potential anti-commensal activity.

2.5 Mechanistic Insights on Xenobiotic-Driven Modulation of Antibiotic Resistance

Co-selection describes the selection of resistance to multiple agents following exposure to a single compound and can arise through three related processes: co-resistance, cross-resistance, and co-regulation. Co-resistance occurs when multiple resistance traits are genetically linked for example on a conjugative plasmid carrying both antibiotic and biocide or metal resistance genes (Chapman 2003; Baker-Austin, et al. 2006; Pal, et al. 2015). Cross-resistance arises when a single mechanism confers reduced susceptibility to multiple compounds. For example, multidrug efflux transporters such as AcrAB–TolC in *E. coli* can export structurally diverse agents including β-lactams and biocides (Poole 2002; Chapman 2003; Baker-Austin, et al. 2006) Co-regulation involves coordinated transcriptional responses in which exposure to one compound induces multiple

resistance-associated pathways (Murray, et al. 2024). For example activation of the *marRAB* regulon by salicylate upregulates efflux and stress-response genes resulting in broader antimicrobial tolerance (Nazaret, et al. 2023; Obaid, et al. 2026).

Xenobiotics can modulate antibiotic efficacy both directly and indirectly through overlapping cellular defense mechanisms (Noto Guillen, et al. 2024). These effects arise through conserved stress responses that are not specific to antibiotics, Ros activation, including modulation of multidrug efflux systems, alterations in membrane permeability and rewiring of regulatory networks (Shi, et al. 2022; Feng, et al. 2023; Noto Guillen, et al. 2024; Roux, et al. 2025). Such responses can shift bacterial cells into adaptive physiological states that potentially alter susceptibility profiles and promote cross resistance.

Combined xenobiotic antibiotic exposure has also been associated with activation of interconnected stress adaptation pathways. In *E. coli,* co-exposure to duloxetine and chloramphenicol enhanced multidrug efflux activity, activated oxidative stress and DNA repair responses promoting more rapid resistance associated adaptation than exposure to duloxetine or chloramphenicol individually (Shi, et al. 2022).

Importantly, these effects do not depend on direct antibacterial activity as sublethal chemical stress can induce physiological changes that modify antibiotic susceptibility. Consistent with this, exposure to commercial formulations of herbicides such as dicamba, 2,4-dichlorophenoxyacetic acid, and glyphosate has been shown to change the antibiotic susceptibility in *E. coli* and Salmonella enterica (Kurenbach, et al. 2015). Comparable effects have been reported for other xenobiotics across diverse bacterial systems, indicating that low-level xenobiotic exposure can broadly modulate cellular physiology (Feng, et al. 2023; Wallace, et al. 2023; Yang, et al. 2023).

In a comprehensive analysis of antibiotics and xenobiotics against *E. coli*, genome-wide fitness profiling showed that chemically unrelated xenobiotics were found to alter bacterial susceptibility profiles through shared cellular processes

rather than compound-specific targets (Noto Guillen, et al. 2024). Further analysis identified multiple drug efflux, envelope stress responses and transport-mediated permeability changes as common mechanisms contributing to altered susceptibility (Noto Guillen, et al. 2024). Similar trends has been observed in large-scale systematic study of human target drugs against gut associated bacteria. Screening of 1197 compounds across 40 representative gut bacterial species, including antibiotics and diverse xenobiotics such as antipsychotics, antidepressants, antidiabetics, proton-pump inhibitors, NSAIDs, hormones and chemotherapeutic agents showed that a substantial fraction of xenobiotics inhibited the growth of at least one strain in vitro (Maier, et al. 2018). Susceptibility to antibiotics and human-targeted drugs correlated across bacterial species, supporting the role of shared cellular defense mechanisms. However, some outlier species, including *C.difficile* and *P. distasonis* showed strong resistance to antibiotics but weaker resistance to human targeted drugs, indicating that certain resistance traits remain compound specific rather than contributing to broader multidrug resistance (Maier, et al. 2018). To further investigate these shared resistance mechanisms, Maier et al. performed a genome wide overexpression screen in *E. coli* using multiple xenobiotics with broad anticommensal activity. Disruption of key multidrug efflux component TolC in *E. coli* increased sensitivity to both antibiotics and xenobiotics while over expression of TolC restored growth under xenobiotic exposure (Maier, et al. 2018). Additional transporter families, including MFS, MATE, SMR, and ABC together with transcriptional regulators (rob) linked to efflux and stress responses were also identified as contributors to resistance against these compounds (Maier, et al. 2018).

Numerous industrial and agricultural xenobiotics have also been reported to exert direct antimicrobial effect on prevalent human gut (Roux, et al. 2025). Chemical genetics analysis in Parabacteroides merdae, for example showed that loss of efflux repressor AcrR conferred resistance to multiple xenobiotics while simultaneously reducing susceptibility to ciprofloxacin (Roux, et al. 2025). AcrR normally represses transcription of the AcrAB-TolC multidrug efflux system; its disruption increases efflux activity, reducing intracellular antibiotic accumulation

and promoting cross-resistance (Roux, et al. 2025). These observations suggest that multidrug efflux systems represent a shared defense mechanism linking bacterial responses to xenobiotics and antibiotics.

Xenobiotic exposure may not only select resistance mechanisms, but such compounds may also bioaccumulate intracellularly. A recent study demonstrated that 38 human gut bacterial strains bioaccumulate per- and polyfluoroalkyl substances (PFAS) across environmentally relevant concentrations ranging from nanomolar to hundreds of micromolar (Lindell, et al. 2025). Cryogenic ion beam–imaging further confirmed intracellular PFAS localization, supporting cellular accumulation (Lindell, et al. 2025). Diverse human targeted drugs have likewise been shown to accumulate within gut bacteria (Klünemann, et al. 2021).

The PFAS study also provided a mechanistic insight into how persistent xenobiotics engage the conserved bacterial defense system(Lindell, et al. 2025). PFAS bioaccumulation was limited by multidrug flux, but deletion of the outer membrane channel TolC in *E. coli* significantly increased PFAS bioaccumulation, indicating active TolC-dependent export (Lindell, et al. 2025). Consistent with chemical-genetic studies showing that enhanced efflux reduced intracellular accumulation of both antibiotics and structurally unrelated xenobiotics and efflux disruption increased susceptibility (Roux, et al. 2025). PFAS exposure additionally induced proteomic and metabolic remodeling and adaptive evolution under prolonged exposure (Lindell, et al. 2025). In *vivo* mice colonized with human gut bacteria exhibited higher fecal PFAS levels than germ-free controls, suggesting that the role microbial bioaccumulation contributes to PFAS persistence within the host (Lindell, et al. 2025).

Xenobiotic compounds that are not intrinsically antibacterial and are commonly encountered as a part of the routine diet can also modulate bacterial drug responses. Dietary molecules such as caffeine have been shown to engage bacterial regulatory networks, including Rob-dependent pathways that reduce porin abundance, limit antibiotic uptake and reduce efficacy (Binsfeld, et al. 2025).

Xenobiotic exposure can reduce microbial diversity and restructure microbial community composition(Köninger, et al. 2026). In some cases, these exposures may also enrich microbes with xenobiotic degrading or resistance associated functions (De Filippis, et al. 2024). Over time such exposures can shift community composition towards stress-tolerant or opportunistic taxa adapted to persistent xenobiotic exposure (Muturi, et al. 2017; Basapuram, et al. 2025; Köninger, et al. 2026).

These studies suggest that antibiotics and xenobiotics can converge on conserved cellular pathways including transport, permeability and global stress regulations network. Across microbial communities such exposures may drive adaptive physiological remodeling and broader restructuring favoring persistence and enrichment of stress tolerant taxa and reduced microbial diversity. Based on these experimentally supported findings, Figure 2 summarizes the proposed links between xenobiotic-induced cellular stress responses, adaptive physiological remodeling and broader community-level restructuring.

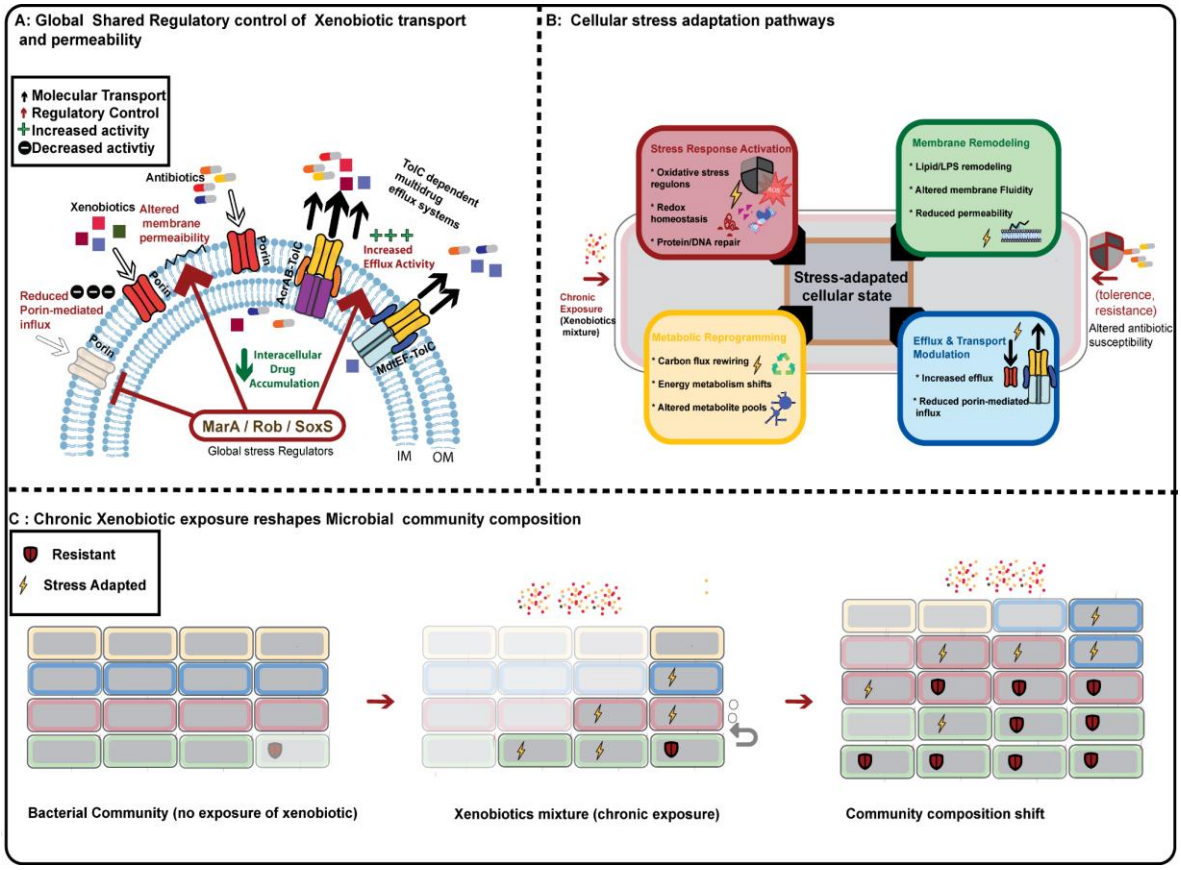


***Figure 2**. A. Xenobiotics and antibiotics activate shared global stress regulators by reducing membrane permeability and increasing multidrug efflux which lowers intracellular drug accumulation. (B) Chronic exposure induces a stress-adapted cellular state that triggers oxidative stress regulation, membrane remodeling, metabolic reprogramming, and transport modulation, leading to tolerance and altered antibiotic susceptibility. (C) At the community level, prolonged xenobiotic exposure reshapes microbial composition by enriching stress-tolerant and resistant taxa while reducing abundance of sensitive members. Horizontal gene transfer further accelerates the spread of adaptive traits*.

2.6 Implications of Co-exposure and Co-selection

Growing evidence suggests that xenobiotic co-exposure can amplify selective pressures and accelerate the emergence of antibiotic resistance (Shi, et al. 2022). Notably, several industrial chemicals exhibit antimicrobial activity against members of the human gut microbiota at concentrations relevant to intestinal exposure, despite not being intended for antibacterial use (Roux, et al. 2025). This indicates that xenobiotic exposures can directly suppress commensal bacteria and potentially disrupt microbiota function even in the absence of antibiotics.

Chemical perturbations can also reshape microbial communities and compromise host defenses (Grießhammer, et al. 2025). As a result, enteric pathogens such as Salmonella enterica can expand more readily in xenobiotic-perturbed communities, leading to increased intentional colonization and accelerated disease onset in vivo (Grießhammer, et al. 2025).

Aquatic systems are major reservoirs of antibiotic resistance genes (ARGs) shaped by human-impacted activities. Global survey of inland waters reveals persistent ARGs co-occurring with mobile genetic elements, suggesting potential for genetic mobilization and environmental dissemination(Wang, et al. 2024). Elevated ARG abundance in freshwater systems, together with the detection of ARG-harboring bacteria including potential pathogens underscores the role of

aquatic environments as interfaces connecting environmental contamination to human exposure (Leonard, et al. 2015; Wang, et al. 2024; Yang, et al. 2025).

Waste water treatment plants (WWTPs) represent critical interfaces between human activities and natural ecosystems. Assessment of European wastewater treatment plant effluents detected hundreds of organic contaminants persistent after treatment. Several of them exceeded ecological risk thresholds when evaluated as mixtures, indicating continuous release of biologically active chemicals into aquatic environments (Finckh, et al. 2022). Wastewater plants function not only as a sink for chemical pollutants and resistant genes but also as a bioreactor that creates conditions conducive to genetic exchange among microbial populations (Finckh, et al. 2022).

Direct experimental evidence indicates that environmental chemicals including herbicides can promote horizontal gene transfer by enhancing conjugative plasmid transfer through intracellular oxidative stress, altered membrane permeability and increased cell-to-cell contact between bacteria (Aboagye, et al. 2023). These stress responses can facilitate dissemination of resistance genes even in the absence of antibiotic selection (Aboagye, et al. 2023).

These observations suggest that the consequences of xenobiotic co-exposure extend beyond environmental contamination. xenobiotics may create conditions that favor the emergence and persistence of difficult to treat infections through disruption of commensal microbial communities, enrichment of tolerant taxa and increasing the mobility of resistance determinants. In this way xenobiotic pressures can amplify the clinical impact of antimicrobial resistance even without direct antibiotic misuse.

The clinical implications of xenobiotic-driven resistance are considerable and contribute to an already growing global health burden. Recent estimates suggest that bacterial antibiotic resistance was associated with approximately 4.95 million deaths in 2019, including 1.27 million deaths directly attributed to resistant infections (Murray, et al. 2022). This number could rise dramatically with projections estimating up to 10 million deaths by 2050 without effective

intervention (Price 2016). These trends emphasize the need to expand AMR mitigation strategies beyond antibiotic stewardship alone. Monitoring and reducing xenobiotic exposures may become equally important for limiting the emergence and spread of resistance across ecological and clinical settings (Fig.4).

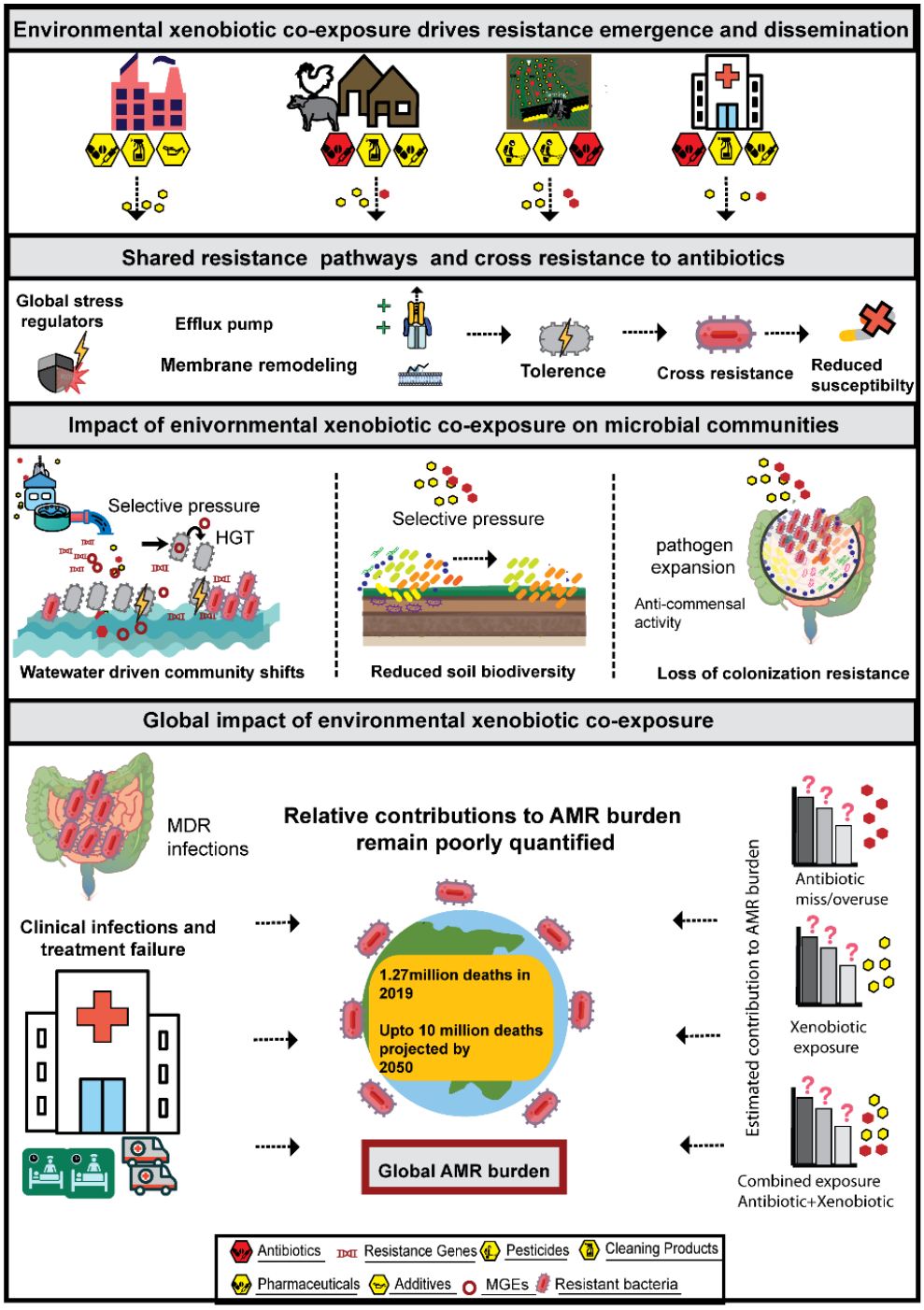


Figure 4: From environmental co-exposure to the global antimicrobial resistance burden: Environmental c*o-exposure across industrial, agricultural, and household sources selects for antibiotic resistance through shared physiological pathways, cross-resistance, and horizontal gene transfer, while simultaneously disrupting microbial communities. Gut exposure leads to loss of colonization resistance and expansion of resistant pathogens, contributing to increased clinical infections and treatment failure. The global antimicrobial resistance burden, which caused an estimated 1.27 million deaths directly attributable to bacterial AMR in 2019, could reach up to 2 million deaths annually by 2050 if current trends continue. The relative contribution of xenobiotic exposure, antibiotic misuse, and their combined effects to the global AMR burden remains unknown.*

2.7 Outlook and Future Directions

Recognizing xenobiotics as pervasive yet often overlooked modulators of bacterial stress responses calls for a reassessment of how antimicrobial resistance is studied, monitored, and addressed. Although an increasing number of studies now examine the effects of xenobiotics across environmental and host-associated microbiomes, most experimental designs still focus primarily on single-compound exposures despite the reality that microbes are routinely exposed to complex and dynamic xenobiotic mixtures. Systematically incorporating this chemical complexity into resistance research represents an important next step.

At the experiment level, future work should build on emerging high-throughput chemical genetic (Roux, et al. 2025) and multi-Omics approaches to identify molecular determinants of xenobiotic responses (Ghosh, et al. 2025) and capture system-level physiological changes. Determining how low-level exposure reshapes bacterial regulatory pathways and evolutionary trajectories remains a major challenge. Long-term evolution experiments (Cousins, et al. 2022) which are widely used to study adaptation to antibiotics and other stressors could be

extended to mixture-based exposures to determine whether stress responses driven by xenobiotics stabilize into heritable resistance phenotypes.

At the ecological scale, a major challenge is translating mechanistic insights from controlled laboratory studies to the complex dynamics of real microbial communities. Natural and host-associated environments are chemically heterogeneous settings where antibiotics coexist with diverse industrial, agricultural, and consumer-derived xenobiotics (Tao, et al. 2023). Microcosm-based models provide an intermediate system for examining how such co-occurring stressors influence community composition, functional interactions and resistance gene dynamics under controlled yet ecologically relevant conditions (Hong, et al. 2022). Combining these systems with functional assays and multi-omics profiling will help clarify whether stress responses observed in single-species studies persist and propagate within complex communities.

Environmental reservoirs such as wastewater treatment plants, agricultural soils, and surface waters represent critical points where xenobiotic mixtures, dense microbial populations, and mobile genetic elements interact. Metagenomic analyses suggest that broadly distributed taxa (“generalist”) together with mobile genetic elements may facilitate gene exchange across habitat boundaries linking environmental and host-associated microbiomes (Kim, et al. 2026). Understanding these interactions may help explain how environmental chemicals contribute to the emergence and spread of antimicrobial resistance.

Environmental and antibiotic resistance surveillance are still often conducted separately, even though microbes in real-world settings are simultaneously exposed to antibiotic and diverse xenobiotic pressure. Integrating these approaches could improve understanding of how environmental chemical exposures influence microbial adaptation and resistance dynamics (Bengtsson-Palme, et al. 2023). Monitoring programs are now increasingly tracking industrial chemicals, pesticides, and pharmaceuticals across different environments (Wilkinson, et al. 2022; Köninger, et al. 2026). But their contribution to resistance outcomes remains insufficiently explored. Expanding resistance surveillance and

risk assessment frameworks to include xenobiotic co-exposures may improve future mitigation strategies across environmental and clinical settings.

Chapter 3

Xenobiotic adaptation and antibiotic cross-tolerance in *E. coli*: multi-omics insights

3.1 Abstract

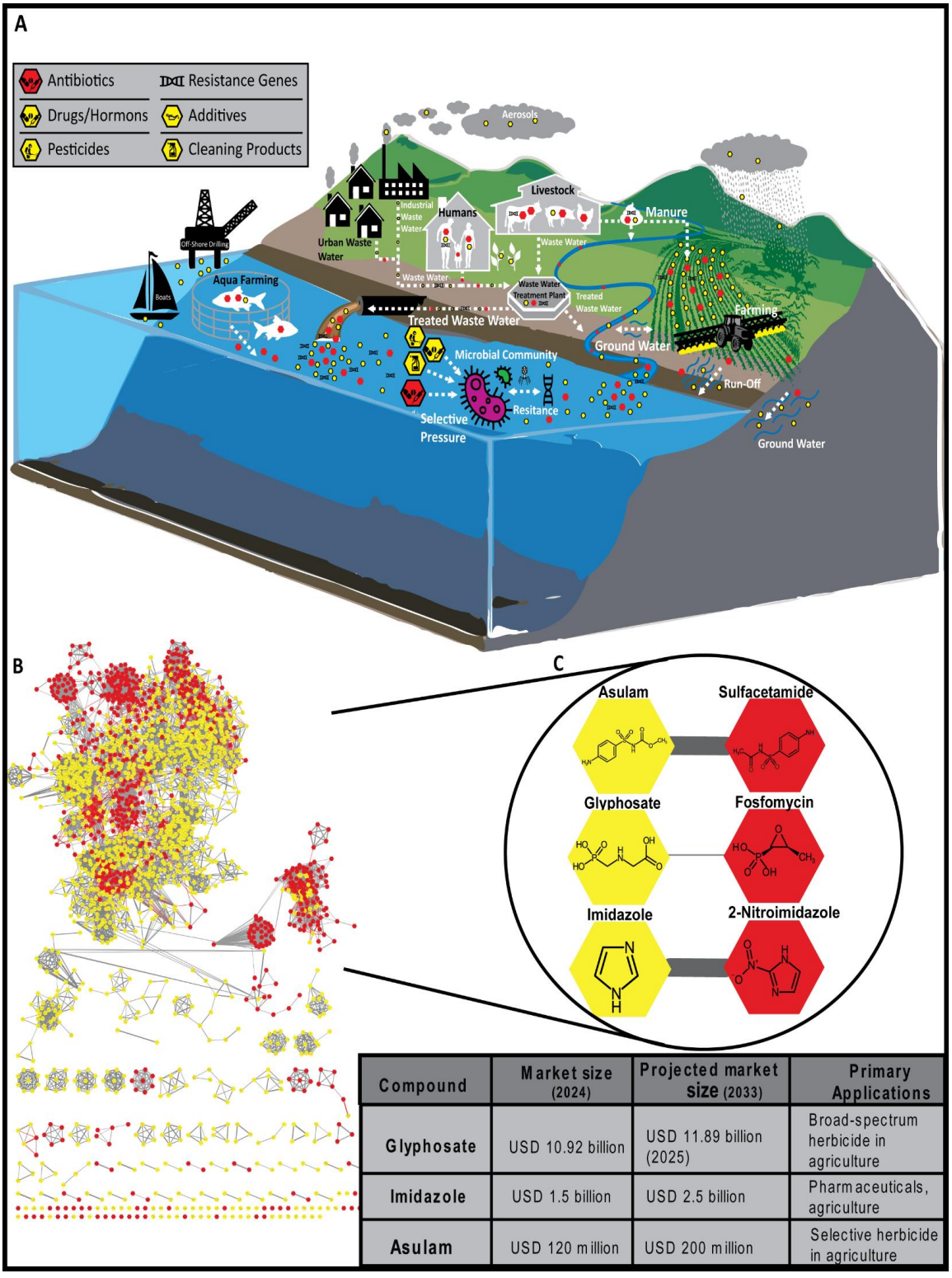
Human activities have increased the occurrence of xenobiotics in marine environments, posing selective pressures on microbial communities. To investigate the potential impact of xenobiotic stress on early bacterial adaptation, we used *Escherichia coli* as a well-characterized model organism in controlled laboratory evolution experiments to examine metabolomic and proteomic responses to long-term xenobiotic exposure. This study investigated adaptive responses in *E. coli* to three widely used xenobiotics, Imidazole, Asulam, Glyphosate and the antibiotic Fosfomycin. Laboratory-based adaptation experiments were conducted under controlled exposure to these compounds. Evolved strains exhibited compound specific phenotypic responses, including increased MIC values and improved growth under sublethal concentrations, whereas Glyphosate evolved strain showed no detectable increase in MIC or tolerance. These adaptations were accompanied by altered biofilm formation and increased tolerance to Kanamycin, indicating that xenobiotic-driven stress responses may also contribute to antibiotic tolerance. Metabolomic profiling revealed 36 metabolite features consistently upregulated across all evolved strains, including annotated metabolites associated with membrane lipids and amino acids metabolism. Proteomic analysis revealed compound specific responses including upregulation of FolC in Asulam evolved strain and MurA in Fosfomycin evolved strain, as well as shared responses, including biofilm associated changes, redox/oxidative stress regulation and membrane associated remodeling across evolved strains. These phenotypic and molecular shifts suggest that adaptation to environmental chemicals can potentiate broader stress tolerance and contribute to the emergence of antibiotic cross-tolerance.

3.2 Introduction

Antibiotic resistance is a major global health threat (Tosas Auguet, et al. 2025). The impact of this crisis is estimated to have caused 1.27 million deaths globally in 2019, with attributable AMR-related deaths projected to rise to 10 million by 2050 (Price 2016). Frequent and excessive use of antibiotics has created intense selective pressure on bacteria, developing resistance and tolerance mechanisms (Andersson and Hughes 2014). While antibiotic resistance has been extensively studied in the context of direct antibiotic exposure, little is known about the effects and impacts of agricultural and industrial chemical components (Lindell, et al. 2022). Xenobiotics are chemical compounds derived from human activities that are not naturally produced or typically encountered by organisms in natural environments. They include pharmaceuticals, pesticides, cosmetics, food additives, and fragrances (Singh, et al. 2023). Some xenobiotics are essential in agriculture and industry, but their widespread use and persistence in the environment have resulted in pollution levels that may have already surpassed ecologically safe thresholds (Cousins, et al. 2022). Bacteria employ various survival strategies when exposed to stressors, including xenobiotics such as commonly used antimicrobial agents, through mechanisms such as genetic mutations, efflux pump activation, enzymatic degradation, and horizontal gene transfer (Abbas, et al. 2024). Cross-resistance arises when a single mechanism confers reduced susceptibility to multiple compounds (Sanders 2001; Naghavi, et al. 2024). Recent studies have shown that exposure to certain pesticides, such as ammonium acetate, pyrethrins, and atrazine, can drive antibiotic resistance in bacterial populations by altering efflux pump activity (Jun, et al. 2019). In another study, adaptation of *E. coli* and *Salmonella enterica* serovar, Typhimurium to Herbicides Dicamba, 2,4-Dichlorophenoxyacetic Acid, and Glyphosate has led to increased Minimum Inhibitory Concentrations (MICs) for clinically significant antibiotics like ampicillin, chloramphenicol, ciprofloxacin, and tetracycline (Kurenbach, et al. 2015). Environmental contamination with xenobiotics is a growing concern, as natural processes and human activities contribute to their accumulation in soil and aquatic systems (Malagón-Rojas, et al. 2020). These findings underscore the urgent need to understand the molecular and

physiological mechanisms that enable bacterial adaptation under these xenobiotic exposures.

We hypothesize that structural similarity between xenobiotics and antibiotics may drive overlapping adaptive and resistance associated responses. Exploring these interactions is crucial for understanding how environmental pollution and xenobiotic exposure may influence microbial adaptation and the emergence of antibiotic cross-resistance. We constructed a combined network of xenobiotics and antibiotics to assess their structural similarity using Tanimoto chemical similarity analysis in Cytoscape with the ChemViz app. This analysis connects similar structures between xenobiotics and antibiotics (Figure 1). We selected three xenobiotic compounds based on their structural similarity to antibiotics: Asulam, Imidazole, and Glyphosate. Asulam, a sulfonamide herbicide, shares a Tanimoto similarity of 0.9 with sulfacetamide and 0.63 with sulfamethoxazole, a sulfonamide antibiotic. Imidazole, a precursor of widely used antifungal and industrial chemicals, exhibits a similarity of 0.889 with 2-nitroimidazole and 0.6 with metronidazole. Glyphosate, the most used herbicide worldwide, shares a similarity of 0.34 with fosfomycin, a broad-spectrum antibiotic. To investigate adaptive responses to these selected compounds, controlled laboratory evolution experiments were conducted in *E. coli.* Phenotypic adaptation was assessed through MIC determination and growth kinetics. To characterize global metabolic and protein level changes associated with adaptive responses to compound exposure, we performed LC-MS/MS-based untargeted metabolomics and proteomics. We observed compound-specific progression of MIC values in evolved strains and enhanced growth under sub-inhibitory conditions, except for glyphosate, where the change was not detectable. Omics analysis revealed broad metabolic and proteomics alterations across multiple cellular processes compared to control strains. Enhanced biofilm formation was observed in some evolved strains, along with increased tolerance to kanamycin, indicating that prolonged xenobiotic stress may enhance bacterial tolerance to antibiotics.



Compound	Market size (2024)	Projected market size (2033)	Primary Applications
Glyphosate	USD 10.92 billion	USD 11.89 billion (2025)	Broad-spectrum herbicide in agriculture
Imidazole	USD 1.5 billion	USD 2.5 billion	Pharmaceuticals, agriculture
Asulam	USD 120 million	USD 200 million	Selective herbicide in agriculture

Figure 1. *A schematic representation illustrating how antibiotic resistance genes and xenobiotics contribute to selective pressure, facilitating the acquisition of antimicrobial resistance. B. Whole network diagram depicting the interactions between xenobiotics and antibiotics. (Red nodes represent antibiotics; yellow*

nodes represent xenobiotics.) C. Magnified view focusing on specific interactions between selected antibiotics and xenobiotics, highlighting their interrelationships.

3.3 Results and Discussion

3.3.1 *E. coli* adaptation to Xenobiotics

We conducted lab-based adaptation experiment involving *E. coli* exposed to sub-inhibitory concentrations of three xenobiotics, Asulam, Glyphosate, Imidazole, and the antibiotic Fosfomycin over 24 days (experimental design shown in Figure 2A). Adaptive responses were assessed by monitoring changes in minimum inhibitory concentration (MIC) over 24 days (Figure 2B). Distinct adaptation patterns were observed across the tested compounds. Asulam exposure induced a gradual and consistent increase in MIC values, whereas Imidazole and Fosfomycin demonstrated a sustained increase over the course of the experiment. In contrast, no measurable change in MIC was observed in response to Glyphosate throughout the experiment. These results suggest that *E. coli* responds differently depending on the type of chemical stressor. Some compounds lead to measurable increases in resistance while others do not produce detectable phenotypic change. This is in line with previous studies showing that exposure to herbicides such as Dicamba and 2, 4-dichlorophenoxyacetic acid (2, 4-D) can induce adaptive response and promote cross-resistance to antibiotics (Kurenbach, et al. 2017).

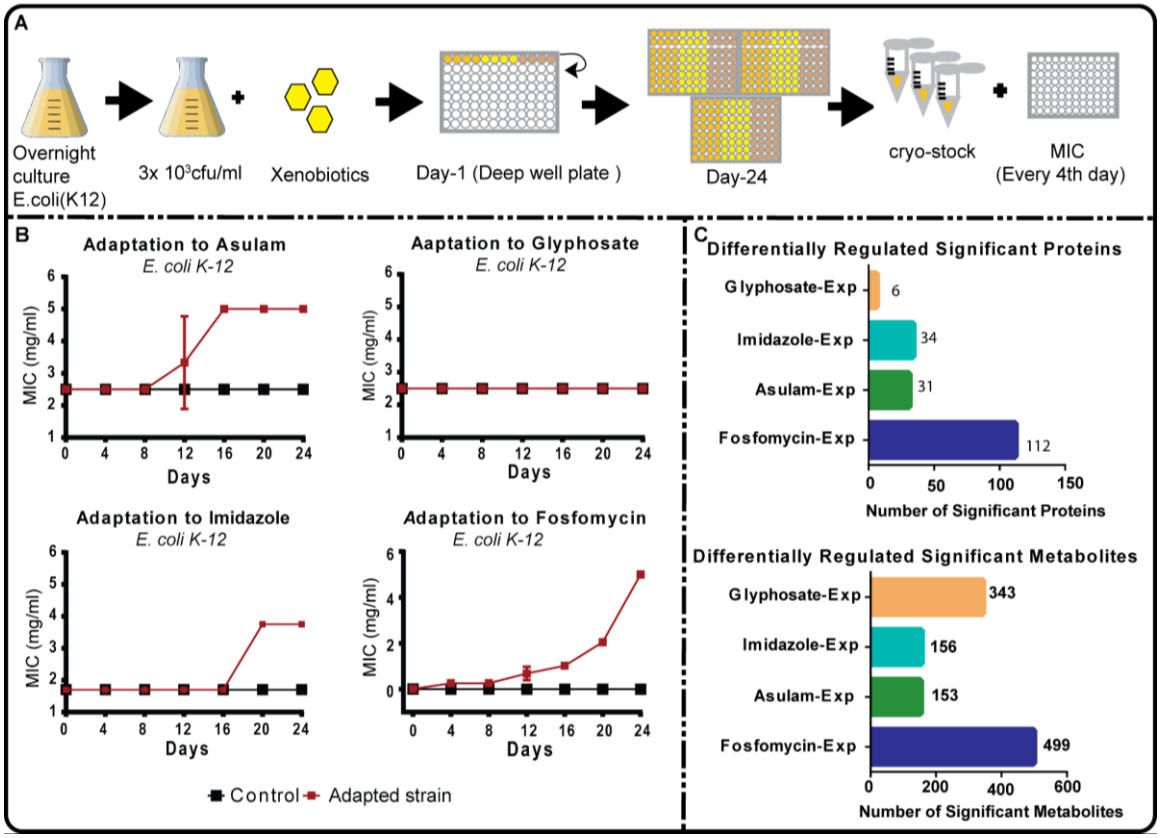


Figure 2. (A) *A graphical representation of the adaptation experimental design.* ***(B)*** *Development of resistance in E. coli (K12) to Asulam, Glyphosate, Imidazole, and Fosfomycin over 24 days. (Data points represent mean values; error bars indicate standard errors.)* ***(C)*** *Total number of significant metabolites and proteins in each adapted strain of E. coli (K12)*

To further examine these, the growth dynamics for each adapted and control strain were evaluated across a range of sub-inhibitory concentrations (Figure 3). Fosfomycin evolved strain showed consistent growth at all tested concentrations, indicating stable tolerance (64 µg/ml and 2.5 mg/ml). Consistent tolerance at all tested subinhibitory concentrations (1.85 mg/ml, 3.4 mg/ml) was observed for Imidazole evolved strain. Although the Asulam evolved strain grew at 2.5 mg/ml during the evolution experiment, it was unable to grow at this concentration upon regrowth from cryostocks and subsequent growth curve analysis, indicating an unstable resistance phenotype. Nevertheless, the strain retained a growth advantage at lower concentrations (1.25 mg/ml and 1.5 mg/ml) compared with the control strain. Antibiotic resistance is often accompanied by fitness costs,

which can result in partial or complete loss of resistance following relaxation of selective pressure, while residual or compensatory adaptations may still confer fitness advantages under lower levels of stress (Andersson and Hughes 2010).

Furthermore, our data indicate that prolonged Glyphosate exposure does not promote resistance in *E. coli.* Instead, the evolved strain showed impaired growth relative to the control strain at sublethal concentrations, suggesting that Glyphosate exposure imposed fitness costs rather than conferring adaptive resistance (Melnyk, et al. 2015). Glyphosate primarily targets the shikimate pathway by inhibiting the EPSP Synthase (Herrmann and Weaver 1999; Dill, et al. 2010) . Its inhibitory effects are known to be strongly media dependent, particularly in nutrient-rich media such as Mueller-Hinton broth, which can buffer pathway specific stress because of the availability of free amino acids yielding comparatively high MIC values and weakening selective pressure for adaptive resistance (Larsson, et al. 2018; Aldehoff, et al. 2025) .

To better understand what might be happening at the cellular level we examined changes in the proteome and metabolome of the adapted strains as shown in Figure 2C. All exposed strains led to substantial differences in the number of significantly differentially regulated proteins and metabolites across all xenobiotic-evolved *E. coli* strains compared to the control. Interestingly changes were also observed in the Glyphosate evolved *E. coli* despite the absence of detectable MIC shift. This suggests that even when resistance is not apparent at the phenotypic level repeated exposure can still drive significant changes at the cellular level.

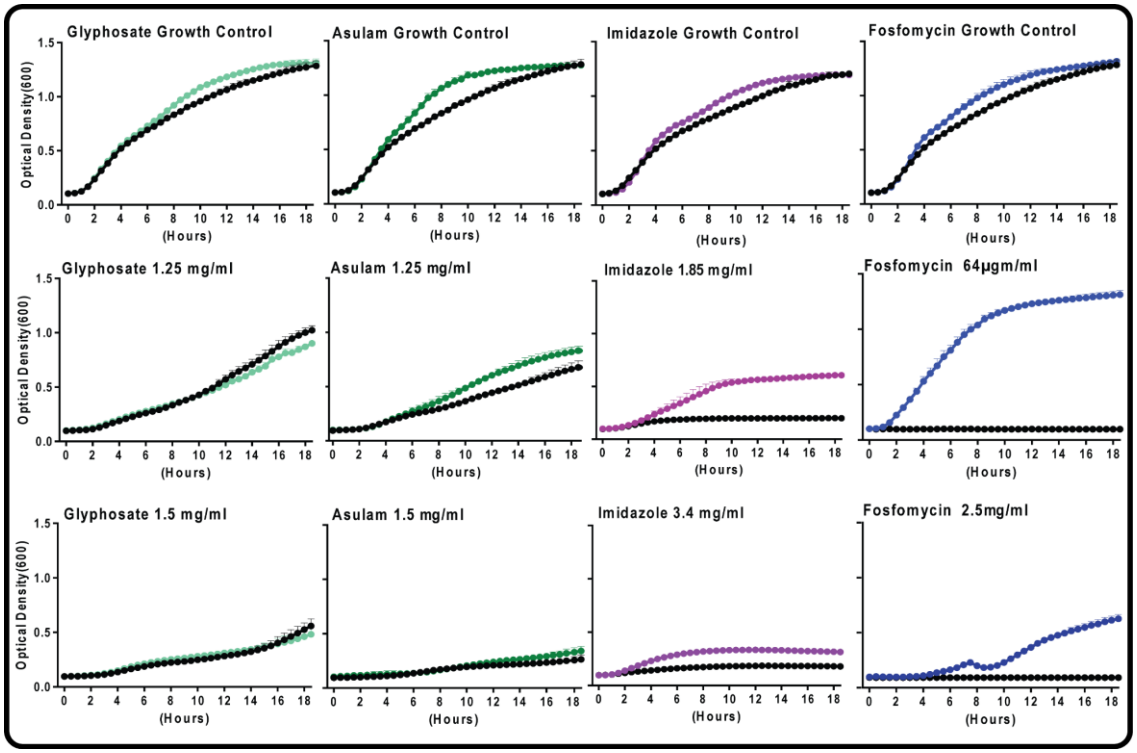


Figure 3. **Growth dynamics of xenobiotic-adapted E. coli under increasing concentrations of Glyphosate, Asulam, fosfomycin, and imidazole.**

Growth curves (OD600 over 18 hours) for xenobiotic-evolved E. coli strains and the control exposed to increasing concentrations of each xenobiotic. Each panel shows the growth of the evolved strain (colored lines) versus control (black line) at the respective concentration. The line represents mean OD values from the replicates (n=3), error bars denote standard deviation. Fosfomycin- and imidazole-evolved strains retain strong growth at concentrations that almost completely inhibit the wild type. In contrast, Asulam-evolved strain exhibits a growth advantage over the wild type at 1.25 mg/ml. Color coding: Green = Asulam evolved strain; blue = Fosfomycin evolved strain. aqua green = Glyphosate evolved strain; magenta = Imidazole evolved strain; black = wild-type control.

3.3.2 Cross-Tolerance of Xenobiotic Evolved *E. coli* to structurally unrelated Compounds

To determine whether adaptation to a specific xenobiotic could confer tolerance to structurally unrelated compounds. *E. coli* strains evolved with Asulam, Fosfomycin, Imidazole, and Glyphosate were tested for cross tolerance. Growth

during xenobiotic exposure was calculated relative to the corresponding untreated control for each strain to account for differences in baseline growth and lag phase between evolved strains and the wild type (WT) strain (Figure 4A). When tested with Asulam (1.25 mg/ml) Fosfomycin, Imidazole and Glyphosate evolved strains showed 59.95%, 40.03% and 17.9% growth at 12 hours respectively, compared to 30.49% in the WT strain. Similarly, when tested with Imidazole (1.85 mg/ml) Fosfomycin, Asulam and Glyphosate evolved strains showed 61.05%, 49.19% and 42.22% growth respectively, whereas WT exhibited only 21.21% growth at 12 hours, indicating increased tolerance across all evolved strains. When tested with Glyphosate (1.25 mg/ml), Fosfomycin, Asulam and Imidazole evolved strains showed 85.50%, 77.58%, and 71.50% growth, respectively compared to 68.80% in the WT strain. Overall, these findings demonstrate that adaptation to individual xenobiotics can confer varying degrees of cross tolerance to structurally unrelated compounds with particularly pronounced cross tolerance observed when strains were tested with imidazole.

Imidazole containing small molecules reportedly disrupt the bacterial cell membrane, leading to membrane defects, loss of membrane potential, and leakage of cell contents in *E. coli* and Salmonella Typhi (Deblais, et al. 2018; Skrzyniarz, et al. 2024). In evolved strains (Fosfomycin, Imidazole and Glyphosate), we have observed an upregulation of various Phosphaditylethanolamines (PE) and L-Tryptophan, particularly in the Fosfomycin and Asulam evolved dataset, suggesting membrane remodeling as a potential contributor to the observed tolerance. Alteration in phospholipid composition and fatty acid content modulate fluidity, influencing antimicrobial susceptibility (Niu, et al. 2024; Godlewska, et al. 2025). Our data suggests that the Fosfomycin evolved *E. coli* strain exhibits broader tolerance to both chemically related compounds such as Glyphosate and chemically unrelated compounds. This pattern suggests the emergence of a generalized stress tolerance, potentially driven by metabolic adaptation and envelope remodeling.

Overall, the xenobiotic evolved *E. coli* strains exhibited variable cross tolerance. These findings are consistent with a previous study showing that antiviral

exposure can induce a broad spectrum of cross resistance responses in *E. coli,* ranging from no detectable effect to strong cross-resistance (Wallace, et al. 2023).

3.3.3 Antibiotic Cross-Tolerance in Xenobiotic Evolved *E. coli*

To assess the possible acquired cross-resistance to antibiotics, evolved *E. coli* strains were tested under sublethal concentrations of Ampicillin, Kanamycin, and Ciprofloxacin for 12-hours, as shown in Figure 4B.

Growth was monitored by measuring the optical density at 600 nm and relative growth was calculated against the corresponding untreated control for each strain. Distinct antibiotic susceptibility profiles were observed among evolved strains. When tested with kanamycin (2 µg/ml) Fosfomycin, Asulam, and Imidazole evolved strains achieved 60%, 52%, and 54% growth respectively at 12 h compared with 42% in the WT strain. When tested with Ampicillin (2 µg/mL) Fosfomycin, Asulam, Imidazole, and Glyphosate evolved strains achieved 58%, 47%, 44% and 51% growth respectively at 12 h compared to 51% in the WT strain. In contrast only minor differences were observed under ciprofloxacin exposure. Although Fosfomycin evolved strain displayed slightly increased tolerance the growth difference relative to the WT strain was less than 10% at 12 h. Similarly, no significant effect was observed for Asulam, Imidazole and Glyphosate evolved strains. (Figure 4B). The strongest tolerance phenotype was observed in the Fosfomycin evolved strain, accompanied by moderate tolerance in Asulam- and Imidazole evolved strains. Kanamycin is known to induce oxidative stress and reactive oxygen species (ROS), which contribute to antibiotic lethality (Van Acker and Coenye 2017). In Fosfomycin evolved *E. coli* we observed upregulation of glutathione in the metabolomics data together with downregulation of γ-glutamyl transpeptidase (Ggt) in proteomics. This combination suggests reduced periplasmic glutathione degradation, contributing to elevated intracellular glutathione (Allocati, et al. 2009).

In imidazole evolved *E. coli*, upregulation of GsiB, the periplasmic glutathione-binding protein of the glutathione-specific ABC importer (GsiABCD), indicating

enhanced glutathione import (Knoke, et al. 2025). This was accompanied by increased expression of NADH dehydrogenase I subunits NuoC and NuoG, suggesting adaptation of respiratory and redox related pathways during xenobiotic stress (Mutalik, et al. 2019; Van den Bergh, et al. 2022). In Asulam evolved *E. coli* increased expression of oxidative-stress–associated proteins (YaaA, HemC) was observed (Liu, et al. 2011; Mancini and Imlay 2015). Additionally, upregulation of YqjG, a glutathionyl-hydroquinone reductase may indicate enhanced detoxification of redox-active intermediates associated with oxidative stress (Green, et al. 2012). Aminoglycoside antibiotics are well known to induce oxidative stress and reactive oxygen species (ROS), which contribute to antibiotic lethality (Van Acker and Coenye 2017). Extracellular glutathione has been shown to contribute to tolerance to these antibiotics (Cameron and Pakrasi 2011).

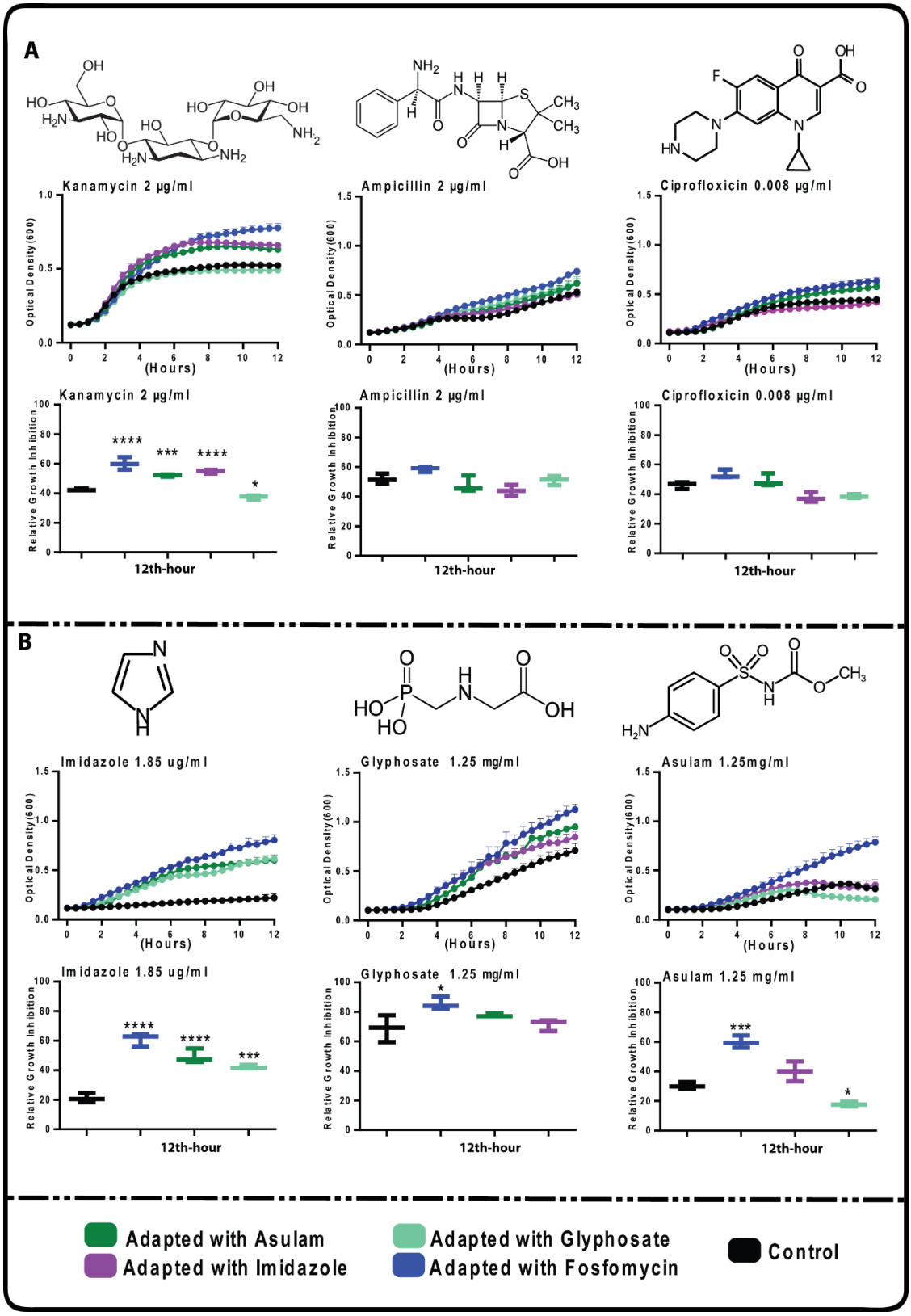


Figure 4. Cross-resistance profiles of xenobiotic-adapted *E. coli*

(A). *Growth-based cross-resistance of E. coli strains adapted under individual xenobiotics (Imidazole, Glyphosate, Asulam) when exposed to other xenobiotics at sublethal concentrations. Curve represents mean growth trajectories over 12-h, and box plots summarize relative growth at 12-h.* ***(B)*** *Antibiotic cross-resistance exhibited by xenobiotic evolved E. coli. Strains evolved under xenobiotics were tested for cross tolerance to the antibiotic's kanamycin, Ampicillin and Ciprofloxacin. (Asterisks (*) indicate statistically significant difference relative to the wild type of control (p <0.05) and ≥ 10% difference at 12-h growth) Bars in the boxplot denote minimum, maximum and mean values from n=3 independent absorbance measurements).*

3.3.4 Metabolomics Profiling Reveals Differential Metabolite Expression in Response to Xenobiotic Exposure.

The metabolomic profiling of *E. coli* evolved with Asulam, Glyphosate, Imidazole, and Fosfomycin revealed unique adaptive responses to these xenobiotics. Using non-targeted LC-MS/MS analysis, followed by feature-based molecular networking, we detected a total of 2,858 metabolite features. From these, 236 compounds were successfully annotated using GNPS2. (Task ID: 2e660c90c8e64bf9b197aa9aa79d26d0). Principal Coordinate Analysis (PCoA) based on the Canberra distance metric showed clustering of evolved and unevolved strains for each compound, indicating significant metabolic changes (Figure 5A).

Statistical analysis was performed using Student's *t*-test, followed by false discovery rate (FDR) correction to account for multiple testing. Metabolites with an adjusted *P* value (FDR-corrected) < 0.05 were considered significantly up- or downregulated in the xenobiotic evolved strains (Figure 5B). 150 metabolites were commonly upregulated in strains adapted with Glyphosate and Fosfomycin, including amino acids such as phenylalanine and arginine, suggesting modification of amino acid metabolism. Furthermore, 36 metabolites were consistently upregulated across all evolved strains, highlighting conserved metabolic adaptations to diverse xenobiotic stresses.

A substantial proportion of significant features could not be confidently annotated, which is expected in untargeted LC-MS/MS datasets due to the limited coverage of microbial metabolites libraries. Nevertheless, some biologically relevant compounds were identified suggesting xenobiotic associated alteration in purine metabolism, tryptophan, indole pathways, glutathione-linked detoxification, membrane lipid composition and cyclic peptides.

In Fosfomycin evolved *E. coli*, the abundance of a metabolite annotated as adenosine was decreased, suggesting altered purine associated metabolism. Previous studies have been shown that exogenous adenosine supplementation can enhance killing of tolerant and persistent cells across multiple antibiotic classes (Kitzenberg, et al. 2022). In addition a large-scale *E.coli* gene perturbation study demonstrated that bottlenecks in purine nucleotide biosynthesis can reduce antibiotic susceptibility in a pathway-specific manner (Lubrano, et al. 2025).

Imidazole evolved *E.coli* also showed decreased xanthosine levels suggesting altered purine metabolism during adaptation (Stevens, et al. 2025). Fosfomycin-evolved *E.coli* showed decreased guanine and guanosine abundance consistent with altered purine homeostasis associated bacterial stress (Grove 2025; Salzer, et al. 2025).

The Fosfomycin evolved *E.coli* showed upregulation of oxidized glutathione (GSSG) together with a feature putatively annotated as S-lactoylglutathione (GNPS spectral match , cosine 0.9074) suggesting altered glutathione associated redox metabolism (Masip, et al. 2006). Increased GSSG abundance is been previously reported under oxidative stress conditions (Imlay 2008). S.lactoylglutathione is associated with the glyoxalase pathway involved in detoxification of methylglyoxal and related electrophilic intermediates generated during oxidative stress (Ozyamak, et al. 2010). In *E. coli* this metabolite has been linked to activate the potassium efflux system, protecting against electrophilic damage (Ozyamak, et al. 2010).

Fosfomycin evolved *E. coli* also showed increased levels of acetyl-CoA, NAD, FMN, and FAD cofactors essential for redox associated metabolism. Changes in NAD(H) have previously been linked to antibiotic tolerance in *E.coli* (Urbaniec, et al. 2023).

Asulam evolved *E. coli* showed increased levels of L-tryptophan together with an indole-containing metabolite (NSC351976) and suggesting altered aromatic amino acids and indole-associated metabolism under xenobiotic pressure. Fosfomycin evolved *E. coli* also displayed elevated L-tryptophan levels. Tryptophan derivatives and indole-related metabolites have previously been linked to stress adaptation and antibiotic tolerance in *E. coli* (Andersson and Hughes 2010; Dill, et al. 2010; Gaimster, et al. 2014; Abe, et al. 2020). Across all four adapted populations, we observed an upregulation of multiple small peptides and cyclic dipeptides (DKPs). These include cyclo(Pro-Trp), cyclo(Phe-Leu, cyclo(Th-Pro), cyclo(Val-Pro), and cyclo(Phe-Pro), as well as various di- and tripeptides (e.g. Pro-Ile, Ile-Pro-Ile, Val-Leu). Though not very commonly documented in *E. coli* they are found in other *E. coli* strains and other species (Rowlett, et al. 2017; Zhang, et al. 2017; Wagner, et al. 2025)

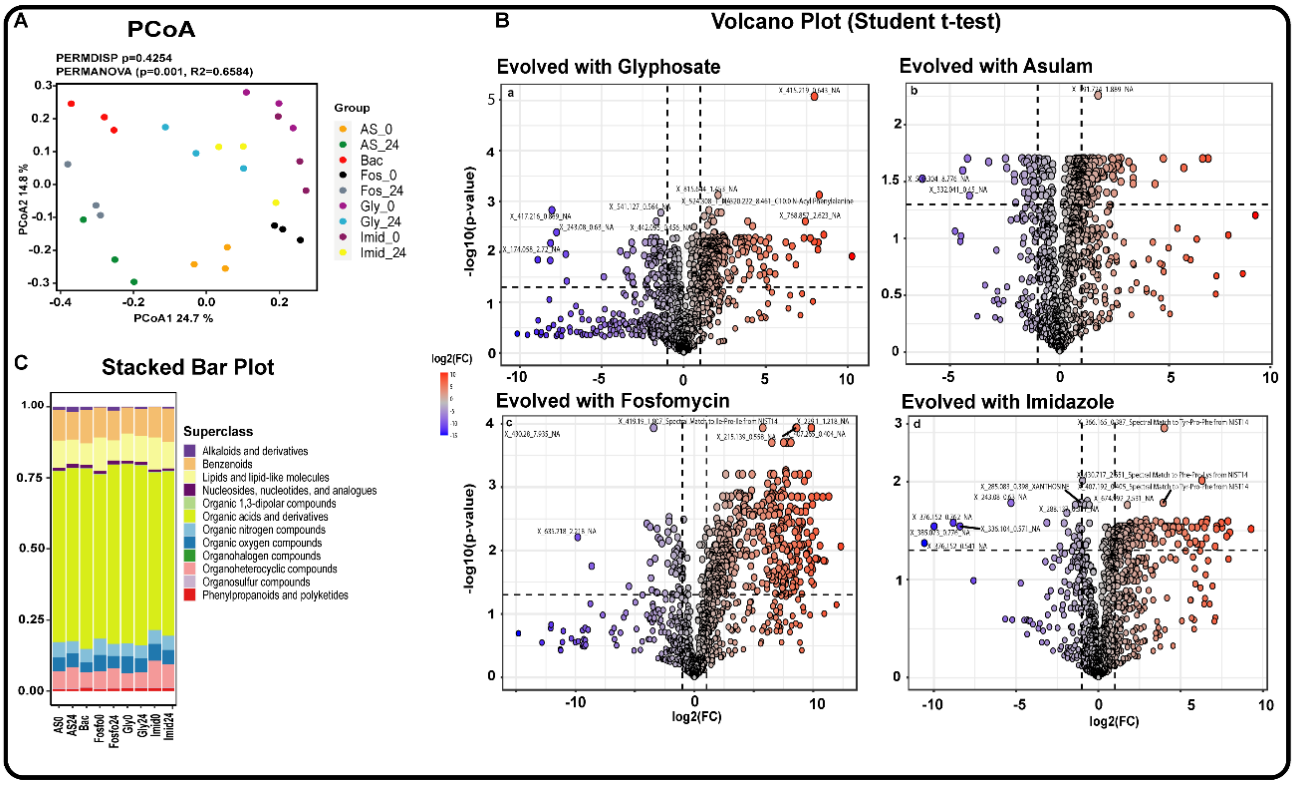


Figure 5: A *PCoA plot based on Canberra distance metric showing clustering of groups exposed to Asulam, Glyphosate, Imidazole, and Fosfomycin. PERMANOVA analysis indicated significant differences in overall metabolic profiles between different groups (p=0.001, R^2 = 0.6542).*

B *Volcano plots displaying paired-wise Student's t-tests, indicating significant differences in metabolite expression levels* ***(p < 0.05).*** *P values were adjusted for multiple testing using Benjamini-Hochberg false discovery rate (FDR) correction following exposure of E. coli to various xenobiotics. (a) Asulam: 119 upregulated and 34 downregulated metabolites. (b) Glyphosate: 277 upregulated and 66 downregulated metabolites. (c) Imidazole: 134 upregulated and 22 downregulated metabolites. (d) Fosfomycin: 449 upregulated and 50 downregulated metabolites* ***C.*** *Stacked Bar plot showing relative abundance of metabolites superclasses in evolved and control strain following exposure to Asulam Imidazole Glyphosate and Fosofomycin treatments*

3.3.5 Proteomic Profiling Reveals Differential Protein Expression in Response to Xenobiotic Exposure.

Our data showed significantly altered protein abundance in *E. coli* after 24 days of exposure to Asulam, Glyphosate, Imidazole, and Fosfomycin. Statistical analysis was performed using Student's t-test with a significance threshold of p< 0.05 (Figure 6A (a-d). Figure 6B presents a box plot highlighting the five most abundant key proteins and their fold changes across adapted strains. Proteins possessing significant changes were further examined to assess their potential roles in cellular function.

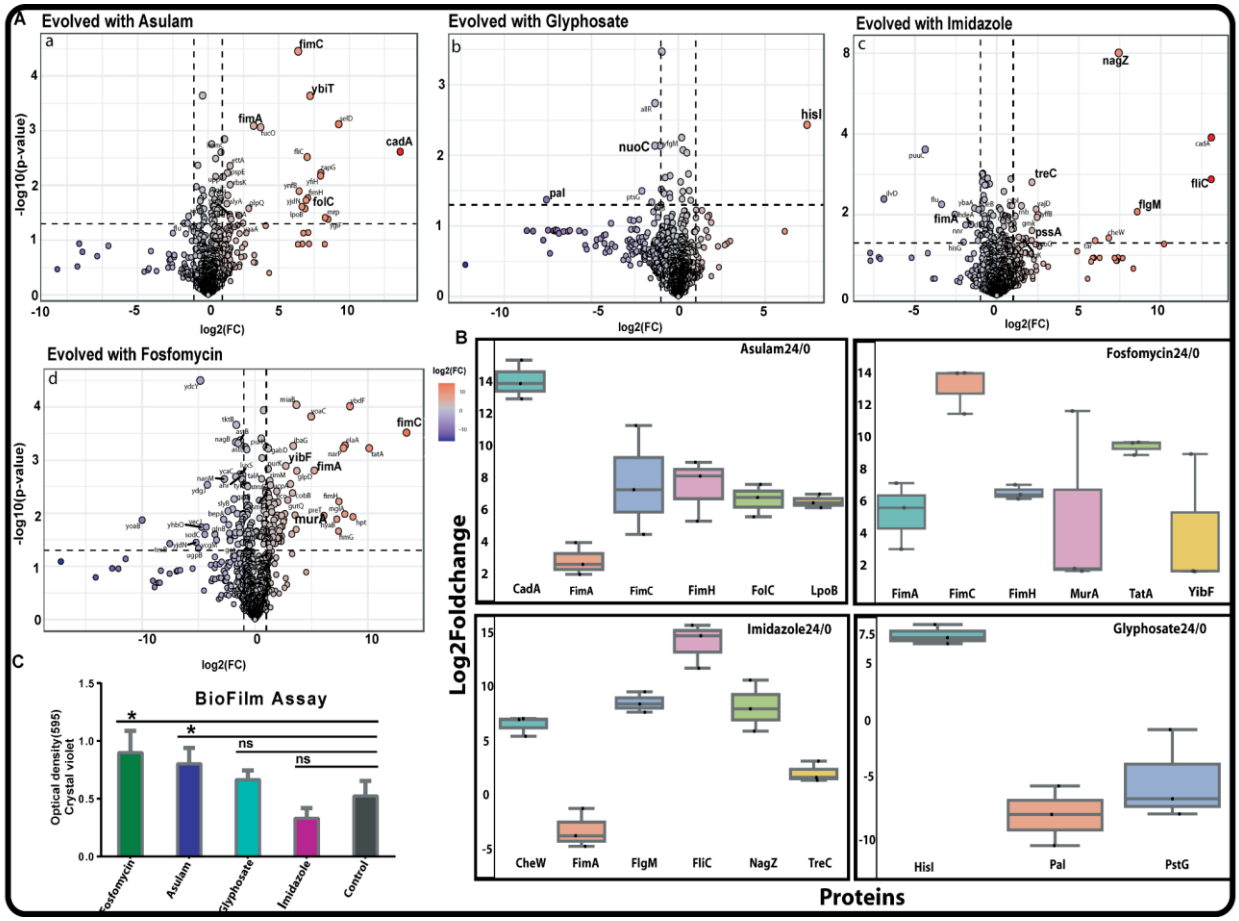


Figure 6.A *Volcano plots depicting pairwise comparisons using Student's t-test ($p < 0.05$), highlighting significant changes in protein expression levels from whole-cell extracts of E. coli following evolution under exposure to various xenobiotics. (a) Asulam: 29 proteins were upregulated, and three downregulated. (bY;) Glyphosate: 1 protein was upregulated, and four were downregulated. (c) Imidazole: 21 proteins were upregulated, and 10 were downregulated. (d) Fosfomycin: 63 proteins were upregulated, and 49 were downregulated. Bar plot showing fold changes of the most abundant proteins in E. coli strains evolved with these xenobiotics. Biofilm assay showing a change in Biofilm production in EE. coli exposed to different xenobiotics.*

We observed significant upregulation of FolC, a bifunctional enzyme in the folate biosynthesis pathway, in *Asulam evolved E. coli*. Asulam, a carbamate herbicide structurally analogous to sulfonamide antibiotics, inhibits the dihydropteroate synthase (DHPS) by mimicking para-aminobenzoic acid (PABA), thereby

disrupting folate biosynthesis (Pallett). Since FolC functions downstream of DHPS in the folate pathway, its increased abundance may reflect a compensatory response to antifolate stress during Asulam adaptation (Green and Matthews 2007). Another significantly upregulated protein was SlyA, a member of the MarR/SlyA family of transcriptional regulators associated with stress adaptation and virulence-associated gene regulation (Ellison and Miller 2006). SlyA has been reported to regulate the genes involved in outer membrane modification, reduced permeability and biofilm formation (McVicker, et al. 2011; Chalabaev, et al. 2014) .Lower abundance of the outer membrane porin OmpF was also apparent in the heat map of Asulam evolved strain. FabG (3-oxoacyl-[acyl-carrier-protein] reductase), an enzyme involved in fatty acid biosynthesis, was significantly upregulated. Altered expression of fatty acid biosynthesis proteins has previously been associated with bacterial membrane stress conditions (Kralj, et al. 2022). In addition YaaA, a member of the OxyR regulon, associated with oxidative stress protection was upregulated (Prahlad, et al. 2020) (Table SI 1).

PssA**,** *a* phosphatidylserine synthase, involved in phospholipid biosynthesis was upregulated in *E. coli evolved with* Imidazole (Figure 6b). Increased PssA abundance has been associated with altered phospholipid composition, enhanced bacterial fitness and improved membrane integrity (Chartier, et al. 2017; Rowlett, et al. 2017; Mutalik, et al. 2019). Imidazole does not have a defined target in *E.coli but* studies have shown that high doses of imidazole containing small molecules affect the membrane integrity (Deblais, et al. 2018).

The upregulation of nuoC and nuoG components of respiratory complex I (NADH:quinone oxidoreductase) suggests altered respiratory and redox associated metabolism under imidazole (Efremov and Sazanov 2011; Sazanov 2015; Kampjut and Sazanov 2022). Respiratory complex I contribute to NADH oxidation and proton motive force generation both of which are important for cellular redox balance and membrane energetics

Several proteins involved in motility and chemotaxis were upregulated, including FliC, the primary structural component of flagella, FlgM a regulator of flagellar gene expression, and CheW, an adaptor protein involved in chemotaxis

signaling. These changes suggest altered motility associated behavior under (Colin, et al. 2021). The glutathione-binding protein GsiB involved transport was also upregulated (Wang, et al. 2018; Knoke, et al. 2024) (Table SI 2).

In the case of Fosfomycin evolved *E. coli,* UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) was significantly upregulated. Overexpression and structural alterations of MurA are well-documented resistance mechanisms that reduce Fosfomycin binding affinity (Horii, et al. 1999). Cra a global transcriptional regulator of carbon metabolism was upregulated together with Glpx (fructose-1,6-bisphosphatase II), a gluconeogenic enzyme involved in carbon flux regulation (Shimada, et al. 2011). Increased abundance of Cra and Glpx may reflect metabolic adaptation involving a shift towards gluconeogenic metabolism under Fosfomycin exposure (Aghamali, et al. 2019). HscB**,** an iron-sulfur cluster co-chaperone, IscA involved in Fe-S cluster assembly and Hmp (flavin hemoglobin) were also upregulated. Hmp has been associated with protection against nitrosative stress through detoxification of nitric oxide and preservation of respiratory enzyme activity (Gardner, et al. 1998; Poole 2020). Similarly, we observed proteins involved in carbohydrate, fatty acid, and amino acid metabolism (Table SI 3), suggesting broader metabolic reprograming during Fosfomycin adaptation.

In Glyphosate evolved strain increased expression of HisI, an enzyme involved in histidine biosynthesis, may indicate altered amino acid metabolism (Table SI 4) (Aldehoff, et al. 2025). Decreased expression of NuoC, a component of the NADH-quinone oxidoreductase complex, (Efremov, et al. 2010) and OppD, a component of the ATP-dependent oligopeptide transport system (Masulis, et al. 2020) indicates perturbation of respiratory and nutrient uptake processes under Glyphosate exposure.

3.3.6 Xenobiotic Exposure Induces Biofilm Formation in *E. coli.*

Ou*r* proteomic analyses have identified significant differential regulation of several proteins associated with biofilm formation. To assess whether these changes translated into phenotypic differences biofilm assays were performed

using evolved *E. coli* strains and the wild type (Figure 2C). Fosfomycin evolved strain demonstrated increased biofilm production accompanied by upregulation of several fimbrial and adhesion associated proteins including FimA, FimB FimC and FimH Similiarly the Asulam evolved strain demonstrated increased Biofilm production together with increased abundance of fimH and SlyA.

In contrast Imidazole evolved strain showed reduced biofilm formation along with decreased abundance of FimA, the major structural subunit of type 1 fimbriae involved in initial surface adhesion during biofilm establishment. A reduction in FimA levels can impair the formation of these fimbriae, leading to decreased biofilm formation capacity (Ballén, et al. 2022). Imidazole derivatives are reported to disrupt quorum sensing and biofilm formation in other bacterial species (Arendse, et al. 2022). In the case of Glyphosate, the change in biofilm formation was not significant.

3.3.7 Integrating metabolites shifts with protein level changes

Integrating the proteomics data with the annotated metabolite profiles revealed links between protein level changes and metabolic adaptation. In Fosfomycin evolved strains we observed an increase in abundance of oxidized glutathione (GSSH), nicotine amide adenine dinucleotide (NAD) and Flavin adenine dinucleotide (FAD). These metabolites are associated with cellular redox balance, electron transfer, and detoxification of electrophilic compounds. Proteomics analysis additionally revealed upregulation of Hmp, a FAD and NADH dependent flavohemoglobin involved in nitro oxide detoxification and protection against nitrosative stress (Hernández-Urzua, et al. 2003; Svensson, et al. 2010; Ezraty, et al. 2017). Metabolomic analyses also revealed increased abundance of glutathione and S-lactoylglutathione, suggesting elevated turnover and activation of glutathione dependent detoxification pathways (Allocati, et al. 2009; Ozyamak, et al. 2013). Consistently, proteomics analysis showed downregulation of γ-glutamyl transpeptidase (ggt), which has previously been associated with elevated intracellular glutathione level upon deletion or reduced expression (Zhang, et al. 2016).

Although glutathione-related metabolites were not significantly detected in imidazole and Asulam evolved *E. coli,* proteomic analysis of imidazole evolved strain revealed upregulation of GsiB, the periplasmic component of a glutathione import system, suggesting altered glutathione flux during xenobiotic adaptation (Wang, et al. 2018). In the Asulam evolved strain, proteomic analysis revealed upregulation of YqjG a glutathionyl-hydroquinone reductase together with YaaA an OxyR regulated protein associated with oxidative stress protection. (Liu, et al. 2011; Green, et al. 2012). These changes suggest activation of glutathione associated and oxidative stress response pathways during adaptation to antibiotic and xenobiotic exposure.

Membrane remodeling emerged as adaptive response across evolved strains. Phosphatidylethanolamine (PE) accounts for 78-80% of *E.coli* membrane phospholipids (Murzyn, et al. 2005). Fosfomycin evolved E. *coli* showed increased levels of phosphatidylethanolamine (PE) and related lipid species, flavin adenine dinucleotide (FAD), L-tryptophan, and several acyl amino acid-related metabolites. Consistently, proteomics analysis revealed upregulation of the membrane-associated, FAD-dependent glycerol-3-phosphate dehydrogenase GlpD. The increase in GlpD abundance together with PE-related lipid species suggests altered glycerol associated membrane metabolism and lipid remodeling during Fosfomycin adaptation (WALZ, et al. 2002; Yeh, et al. 2008). Additionally changes in fatty acid associated proteins including FabH and TesB, further support altered membrane lipid metabolism in Fosfomycin evolved strain.

Similarly, Asulam evolved *E. coli* showed increased upregulation of GlpQ, a periplasmic glycerophosphodiester phosphodiesterase involved in Glycerophospholipid (Larson, et al. 1983; Larson and van Loo-Bhattacharya 1988) together with FabH, the initiating enzyme of fatty acids associated with phospholipid synthesis. Metabolomic analysis additionally revealed upregulation of L-tryptophan together with significant class level annotation of ester-related metabolite feature further supporting altered membrane associated lipid metabolism. L-tryptophan have previously been reported to have a role in

stabilizing protein–lipid and protein–protein interactions at membrane surfaces, potentially supporting membrane protein integrity during bilayer remodeling (Killian and Von Heijne 2000; Sanchez, et al. 2011; Khemaissa, et al. 2022). The imidazole evolved strain exhibited significant accumulation of PE related species in metabolomic data. Correspondingly, proteomic analysis showed upregulation of PssA, involved in phosphatidylethanolamine (PE) biosynthesis (Zhang and Rock 2008). Respiratory complex I subunits NuoC and NuoG, core components required for NADH oxidation and proton translocation across the inner membrane were also upregulated in the proteomics analysis. In addition to the shared redox associated adaptations these findings indicate that membrane remodeling and altered phospholipid turnover also represent common adaptive features associated with antibiotic and xenobiotic exposure. MS/MS mirror plots corresponding to the GNPS library matches are provided in Appendix Figures A1-A6.

3.4 Conclusion

Adaptation to xenobiotics, such as Asulam, Imidazole, Glyphosate, and Fosfomycin, induced adaptive responses that increased tolerance, resulting in detectable, though limited cross-tolerance to antibiotics. These findings suggest that environmental xenobiotics may act as a selective pressure with the potential for antimicrobial resistance evolution. Metabolomic profiling revealed shifts in metabolite abundance, and class-level-based annotation showed shifts in lipid and lipid-like molecules, organo-heterocyclic compounds, alkaloids, organic oxygen compounds, and organic acids. Proteomics analysis further revealed changes in protein abundance, particularly among proteins involved in stress responses, biofilm formation, and redox homeostasis, membrane remodeling, indicating adaptive responses that may enhance survival under xenobiotic stress.

Integration of metabolic and proteomic datasets further highlighted recurring adaptive features, including redox associated responses and membrane remodeling linked to altered Phospholipid turnover. Collectively these findings demonstrate that xenobiotic exposure can induce broad physiological and

metabolic adaptations in *E. coli*. These results further emphasize the importance of considering environmental xenobiotics as contributors to microbial adaptive evolution. Determining whether these responses translate into stable and heritable antimicrobial resistance phenotypes will require longer-term evolution experiments with mechanistic validation studies. Importantly, future work should additionally investigate mixed compound exposure scenarios to better reflect environmentally relevant conditions where microbes encounter complex chemical mixtures.

3.5 Methods

3.5.2 Determining Minimum Inhibitory Concentration

The first step in performing adaptation experiments was to evaluate the minimum inhibitory concentrations for the xenobiotic compound. Xenobiotic compounds from molecular networks in Cytoscape with similarity in structures with relevant antibiotics were selected to determine the MIC value against *E. coli*. i.e., Imidazole, glyphosate, Asulam, and Fosfomycin as positive control.

To determine the minimum inhibitory concentration (MIC) necessary for our adaptation experiments, we performed MIC assays using the previously reported method with some modifications (Wiegand, et al. 2008). Three compounds: Glyphosate, Imidazole and Asulam and Fosfomycin served as the positive control. Glyphosate was chosen due to its structural similarity with Fosfomycin. Stock solutions of the compounds were prepared, with concentrations of 10 mg/ml for Glyphosate, 5 mg/ml for Asulam, 30 mg/ml for Imidazole, and 10 mg/ml for Fosfomycin, and filtered with a 0.22 µm syringe filter. Using a 96-well plate, Mueller-Hinton broth was added to all wells, with compound stock solutions added to designated wells and subjected to two-fold serial dilutions. Bacterial dilutions were added to appropriate wells, with columns without bacteria serving as contamination indicators. Plates were incubated at 37°C for 16-24 hours and were properly sealed to prevent evaporation. Minimum inhibitory concentrations were determined, i.e. lowest compound concentrations at which bacterial growth was inhibited, to identify optimal concentrations for evolutionary experiments.

3.5.3 Lab-based evolution experiments under xenobiotic stress of individual strains

Adaptation experiments were performed following a previously reported method with minor modifications (El Shazely, et al. 2020). The experiments were designed to assess bacterial adaptation under prolonged exposure to sublethal compound concentrations. *E. coli* was serially passaged for 24 days in deep-well plates containing either xenobiotics or no compound (control), using concentrations below the minimum inhibitory concentration (MIC).

At initiation, wells in the first row were filled with Mueller–Hinton broth supplemented with the appropriate sublethal compound concentration and inoculated with a bacterial pre-culture (5 × 10^5 CFU/ml). Sublethal concentrations were determined by MIC assays conducted prior to the experiment and used as follows: imidazole (0.75 mg/ml), Asulam (0.75 mg/ml), Glyphosate (0.75 mg/ml), and Fosfomycin (0.5 µg/ml). Cultures were incubated at 37 °C with shaking at 150 rpm. After 24 hours, 100 µl of the culture was transferred into fresh medium containing the same compound concentration in the subsequent row. This daily transfer was repeated for 24 consecutive days.

MIC values were determined every fourth day throughout the experiment to monitor changes in susceptibility. All conditions were performed in three independent biological replicates. Triplicate cultures grown in the absence of xenobiotics were used as untreated controls.

3.5.4 Growth Curve Assays for Cross-Resistance to Antibiotics

Bacterial strains were inoculated in Muller-Hinton Broth (MHB) in 100 ml glass flasks from their cryostocks and incubated overnight at 37°C. At 180 rpm. For growth curve assays, these cultures were diluted in fresh media to an initial OD600 of 0.2. 96-well microtiter plates were used to perform the assay, using a final volume of 200 µl per well. Each well was prepared by adding 90 µl of growth medium, 100 µl of the antibiotic solution at the desired final concentration, and 10 µl of the evolved *E. coli* culture adjusted to an OD600 of 0.2. So, the final starting OD600 was approximately 0.01 in each well. Growth kinetics were

documented at 37 °C for 12 h in a microplate reader with continuous shaking, measuring at OD at 30 min intervals.

Growth of E. *coil* evolved strains with Asulam, Imidazole, Glyphosate and Fosfomycin were assessed in the presence of sublethal concentrations of these compounds. Fosfomycin evolved strain was tested at 64 µg/L and 2.5 mg/ml. Imidazole evolved strain was tested at 1.85 mg/ml and 3.4 mg/ml. Asulam evolved strain was tested at 1.25 mg/mL and 1.5 mg/mL and Glyphosate evolved strain was tested at 1.25 mg/mL and 1.5 mg/ml. Growth was monitored for 18h by measuring optical density at 600nm (OD600).

Growth curves were also performed to assess the cross tolerance of evolved *E. coli* strains to other xenobiotics and antibiotics. These experiments were carried out under standardized sublethal conditions to assess potential cross-resistance or tolerance without imposing strong lethal selection pressure.

These concentrations were used based on assessing the MIC values obtained for the parental (unexposed) and evolved strain under identical experimental conditions. Testing at 0.5 of MIC allows detection of subtle growth improvement or tolerance that may not be visible under full inhibitory conditions. Parallel control wells without antibiotics were included for each strain as growth control, and without bacteria and antibiotics for media control. Each experimental condition was tested in biological triplicate (Atolia, et al. 2020).

3.5.5 Crystal Violet Biofilm Assay (Indirect Measurement of Biofilm Biomass)

The Biofilm experiments were conducted using the methods previously reported with some modifications, using indirect measurements of Biofilm biomass (Wilson, et al. 2017). Biofilm formation was evaluated using a crystal violet staining assay in a 96-well polystyrene microtiter plate. Overnight cultures of xenobiotic-adapted *E. coli* were grown in LB broth at 37°C with shaking at 180 rpm. 100 µl of each diluted culture (1 x 10^7 CFU/ml) was added to wells, with sterile LB serving as a blank control. Plates were incubated at 37°C for 48 hours to allow biofilm to develop, while the media was replaced after 24 hours. After

incubation, planktonic cells were removed, and wells were washed three times with PBS. Biofilms were stained with 125 µl of 0.1% crystal violet for 30 minutes, bathed with distilled water until excess stain was removed, and air-dried overnight. The dye was solubilized with 200 µl of 30% acetic acid, and 125 µl of the solution was transferred to a new plate for absorbance measurement at 595 nm using a microplate reader. Absorbance values were blank-corrected. The dye was solubilized with 200 µl of 30% acetic acid, and 125 µl of the solution was transferred to a new plate for absorbance measurement at 595 nm using a microplate reader. Absorbance values were blank-corrected.

3.5.6 Sample Preparation of Bacterial Cultures for metabolomics and proteomics

The protocol for metabolomics and proteomics analysis of evolved bacterial strains involved culturing the strains (D0, D24) under conditions identical to those of the respective day in 5 ml volumes, resulting in a total of 27 samples (3 biological replicates × 2 time points × 4 compounds + control bacteria). The specified concentrations for culturing *E. coli* with Glyphosate, Asulam, Imidazole and Fosfomycin were 0.75 mg/ml, 0.75 mg/ml, 0.75 mg/ml, and 1 µg/ml respectively, for unevolved bacteria and 2 mg/ml, 2 mg/ml, 2.5 mg/ml and 0.5 mg/ml respectively, for evolved strains. Negative controls consisted of bacteria cultured without the addition of any compounds. 100 ml flasks were prepared with compound and medium. Cultures both evolved and unevolved strains were diluted 1:100 into 100 ml of fresh media containing sub-lethal concentrations of compounds (Glyphosate 0.3 mg/ml, Imidazole 0.3 mg/ml, Asulam 0.3 mg/ml, and Fosfomycin 0.5 µg/ml) or without any compound (negative control). Flasks were incubated at 37°C 220 rpm for 6 hours, and metabolomics analysis was conducted on samples along with control bacteria. The procedure involved transferring 50 ml of culture into Falcon tubes for metabolomics and proteomics experiments.

3.5.7 Metabolites Extraction

Metabolites were extracted from washed cell pellets using liquid-phase extraction (LPE). The cell pellets were suspended in ice-cold 80% MeOH based on their dry weight (10 ml of MeOH per 1 g of pellet) to ensure matching metabolite concentrations. This was followed by 20 minutes of sonication in an Ultrasonic Cleaner (USC-T, VWR International GmbH, Darmstadt, Germany), after which the samples were centrifuged at 4000 x g for 10 minutes at 4°C. The supernatant was collected, transferred to glass vials, and dried overnight in a speed vacuum. The dried samples were solubilized relative to their dry weight with 50% Methanol.

3.5.8 Proteins Extraction

The cell pellets were washed with PBS and centrifuged at 4000 x g for 20 minutes at 4°C, and the cells were then resuspended in 2 ml of lysis buffer along with a protease inhibitor at a concentration of 10 µl per ml of lysis buffer. After that, ultrasonication was performed in seven 30-second cycles with 1-minute pauses between cycles. After ultrasonication, the samples were centrifuged at 12000 x g for 20 minutes to remove cell debris. To assess the protein concentrations, the Bradford assay was performed, and a standard curve was prepared using BSA stock solutions ranging from 0.1 mg/ml to 1 mg/ml. The samples were diluted 1:10, and 4 µl of each sample was mixed with 196 µl of Bradford dye in a 96 -well plate. The plate was measured in the dark for 5 minutes in the dark before measuring absorbance at 595 nm using a spectrophotometer. PBS was used for dilution of the concentration if it is higher than 0.5 mg/ml. 100 µl of 8 M urea and 10µl of 100 mM TCEP were added to the samples to reduce the proteins, followed by vortexing and incubation for 20 min at room temperature. 21 ul of 500 mM IAA was used for alkylation, followed by 15 min incubation in the dark. The digestion buffer consisting of 230 µl of 0.5 mM TRIS, 4.6 µl of 100 mM CaCl2, and 4 µl of trypsin (0.5 µg/ml) was added to the samples, which were incubated overnight at 37 °C for at least 14 hours to complete the digestion. After digestion, the samples were centrifuged at 12,000 x g for 20 minutes to remove any remaining particles. The supernatant was then collected for C18 column cleanup. The columns were

cleaned with 1 ml of 100% methanol, then activated with 1 ml of 5% methanol, and washed twice with water containing 0.5% formic acid and 5% acetonitrile. The peptides were poured into the column and allowed to sit for 10 min before being centrifuged at 760x g for 10-20 min. The column was washed three times with water containing 0.5% formic acid and 5% acetonitrile. Finally, peptides were eluted using 1.5 ml of 70% acetonitrile in glass vials. (Eppendorf, Fisher Scientific GmBH, Schwerte, Germany.) Finally, the elutes were evaporated in a SpeedVac and dissolved in 50 µL of water containing 0.5% formic acid and 5% acetonitrile for subsequent analysis.

3.5.9 Metabolomics UHPLC-MS/MS Analysis

Untargeted metabolomic profiling was performed using ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC). Samples (5 µL) were analyzed on a UHPLC system (Thermo Scientific, Bremen, Germany) coupled to a Q-Exactive HF hybrid quadrupole–Orbitrap mass spectrometer (Thermo Scientific). Chromatographic separation was achieved on a C18 core-shell column (Kinetex, 50 × 1.0 mm, 1.7 µm, 100 Å; Phenomenex) at a flow rate of 0.15 mL min^{-1}. Mobile phases consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The linear gradient was 5% B (0–4 min), 5–99% B (4–5 min), followed by a washout at 99% B (5–7 min) and re-equilibration at 5% B for 3 min.

Ionization was performed using heated electrospray ionization (HESI) in positive mode with the following parameters: spray voltage 3.5 kV, sheath gas 30 L min^{-1}, auxiliary gas 10 L min^{-1}, sweep gas 2 L min^{-1}, capillary temperature 250 °C, auxiliary gas temperature 200 °C, and S-lens RF level 50. Full MS scans were acquired over an m/z range of 150–2000 at a resolution of 45,000 (at m/z 200), with one microscan, a maximum injection time of 100 ms, and an AGC target of 1×10^6. Data-dependent MS/MS (DDA) acquisition targeted the five most intense precursor ions per duty cycle, acquired at a resolution of 15,000 with one microscan, a maximum injection time of 100 ms, and an AGC target of 5×10^5. MS/MS scans were triggered at peak apex (2–15 s), with dynamic exclusion set

to 5 s; unassigned charge states were excluded, and isotope peaks were included.

3.5.10 Proteomic UHPLC–MS/MS analysis

Proteomic analyses were performed using µ-flow UHPLC–MS/MS on the same UHPLC platform coupled to a Q-Exactive quadrupole–Orbitrap mass spectrometer (Thermo Scientific). Peptide separation was achieved on a C18 column (Acclaim PepMap RSLC, 1 mm × 15 cm, 2 µm, 100 Å) at a flow rate of 100 µl min^{-1}, using the same mobile phases as above. The gradient consisted of 5% B (0–5 min), followed by a washout to 99% B (5–7 min), re-equilibration to 99% B (5–7 min), and re-equilibration to 5% B for 3 min.

Full MS scans were acquired in positive mode over an m/z range of 150–1500 at a resolution of 120,000, with one microscan, a maximum injection time of 100 ms, and an AGC target of 1×10^6. MS/MS acquisition was performed in DDA mode targeting the five most abundant precursors per cycle, acquired at a resolution of 15,000 with one microscan, a maximum injection time of 50 ms, and an AGC target of 5×10^5. MS/MS scans were triggered at the chromatographic peak apex (2–15 s), with dynamic exclusion set to 5 s; isotope peaks were excluded from precursor selection.

The raw datasets were processed by converting them into .mzXML files using MSConvert (Chambers, et al. 2012). Further analysis and annotation of the MS/MS spectra for the metabolomics data were performed using Global Natural Products Social Molecular Networking (GNPS) (Wang, et al. 2016). Additionally, detected spectra were annotated with the in-silico application SIRIUS, utilizing the tools ZODIAC and CANOPUS to predict the molecular formulas of compounds not found in databases (Dührkop, et al. 2019; Ludwig, et al. 2020; Dührkop, et al. 2021).

For the proteomic data, raw datasets were similarly converted into. mzXML files using MSConvert. Further analysis and annotation of the MS/MS spectra were carried out using FragPipe, along with its built-in tool MSFragger (Version 3.8)

(Kong, et al. 2017; da Veiga Leprevost, et al. 2020; Geiszler, et al. 2021). Statistical analysis was carried out on the blank removed and imputed dataset using the Hitchhiker's Guide to Statistical Analysis of Feature-based Molecular Networks https://github.com/Functional-Metabolomics-Lab/FBMN-STATS.

3.6 Author Contributions

ST, KS and DP conceptualized and planned the research. ST performed the culturing, and adaptation experiments. ST and TC performed the MIC and bacterial phenotyping experiments. ST and PS performed the metabolomics and proteomics experiments. ST, KS, PS, TC, and DP analyzed the data. AH and DP supervised the study. ST wrote the manuscript. All authors edited and approved the manuscript.

3.7 Acknowledgement

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3.8 Use of Artificial Intelligence

ChatGPT 5 was used for proofreading and improving the clarity of language in the manuscript. No generative artificial intelligence (e.g. large language models was used to generate original content of the manuscript. The authors take full responsibility for the content of the manuscript

Chapter 4

Exploring the association between proteome-wide off target effects and the rate of resistance evolution in *E. coli*

4.1 Introduction

Antibiotic resistance is a major global health challenge and one of the leading causes of death worldwide. In 2019, antimicrobial resistance was directly responsible for an estimated 1.27 million deaths globally (Murray, et al. 2022). This escalating crisis is mainly driven by the rapid adaptation of bacteria under antimicrobial selective pressure. Experimental evolution has emerged as a powerful framework to investigate these adaptive processes in real time, enabling the systematic characterization of resistance trajectories and the identification of factors that govern the rate and extent of adaptation (Toprak, et al. 2012; Hughes and Andersson 2017).

The evolution of resistance is shaped by the fitness costs of resistance mutations, compensatory mutations, and the selective pressure imposed by that antibiotic (Melnyk, et al. 2015; Chigozie, et al. 2026). Evolution studies in *E.coli* have demonstrated that resistance development is highly variable across antibiotics, with some compounds permitting rapid evolution of high-level resistance, whereas others produce slower or more constrained adaptive responses (Maeda, et al. 2020; Iwasawa, et al. 2022).

In chapter 3, we demonstrated that *E. coli* evolved under fosfomycin and other xenobiotics exhibit context-dependent resistance, accompanied by broad proteomic and metabolic remodeling. Those findings suggest that antibiotic exposure has broader impact on the metabolome and proteome of bacteria. Recently, fosfomycin, an antibiotic discovered in the 1970s, has gained new

attention due to its broad-spectrum activity against resistant pathogens (Aghamali, et al. 2019). Fosfomycin inhibits UDP-N-acetylglucosamine enol pyruvyl transferase (MuA), which catalyzes the first cytoplasmic step of bacterial cell wall biosynthesis (Borisova, et al. 2014; Aghamali, et al. 2019).

However, advances in proteome-wide interaction studies, increasingly challenge the traditional one drug one target drug paradigms of antibiotic activity (Kabir and Muth 2022).

Although antibiotics are designed to engage a specific essential target, it is now well established that most small-molecule compounds interact with multiple proteins within the cell, a phenomenon called drug promiscuity or off-target engagements (Xie, et al. 2011; Chartier, et al. 2017).

We propose that the extent of this off-target engagement is one of the key determinants of resistance evolution rate. Broad off target perturbations may constrain bacterial adaptation because single resistance mutations may be insufficient to compensate for widespread drug induced physiological disruption and may impose pleiotropic fitness costs that limit further evolution (Kohanski, et al. 2010; Chevereau, et al. 2015). In contrast, antibiotics with limited number of targets may permit more rapid resistance evolution because mutation affecting single target or pathway can substantially reduce susceptibility such as DNA gyrase or penicillin binding proteins (Hooper and Jacoby 2015; Munita and Arias 2016). We therefore hypothesize that antibiotics with broader off-target protein interactions will exhibit significantly slower rates of resistance evolution than those with more restricted target specificity.

To test this hypothesis, we subjected *E. coli* to laboratory evolution under subinhibitory exposure to twelve antibiotics of different classes over sixteen days, measuring minimal inhibitory concentrations (MICs) every fourth day to quantify resistance progression. We observe variability in the rate and magnitude of resistance evolution across compounds. To map the off-target interaction of each antibiotic, we will employ thermal proteome profiling combined with liquid chromatography tandem mass spectrometry (LC-MS/MS). In this approach,

antibiotic-treated bacterial lysates are heated across a temperature gradient, and proteins whose thermal stability is shifted by drug binding are identified by quantitative mass spectrometry (Savitski, et al. 2014; Franken, et al. 2015; Mateus, et al. 2020). This unbiased proteome-scale method enables simultaneous detection of primary and off-target interactions without prior knowledge of binding sites.

By integrating resistance evolution kinetics with proteome-wide drug target interactions, this study aims to establish a mechanistic framework linking antibiotics' off-target engagement to evolutionary outcomes. If validated, this framework could help explain why certain antibiotics are more prone to rapid resistance evolution and may provide insights relevant to antibiotic stewardship and therapeutic design and combination treatment strategies that impose stronger evolutionary constraints on bacterial adaptation.

4.2 Hypothesis

Based on these considerations, we propose the following hypothesis. Antibiotics that interact with a wider range of proteins across the bacterial proteome (off-target effects) disrupt multiple core cellular processes. This creates stronger constraints on bacterial adaptation, thereby slowing the evolution of resistance. In contrast, antibiotics with more limited interaction profiles allow bacteria to adapt more easily and develop resistance more rapidly.

To test this hypothesis, we monitored resistance evolution using laboratory evolution experiments. To test the second part of the hypothesis, we are planning to analyze the off-target effect using Thermal Proteome Profiling.

4.3 Methods

4.3.1 Minimum Inhibitory Concentration

The first step in performing adaptation experiments was to evaluate the minimum inhibitory concentrations for the antibiotics listed in Table 1. To determine the minimal inhibitory concentration (MIC) necessary for our adaptation experiments,

we performed MIC assays of 12 antibiotics from different classes (Table 1). Stock solutions of the antibiotics were prepared and filtered with a 0.22 µm syringe filter. Using a 96-well plate, Mueller-Hinton broth was added to all wells, with compound stock solutions added to designated wells and subjected to two-fold serial dilutions. Bacterial inoculum was added to appropriate wells, with columns without bacteria serving as contamination indicators, while wells with just bacteria without antibiotic pressure were the growth control. Plates were sealed to prevent evaporation and incubated at 37°C for 16-24 hours. MICs were determined as the lowest compound concentrations inhibiting bacterial growth, aiding in identifying optimal concentrations for subsequent evolutionary experiments. This methodology aligns with established literature practices, ensuring consistency and reliability in our experimental approach.

4.3.2 Lab-based evolution experiments under xenobiotic stress of individual strains

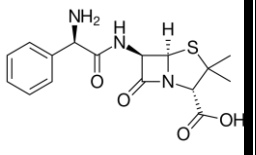
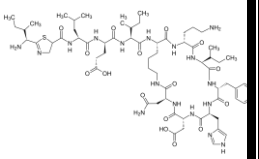
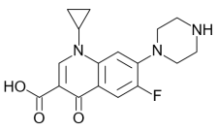
Adaptation experiments were performed following the reported methods with minor modifications (El Shazely, et al. 2020). The experiments were designed to assess bacterial adaptation under prolonged exposure to sublethal compound concentrations. *E. coli* was serially passaged for 16 days in deep-well plates containing either antibiotic or no compound (control), using concentrations below the minimum inhibitory concentration (MIC).

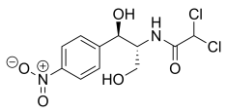
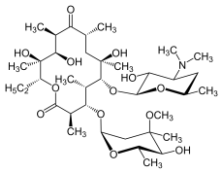
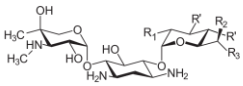
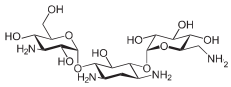
Wells in the first row were filled with Mueller–Hinton broth supplemented with the appropriate sublethal compound concentration and inoculated with bacterial pre-culture approximately 5 × 10^5 CFU/ml. Sublethal concentrations were determined by MIC assays conducted before the experiment and used as follows: Ciprofloxacin (0.001 µg/ml), Novobiocin (100 ug/ml), Rifampicin (0.5 ug/ml), Gentamicin (0.2 µg/ml), Ampicillin (0.5 µg/ml), Spectinomycin (1 µg/ml), Erythromycin (4 µg/ml), Chloramphenicol (0.5 µg/ml), Kanamycin (0.2 ug/ml), Polymyxin B (0.1 µg/ml), Vancomycin (32 µg/ml), Bacitracin (128 µg/ml), and Lincomycin (32 µg/ml). Cultures were incubated at 37 °C with shaking at 150 rpm. After 24 h, 100 µl of the culture was transferred into fresh medium containing the

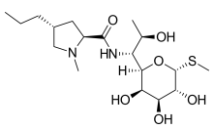
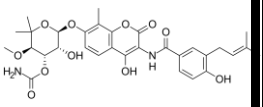
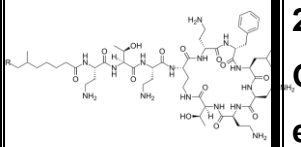
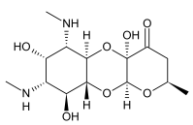
same compound concentration in the subsequent row. This daily transfer was repeated for 16 consecutive days.

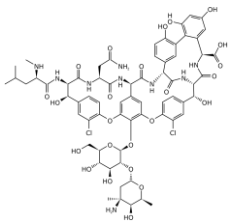
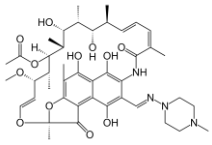
MIC values were determined every fourth day throughout the experiment to monitor changes in susceptibility. All conditions were performed in three independent biological replicates. Triplicate cultures grown in the absence of xenobiotics were used as untreated controls.

Table 1: List of Antibiotics and their mode of action and structures.

Antibiotics	Class	MOA	Structure	References
Ampicillin	β-Lactam	Inhibits PBPs → cell wall synthesis		(Walsh 2000; Bereda 2022)
Bacitracin	Polypeptide	Blocks bactoprenol recycling → cell wall synthesis		(Smith and Weinberg 1962; Dowling 2024)
Ciprofloxacin	Fluoroquinolone	Inhibits DNA gyrase/topo IV		(Drlica and Zhao 1997; Walsh 2000;

				Xue, et al. 2025)
Chloramphenicol	**Amphenicol**	**Inhibits 50S peptidyl transferase**		**(Xue, et al. 2025)**
Erythromycin	**Macrolide**	**Binds 50S ribosome → blocks elongation**		**(Dunkle, et al. 2010)**
Gentamicin	**Aminoglycoside**	**Binds 30S ribosome → mistranslation**		**(Krause, et al. 2016)**
Kanamycin	**Aminoglycoside**	**Binds 30S ribosome → translation errors**		**(Krause, et al. 2016)**

Lincomycin	**Lincosamide**	**Binds 50S ribosome → inhibits peptide bond formation**		**(Wilson 2014)**
Novobiocin	**Aminocoumarin**	**Inhibits DNA gyrase (GyrB ATPase)**		**(Collin, et al. 2011)**
Polymyxin B	**Polymyxin**	**Disrupts outer membrane by binding LPS**		**(Poirel, et al. 2017; Goode, et al. 2021)**
Spectinomycin	**Aminocyclitol**	**Binds 30S ribosome → inhibits translocation**		**(Borovinskaya, et al. 2007)**

Vancomycin	**Glycopeptide**	**Binds D-Ala-D-Ala → inhibits cell wall synthesis**		**(Walsh 2000; Gardete and Tomasz 2014)**
Rifampicin	**Rifamycin/ ansamycin**	**Inhibits DNA-dependent RNA polymerase → blocks transcription**		**(Campbell, et al. 2001; Nagarajan 2024)**

4.4 Results

4.4.1 Lab-based antibiotic adaptation of *E. coli K-12*

To investigate adaptive responses under sublethal antibiotic exposure, *E. coli* evolved for 16 days in the presence of 13 antibiotics representative of different classes MIC was performed every fourth day to observe the change in MIC during evolution (0, 4, 8, 12, 16 days). Control populations maintained without antibiotic pressure showed no significant change in susceptibility over time.

Overall, evolved strains exhibited a significant increase in MIC during 16 days of exposure, although the magnitude and dynamics of adaptation varied among different antibiotics (Figure 1).

Several antibiotics targeting protein synthesis showed a significant increase in MIC over the adaptation period. For example, *E. coli* under spectinomycin

exposure displayed an increase from 2 µg/ml to 64 µg/ml on day 12, which remained stable through day 16. Similarly, *E. coli* under kanamycin exposure showed a strong increase after day 8th, ranging between 32-64 µg/ml. Chloramphenicol also displayed a progressive increase reaching 32 µ/ml. Rifampicin evolved strain exhibited an increase in resistance after day 4th of exposure and increased from 0.5 µg/ml to 64 µg/ml on day 12th. A pronounced increase was observed for lincomycin, though it is an antibiotic for gram-positive bacteria and not effective against gram-negative bacteria. Its exposure still increases the resistance, and the MIC reached up to 1024 µg/ml

In contrast, gentamicin showed only a modest increase in MIC, suggesting more constrained adaptation compared to other translation inhibitors.

Antibiotic targeting cell wall synthesis exhibited heterogeneous adaptive responses. Ampicillin showed an early increase during the first 4 days, followed by moderate fluctuations over 16 days. Bacitracin and vancomycin are not effective against gram-negative bacteria, but over the span of 16 days, they still showed an increase in resistance from an initial 512 µg/ml to 2000 µg/ml for bacitracin and from 64 µg/ml to 512 µg/ml for vancomycin.

For polymyxin B, which disrupts the outer membrane, adaptation occurred gradually, ranging from 0.025 µg/ml to 32 µg/ml by day 12, after which it remained relatively stable.

Antibiotics targeting DNA processes showed distinct dynamics. Ciprofloxacin showed a steady increase across adaptation experiments, reaching approximately 0.5 µg/ml. In the case of novobiocin, the strain was quite resistant, but over the span of 16 days there was increase in MIC.

These results show that *E. coli* can adapt to antibiotic pressure, although the rate and magnitude of adaptation vary depending on the antibiotic. Some antibiotics induced a rapid increase of MIC within the first 8 days, while others exhibited slower and more gradual progress. To understand this complexity pattern, we have planned to do Thermal proteome profiling (TTP) to see the off-target effects

of these antibiotics. We hypothesize that bacteria take a longer time to adapt to the antibiotics that have more off-target effects, while the antibiotics that have fewer off-target effects, bacteria adapt to them quickly.

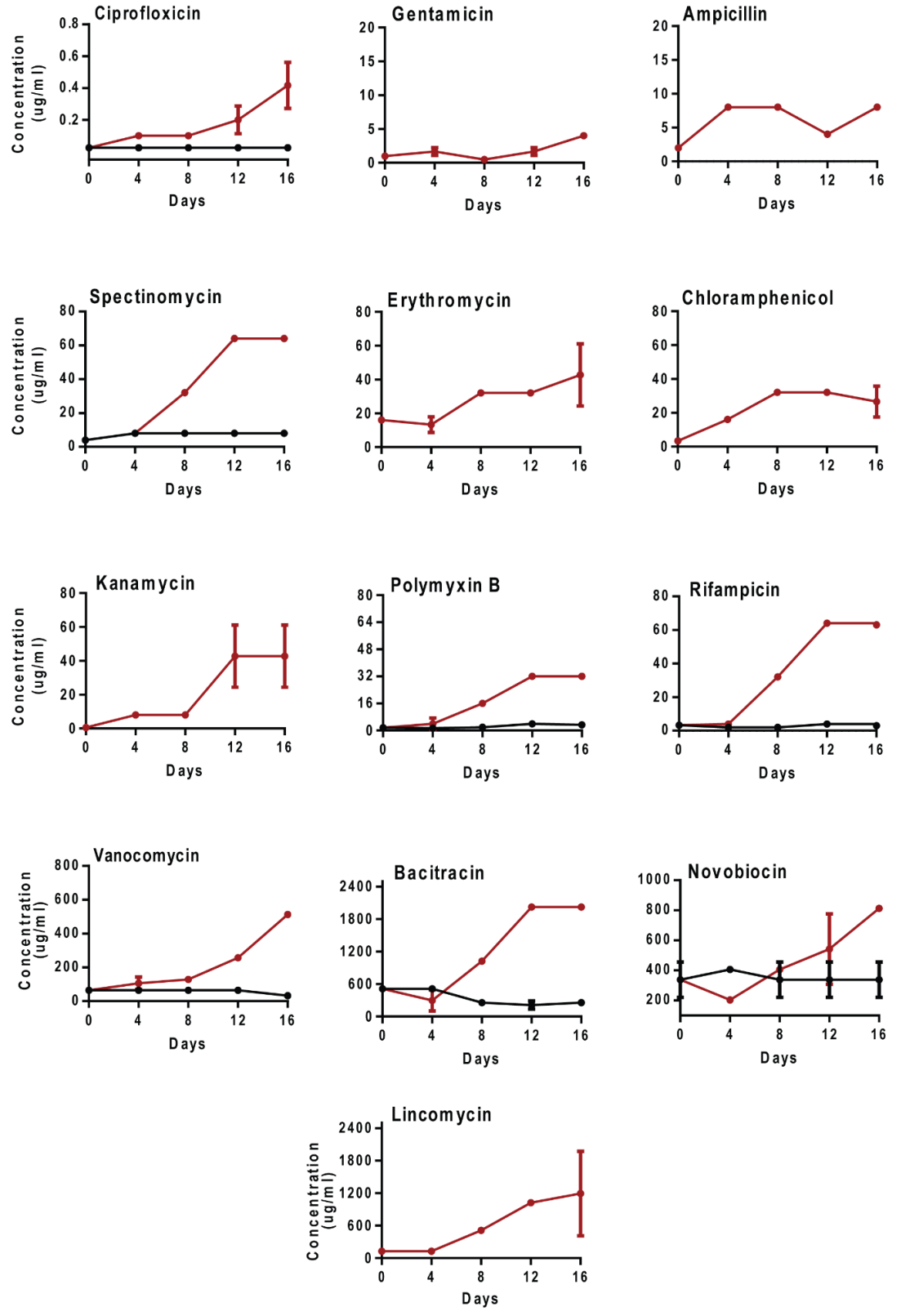

Ciprofloxicin
Gentamicin
Ampicillin
Spectinomycin
Erythromycin
Chloramphenicol
Kanamycin
Polymyxin B
Rifampicin
Vanocomycin
Bacitracin
Novobiocin
Lincomycin
Concentration (ug/ml)
Days
E.coli (K12) adapted with antibiotics
Control

Figure 1: Evolution of *E. coli K-12* against different classes of antibiotics over 16 days.

E. coli K-12 was exposed to different classes of antibiotics. MIC was taken every 4th day. These graphs showed the adaptation over 16 days against each antibiotic.

4.5 Discussion

The adaptation experiment showed that *E. coli* can rapidly increase MIC to several antibiotics under continuous selection pressure, although the magnitude and adaptation varied considerably across different compounds. Such variability has been widely reported in experimental evolution studies, reflecting the fact that the occurrence of resistance depends not only on the existence of resistance mutations but also on how easily these mutations can arise and spread in bacterial populations. In addition, resistance mutations often impose fitness costs on bacteria, which can influence the progression of antibiotic resistance (Andersson and Hughes 2010). Although such costs may reduce bacterial fitness in the absence of antibiotics, compensatory mutations can arise that mitigate these disadvantages. Consequently, fitness costs and compensatory evolutions play an important role in shaping the rate, trajectory, and stability of antibiotic resistance (Andersson and Hughes 2010).

Polymyxin B, which disrupts the bacterial outer membrane by interacting with LPS, exhibited a gradual increase in MIC during the experiment. Resistance to polymyxin B has been documented previously associated with modification of lipid A that reduces antibiotic binding to the outer membrane (Munoz-Escudero, et al. 2023). A gradual increase observed here suggests that adaptive changes may emerge more slowly compared to antibiotics targeting intracellular mechanisms.

Vancomycin, bacitracin, and lincomycin are generally poorly active against Gram-negative bacteria due to the presence of the outer membrane barrier.

Antibiotics targeting intracellular processes, particularly protein synthesis and DNA replication, showed a pronounced increase in resistance during evolution experiments. For example, kanamycin and chloramphenicol displayed a progressive increase in MIC over the course of the experiment.

In contrast, gentamicin showed only a modest increase in MIC over the course of experiment. Gentamicin evolved strain exhibited impaired growth relative to control suggesting that the initial gentamicin exposure may have impaired overall fitness. This growth impairment may have limited the extent of resistance development (Chowdhury and Findlay 2023). Resistance to these compounds can arise through several mechanisms, including enzymatic modification of the antibiotic, Target protection, change in drug uptake or efflux that reduces intracellular antibiotic concentration and mutation in ribosomal components (Schwarz, et al. 2004; Hollenbeck and Rice 2012; Wilson 2014; Darby, et al. 2023). As multiple pathways can induce susceptibility to these antibiotics, adaptation can often occur relatively rapidly under sustained selection pressure.

Similarly, antibiotics targeting DNA replication exhibited clear adaptive responses. Ciprofloxacin, a fluoroquinolone antibiotic, inhibits bacterial DNA gyrase and topoisomerase IV, an enzyme essential for DNA replication and chromosome segregation (Drlica and Zhao 1997). Resistance to fluoroquinolones frequently arises due to target mutation (mutation in gyrA and parC genes), porin loss, membrane alteration, and efflux (Redgrave, et al. 2014). Novobiocin, an aminocoumarin antibiotic, inhibits the ATPase activity of DNA gyrase, thereby preventing DNA supercoiling and replication (May, et al. 2017). Its activity against *E. coli* is limited by the permeability of the outer membrane barrier. However, prolonged exposure may still allow sufficient intracellular antibiotic accumulation to exert selective pressure, potentially promoting adaptive changes affecting DNA gyrase function or membrane permeability (May, et al. 2017).

In contrast to novobiocin, which may still enter Gram-negative cells at lower levels, vancomycin, bacitracin, and lincomycin are large molecules whose entry into *E. coli* is strongly restricted by the outer membrane permeability barrier

(Nikaido 2003). Bacitracin and vancomycin inhibit cell wall synthesis, while lincomycin inhibits protein synthesis by binding 50S ribosomal subunit (Wilson 2014; Buijs, et al. 2024; Tabarzad, et al. 2025). Continuous exposure to these compounds may influence bacterial membrane properties or regulatory pathways that affect antibiotic entry and accumulation, thereby contributing to a gradual increase in MIC during the experiment (Munita and Arias 2016).

Resistance mechanisms impose physiological burdens on bacteria, such as reduced growth rate, altered metabolism, or impaired cellular processes (Andersson and Hughes 2010) .The evolutionary success of resistance mutations depends not only on their ability to confer resistance but also on their impact on overall cellular fitness. Experimental evolution studies have shown that compensatory mutations can also arise to reduce these fitness costs and further shape the adaptive pathways of resistant bacteria (Andersson and Hughes 2010).

Recent laboratory evolution studies have further shown that bacteria may follow multiple adaptive pathways shaped by genetic limitation and physiological trade-offs under antimicrobial stress (Maeda, et al. 2020; Pinheiro, et al. 2021).

These adaptation experiments over 16 days under antibiotic exposure provide a phenotypic overview of how *E. coli* adapts to antibiotics. However, antibiotics can also interact with broader cellular processes beyond their primary molecular targets, affecting multiple physiological pathways (Meylan, et al. 2017)´. Our Future work is currently underway to investigate these potential off-target effects using proteome-wide approaches including Thermal proteome profiling (TPP) through high-throughput proteome Integral solubility Alteration (PISA), which enables detection of drug-protein interactions by quantifying changes in protein thermal stability across the proteome (Savitski, et al. 2014; Gaetani, et al. 2019). Characterization of these cellular interactions may provide insights into off-target mechanisms underlying bacterial adaptation and could further help identify potential synergistic interactions between different antibiotics that enhance antimicrobial efficacy.

4.6 Future Directions

The present evolution experiments provide a quantitative framework for comparing the patterns of antibiotic adaptation across compounds with distinct cellular targets and physicochemical properties. Ongoing work is focus on integrating PISA based thermal proteome profiling with laboratory evolution data to investigate how antibiotic target breadth influences resistance evolution (Savitski, et al. 2014; Gaetani, et al. 2019; Van Vranken, et al. 2021). We hypothesized that antibiotics interacting with a broader range of cellular proteins along with their primary targets may exhibit greater resilience to resistance development. Broader cellular perturbation may reduce the accessibility of adaptive mutational pathways accessibility of adaptive mutational pathways and potentially slow down resistance evolution under sustained selective pressure.

To evaluate this hypothesis, evolutionary stability defined as the rate of change in MIC during laboratory evolution experiment could be quantitatively correlated with the number of antibiotic-protein interactions identified through proteome wide profiling. This framework would enable a direct comparison between phenotypic adaptation and proteome-level interactions.

In this context, antibiotics with highly specific mode of action targeting limited number of proteins may permit more rapid adaptation, whereas antibiotics with broader cellular interactions may constrain resistance development. Comparing these interaction profiles may therefore help clarify how target breadth shapes the rate of resistance evolution.

Extending this framework to antibiotic combinations may help identify synergistic interactions that improve antimicrobial efficacy and reduce the likelihood of resistance. A conceptual model illustrating the proposed relationship between antibiotics' target breadth and the rate of resistance evolution is presented in

Figure 3.

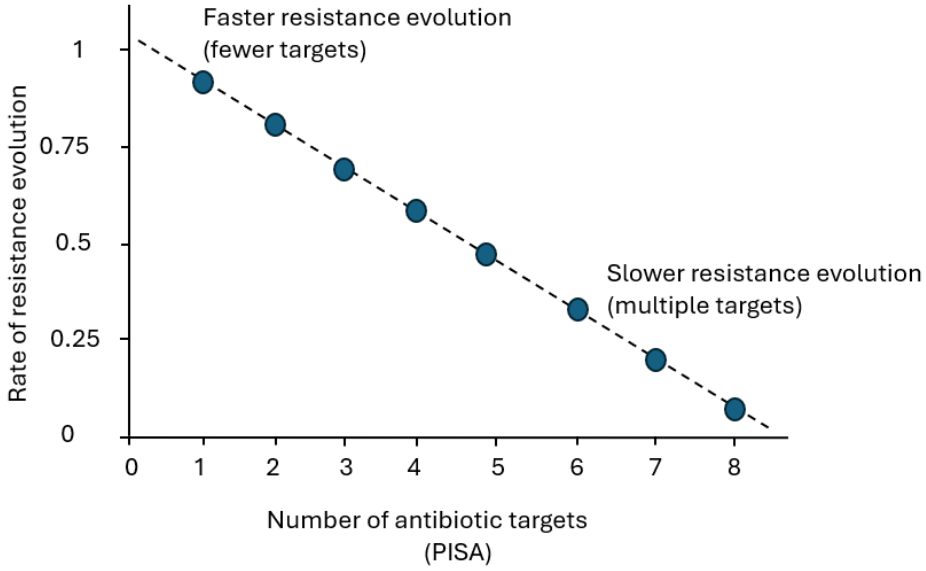


Figure *3. Conceptual graph showing the relationship between antibiotic target breadth and resistance evolution.*

Resistance evolution is represented as the rate MIC increase during laboratory evolution experiment. Each point represents an antibiotic plotted against the number of cellular protein interactions identified through proteome wide profiling (PISA). The dashed line illustrates the proposed inverse relationship where antibiotics interacting with fewer cellular off targets permit rapid adaptation whereas broader interaction profiles may constrain adaptive pathways and slow resistance evolution.

Chapter 5

General Discussion

An emerging challenge in antimicrobial resistance research is understanding how bacterial adaptation to xenobiotics may contribute to resistance evolution. This PhD work investigated bacterial adaptation through *E. coli* experimental evolution under diverse chemical stressors, including antibiotics, herbicides Asualm and Glyphosate, and fungicidal scaffold imidazole. This work explores how structurally related and chemically diverse compounds influence bacterial adaptation and contribute to cross tolerance phenotypes. The experimental work builds upon the conceptual framework outlined in the review chapter, which highlights the potential role of xenobiotics in shaping resistance evolution (Kurenbach, et al. 2015; Noto Guillen, et al. 2024).

In this work, laboratory-based experimental evolution of *E. coli* under xenobiotic stress was performed. Fosfomycin was included as an antibiotic condition within the same experimental framework. LC-MS/MS-based untargeted proteomics and metabolomics analysis were integrated with phenotypical assays, including minimum inhibitory concentration, cross-tolerance growth curves, and biofilm quantification to characterize adaptive responses at both the molecular and phenotypic levels.

Across the xenobiotic evolution experiment, a consistent pattern was observed in which adaptation to xenobiotics was associated with widespread proteomic and metabolomic remodeling. These responses reflect broader cellular adaptation rather than confined target specific effects. The untargeted nature of omics analyses enabled the detection of coordinated changes across interconnected cellular pathways providing insights into broader physiological adjustments associated with adaptation (Fiehn 2002; Hasin, et al. 2017).

Chapter 3, therefore supports the broader thesis concept that bacterial adaptation to chemical stressors, including xenobiotics, is shaped by interconnected cellular processes associated with widespread changes in protein and metabolite profiles with potential implications for antibiotic tolerance. Chapter 4. extends this investigation to experimental evolution of different classes of antibiotics. Further characterization of off target interaction using thermal protein profiling is currently ongoing and remains beyond the scope of this thesis. Together, these efforts provide a broader framework for understanding how both antibiotic and xenobiotic exposures shape bacterial adaptation.

5.1 Phenotypic adaptation and cross tolerance to Xenobiotics and antibiotics.

Phenotypic adaptation in *E. coli* evolved strains under xenobiotic pressure was consistently modest. Tolerance has been conceptually distinguished from resistance on the basis that it reduces susceptibility without requiring target modification or the acquisition of specific resistance determinants (Brauner, et al. 2016) and is often associated with broad physiological stress responses rather than discrete genetic changes (Poole 2012). The MIC profiles observed here are consistent with this distinction. Across all xenobiotic-evolved strains, MIC increases were generally limited to approximately two-fold, suggesting that the adaptations during these experiments primarily reflected enhanced tolerance rather than strong target-mediated resistance. Fosfomycin evolved strains represented a clear exception, exhibiting pronounced increases in MIC consistent with strong directional selection under direct antibiotic pressure. This strain therefore provided a useful comparative reference for modest xenobiotic-driven phenotypes observed throughout this study.

Cross-tolerance profiling further extended the phenotypic characterization beyond the evolving compound. The resulting patterns varied considerably depending on both the compound tested and the selective condition under which strains evolved. Broad tolerance to Imidazole was observed across all evolved strains, suggesting a common adaptive response to diverse chemical stresses

rather than an effect specific to Imidazole exposure. This shared tolerance may be associated with general changes in cell envelope properties commonly altered during adaptation, though this interpretation remains inferential in the absence of direct experimental validation. In contrast, cross-tolerance to Glyphosate and Asulam was largely restricted to Fosfomycin-evolved strains, indicating a more compound-specific adaptation.

Antibiotic cross tolerance also followed a compound specific pattern. Cross-tolerance to kanamycin was detected in strains evolved under Asulam, Imidazole, and Fosfomycin, but not in Glyphosate evolved strain. Aminoglycoside uptake is known to depend on membrane potential and energy-dependent transport systems (Lang, et al. 2023; Lang, et al. 2025) while bactericidal activity has been associated with reactive oxygen species production (Lang, et al. 2023). The observed Kanamycin cross-tolerance is possibly linked to membrane and redox-related proteomic changes identified in the evolved strains. These cross-tolerance patterns indicate that adaptation under xenobiotic pressure can influence responses to structurally unrelated compounds including clinically relevant antibiotics. However cross tolerance phenotypes remained compound specific rather than conferring broad tolerance across all tested compounds. Biofilm formation also varied in a compound-specific manner. Fosfomycin and Asulam evolved strains exhibited enhanced biofilm formation relative to the wild type. In contrast Imidazole evolved strains showed reduced biofilm formation and Glyphosate evolved strains displayed no significant change.

5.2 Molecular basis of Xenobiotic adaptation

The proteomics and metabolomics data presented in Chapter 3 reveal that xenobiotic adaptation to *E. coli* involves both compound-specific changes linked to primary target pathways and broader shared responses associated with redox homeostasis and membrane remodeling.

The upregulation in FolC in the Asulam evolved strain represents a target proximal adaptive response, given its sulfonamide related structure and similarity to para-aminobenzoic acid (Pallett ; Venkatesan, et al. 2023; 2024). As FolC

functions downstream in folate metabolism, its increased abundance suggests compensatory flux adjustment within this pathway (Green and Matthews 2007). Similarly, *MurA* upregulation in the Fosfomycin evolved strain is consistent with a well-characterized resistance mechanism that reduces Fosfomycin binding affinity (Horii, et al. 1999).

Redox and Glutathione management emerged as a common adaptive response across evolved strains. Elevated glutathione pools, S-lactoylglutathione, and FAD-linked cofactors in Fosfomycin evolved strain together with Hmp upregulation and ggt downregulation, indicate coordinated regulation of oxidative and nitrosative stress. Increased S-lactoylglutathione abundance further suggests activation of glutathione-dependent detoxification pathways, potentially reflecting enhanced processing of reactive carbonyl intermediates under antibiotic pressure (Thornalley 1990; Jain, et al. 2018; Kralj, et al. 2022; Kuczyńska-Wiśnik, et al. 2025). Similar redox associated responses were also observed in Imidazole and Asulam evolved strains. GsiB, the periplasmic binding protein of the glutathione ABC import system responsible for glutathione uptake in *E. coli* was upregulated under Imidazole adaptation, suggesting altered glutathione flux (Poole 2012). Asulam evolved strain additionally exhibited upregulation of glutathione and oxidative stress associated proteins YqjG and YaaA , further supporting the involvement of redox-associated adaptation across xenobiotic and antibiotic exposure.

Membrane remodeling represented one of the most consistent adaptive responses across all evolved strains and it is particularly relevant given its broader relevance to antimicrobial susceptibility (Delcour 2009; Godlewska, et al. 2025). Changes in proteins associated with fatty acid synthesis (FabG), phospholipid turnover (GlpQ), phospholipid biosynthesis (PssA) and glycerol phosphate metabolism collectively reflect coordinated remodeling of membrane lipid homeostasis. These protein shifts together with alterations in phospholipids related metabolites collectively suggest active restructuring of envelope lipid composition under xenobiotic stress (Mitchell and Silhavy 2019).

Proteomic changes further indicated structural and functional adjustments of the cell envelope. Upregulation of LpoB, a membrane lipoprotein required for activation of peptidoglycan synthesis by PBP1B may reflect adaptation associated with maintenance of outer membrane integrity (Typas, et al. 2010). Concurrently increased level of TatA, a core component of the twin arginine translocation responsible for protein export to the cell envelope, suggests broader alteration in membrane associated processes (Palmer and Berks 2024).

Membrane composition is a critical determinant of passive drug permeability, efflux transporter function and outer membrane integrity (Delcour 2009; Mitchell and Silhavy 2019). Accordingly, membrane and redox associated changes observed across evolved strains may contribute to cross tolerance phenotypes.

Biofilm formation assays revealed both shared and condition-specific responses across evolved strains. Increased biofilm formation in Fosfomycin and Asulam-evolved strains was consistent with the upregulation of FimH and associated fimbrial components detected in the proteomics data, supporting enhanced surface attachment. Increased abundance of an indole-related metabolite feature in Asulam evolved strain may further reflect altered signaling associated with biofilm regulation and surface attachment (Cassat, et al. 2007; Zhang, et al. 2024).

In contrast, reduced biofilm formation in the imidazole evolved strain aligned with downregulation of FimA, together with changes in motility-associated proteins, including FliC, FlgM, suggesting altered transitions between motile and surface-attached states. Reduced biofilm formation is consistent with the reported antibiofilm activity of imidazole derivatives. (Miglani, et al. 2022; Aboagye, et al. 2023).

Collectively, these findings indicate that xenobiotic adaptation involved multiple physiological processes, including redox associated responses, membrane remodeling and biofilm formation.

5.3 Broader Implications and Integrative Synthesis

Environmental chemical exposures can influence antimicrobial resistance in ways that overlap with antibiotic responses (Noto Guillen, et al. 2024). The xenobiotic evolution experiments presented in Chapter 3 revealed molecular and phenotypic changes associated with stress adaptation including cross tolerance, membrane remodeling, redox associated responses and altered biofilm formation. Several of them overlapped with responses observed during antibiotic adaptation. These findings support the idea that xenobiotics may contribute to tolerance phenotypes. This aligns with the broader framework discussed in Chapter 2, highlighting xenobiotics as potential co-selective pressures in antimicrobial resistance (Murray, et al. 2024).

Enhanced biofilm forming capacity observed in Fosfomycin and Asualm evolved strains further supports this interpretation. Biofilm-associated populations are known to tolerate a wide range of antimicrobials (Liu, et al. 2024). They also create conditions that facilitate horizontal gene transfer, a key mechanism for the spread of resistance in both environmental and clinical settings (Abe, et al. 2020). Cross tolerance was not symmetric between Fosfomycin and xenobiotics evolved strains. Fosfomycin adaptation resulted in broader cross-protection, whereas xenobiotic adaptation produced comparatively limited phenotypes. This pattern is consistent with the idea that direct antibiotic selection remains the primary driver of strong resistance associated phenotypes whereas xenobiotic may act more as a modifying or co-selective influence.

In natural environments where multiple chemical stressors coexist, such responses may collectively shape antimicrobial resistance dynamics. This work contributes to the broader shift towards a more integrated understanding of how chemical exposures may influence antimicrobial resistance.

5.4 Limitations

Experiments were conducted in a single laboratory *E. coli K-12* strain under controlled monoculture conditions. While this provides a reproducible experimental system, it does not capture the genetic diversity, horizontal gene transfer or ecological complexity of natural microbial communities. The findings should therefore be interpreted as mechanistic observations generated under simplified conditions and extrapolation to environmental settings should be made cautiously.

Proteomics and metabolomics analyses identify candidate proteins, metabolites and pathways associated with adaptation and provide a mechanistic insight into observed phenotypes. However, functional validation is required to establish direct causal relationships. Interpretation involving proteins such as FabH, GlpQ, FolC and PssA together with glutathione associated pathway should therefore be considered inferential.

The relatively short duration of the evolution experiments limits the extent to which longer-term adaptive trajectories can be assessed. Incorporation of whole genome sequencing and extended evolution experiments would provide deeper insights into the genetic basis and stability of observed adaptations.

In addition, adaptation under single compound conditions does not fully reflect the complexity of real-world environments where microbes are simultaneously exposed to mixtures of xenobiotics, antibiotics and additional environmental stressors. Future studies incorporating combined or sequential chemical exposures would improve the ecological relevance of these findings.

5.5 Future Direction

The findings of this study highlight several directions for future investigations aimed at strengthening the mechanistic and ecological understanding of xenobiotic driven adaptation. Omics analyses identified candidate pathways and molecular targets for follow up investigation.

A key next step would be to move from association to causality. Proteomic and metabolomic analyses highlighted proteins and pathways associated with membrane remodeling, detoxification, oxidative stress responses and general stress adaptation, suggesting that these processes may contribute to the observed tolerance and cross tolerance phenotypes. Candidate proteins linked to membrane lipid remodeling including PssA, FabG, GlpQ as well as proteins linked to glutathione metabolism and oxidative stress responses Ggt, GsiB, YaaA, represent suitable targets for functional validation through gene deletion, complementation or overexpression.

Further studies should incorporate more direct approaches for identifying protein compound interactions, including thermal proteome profiling. Such approaches would help to distinguish direct compound interactions from indirect stress responses and are particularly relevant to the ongoing work described in Chapter 4.

Extending these approaches to environmental microbial communities would further improve ecological relevance and allow assessment of how ecological interactions including competition and horizontal gene transfer influence adaptation under xenobiotic exposure.

Related environmental work connected to the TREC expedition provides an additional direction for future investigation. This large-scale effort involves sampling coastal ecosystems across different regions of Europe, where environmental sampling is integrated with molecular analyses. Coastal water samples have already been collected and processed, and LC-MS/MS-based untargeted mass spectrometry is being used to profile xenobiotic compounds, including antibiotics. At the same time metagenomics analyses are underway to assess the abundance of antibiotic resistance genes. Although this work is still ongoing and not part of this thesis, it provides an opportunity to examine the potential association between xenobiotic abundance and antibiotic resistance genes in natural environments.

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Appendix

Chapter 3

Table 1 Supplementary: Differentially regulated significant Proteins in Asulam evolved *E. coli* strain

Upregulated Proteins in *E. coli (K12)* Evolved with Asulam		
Proteins	**Function**	**Function category**
PspE	Thiosulfate sulfurtransferase PspE	Sulfur transferase activity
YqjG	glutathionyl-hydroquinone reductase	Oxidoreductase activity
FucO	Lactaldehyde reductase	Carbohydrate metabolism
HemC	Hydroxymethylbilane synthase	Heme Biosynthesis process
FolC	Bifunctional folylpolyglutamate synthetase / dihydrofolate synthetase	Folate synthesis
SelD	Selenide, water dikinase	Amino acid biosynthesis

FabG	3-oxoacyl-[acyl-carrier-protein] reductase fatty acid biosynthetic process	Fatty acid biosynthesis
YaaA	DNA binding and peroxide stress response protein YaaA	Stress response
SlyA	DNA-binding transcriptional dual regulator	Stress response
CadA	Lysine decarboxylase 1	Secondary metabolite Biosynthesis
LpoB	outer membrane lipoprotein	Peptidoglycan Biosynthesis/Cell Structure
ZapG	Inner membrane protein	Cell Struture
FliC	Flagellar filament structural protein	Cell Struture
FimH	Cell adhesion/Biofilm formation	Cell structure
FimC	Type 1 fimbriae periplasmic chaperone	Cell Struture
YnfB	cell outer membrane	Cell Structure
FimH	Cell adhesion/Biofilm formation	Cell structure

FimA	Cell adhesion/Biofilm formation	Cell Struture
YfiH	Purine nucleoside phosphorylase	Catalytic activity
GlpQ	Glycerophosphodiester phosphodiesterase activity	Catalytic Activity/Transport
EttA	Energy-dependent translational throttle protein EttA	Regulatory Protein
AlsB	D-allose ABC transporter periplasmic binding protein	ABC Transport subunit
	Downregulated Proteins in *E.coli K12* Evolved with Asulam	
FadA	3-ketoacyl-CoA thiolase	Fatty acid metabolism
Flu	CP4-44 prophage; self-recognizing recognizing antigen 43 (Ag43) autotransporter	Cell structure

Table 2 Supplementary: upregulated significant Proteins in Imidazole evolved *E. coli* strain

Upregulated Proteins in *E.coli (K12)* Evolved with Imidazole		
Proteins	**Function**	**Function category**
CadA	Lysine decarboxylase 1	Secondary Metabolite Biosynthesis
FliC	Flagellar filament structural protein	Cell structure
FlgM	Anti-sigma factor for FliA	Cell structure
NagZ	β-*N*-acetylglucosaminidase	Peptidoglycan metabolic process
CheW	Chemotaxis protein	Cellular process
Tar	Methyl-accepting chemotaxis protein	Cellular process
YffB	Putative reductase YffB	Cellular anatomical entity
YajD	HNH nuclease family protein	Cellular anatomical entity
Gmk	Guanylate kinase	Kinase activity

PssA	Phosphatidylserine synthase	Phospholipid Biosynthesis
NuoC	NADH:quinone oxidoreductase subunit CD	ATP Synthesis Coupled Electron Transport
NuoG	NADH:quinone oxidoreductase subunit G	ATP Synthesis Coupled Electron Transport
TreC	Trehalose-6-phosphate hydrolase	Carbon metabolism
NusB	transcription antitermination protein	Ribosome Biogenesis
GsiB	Glutathione ABC transporter periplasmic binding protein	Cellular response to stress
MalE	maltose ABC transporter periplasmic binding protein	Cellular response to stress
CspI	Cold shock-like protein	Regulation of gene expression
GarR	tartronate semialdehyde reductase	Carbohydrate metabolism

Table 3 Supplementary: upregulated significant Proteins in Imidazole evolved *E. coli* strain

Downregulated Proteins in *E.coli (K12)* Evolved with Imidazole		
Proteins	**Function**	**Function category**
nanM	*N*-acetylneuraminate mutarotase	acetylneuraminate catabolic process
gadB	glutamate decarboxylase B	Glutamate metabolic process
hisG	ATP phosphoribosyltransferase	Transferase activity
hisC	histidinol-phosphate aminotransferase	Transferase activity
hdeB	periplasmic acid stress chaperone HdeB	Part of Cell envelope/Cellular process
hdeA	periplasmic acid stress chaperone HdeA	Part of Cell envelope/Cellular process
sufA	[Fe-S] cluster insertion protein SufA	Cellular process
fimA	Cell adhesion/Biofilm foration	Cell Struture
flu	CP4-44 prophage; self recognizing antigen 43 (Ag43) autotransporter	Cell structure

copA	soluble Cu+ chaperone	Tranport
ilvD	dihydroxy-acid dehydratase	Valine metabolic process
yhbO	protein/nucleic acid deglycase 2	Catalytic activity
puuC	γ-glutamyl-γ-aminobutyraldehyde dehydrogenase	Catalytic activity
yahK	aldehyde reductase, NADPH-dependent	Catalytic activity
nnr	NAD(P)HX epimerase / NAD(P)HX dehydratase	Catalytic activity

Table 3 Supplementary: Upregulated significant Proteins in Fosfomycin evolved *E. coli* strain

Upregulated Proteins in *E. coli* (K12) Evolved with Fosfomycin		
Proteins	**Functions**	**Function category**
ElaA	Acetyltransferase	Transferase activity
Eco	serine protease inhibitor ecotin	Stress response

Hmp	nitric oxide dioxygenase	Stress response
HscB	[Fe-S] cluster biosynthesis	Stress response
IbaG	acid stress protein	Stress response
LigA	DNA ligase	Stress response
MurA	UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase	Peptidoglycan Biosynthesis
Ndk	nucleoside diphosphate kinase	Purine biosynthesis
PurC	phosphoribosylaminoimidazole-succinocarboxamide synthase	Purine biosynthesis
PurK	5-(carboxyamino)imidazole ribonucleotide synthase	Purine biosynthesis
ParC	DNA topoisomerase IV subunit A	DNA Replication
PyrF	orotidine-5'-phosphate decarboxylase	Pyrimidine biosynthesis
MukB	chromosome partitioning protein	Cellular process

IscA	[Fe-S] cluster insertion protein	Cellular process
UspF	universal stress protein F	Cellular process/protection
RdgB	XTP diphosphatase activity	Cellular process/protection
NfsB	NAD(P)H-dependent nitroreductase	Cellular process/Drug resistance
NarP	DNA-binding transcriptional dual regulator	Regulatory Protein
Cra	DNA-binding transcriptional dual regulator	Regulatory Protein
Rnk	nucleoside diphosphate kinase regulator	Regulatory protein
AnsA	asparaginase activity|	Amino acid Metabolism
GlaH	glutarate dioxygenase	Amino acid Metabolism
Der	50S ribosomal subunit stability factor	Ribosome biogenesis
LepA	30S ribosomal subunit biogenesis factor	Ribosome biogenesis

RplD	50S ribosomal subunit protein L4	Cell structure/Regulatory protein
RplI	50S ribosomal subunit protein L9	Cell structure/Protein synthesis
RplV	50S ribosomal subunit protein L22	Cell structure/Protein synthesis
RplW	50S ribosomal subunit protein L23	Cell structure/Protein synthesis
TatA	twin arginine protein translocation system	Cell structure/transport
RimM	ribosome maturation factor	Cell structure
FimA	cell adhesion/ biofilm formation/ pilus	Cell structure
FimC	cell wall organization, protein folding chaperone	Cell structure
FimG	cell adhesion/ biofilm formation/ pilus	Cell structure
FimH	cell adhesion/ biofilm formation/ pilus	Cell structure

GutQ	D-arabinose 5-phosphate isomerase	Cell structure
RsmC	16S rRNA m2G1207 methyltransferase	RNA modification
MiaB	isopentenyl-adenosine A37 tRNA methylthiolase	RNA modification
GlpD	aerobic glycerol 3-phosphate dehydrogenase	Carbohydrate metabolic process
TreC	trehalose-6-phosphate hydrolase	Carbon metabolism
UcpA	oxidoreductase	Carbon metabolism
MaeA	malate dehydrogenase	Pyruvate metabolic process
UxuB	D-mannonate oxidoreductase	D-glucuronate metabolic process
Fbp	fructose-1,6-bisphosphatase 1	Gluconeogenesis
GlpX	fructose-1,6-bisphosphatase 2	Gluconeogenesis

GabD	succinate-semialdehyde dehydrogenase (NADP+)	Enzymatic process
HcxB	hydroxycarboxylate dehydrogenase B	Enzymatic process
PncB	nicotinate phosphoribosyltransferase	Enzymatic process
YibF	glutathione transferase-like protein	Enzymatic process
MglA	D-galactose/methyl-galactoside ABC transporter	Active transporter

Table 3 Supplementary: Upregulated significant Proteins in Fosfomycin evolved *E. coli* strain

Downregulated Proteins in *E.coli (K12)* Evolved with Fosfomycin		
Proteins	**Function**	**Function category**
AstB	arginine catabolic process	Amino acid Metabolism
GdhA	glutamate dehydrogenase	Amino acid Metabolism
AstD	arginine catabolic process	Amino acid Metabolism

DcyD	|D-cysteine catabolic process	Glutathione metabolic process
Ggt	glutathione hydrolase proenzyme	Carbohydrate metabolic process
NanM	N-acetylneuraminate catabolic process	Carbohydrate metabolic process
NagA	N-acetylgalactosamine-6-phosphate deacetylase activity	Carbohydrate metabolic process
NagB	carbohydrate metabolic process	Carbohydrate metabolic process
NagD	phosphatase activity, UMP catabolic process	Carbohydrate metabolic process
TalA	pentose-phosphate shunt	Carbohydrate metabolic process
TktB	pentose-phosphate shunt	Carbohydrate metabolic process
MtlD	mannitol-1-phosphate,5-dehydrogenase	Carbohydrate metabolic process
GarR	D-glucarate catabolic process	Carbohydrate metabolic process

PflB	glucose metabolic process	Fatty acid metabolism and biosynthesis
FabH	fatty acid biosynthetic process	Fatty acid metabolism and biosynthesis
GlnB	regulation of nitrogen utilization	Fatty acid metabolism and biosynthesis
TesB	fatty acyl-CoA hydrolase activity	Fatty acid metabolism and biosynthesis
SlyB	outer membrane lipoprotein	Structural protein
TyrS	tyrosyl-tRNA aminoacylation	Aminoacyl-tRNA synthetases
PoxB	thiamine diphosphate	Pyruvate metabolic process
IlvD	dihydroxy-acid dehydratase	Amino acid biosynthesis
Map	methionine aminopeptidase	Catalyze activity
TreA	cellular hyperosmotic response	Cellular response

AhpC	peroxidase, response tooxidative stress	Cellular response
AppA	Phosphotransferase	Cellular response
SodC	removal of superoxide radicals	Cellular response

Table 3 Supplementary: Differentially regulated significant Proteins in Glyphosate evolved *E. coli* strain

Upregulated Proteins in *E. coli (K12)* Adapted with Glyphosate		
Gene	Function	Function category
HISI	Phosphoribosyl AMP cyclohydrolase	Histidine Biosynthesis
Downregulated Proteins in *E. coli* (K12) Adapted with Glyphosate		
ALLR	HTH-transcriptional repressor AllR	Regulatory protein
NUCO	NADH:quinone oxidoreductase subunit CD	ATP Synthesis Coupled Electron Transport

NRDA	Ribonucleoside-diphosphate reductase 1 subunit alpha	Metabolic process
PAL	Peptidoglycan-associated protein	Cell structure
OPPD	Oligopeptide transport ATP-binding protein	Transport

Figure A1-A6 MS/MS Spectral Matching: The identities of selected metabolites were supported by GNPS library matching. Mirror plots comparing experimental and reference MS/MS spectra are shown below. Cosine similarity scores indicate the degree of spectral agreement between the detected feature and the library spectrum.

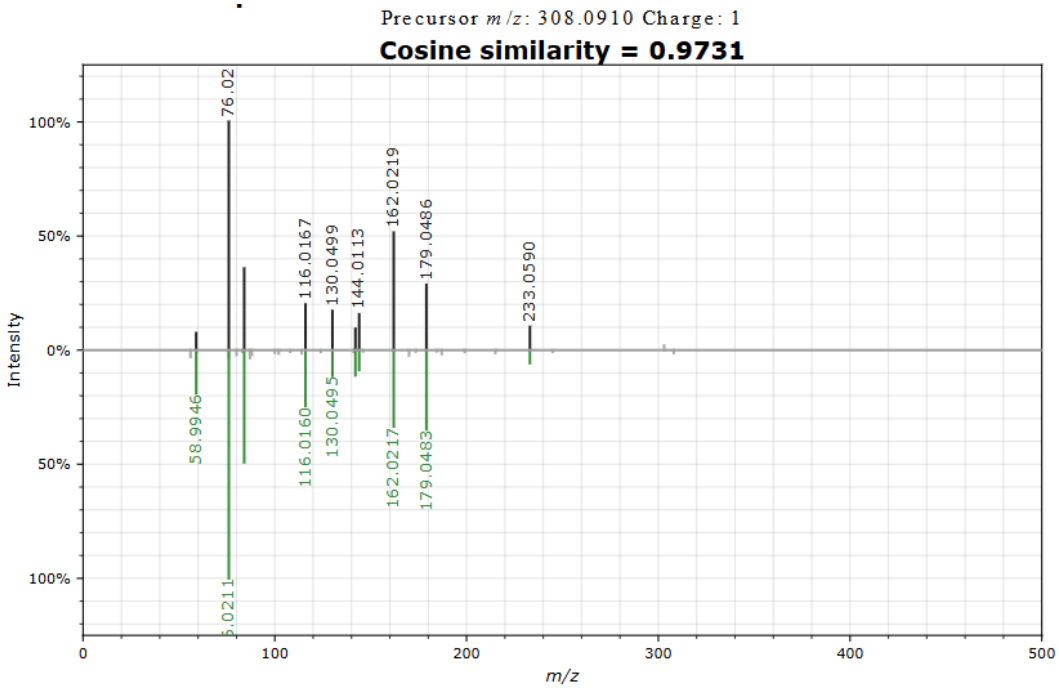


Figure A1. GNPS mirror plot supporting the annotation of Glutathione.

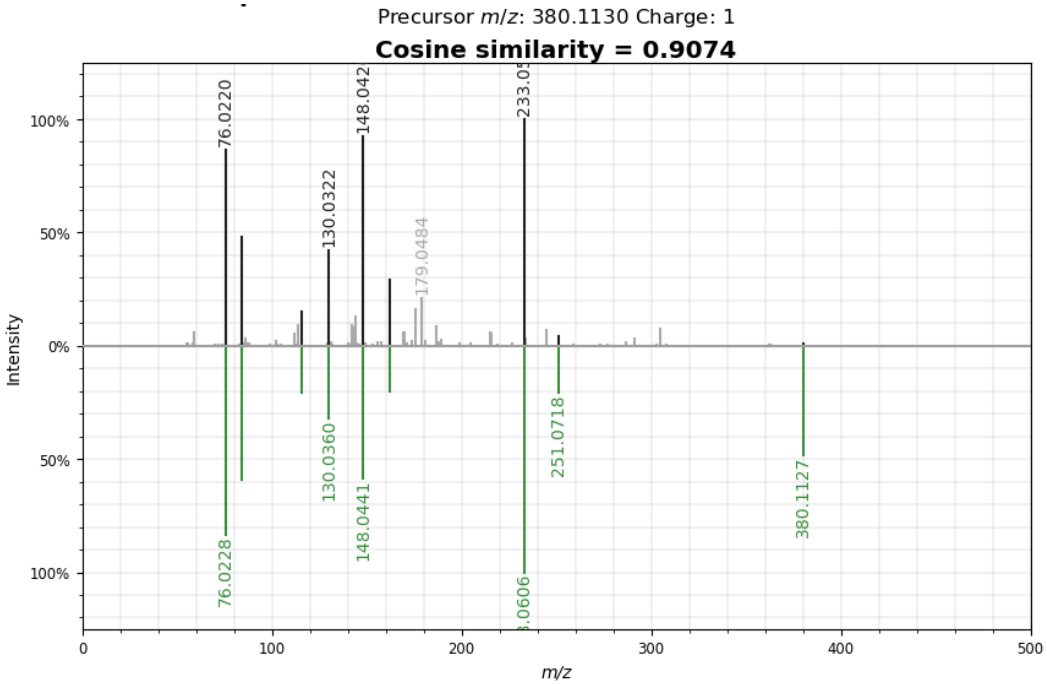


Figure A2. GNPS mirror plot supporting the annotation of S-Lactoylglutathione.

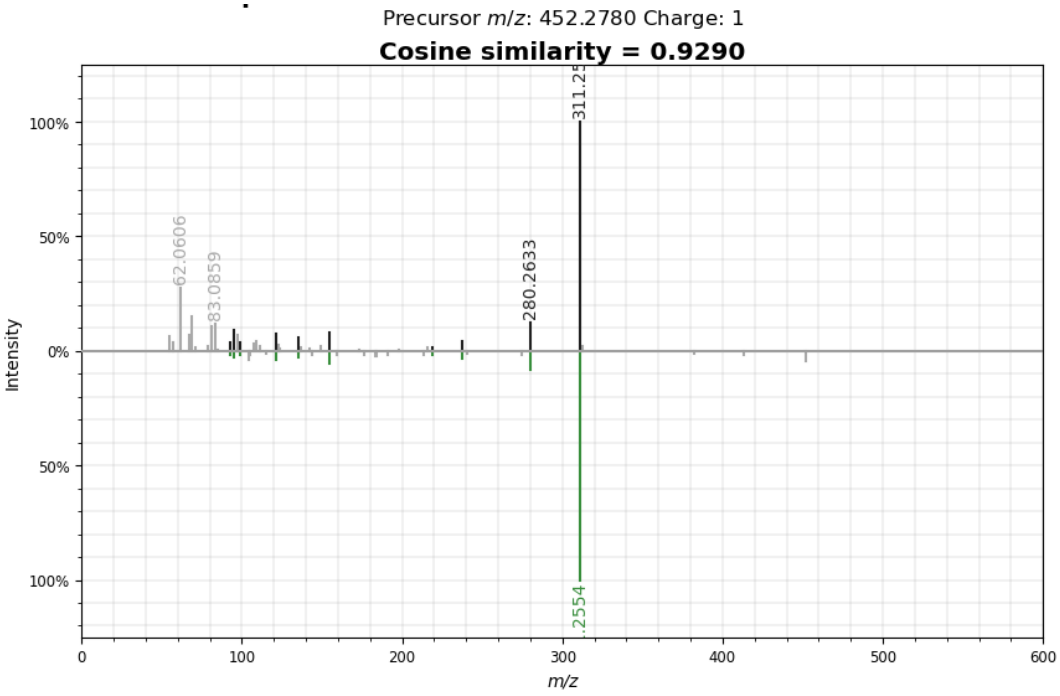


Figure A3. GNPS mirror plot supporting the annotation of PE.

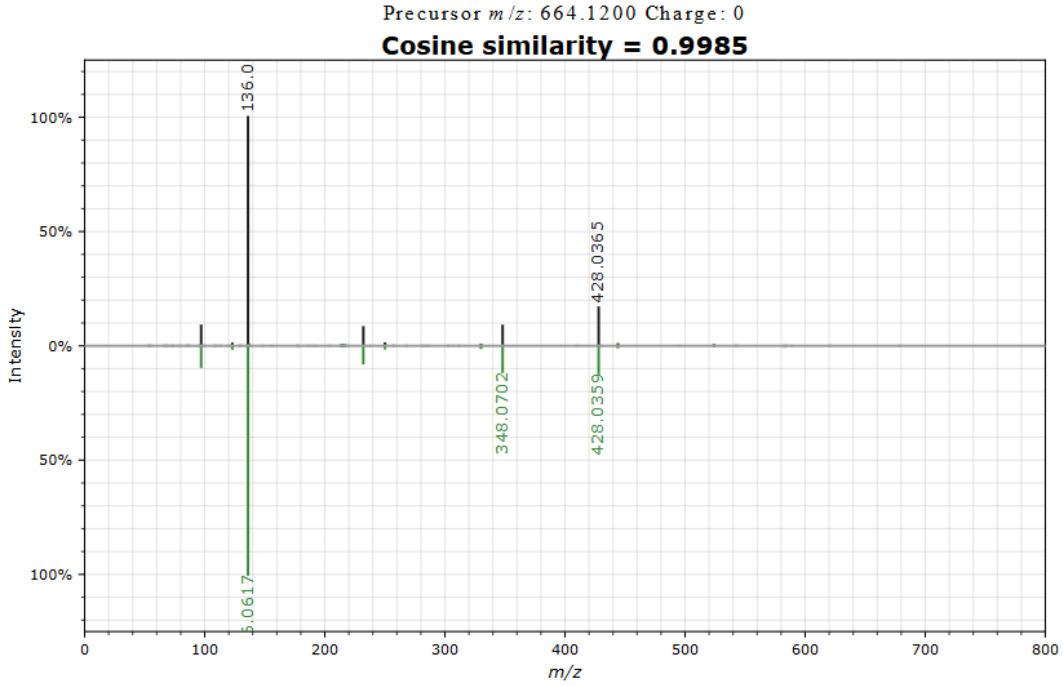


Figure A4. GNPS mirror plot supporting the annotation of NAD.

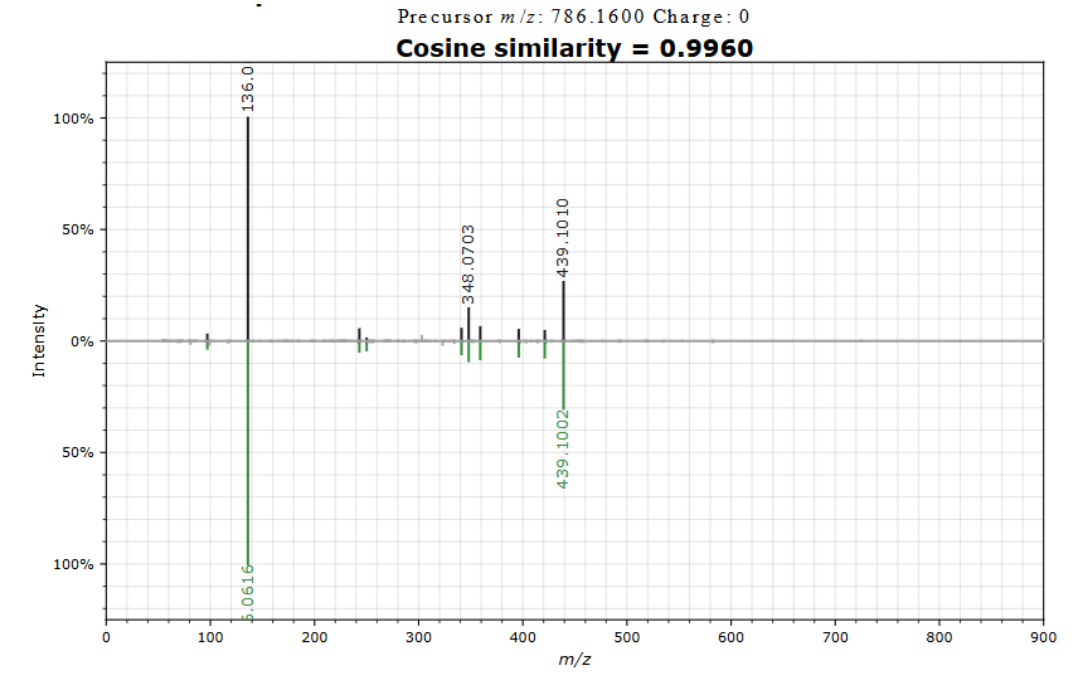


Figure A5. GNPS mirror plot supporting the annotation of FLAVIN ADENINE DINUCLEOTIDE

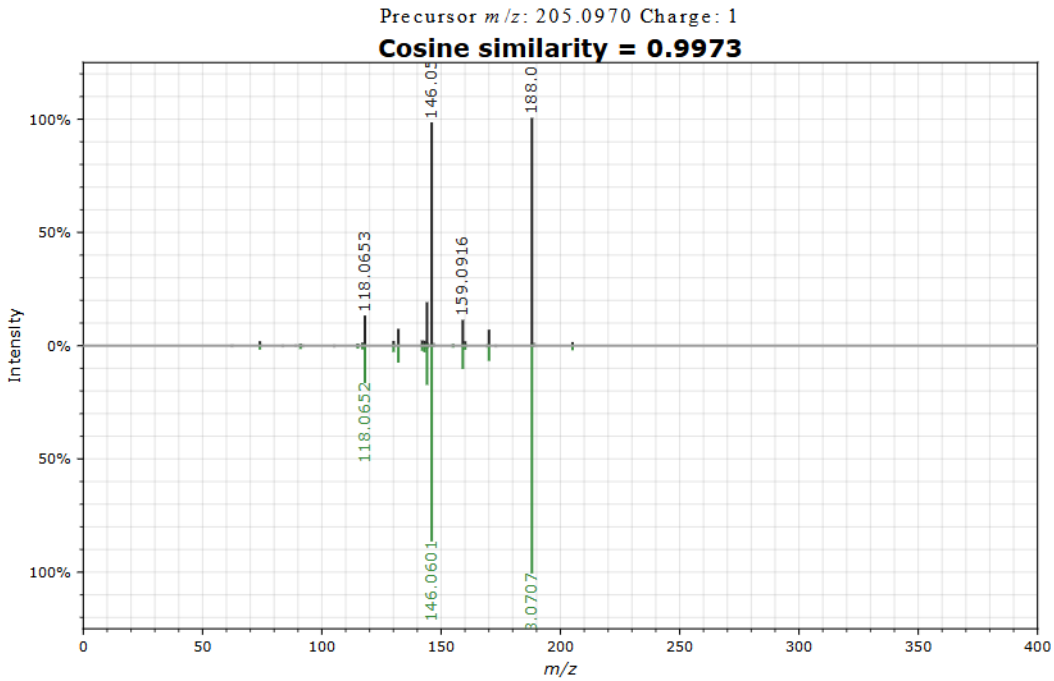


Figure A6. GNPS mirror plot supporting the annotation of L Tryptophan

Materials

1. Bacteria

Phylum	**Class**	**Order**	**Specie**	**Strain**	**Catagory**
Proteobacteria	Gamma proteobacteria	Enterobacterial	*Escherichia coli*	*K-12*	*Gram-negative*
Firmicutes	Bacilli	Bacillales	*Staph aureus*	USA300	Gram-positve

2. Solid and Liquid Media

2.1 Solid Media

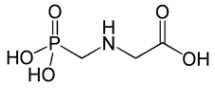
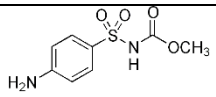
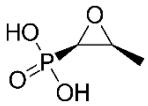
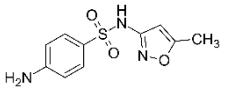
Media	Substance	Amount	Company
Luria-Bertani LB (agar)	Tryptone	10g	Sigma Aldrich
	NaCl	5g	Sigma Aldrich
	Yeast extract	4g	Sigma Aldrich
	Agar	15g	Sigma Aldrich
	ddH20	1L	Sigma Aldrich
Müller-Hinton (MH) Broth	Müller-Hinton 16.8 g VWR	16.8 g	VWR
	ddH_2O	0.8L	
	Agar	15g	Sigma Aldrich

2.2 Liquid Media

Media	Substance	Amount	Company
Luria-Bertani LB (agar)	Tryptone	10g	Sigma Aldrich
	NaCl	5g	Sigma Aldrich
	Yeast extract	4g	Sigma Aldrich
	ddH20	1L	Sigma Aldrich
Müller-Hinton (MH) Broth	Müller-Hinton 16.8 g VWR	16.8 g	VWR
	ddH_2O	0.8L	

3.Xenobiotics and Antibiotics

Xenobiotics/Antibiotics	**Solvent**	**Structures**	**M.W**

Imidazole	Water		68.07g/mol
Glyphosate	Water		169.07g/mol
Asulam	Water		230.24g/mol
Fosfomycin	Water		138.03g/mol
Sulfamethoxazole	DMSO+Water		253,28 g/mol

Author Contribution

Chapters 2 and 3 of this thesis are prepared as manuscripts for submission to peer-reviewed journals.

Chapter 2: Review Article (Manuscript prepared)

Title: *Xenobiotic abundance in the environment contributes to antibiotic resistance*

Personal Contribution

The review presented in Chapter 2 was conceptualized together with my supervisor. I conducted the literature search and synthesized current research on xenobiotics, antibiotic resistance, and microbial adaptation. I developed the structure of the manuscript and wrote the main text under the guidance of my supervisor. The manuscript was refined through regular discussions with my supervisor, who provided feedback and guidance on the content, interpretation, and overall organization.

Chapter 3: Experimental Research Article (Manuscript will be submitted soon)

Title: *Xenobiotic adaptation and antibiotic cross-tolerance in E. coli*: multi-omics insights

Authors:

Sibgha Tayyab[1], Paolo Stincone[1], Karoline Steuer-Lodd[2], Thomas Cummins[3], Ansel Hsiao[3], Daniel Petras[1,2]

Personal Contribution

I conceptualized and planned the study together with Daniel Petras (main supervisor for the project and thesis, and corresponding author). I designed and performed experimental evolution studies in *Escherichia coli* K-12 under different

chemical stress conditions. I carried out the bacterial culturing, adaptation experiments, minimum inhibitory concentration (MIC) assays, phenotypic characterization, and cross-resistance growth curve analyses.

For the metabolomics and proteomics workflows, I received methodological guidance from Paolo Stincone. I performed the sample preparation and coordinated the experiments for mass spectrometry analysis. Together with Paolo Stincone, I conducted the LC-MS/MS measurements, while Karoline Steuer-Lodd contributed to the proteomics protocol.

I performed the primary data analysis and interpretation of the proteomic and metabolomic datasets, focusing on identifying stress-response mechanisms and patterns of antibiotic cross-tolerance under the guidance of my supervisor. I prepared the figures and wrote the manuscript. All authors contributed to reviewing and editing the manuscript and approved the final version.

Chapter 4: Additional Research Work (Project in progress)

Title: *Exploring the association between proteome-wide off-target effects and the rate of resistance evolution in E. coli*

Personal Contribution

Chapter 4 is part of a broader project conceptualized by Daniel Petras. Within this project, I conducted experimental evolution studies to investigate bacterial adaptation under antibiotic pressure. I designed and performed the experiments and carried out the initial phenotypic characterization.

Further analyses and downstream work related to this project are ongoing and will be completed beyond the timeframe of this thesis. The current chapter presents the experimental evolution framework and preliminary findings generated during my contribution to the project.

Other Publications

Authur Contribution

The following publication is not part of the dissertation chapters but represents collaborative work to which I contributed during my PhD studies.

Peer-reviewed Research Article

Pakkir Shah, Abzer K.; Walter, Axel; …; Sibgha Tayyab; …; Ernst, Madeleine; & Petras, Daniel (2025). *Statistical analysis of feature-based molecular networking results from non-targeted metabolomics data*. *Nature Protocols, 20*(1), 92–162. https://doi.org/10.1038/s41596-024-01046-3

Personal Contribution

I tested and validated the statistical analysis protocol on my untargeted metabolomics datasets, including assessment of both the computational code and the app interface.

I confirm that the above statement is correct.

May16,2026
Date, Signature of the Candidate

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I certify that the above statement is correct.

May18,2026
Date, Signature of the Supervisor

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